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Epigenetic remodeling in colorectal cancer results in coordinate gene suppression across an entire chromosome band

Jordi Frigola[1,2], Jenny Song[1], Clare Stirzaker[1], Rebecca A Hinshelwood[1], Miguel A Peinado[2] & Susan J Clark[1]

We report a new mechanism in carcinogenesis involving coordinate long-range epigenetic gene silencing. Epigenetic silencing in cancer has always been envisaged as a local event silencing discrete genes. However, in this study of silencing in colorectal cancer, we found common repression of the entire 4-Mb band of chromosome 2q.14.2, associated with global methylation of histone H3 Lys9. DNA hypermethylation within the repressed genomic neighborhood was localized to three separate enriched CpG island 'suburbs', with the largest hypermethylated suburb spanning 1 Mb. These data change our understanding of epigenetic gene silencing in cancer cells: namely, epigenetic silencing can span large regions of the chromosome, and both DNA-methylated and neighboring unmethylated genes can be coordinately suppressed by global changes in histone modification. We propose that loss of gene expression can occur through long-range epigenetic silencing, with similar implications as loss of heterozygosity in cancer.

Cancer is a disease of the DNA, with both genetic and epigenetic lesions contributing to changes in gene expression. Genetic changes include gene mutations in critical tumor-associated genes and gene deletion or loss of heterozygosity (LOH). In addition to genetic changes, it is clear that epigenetic changes are also a common hallmark of cancer, with changes in both DNA methylation and histone modification of CpG island–associated promoters of tumor suppressor genes[1,2].

DNA methylation in mammals occurs mainly at cytosine residues in CpG dinucleotide pairs. The bulk of the mammalian genome is depleted of CpG, but short stretches of CpG-dense DNA, known as CpG islands, also exist, and these are typically found associated with the 5′ promoter end of all housekeeping genes and many tissue-specific genes and with the 3′ end of some tissue-specific genes[3]. There are at least 29,000 CpG islands in the human genome, according to DNA sequencing estimates[4]. Most CpG island promoters are unmethylated, a state associated with active gene transcription in a normal cell. In contrast, CpG island promoters can become *de novo* methylated in a cancer cell; this methylation is associated with gene silencing. DNA hypermethylation is also accompanied by local changes in histone modification, including deacetylation and methylation of the Lys9 residue of histone H3 (K9-H3).

One of the major questions still to be resolved is the mechanism responsible for epigenetic change in cancer; in particular, what determines which CpG island–associated genes are susceptible to hypermethylation and histone modification in different cancer types[5–8]. Thus far, studies using either candidate gene approaches or global array surveys[9–13] have demonstrated that hundreds of different and discrete CpG islands can be methylated in any one tumor; some of these are commonly methylated in several tumor types, whereas others are methylated in a tumor-specific manner. Thus, despite numerous examples of methylation-associated gene silencing events in human cancer cells, it is not clear what dictates epigenetic silencing and what protects some islands from methylation.

Using a global methylation approach (amplification of intermethylated sites, or AIMS[14]) and chromatin immunoprecipitation (ChIP)[15,16], we now show that epigenetic changes in cancer are not simply restricted to individual, discrete CpG island–associated genes but can also encompass multiple neighboring CpG islands and genes. Our results demonstrate for the first time that epigenetic gene silencing in cancer can encompass a large chromosomal region, with the equivalent implications for global gene silencing as in the case of gross genetic changes.

RESULTS

Identification of a differentially methylated region

To screen for common methylation changes in colorectal cancer, we used a global methylation approach (AIMS)[14,17] that allows for amplification of DNA only if it is flanked by two methylated *Sma*I sites (**Supplementary Fig. 1** online). We identified a number of fragments methylated differently in the cancer cells compared with matched normal colon cells (**Supplementary Fig. 1**). One fragment

[1]Cancer Program, Garvan Institute of Medical Research, 384 Victoria Street, Darlinghurst, Sydney 2010, New South Wales, Australia. [2]Institut d'Investigacio Biomedica de Bellvitge (IDIBELL)-Institut de Recerca Oncologia, Hospital Duran i Reynals, 08907 L'Hospitalet, Barcelona, Spain. Correspondence should be addressed to S.C. (s.clark@garvan.org.au).

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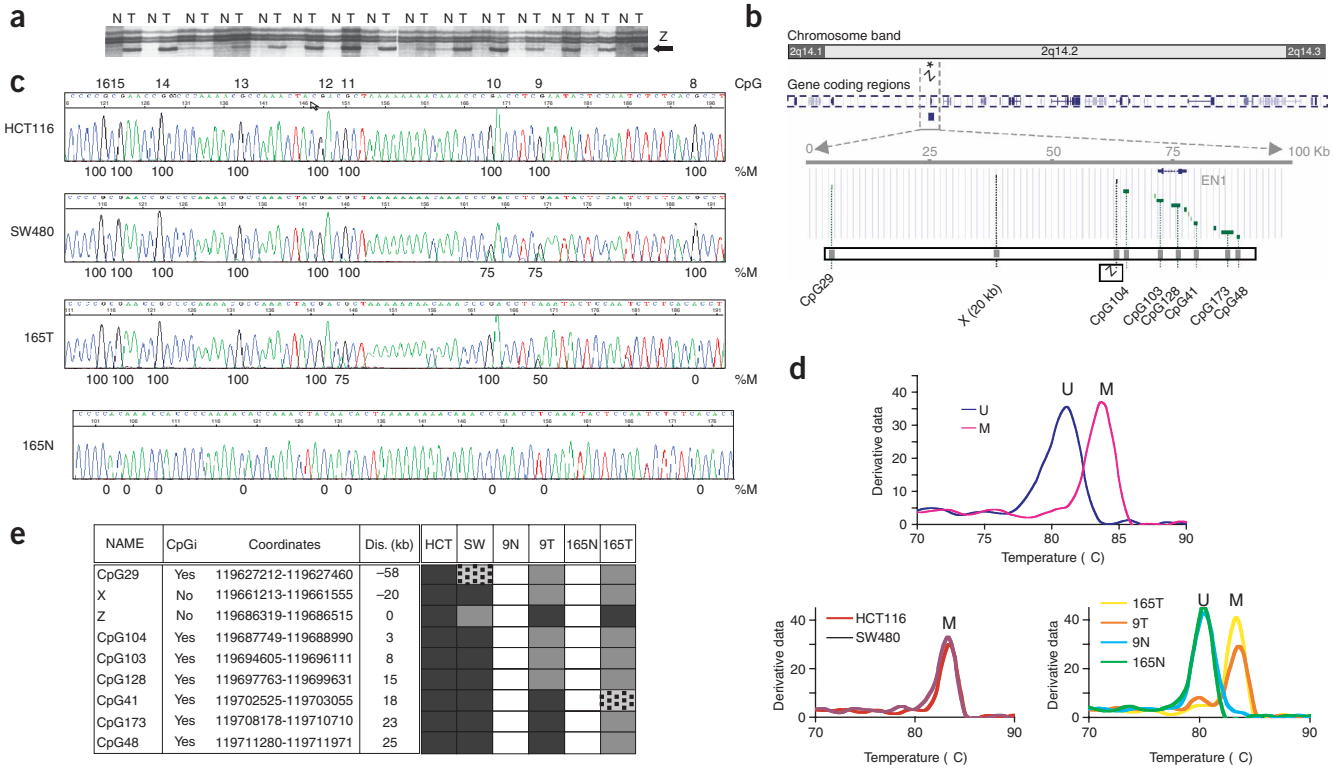
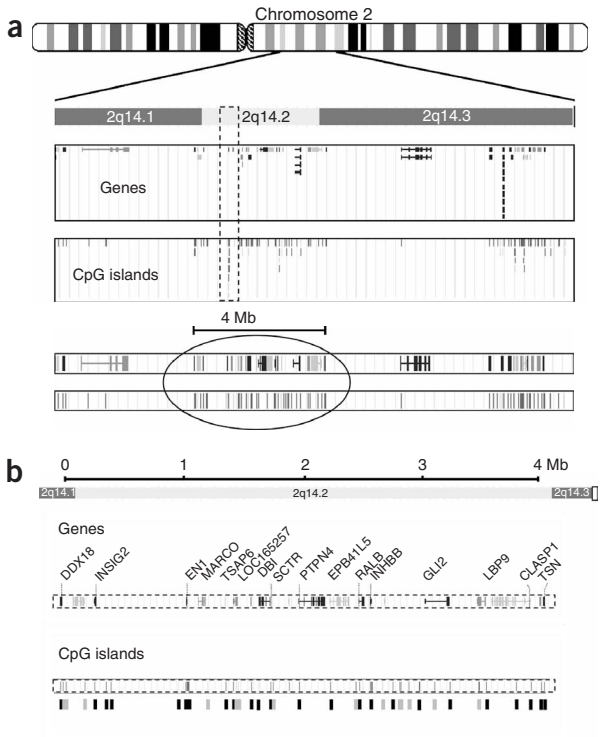


