

Genetic unmasking of epigenetically silenced tumor suppressor genes in colon cancer cells deficient in DNA methyltransferases

Maria F. Paz¹, Susan Wei², Juan C. Cigudosa³, Sandra Rodriguez-Perales³, Miguel A. Peinado⁴, Tim Hui-Ming Huang² and Manel Esteller^{1,*}

¹Epigenetics Laboratory, Molecular Pathology Program, Spanish National Cancer Centre (CNIO), Madrid 28029, Spain, ²Department of Pathology and Anatomical Sciences, Ellis Fischel Cancer Center, University of Missouri School of Medicine, Columbia 65203, USA, ³Cytogenetics Unit, Spanish National Cancer Center (CNIO), Madrid 28029, Spain and ⁴Research Institute of Oncology (IRO), L'Hospitalet 08907, Catalonia, Spain

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Hypermethylation associated silencing of the CpG islands of tumor suppressor genes is a common hallmark of human cancer. Here we report a functional search for hypermethylated CpG islands using the colorectal cancer cell line HCT-116, in which two major DNA methyltransferases, DNMT1 and DNMT3b, have been genetically disrupted (DKO cells). Using two molecular screenings for differentially methylated loci [differential methylation hybridization (DMH) and amplification of inter-methylated sites (AIMS)], we found that DKO cells, but not the single DNMT1 or DNMT3b knockouts, have a massive loss of hypermethylated CpG islands that induces the re-activation of the contiguous genes. We have characterized a substantial number of these CpG island associated genes with potentially important roles in tumorigenesis, such as the cadherin member FAT, or the homeobox genes LMX-1 and DUX-4. For other genes whose role in transformation has not been characterized, such as the calcium channel $\alpha 11$ or the thromboxane A2 receptor, their re-introduction in DKO cells inhibited colony formation. Thus, our results demonstrate the role of DNMT1 and DNMT3b in CpG island methylation associated silencing and the usefulness of genetic disruption strategies in searching for new hypermethylated loci.

INTRODUCTION

The inactivation of tumor suppressor genes is one of the main events leading to the development and progression of all common forms of human cancer (1). This inactivation occurs through intragenic mutations, genomic deletions and also very often by epigenetic silencing associated with the hypermethylation of the CpG islands located in the promoter regions of these genes (2,3). Examples of widely recognized tumor suppressor genes undergoing CpG island promoter hypermethylation in sporadic tumors include the cell cycle inhibitor p16^{INK4a}, the DNA mismatch repair gene hMLH1 or the breast cancer gene BRCA1 (2,3). However, the mechanisms and molecular players involved in generating these specific hypermethylated DNA loci remain unclear. Global cytosine methylation patterns in mammals appear to be established by a complex interplay of at least three independently encoded

DNA methyltransferases (DNMTs): DNMT1, DNMT3a and DNMT3b (4,5). DNA methyltransferases are commonly classified as *de novo* (DNMT3a and DNMT3b) or maintenance (DNMT1) enzymes (4,5). Most interesting, overexpression of DNMT1 and DNMT3b is a common finding in human tumors (4,5). However, their role in the epigenetic silencing of tumor suppressor genes is still not well characterized.

The generation of somatic cell knockouts through homologous recombination is a powerful tool for clarifying the function of any candidate gene in human cancer (6). Recently, homologous recombination has been used in the colorectal cancer cell line HCT-116 to disrupt DNMT1, DNMT3b and both enzymes together (the double knockout, DKO) (7,8). These studies showed that, while the lack of each DNMT had little effect on DNA methylation patterns, in the DKO cells DNA methyltransferase activity was almost completely eliminated and there was a 95% reduction in 5-methylcytosine

*To whom correspondence should be addressed at: Cancer Epigenetics Laboratory, Spanish National Cancer Centre (CNIO), Melchor Fernandez Almagro 3, 28029 Madrid, Spain. Tel: +34 912246940; Fax: +34 912246923; Email: mesteller@cnio.es

content, demethylation of repeated sequences, loss of imprinting at the IGF2 locus and abrogation of the methylation-mediated silencing of two genes, p16^{INK4a} and TIMP-3 (8). However, it has also recently been proposed that the mere depletion of DNMT1 alone by antisense or siRNA approaches is able to release methylation-mediated silencing, differences that probably arise from the distinct methodologies used (9).

The search for new genes that undergo methylation associated inactivation in cancer cells has taken candidate gene (10), genomic (11) and transcriptional (12,13) approaches, but the DNMT genetic avenue has not yet been explored. We wondered about the extent of epigenetic release in the single DNMT knockout cells, the DKO cells and whether these last cells could be used to find new genes with hypermethylation associated inactivation in human cancer. To accomplish this aim we combined, among others, two complementary approaches to study DNA methylation changes at a global genomic scale: differential methylation hybridization (DMH), that uses a CpG island microarray enriched in single-copy genes (14,15); and amplification of inter-methylated sites (AIMS), a PCR based assay ideal for characterizing anonymous DNA sequences with differential methylation (16). Our results demonstrate that the cancer cells lacking DNMT1 and DNMT3b, but not the single DNMT knockouts, undergo a massive release of their epigenetic silencing exemplified for the unveiling of a myriad of demethylation events in promoter CpG islands through the entire genome. These newly unmasked targets of epigenetic inactivation have not only methylation mediated silencing and tumor suppressor-like growth inhibitory effects in cancer cell lines, but are also a common epigenetic aberration present in cancer patients.

RESULTS

All known tumor suppressor genes that had hypermethylation associated silencing in HCT-116 became demethylated in DKO cells

First, we determined CpG island methylation status by bisulfite genomic sequencing and methylation-specific PCR of 34 well-known genes in which promoter hypermethylation had been previously described in human tumorigenesis (2,3) (Fig. 1A). Of these, eight were unmethylated in the wild-type HCT-116 cell line, and thus were considered uninformative for our purpose. All of the 26 genes hypermethylated in the original HCT-116 cell line became demethylated in the DKO cell line (Fig. 1A). This unmethylated state was not observed in the single DNMT1 or DNMT3b knockout cells (Fig. 1A). We also analyzed the transcriptional profile of seven genes (CRBP-1, AR, CDH13, SYK, ER, PR and HIC-1) and in all cases the demethylation of CpG islands observed in the DKO cells was associated with the re-expression of the corresponding gene, which was transcriptionally silenced in the wild-type HCT-116 cell line (Fig. 1B). No re-expression was observed in the single DNMT1 or DNMT3b knockout cells. As expected, when the original HCT-116 cells were treated with the demethylating agent 5-aza-2'-deoxycytidine (DAC), DNA hypomethylation found in the respective islands was also associated with gene re-expression.

Identification of new targets of epigenetic inactivation in colon cancer by DMH of DKO versus HCT-116 cells

We next determined how widely distributed these CpG island demethylation events were in the DKO cells. Our first approach was to use DMH, a CpG island microarray-based technique (14,15), to evaluate hypomethylation changes in the single and double knockouts and HCT-116 cells treated with DAC, in relation to the wild-type HCT-116 (Fig. 2A). We observed demethylation in a multitude of CpG island loci and greater overall levels of DNA hypomethylation in DKO and DAC-treated cells in comparison to the single knockout cells. There were respectively 27, 43, 343 and 202 demethylated CpG island loci in the DNMT1^{-/-}, DNMT3b^{-/-}, DKO and DAC-treated cells relative to the HCT-116 cells (Fig. 2B). These loci included multiple-copy (e.g. Alu, rDNA and α -satellites) and 253 single-copy loci in the DKO cells (Fig. 2B). The Cy5(test):Cy3(wild-type) values of these same loci among the cells were compared (Fig. 2C). An average value of zero for log₂ Cy5/Cy3 ratio indicates equal or similar methylation in tests and the wild-type, whereas an average value of less than zero would suggest demethylation (Fig. 2C). As shown by the loci analyzed, there was little change in methylation between the wild-type and single DNMT knockout cells but a marked difference in demethylation levels in the DKO and DAC-treated cells.