NAME	CpGi	Coordinates	Dis. (kb)	HCT	SW	9N	9T	165N	165T
CpG29	Yes	119627212-119627460	–58						
X	No	119661213-119661555	–20						
Z	No	119686319-119686515	0						
CpG104	Yes	119687749-119688990	3						
CpG103	Yes	119694605-119696111	8						
CpG128	Yes	119697763-119699631	15						
CpG41	Yes	119702525-119703055	18						
CpG173	Yes	119708178-119710710	23						
CpG48	Yes	119711280-119711971	25						

Figure 1 Differential methylation of a region in colorectal cancer identified by AIMS. (**a**) Example of differential methylation from 15 sets of cancer and matched normal colorectal samples. The Z AIMS fragment (196 bp) is indicated by an arrow. (**b**) Chromosomal location of the Z fragment (*) on 2q14.2, in context of the surrounding gene coding regions and CpG islands identified via the Genome Browser. Expanded view shows the position of the CpG islands, the CpG-depleted region (X) and Engrailed-1 (*EN1*). Dark green lines: CpG islands >300 bp. Light green lines: CpG islands <300 bp. (**c**) Direct PCR bisulfite sequencing electrophoretograms of *EN1* (CpG128). Examples from colorectal cell lines HCT116 and SW480 and matched normal (165N) and tumor samples (165T). CpG sites are numbered relative to the start of the PCR fragment. The percentage of methylation (%M) at each CpG site was determined by the relative C-to-T peak heights. (**d**) Real-time PCR dissociation analysis. Upper panel: unmethylated control DNA (U); methylated control DNA (M). Lower panels: dissociation curves of *EN1* (CpG128) from HCT116, SW480 and two normal (9N/165N) and matching tumor samples (9T/165T). (**e**) Summary of the DNA methylation profile across the 83-kb region encompassing the Z fragment. Coordinates from the Genome Browser and distance from the Z fragment are indicated. CpGi denotes presence or absence of a CpG island. The degree of methylation from HCT116, SW480 and 9N/T and 165N/T was determined by direct PCR sequencing: 0–25% (white); 25–50% (dotted gray); 50–75% gray; 75–100% (black). (**f**) Genomic bisulfite sequencing of individual clones across the 83-kb region encompassing the Z fragment. Each square denotes a CpG site across PCR fragments amplified from HCT116, SW480, 9N/T and 165N/T. Filled squares: methylated; open squares: unmethylated.



c

Name	CpGi	Dist (Kb)	Gene	Status	HCT	SW	9N	9T	165N	165T
CpG48	Yes	−1020	*DDX18*	Reviewed						
CpG49	Yes	−745	*INSIG2*	Reviewed						
CpG41.2	Yes	−650	–							
CpG61	Yes	−610	–							
CpG29	Yes	−590	–							
X	No	−25	–							
Z	No	0	–							
CpG104	Yes	2	–							
CpG103	Yes	8	–							
CpG128	Yes	11	*EN1*	Reviewed						
CpG41	Yes	16								
CpG173	Yes	23	–							
CpG48	Yes	25	–							
5′-MARCO	No	110	*MARCO*	Reviewed						
CpG229	Yes	320	*LOC165257*	Predicted						
CpG85	Yes	390	*TSAP6*	Provisional						
CpG85	Yes	540	*DBI*	Provisional						
CpG85	Yes	600	–							
CpG67	Yes	690	*SCTR*	Reviewed						
CpG86	Yes	930	*PTPN4*	Reviewed						
CpG102	Yes	1180	*EPB41L5*	Predicted						
CpG115	Yes	1400	*RALB*	Reviewed						
CpG285	Yes	1500	*INHBB*	Reviewed						
CpG26	Yes	1700	–							
CpG206	Yes	1900	–							
CpG22	Yes	2150	*GLI2*	Reviewed						
CpG112	Yes	2500	*LBP9*	Provisional						
CpG51	Yes	2700	–							
CpG104	Yes	2800	*CLASP1*	Provisional						
CpG37	Yes	2900	*MKI671P*	Provisional						
CpG59	Yes	2920	*TSN*	Reviewed						

1 Mb

Figure 2 DNA methylation profile of the 4-Mb region in colorectal cancer on chromosome 2q14.2. (**a**) Chromosomal location of 2q14.2 on chromosome 2. The locations of the genes and CpG islands are indicated in the panels (as determined from the Genome Browser). The dotted lines indicate the region spanning the Z fragment that was sequenced in **Figure 1**. (**b**) Detailed analysis of the location of the genes and associated CpG islands across the 4-Mb region, based on SWISS-PROT. Lower panel shows the location of the CpG islands. Bisulfite-sequenced islands ($n = 31$) are black, and CpG islands not analyzed are gray ($n = 15$). (**c**) Summary of the DNA methylation profile across the 4-Mb region of chromosome 2q14.2. Direct PCR sequencing was used for methylation analysis of the CpG islands and CpG-depleted regions in two colorectal cell lines (HCT116 and SW480) and two pairs of cancer and matched normal samples (9N/9T, 165N/165T). CpGi denotes presence or absence of a CpG island. Shown are the distance from the Z fragment and the status of the defined and provisional and predicted genes. Average overall methylation of the CpG island is indicated: 0–25% (white); 25–50% (dotted gray); 50–75% gray; 75–100% (black).

(termed the 'Z fragment') was commonly hypermethylated in 71 out of 112 (63%) colorectal tumors (**Fig. 1a**). The Z fragment was gel purified, sequenced and found to encompass a pair of *Sma*I sites 196 bp apart. It was CpG rich, but did not meet the standard definition of a CpG island[3]. However, the Z fragment was located on chromosome 2q14.2, 1.2 kb upstream from a region enriched with multiple CpG islands (**Fig. 1b**). The CpG-dense region spanned 25 kb and contained 11 discrete CpG islands. However, only one island, CpG128 (containing 128 CpG sites), was associated with a reviewed gene; this island spanned the promoter and first exon of a developmentally regulated homeobox-containing gene, Engrailed-1 (*EN1*; **Fig. 1b**).