To gain further knowledge of the type of genes that are hypomethylated in the DKO cells, we selected five random CpG island clones that were demethylated in these cells for further characterization by nucleotide sequencing. The sequence data were used to search for known sequences in the GenBank database. These clones matched five known genes: FAT tumor suppressor homolog 1 (FAT) (a member of the cadherin super-family), Lim/homeobox protein-1 (LMX-1), Thrombomodulin (THRM), Collagen XIV a1 or undulin (UND), and the zinc finger protein gene-37 (ZFP37) (Table 1). Bisulfite genomic sequencing spanning their corresponding CpG islands demonstrated hypermethylation in the HCT-116 wild-type cells and hypomethylation in the DKO cells (Fig. 3). Methylation-specific PCR corroborated these results (Fig. 3). The single knockouts (DNMT1 or DNMT3b) did not show any demethylation events, while HCT-116 cells treated with DAC were also hypomethylated (data not shown). Most importantly, the demethylation of the five genes described in the DKO cells was associated with the re-expression of the transcripts that were silenced in the wild-type HCT-116 (Fig. 3). No re-expression was observed in the single DNMT1 or DNMT3b knockout cells. To establish whether the hypermethylation associated inactivation of these genes was merely a feature of this particular cell line, we analyzed a large set of colorectal carcinoma cell lines and primary tumors. While CpG island hypermethylation did not occur in any normal colon tissue analyzed, aberrant DNA methylation was observed in a significant proportion of cell lines and primary tumors (Fig. 3 and Table 1). Most importantly, in 11 colon tumors where RNA was available, the methylation of LMX-1 and FAT was associated with the loss of their transcripts (Fig. 3). There was also CpG island hypermethylation of these genes in colorectal adenomas (Fig. 3 and Table 1), demonstrating that these epigenetic lesions occur early in colon tumorigenesis.

A

Summary of the CpG island methylation status of 34 known genes that undergo promoter hypermethylation in human cancer.

Samples	Genes																																			
	CXXI	DeR1	FHIT	HTLF	p14 ^{ARF}	RASSF1A	TIMP2	Stratfin	AR	Caspase8	CDH13	CHFR	CRBP1	CYCD2	DeR2	DR4	ER	FOLH1	HIC1	HIN	LKB1	LPHH1	MGMT	PR	p73	RARβ2	RIZ	RUNX3	SEZ6L	SFRP1	SOCS1	SYK	TMS1	TWIST		
Normal Colon																																				
HCT-116																																				
DNMT1 ^{-/-}																																				
DNMT3b ^{-/-}																																				
DKO																																				
DAC																																				

B

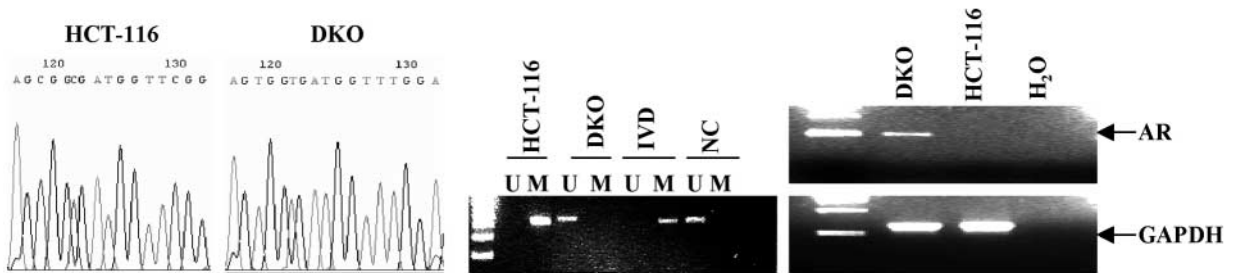


Figure 1. (A) Summary of the methylation-specific PCR (MSP) analyses of the CpG island methylation status of 34 known genes that undergo promoter hypermethylation in human cancer. Those genes hypermethylated in the HCT-116 wild-type cell line only achieve an unmethylated state in the DKO line or in the DAC-treated cells. Light and dark shading indicate methylation or no methylation, respectively. (B) Example of the DNA methylation and expression analysis of the AR gene. Left, bisulfite genomic sequencing of the AR CpG island demonstrating hypermethylation in the HCT-116 wild-type cell line (persistence of ‘C’ preceding ‘G’) and demethylation in the DKO cells (‘C’ preceding ‘G’ have been transformed in ‘T’). Middle, example of the MSP analysis of the AR gene. The presence of a PCR band under the ‘U’ or ‘M’ lane indicates unmethylated or methylated alleles, respectively. *In vitro* methylated DNA (IVD), used as positive methylated control and normal colon (NC), used as unmethylated control. Right, example of the RT-PCR analysis of the AR gene. Restoration of gene expression is observed in the DKO line when compared to the wild-type HCT-116. GAPDH expression is shown as an internal control.

Table 1. Target genes of methylation associated silencing in human cancer identified in DKO cells using DMH and AIMS strategies

Gene	Function	Found by	Chr Loc	% CpG island hypermethylation			
				Carcinomas	Adenomas	Cell lines	Normal colon
FAT	Cadherin super-family	DMH	4q34–q35	45% (14/31)	50% (6/12)	87% (7/8)	0% (0/7)
LMX-1	Homeobox transcription factor	DMH	1q23.1	55% (17/31)	42% (5/12)	75% (6/8)	0% (0/7)
THRM	Thrombomodulin	DMH	20p12	67% (18/27)	—	100% (8/8)	0% (0/7)
UND	Collagen XIV α1	DMH	8q23	56% (14/25)	—	100% (8/8)	0% (0/7)
ZFP37	Zinc finger transcription factor	DMH	9q32	60% (15/25)	—	37% (3/8)	0% (0/7)
DUX-4	Double homeobox protein 4	AIMS	4q35	52% (13/25)	—	75% (6/8)	0% (0/7)
SURF-1	Biogenesis of the COX complex	AIMS	9q34.2	48% (12/25)	42% (5/12)	37% (3/8)	0% (0/7)
SURF-2	Biogenesis of the COX complex	AIMS	9q34.2	48% (12/25)	42% (5/12)	37% (3/8)	0% (0/7)
TBXA2R	Thromboxane A2 receptor	AIMS	19p13.3	64% (16/25)	—	87% (7/8)	0% (0/7)
COL5A	Collagen V α1	AIMS	9q34.2–q34.3	64% (23/36)	—	87% (7/8)	0% (0/7)
CALCAII	Calcium channel α1I	AIMS	22q13.1	44% (16/36)	50% (6/12)	100% (8/8)	0% (0/7)
MICCC-1	Fibronectin homologies	AIMS	9q21.11	40% (10/25)	—	87% (7/8)	0% (0/7)

AIMS, amplification of intermethylated sites; COX, cytochrome C oxidase; Chr Loc, chromosomal location; DMH, differential methylation hybridization; MICCC-1, methylated in colon cancer cells-1.

Unmasking of a new set of genes with methylation associated silencing in colon cancer by the AIMS of DKO versus HCT-116 cells

In a third approach to define the release of DNA hypermethylation present in DKO cells, we analyzed these cells and the single knockouts using the AIMS technique (16). The AIMS

approach, based on similarly established methods (17,18), exploits the differential cleavage of methyl-isoschizomers linked to PCR amplification using adaptor-specific primers extended with arbitrary primers. The methylation fingerprint consists of multiple anonymous bands or tags, representing DNA sequences flanked by two methylated sites, which can be isolated (Fig. 2D). The AIMS analysis of the different cell lines