DNA hypermethylation of CpG islands neighboring Engrailed-1

We determined if the 25-kb cluster of CpG islands was also differentially methylated in colorectal cancer using a combination of direct bisulfite PCR sequencing (**Fig. 1c**) and PCR melting dissociation temperature (**Fig. 1d**). **Figure 1e** summarizes the methylation status of the Z fragment, 6 of the 11 CpG islands from two colorectal cell lines, HCT116 and SW480, and two matched pairs of tumor and normal colorectal tissue samples (9N/T and 165N/T). The Z fragment and neighboring CpG islands, including the *EN1* CpG island (CpG128), were all unmethylated in the normal colorectal tissue but were extensively methylated in the colorectal cancer cell lines and cancer tissue samples (**Fig. 1e**). Even though there was a distinct difference in methylation between the normal and tumor cells, not all CpG islands were methylated to the same extent. Clonal sequencing data of a subset of CpG islands (**Fig. 1f**) showed that the lesser degree of methylation observed in the tumor tissue samples versus the cell lines was due to both unmethylated and heterogeneously methylated molecules. It is possible that the unmethylated molecules were derived from the presence of normal cells in the cancer sample. Clonal sequencing analysis also detected a low level of methylation in some of the molecules in the matched normal samples, reflecting either minor cancer cell contamination or a low level of methylation in the normal colon. In contrast, direct PCR sequencing, which is less sensitive, showed limited detectable methylation, or none at all, in the normal samples, compared with extensive methylation in the cancer samples (**Fig. 1c**).

To determine if differential methylation also extended upstream of the Z fragment, we analyzed the CpG-depleted region 20 kb upstream from the Z fragment, termed 'X' (a 182-bp region; GC content: 63%; CpG O/E = 0.6), and the next upstream island (CpG29) that was located 58 kb from the Z fragment (**Fig. 1b**). The CpG sites in X and in CpG29 were hypermethylated in HCT116 and in the two cancer samples, compared with the matched normal samples (**Fig. 1e**). These results demonstrate contiguous hypermethylation of neighboring CpG islands in the cancer cells across a region that spanned 83 kb from CpG29 to CpG48. Differential hypermethylation also occurred in regions that were not CpG islands, indicating that hypermethylation is not restricted to CpG island regions in cancer cells.

DNA methylation analysis across chromosome 2q14.2

We extended the methylation analysis on either side of the 83-kb methylated region to define the length and boundaries of the differentially methylated region across the 14.2 cytogenetic band on chromosome 2. Chromosome 2q14.2 is rich in genes and associated CpG islands and spans a 4-Mb region (**Fig. 2a**). The locations of the reviewed and predicted genes and associated CpG islands are shown in **Figure 2b**. Ten reviewed genes reside in 2q14.2; of these, eight have CpG island–associated promoters (**Supplementary Fig. 2** online), one (*GLI2*) has a 3′ CpG island and *MARCO* has no associated CpG island. We sequenced the CpG islands from eight genes and a number of the intervening CpG islands, many of which are associated with predicted genes, using direct bisulfite PCR (**Supplementary Fig. 3** online) and PCR clonal analysis (**Supplementary Fig. 4** online). **Figure 2c** summarizes the DNA methylation profile for all CpG island and non-island regions sequenced across the 4-Mb region on 2q14.2. In DNA from the cancer cells, we found contiguous hypermethylation of all the neighboring CpG islands in a region that spanned nearly 1 Mb, from CpG61, 610 kb upstream of the Z fragment, to CpG229, 325 kb downstream of the Z fragment. The hypermethylated 1-Mb region contained 15 CpG islands, but only two islands, CpG128 and CpG229, were associated with either a known gene (*EN1*) or a predicted gene (*LOC165257*, encoding a C1q domain–containing protein).

We identified two additional regions of extensive hypermethylation in the colorectal cancer cells along the 14.2q cytogenetic band from chromosome 2 (**Fig. 2c**). The first hypermethylated region was located 690 kb downstream of the Z fragment and included the CpG island (CpG67) spanning the promoter of the *SCTR* gene (coding for the secretin receptor). The second hypermethylated region was located 1.5–2.15 Mb downstream of the Z fragment, spanning 650 kb in length and encompassing four CpG islands: CpG285, which spanned the promoter of the *INHBB* gene (inhibin beta B), CpG26 and CpG206, which were not associated with gene promoter regions, and CpG22, which was located at the 3′ end of the *GLI2* gene (encoding a C2H2-type zinc finger protein).

Each of the three hypermethylated regions within the 14.2q cytogenetic band was flanked by unmethylated CpG islands. Three unmethylated CpG islands were located upstream of the 1-Mb hypermethylated region, overlapping the junction to the 14.1 band, and included the CpG islands associated with the genes *INSIG2* (encoding insulin induced protein 2) and *DDX18* (encoding DEAD box polypeptide18). Similarly, the CpG islands that were located at the 14.3q band junction remained unmethylated in the colorectal cancer cells and were associated with two genes, *SFRS16* (also known as *CLASP*), encoding the CLIP-associating protein, and *TSN* (translin). Two sets of CpG island clusters between the three methylated regions also remained unmethylated in both cancer cell lines and cancerous and normal tissue samples. The first set of islands was associated with the genes *TSAP6* (coding for hypothetical protein Dudulin2) and *BZRP* (also known as *DBI*, encoding diazepam binding inhibitor). The second set was associated with the *PTPN4* gene (encoding protein tyrosine phosphatase, non-receptor type 4) and *RALB* (also known as *RALBB*; encoding ras-related v-ral simian leukemia viral oncogene homolog B; ras related; GTP binding protein; **Fig. 2c**). Notably, the CpG islands that were protected from DNA hypermethylation in the cancer cells also seem to be less susceptible to *de novo* methylation in the normal cells (**Supplementary Fig. 4**). For example, we observed only single sites of CpG methylation, or no sites at all, in the *INSIG2*- or *RALBB*-associated CpG islands from 9N and 165N DNA, whereas we often observed a low level of methylation in the same normal samples from the CpG islands associated with *EN1* and *SCTR*, suggesting that seeding methylation may have a role in promoting subsequent hypermethylation in the cancer cell.

Only two genes across 2q14.2 contained promoters that were not associated with CpG islands: *MARCO* (a type II transmembrane protein of class A scavenger receptor) and *GLI2* (a GLI-Kruppel family member)[18]. The upstream promoter region of *MARCO* contained only four CpG sites, and these were methylated in both normal and cancer cells (**Fig. 2c**). The *GLI2* promoter was also depleted of CpG sites, but a CpG-rich region was located at the 3′ end of GLI2 (CpG22), and this region was also found to be methylated in both the normal and cancer cells (**Fig. 2c**).

DNA methylation in colorectal tumors

To determine if hypermethylation across chromosome 2q14.2 was as common in colorectal cancer as we initially found for the Z fragment (63%), we analyzed the methylation status of three CpG islands associated with *EN1* (CpG128), *SCTR* (CpG67) and *INHBB* (CpG285) from 26 colorectal cancers (**Fig. 3a**) and 12 cell lines (**Fig. 3b**). The three genes were commonly methylated in the cell lines and clinical samples: *EN1* was hypermethylated in 18/26 (70%), *SCTR* in 23/26 (88%) and *INHBB* in 15/26 (58%) of colorectal cancers (**Fig. 3a**). However, 25/26 (96%) cancer samples showed methylation of at least one of these genes, demonstrating that DNA hypermethylation of genes in three neighboring regions within chromosome 2q14.2

a

Sample	*EN1*	*SCTR*	*INHBB*	Age	Sex	Duke's
17	■	■		31	W	C2
127		■		31	W	C2
143	■	■	■	37	W	C1
175		■		46	W	B2
19		■		49	W	B1
69	■	■		52	M	B2
108		■		62	M	C2
122	■	■	■	62	M	C1
78	■	■		62	M	B2
103	■	■	■	66	M	B2
9	■	■	■	67	M	B2
21	■		■	68	W	B2
144		■	■	72	W	B2
72	■	■	■	73	M	C2
113	■	■	■	73	W	B2
63	■	■	■	74	W	C2
223	■	■	■	75	M	B2
147	■	■	■	76	W	C2
165	■	■	■	76	M	C2
75	■	■	■	77	M	B2
102	■			77	M	B2
151	■	■	■	79	M	B2
138		■		80	W	B2
171	■	■	■	85	M	C2
74		■		87	M	C2
90				88	W	C2

b

Cells	*EN1*	*SCTR*	*INHBB*
HCT116	■	■	■
SW480	■		
LoVo	■	■	
KM12sm	■	■	■
CaCo2	■	■	
DLD	■	■	
HT29	■		
KM12c	■	■	
LS174	■	■	
LS411N	■	■	■
LISPI	■	■	■
HCT15	■	■	

Figure 3 DNA methylation of the CpG islands associated with *EN1*, *SCTR* and *INHBB*. (**a**) Summary of methylation for 26 colorectal samples, as determined by real-time PCR dissociation. (**b**) DNA methylation of *EN1* (CpG128), *SCTR* (CpG67) and *INHBB (CpG285)*, determined by real-time PCR dissociation, in 12 colon cancer cell lines. Methylation: filled boxes. Lack of methylation: open boxes.

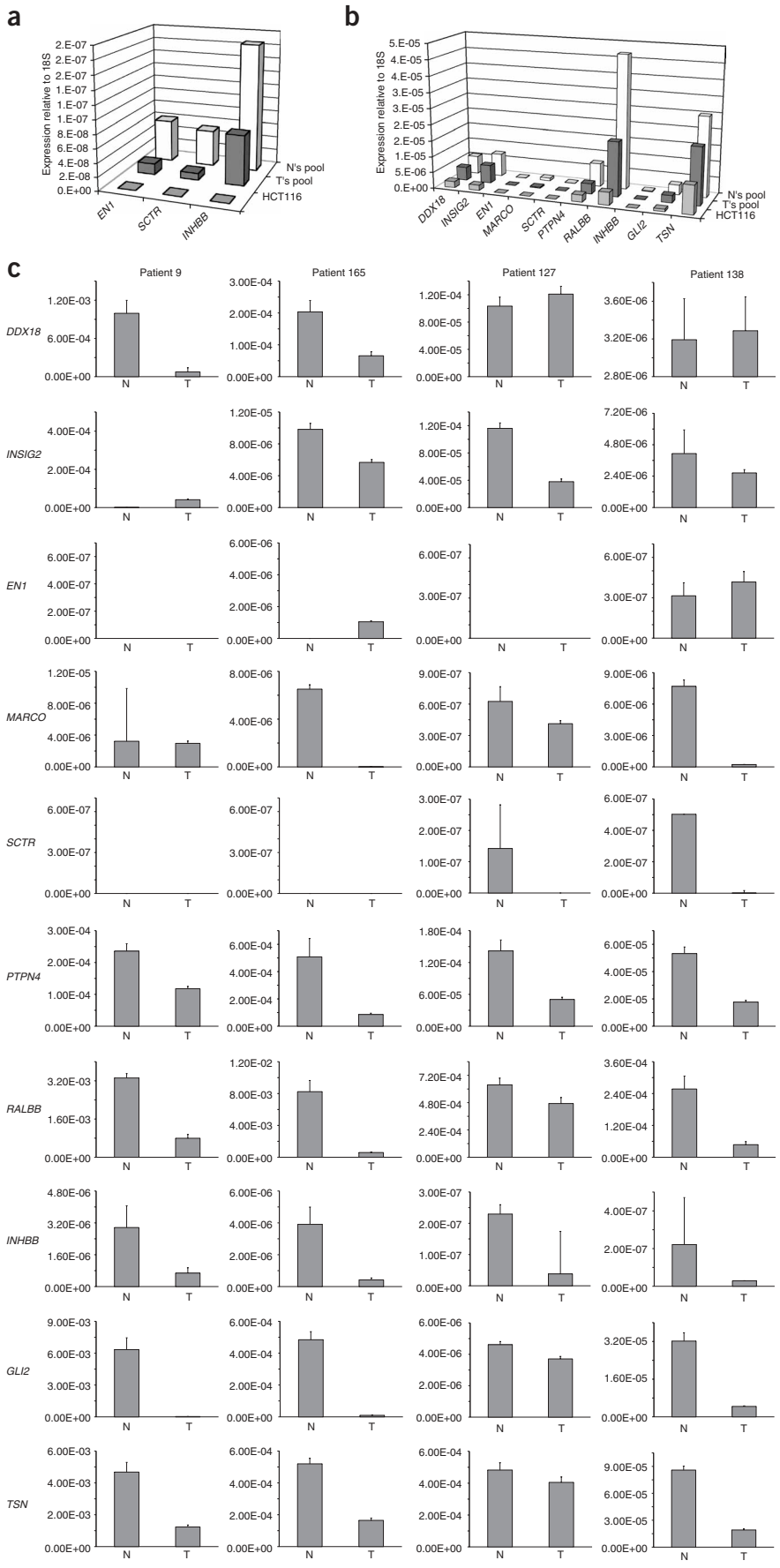


Figure 4 Gene suppression across 2q14.2 in colorectal cancer. (**a**) mRNA expression levels of *ENI*, *SCTR* and *INHBB* from HCT116 cells was compared with the expression levels of RNA isolated from ten pooled colorectal tumor tissue samples and the corresponding ten pooled normal tissues from patients 17, 63, 69, 127, 138, 143, 147, 151, 171 and 175. cDNA was prepared from RNA isolated from each individual sample, pooled and amplified in triplicate using real-time PCR, and the expression of each gene was measured relative to expression of 18S rRNA. (**b**) mRNA expression of all the defined genes across the 2q14.2 cytogenic band in HCT116 cells was compared with expression of the same genes in pooled tumor and pooled matched normal samples. (**c**) mRNA expression of each of the genes located on chromosome 2q14.2, from individual tumor and matched normal colorectal samples, normalized to 18S rRNA expression.

occurs commonly in colorectal cancer. We found the extent of hypermethylation and number of genes methylated to be independent of sex, age and Dukes stage, indicating it may be an early event in colorectal cancer.

Global gene suppression across chromosome 2q14.2

We then determined if hypermethylation of the CpG islands in the three hypermethylated regions correlated with suppression of gene expression. We compared expression of the defined genes across 2q14.2 between HCT116 cells, ten pooled colorectal tumor samples and ten pooled normal samples (**Fig. 4**; **Supplementary Fig. 5** online). Expression of the genes associated with CpG island hypermethylation, *EN1*, *SCTR* and *INHBB*, was significantly reduced in the pooled primary cancer samples relative to the matched normal samples and was completely inactivated in HCT116 cells (**Fig. 4a**). Additionally, we found that expression of genes that were associated with unmethylated CpG islands was also suppressed in HCT116 cells and pooled tumor samples compared with normal samples (**Fig. 4b**). We observed higher levels of suppression for the unmethylated genes directly flanking the hypermethylated regions than for the unmethylated genes located at a greater distance. For example, *PTPN4* and *RALBB*, which are surrounded by methylated domains, showed a greater reduction in expression than *TSN*, *INSIG2* and *DDX18*, which are at the boundaries of the 14.2q cytogenetic band.

We then analyzed gene expression individually from four matched pairs of normal and cancer samples that showed differing levels of DNA methylation (**Fig. 4c**). Substantial gene suppression occurred across the

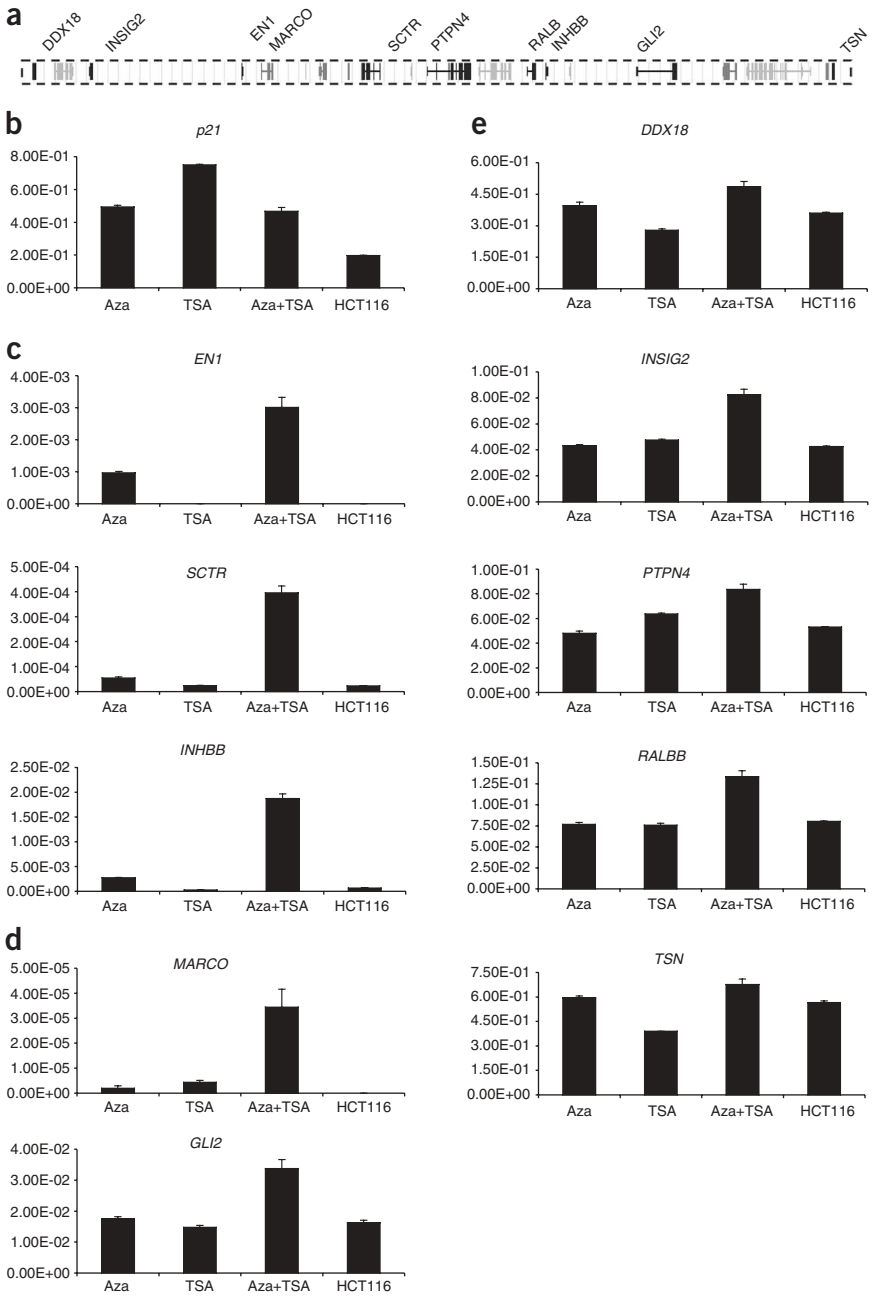


Figure 5 Effect of 5AzaC and TSA on gene expression across region 2q14.2. RNA was isolated from untreated HCT116 cells, and HCT116 cells that were treated with 5AzaC, TSA or a combination of 5AzaC and TSA (AZA+TSA). RNA was reverse-transcribed, and expression was quantitated by real-time PCR and normalized using 18S rRNA expression. (**a**) Chromosomal location on 2q14.2 of the analyzed genes. (**b**–**e**) Levels of gene expression from different gene classes. Expression of a control gene, *p21* (**b**); methylated CpG island–associated genes *EN1*, *SCTR* and *INHBB* (**c**); methylated non–CpG island genes *MARCO* and *GLI2* (**d**); and unmethylated CpG island–associated genes *DDX18*, *INSIG2*, *PTPN4*, *RALBB* and *TSN* (**e**) from treated or untreated HCT116 cells.

4-Mb region in the tumor samples (9T & 165T) in which the three loci containing *EN1*, *SCTR* and *INHBB* were hypermethylated (**Fig. 3a**), and this suppression also included the boundary genes *TSN* and *DDX18*. We also observed gene suppression of the neighboring unmethylated genes in tumor samples (127T & 138T) in which only *SCTR* was hypermethylated (**Fig. 3a**). However, in sample 127, the level of suppression was less pronounced for the boundary genes *TSN* and *DDX18* (**Fig. 4c**). Therefore, the degree of hypermethylation in the tumor DNA correlates with suppression of the flanking unmethylated genes.

In considering the absolute levels of expression in normal colon tissue of genes in the 14.2q region, it is evident that genes associated with CpG islands that remained unmethylated in the cancer cells (*DDX18*, *INSIG2*, *PTPN4*, *RALBB* and *TSN*) were expressed at a higher level in the normal colorectal cells, whereas the CpG island–associated genes (*EN1*, *SCTR*, *INHBB*) that were commonly hypermethylated in the cancer cells were minimally expressed (basal levels) or not expressed at all in normal cells. These data show that there is an overall suppression of gene expression across 2q14.2 in colorectal cancer, even in the genes that remain unmethylated.

Chromatin modification occurs across the entire 2q14.2 band

To address if the gene suppression observed was associated with chromatin modification, we treated HCT116 cells separately with either the demethylating agent 5-aza-2′-deoxycytidine (5AzaC), an inhibitor of histone deacetylase, trichostatin A (TSA), or with a combination of both (**Fig. 5**). As a positive control, we tested the expression of *p21* (refs. 19–21) and found that *p21* expression was activated in HCT116 cells under all treatment conditions. (**Fig. 5b**). For the genes that were hypermethylated in HCT116 (*EN1*, *SCTR* and *INHBB*), treatment with 5AzaC or TSA alone resulted in minimal reactivation, whereas a combination resulted in substantial reactivation of all of these genes (**Fig. 5c**). Likewise, *MARCO* and *GLI2*, which do not have CpG island–associated promoters but are either methylated in the promoter region or in the 3′ downstream associated island, also showed an increase in expression, but only with a combination treatment of 5AzaC and TSA (**Fig. 5d**). These data suggested that the chromatin encompassing the hypermethylated genes required a combination of both demethylation and deacetylation before the genes could be reactivated.

The CpG island–associated genes that were unmethylated and transcriptionally repressed in HCT116 also showed little reactivation after treatment with 5AzaC or TSA alone, but they all showed some activation after treatment with a combination of 5AzaC and TSA (**Fig. 5e**). Notably, we observed the greatest activation for the unmethylated genes (*INSIG2*, *PTPN4* and *RALBB*) that were closest to the methylated CpG-rich regions. The weakest activation was

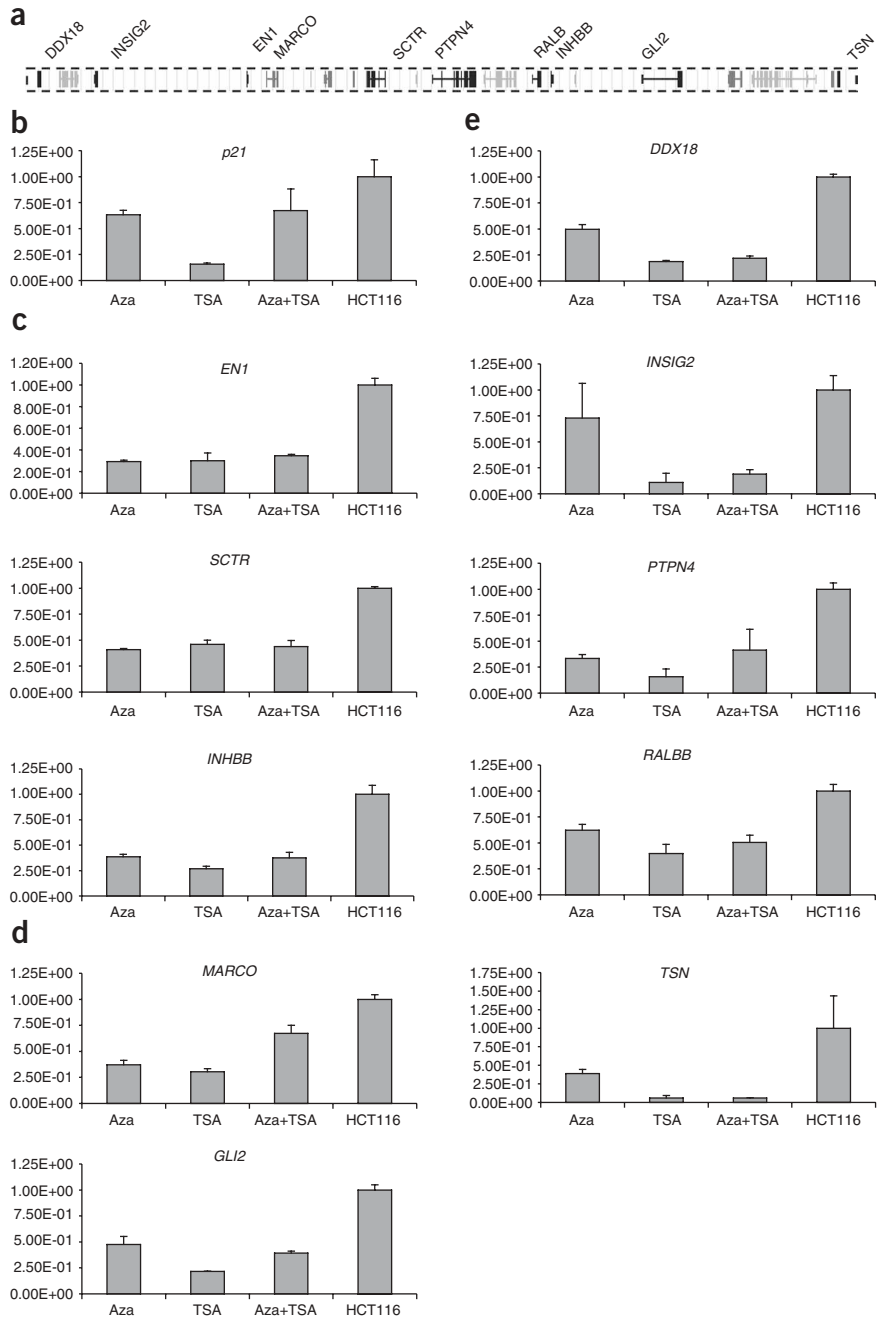


Figure 6 Chromatin immunoprecipitation (ChIP) across the 2q14.2 region. (**a**) Chromosomal location on 2q14.2 of the genes analyzed by ChIP. Chromatin from HCT116 cells that were either untreated or treated with 5AzaC, TSA or a combination of 5AzaC and TSA (AZA+TSA) was immunoprecipitated with an antidimethylated H3K9 antibody. The amount of target that was immunoprecipitated was quantified by real-time PCR, and the amount of immunoprecipitated target DNA was calculated as the ratio of immunoprecipitated DNA to the total amount of input DNA used for the immunoprecipitation. All the results in the graph are expressed relative to untreated HCT116 cells. The relative binding of dimethylated H3-K9 is shown for a control gene *p21* (**b**); methylated CpG island–associated genes *EN1*, *SCTR* and *INHBB* (**c**); methylated non–CpG island genes *MARCO* and *GLI2* (**d**); and unmethylated CpG island–associated genes *DDX18*, *INSIG2*, *PTPN4*, *RALBB* and *TSN* (**e**) from treated or untreated HCT116 cells.

associated with the genes (*DDX18* and *TSN*) that were farthest from the methylated CpG islands and were located at either boundary of band 2q14.2. These results suggest that chromatin modification was associated with the suppression of genes across the entire band in cancer, regardless of their DNA methylation status.

To assess for chromatin repression, we measured the level of methylated K9-H3 in the HCT116 cells before and after treatment with 5AzaC and TSA (**Fig. 6**; **Supplementary Fig. 6** online). First, we found that methylated K9-H3 histones were bound to the promoter region of the DNA-methylated CpG island–associated genes (*EN1*, *SCTR* and *INHBB*; **Fig. 6c**), the methylated 3′ CpG island (*GLI2*) and the methylated gene lacking CpG islands (*MARCO*; **Fig. 6d**). This binding was relieved by treatment with 5AzaC and TSA, either alone or in combination. These data demonstrated a correlation between DNA demethylation and demethylation of the associated H3-K9 histones and elevated gene transcription (**Fig. 5c**,**d**). Second, we determined the binding of methylated H3-K9 histones on the unmethylated gene regions. Similar to the unmethylated control gene *p21* (**Fig. 6b**), we found that after TSA treatment, there was substantial demethylation of the H3-K9 histones associated with all the unmethylated genes (*DDX18*, *INSIG2*, *PTPN4*, *RALBB*) that are suppressed across 2q14.2 (**Fig. 6e**). In addition, treatment with 5AzaC alone or in combination with TSA also resulted in demethylation of the histones at H3-K9. Although we could not perform ChIP studies on normal colorectal epithelial cells, we have analyzed the chromatin state of the genes across this region (J.S., L. Huschtscha, R. Reddel, S.J.C., unpublished data) in normal breast epithelial cells before and after transformation in culture[22] and found an increase in association of methylated H3-K9 histones in this region after transformation. Therefore, we conclude that the long-range genomic gene suppression across the 4-Mb DNA region, encompassing the entire 14.2q band on chromosome 2, is associated with dimethylation of the H3-K9 residue of the associated chromatin regardless of the DNA methylation status.

DISCUSSION

We report a new mechanism in carcinogenesis involving long-range epigenetic silencing (LRES) by heterochromatinization of genes within a 'neighborhood'. Epigenetic silencing of individual tumor suppressor genes involving both DNA and histone (H3-K9) methylation is a common signature of cancer and can affect multiple genes within the one cancer cell. However, abnormal methylation has always been envisaged as a local event silencing discrete genes. It is not yet

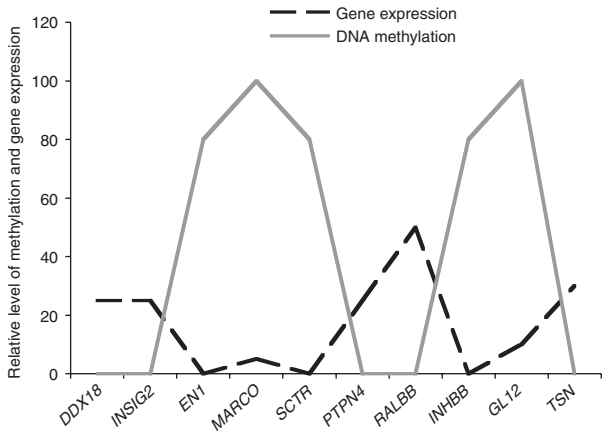


Figure 7 Relationship between DNA methylation and normal gene expression levels. The degree of methylation in colorectal cancer for each CpG island–associated gene, located across chromosome 2q14.2, is plotted relative to the level of expression observed in the normal colorectal cells. There is an inverse correlation with the degree of methylation observed in the cancer cell versus the level of gene expression in the normal cell.

understood why certain genes in cancer are susceptible to DNA methylation and epigenetic inactivation and why other genes are protected from DNA methylation. Moreover, the extent and spread of DNA hypermethylation and its potential influence on the expression of neighboring genes has not been addressed before. This is an important question to resolve, as it has implications for the discovery of epigenetic markers, as well as defining potential targets for epigenetic therapy.

To gain a better understanding of the genes susceptible to hypermethylation in cancer, we used a global methylation screening approach and identified a 4-Mb region located on chromosome 2q14.2 that was commonly hypermethylated in colorectal cancer cells. DNA hypermethylation was found in three distinct zones or 'suburbs', with the largest continuously methylated region encompassing 12 CpG islands, spanning nearly 1 Mb. Thus, in cancer, DNA hypermethylation is not always localized to discrete CpG islands or single genes but can span larger chromosomal domains. Other global approaches have identified several genomic regions up to 20 Mb in size with marked differential hypomethylation, but they failed to find large-scale differences in the chromosome-wide hypermethylation profile[23]. However, concordant methylation of adjacent CpG island gene promoters has been reported for a limited number of functionally related genes[24,25,26], suggesting that LRES may be a more common phenomenon in cancer. However, long-range epigenetic regulation does occur in normal cells; for example, in X chromosome inactivation[27,28] and imprinted gene clusters[29]. As both X inactivation and regulation of imprinting are associated with noncoding RNA or microRNA transcripts[30,31], it is possible that similar mechanisms may be involved in the long-range epigenetic silencing observed across 2q14.2 in colorectal cancer.

In a survey of 26 colorectal cancer samples, we found that 96% of cancers showed methylation of at least one gene from the three hypermethylated zones. Moreover, aberrant methylation was found to occur in adenomas (data not shown), indicating the potential application of this region as an early epigenetic tumor biological marker. The clinical significance of hypermethylation across chromosome 2q14.2 is unclear, but the fact that it is a common event suggests that regions within the cytogenetic band may encode possible tumor suppressor gene(s). Chromosome 2q has been reported to be relatively unstable in colorectal primary tumors[32], but we did not find any evidence of LOH of 2q14.2 in the cell lines or colorectal samples we studied (data not shown). Nonetheless, there are many interesting candidate genes residing in this region, including the three hypermethylated genes *EN1*, *SCTR* and *INHBB*. Engrailed-1 (*EN1*) is a homeobox transcription factor and interacts directly with the canonical Wnt signaling pathway[33]. *EN1* also is essential in development[34], and thus its inactivation may be significant in colorectal cancer progression. *SCTR*, coding for the secretin receptor, is an important glycoprotein receptor and is hypermethylated[35,36] and downregulated in pancreatic cancer[37]. Inhibin is a growth factor involved in cell differentiation and proliferation and has been considered as a tumor suppressor gene[38]. *INHBB* encodes for the inhibin β B subunit and is downregulated in breast metastatic lymph nodes[39], and the inhibin α subunit has been reported to be hypermethylated in 42% of prostate cancers.

We have demonstrated that aberrant histone lysine methylation is associated not only with the repression of chromatin of specific genes (that is, 'gene-level' repression) in cancer, but also with the repression of large chromosomal regions (that is, 'chromosome-level' repression)[40]. Global gene suppression across 2q14.2 could be relieved by treatment with DNA methylation and histone deacetylase inhibitors, and this correlates with release of the associated methylated H3-K9

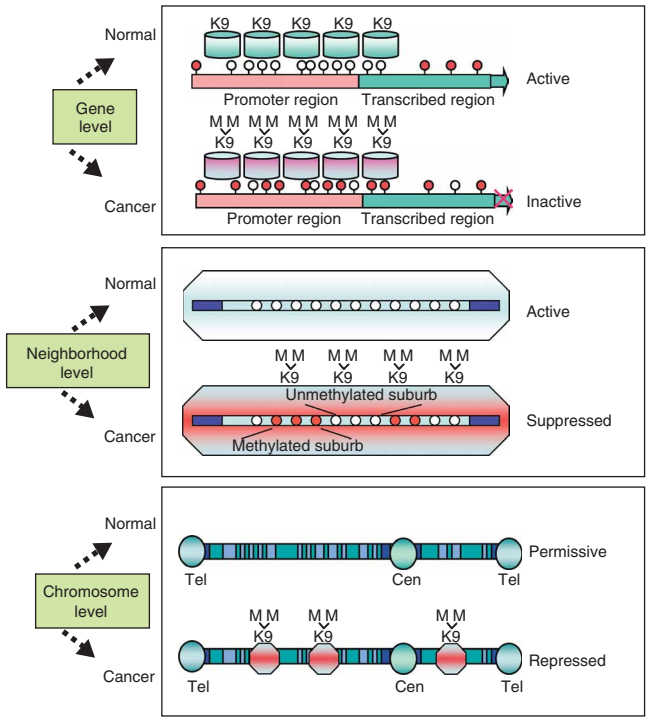


Figure 8 Epigenetic lesions in cancer. A summary of the proposed epigenetic changes that can occur between normal and cancer cells (i) at the 'gene level', through DNA methylation and histone H3-K9 methylation of the CpG island promoter region resulting in gene inactivation; (ii) at the 'neighborhood level', through long-range epigenetic silencing, resulting in suppression of both unmethylated and methylated DNA 'suburbs' of genes; and (iii) at the 'chromosome level', where we propose that suppressed neighborhoods encompass large regions of the chromosome, converting permissive chromatin to repressed chromatin resulting in an apparent LOH in the cancer cells.

histones. Thus, histone lysine methylation can occur independently of DNA methylation, and, unlike reports for some genes, may not always be a trigger for DNA methylation[41]. There are an increasing number of reports in the literature of CpG island genes such as *p21* (ref. 19) that are unmethylated and silent in cancer cells but can be reactivated by treatment with 5AzaC and TSA, suggesting that this class of genes may be more common than previously thought. This is an important consideration, especially in light of the current clinical trials using HDAC inhibitors. Activation of both methylated and unmethylated genes should now be evaluated. Indeed, a 75-kb gene cluster belonging to the metallothionein family was recently reported to be coordinately upregulated after 5AzaC treatment, even though not all genes in the family are regulated by DNA methylation[42].

An important question highlighted by this study is what dictates why the CpG islands within the three methylated zones are highly susceptible to DNA methylation and epigenetic inactivation, and yet the other CpG-associated genes are protected from DNA methylation. In a normal cell, most CpG islands remain unmethylated and are actively transcribed[43]. We previously proposed that active transcription not only protects the islands from methylation but promotes demethylation if random sites are stochastically methylated[6]. In this study, we find that there is an inverse correlation between susceptibility to DNA methylation across 2q14.2 in colorectal cancer and the level of gene expression in the normal cell (**Fig. 7**), supporting the hypothesis that the level of active transcription is a key determinant in protecting CpG islands from hypermethylation in cancer. Notably, CpG islands that became methylated in the cancer cells also showed a low level of methylation ('seeds' of methylation) in the normal cells, whereas the CpG islands that were not methylated in cancer were essentially devoid of 'seeding' methylation. Together, these findings support the model that genes that are methylated in cancer are those that are silent and seeded and that gene repression alone is insufficient to promote DNA methylation[5,6]. In addition, a critical density of seeded DNA methylation may be important to permit subsequent spreading[44]. Hypermethylation of nonpromoter exonic CpG islands has also been linked to the spread of methylation to the CpG island promoter regions[45], and hypermethylation of intervening sequences has been shown to affect heterochromatin and local gene expression, supporting our findings that methylation of non–CpG islands can also contribute to gene suppression via chromatin remodeling[46]. In contrast to the 'silencing and seeding' model for promoting DNA hypermethylation, others have suggested[12] that the propensity for methylation is based on a 'sequence signature'; however, we found that the seven signature sequence patterns reported showed no significant discrimination for methylation in the CpG islands across chromosome 2q14.2 (data not shown).

Our results have highlighted a new mechanism in carcinogenesis that results in long–range epigenetic silencing of genes, with epigenetic lesions in cancer occurring at the 'gene level', the 'neighborhood level' and the 'chromosome level' (summarized in **Figure 8**). We propose that the expression of genes residing in the same neighborhood is affected by hypermethylation of their flanking genes and can become coordinately silenced by chromatin remodeling involving H3K9 methylation. In cancer, the chromosome neighborhood can be viewed as being divided into two classes of suburbs: suburbs with methylated DNA and suburbs with unmethylated DNA, with both suburbs repressed by the chromatin modification encompassing the entire neighborhood. Whereas in a normal cell, the neighborhood of genes is permissive for expression, in a cancer cell, all genes within the neighborhood are suppressed. LRES spanning large chromosomal regions provides the cancer cell with a global gene silencing mechanism with equivalent effects as genetic deletion through LOH; that is, suppression or inactivation of all genes and noncoding transcripts within the cytogenetic region. Thus, changes in gene expression due to modifications of chromatin structure could be as important in malignant transformation as are genetic changes that can affect the DNA sequence. Whether the long-range epigenetic silencing that we observe at 2q14.2 is a general phenomenon or a unique example is an open question, but further genomic approaches to epigenetic analysis should elucidate the extent of this mechanism in cancer.

METHODS

Samples. We collected 112 colorectal carcinomas, with paired nonadjacent areas of normal colonic mucosa, as fresh-frozen tissues within 2 h of removal and stored them at –80 °C. All samples were obtained from the Hospital de la Santa Creu i Sant Pau (Barcelona, Spain). The study protocol was approved by the Ethics Committee of Hospital Duran i Reynals. All subjects provided written informed consent. Human colorectal carcinoma cell lines were obtained from the American Type Culture Collection (ATCC).

Amplification of intermethylated sites (AIMS). The AIMS method was used to screen for tumor-specific alterations in DNA methylation using a set of primers (set B) previously described[14]. Results on global profiles of DNA methylation have been described elsewhere[17]. The AIMS fragments were excised from the fresh dried polyacrylamide gels, PCR amplified, cloned and sequenced to determine the identity of the isolated bands. The chromosomal position of the AIMS fragments was identified using the Santa Cruz Genome Browser (July 2003).

5-Aza-2′-deoxycytidine and trichostatin A treatment of cells. The colon cancer cell line HCT116 was cultured in D-MEM/F12 (Gibco/BRL) supplemented with MEM sodium pyruvate, L-glutamine and 10% fetal calf serum. We seeded 100-mm tissue culture dishes with 0.5×10^6 cells, treated them 24 h later with 0.5 μM 5-aza-2′-deoxycytidine (5-AzaC; Sigma) for 24 h and cultured them with fresh medium for another 48 h. Cells were treated with trichostatin A (TSA; Sigma) at 100 nM for 24 h. For cotreatment of cells with 5-AzaC and TSA, 5-AzaC was added initially for 24 h, after which it was removed and TSA added for an additional 24 h.

RNA extraction and quantitative real-time RT-PCR. RNA was extracted from tumor and matched normal samples and from the cell lines using TRIZOL reagent (Invitrogen), and cDNA was reverse transcribed with 200 ng of random hexamers (Roche) from 2 μg of total RNA using SuperScript III RNase H^- reverse transcriptase (Invitrogen Life Technologies) according to the manufacturer's instructions. Expression was quantified using the ABI PRISM 7000 Sequence Detection System. We used 5 μl of a 1:20 dilution of the reverse transcription reaction in a quantitative real-time PCR reaction, using 2× SYBR Green 1 Master Mix (P/N 4309155) with 50 ng of each primer. The primers used for RT-PCR amplification are listed in **Supplementary Table 1** online. To control for the amount and integrity of the RNA, we used the Human 18S Ribosomal RNA (rRNA) Kit (Applied Biosystems). We used 5 μl of the reverse transcription reaction in a 20-μl reaction in TaqMan Universal PCR Master Mix with 1 μl of the 20× human 18S rRNA mix. The reactions were performed in triplicate, and standard deviation was calculated using the comparative method (ABI PRISM 7700 Sequence Detection System, user bulletin #2, 1997).

Genomic bisulfite sequencing. DNA was extracted from the HCT116/SW480 cells using the Puregene Extraction Kit (Gentra Systems) and TRIZOL reagent (Invitrogen) from the cancer and normal samples according to the manufacturer's protocol. The bisulfite reaction was carried out on 2 μg of restriction-digested DNA for 16 h at 55 °C under conditions as previously described[15,47]. Three independent PCR reactions were performed and products were pooled to ensure a representative methylation profile. The primers used for the bisulfite PCR amplifications are listed in **Supplementary Table 1**. Methylation analysis was carried out on the pooled PCR products by direct PCR sequencing, real-time PCR melting temperature dissociation and PCR clonal analysis. For details, see **Supplementary Methods** online.

Chromatin immunoprecipitation (ChIP) assays. ChIP assays were carried out on 1×10^6 HCT116 cells in a 10-cm dish according to the manufacturer's instructions (Upstate Biotechnology) and as described in ref. 15. The complexes were immunoprecipitated with an antibody specific for dimethyl-histone H3(Lys9) (Upstate Biotechnology) according to the manufacturer's instructions. We did not include any antibody controls for each ChIP assay and did not observe any precipitation. The amount of target immunoprecipitated was measured by real-time PCR using the ABI PRISM 7900HT Sequence Detection System. Amplification primers are listed in **Supplementary Table 1**. PCR was set up according to the Sequence Detection System compendium (V 2.1) for the 7900HT Applied Biosystems Sequence Detector, as described previously[15], and was performed in triplicate. For each sample, an average C_T value was obtained for immunoprecipitated material and for the input chromatin. The difference in C_T values (ΔC_T) reflects the difference in the amount of material that was immunoprecipitated relative to the amount of input (ABI PRISM 7700 Sequence Detection System, user bulletin #2, 1997). Standard deviation was calculated using the comparative method (ABI PRISM 7700 Sequence Detection System, user bulletin #2, 1997).

URLs. University of California Santa Cruz Human Genome Browser: http://genome.ucsc.edu (data from July 2003).

Note: Supplementary information is available on the Nature Genetics website.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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