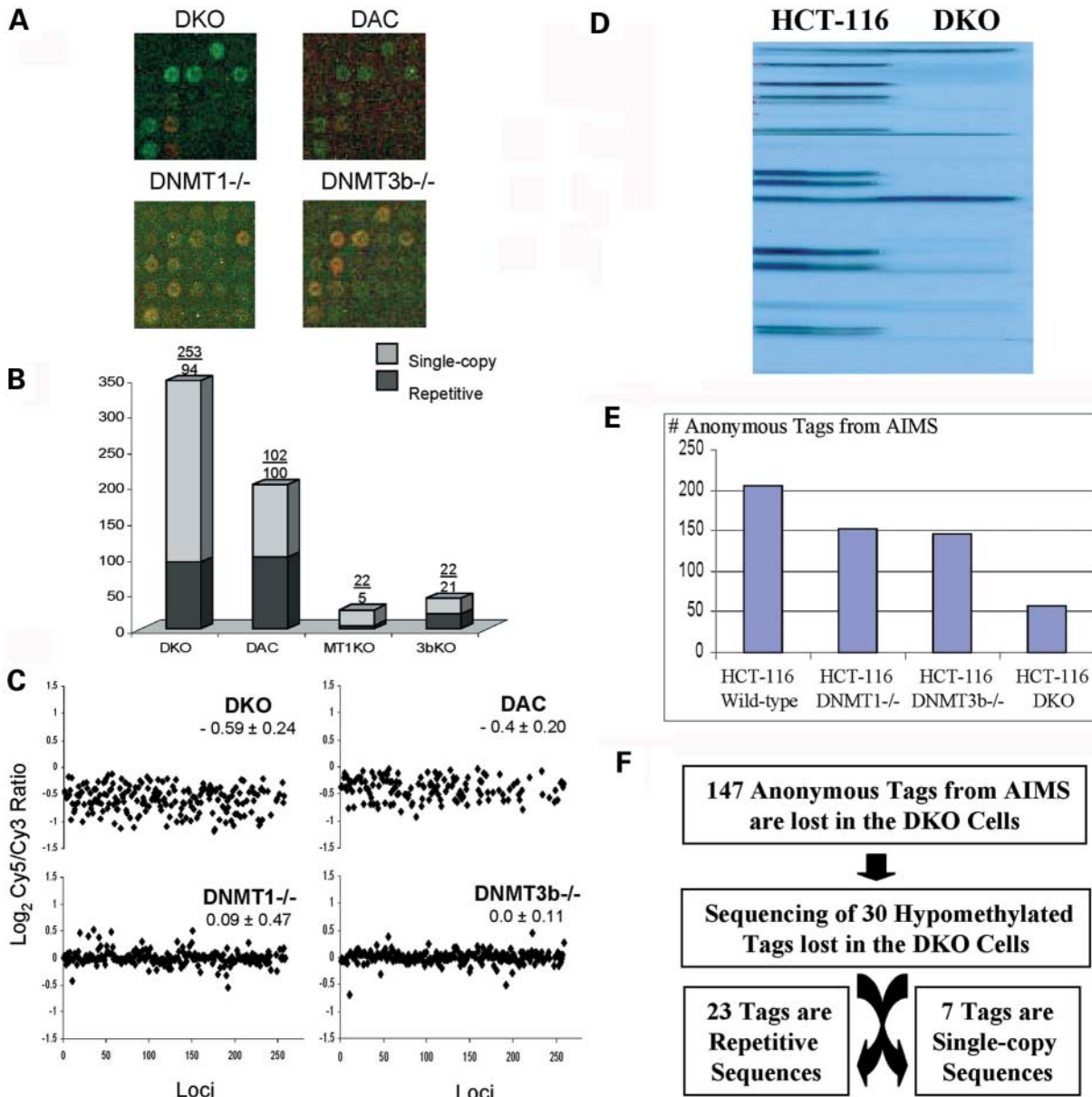


Figure 2. (A–C) DMH analysis using CpG island microarray in the DKO cells. Methylation amplicons from test and control DNA samples were prepared as described in the text and labeled with Cy5 (red) and Cy3 (green) dyes, respectively. The labeled samples were co-hybridized to the microarray panel. (A) Representative microarray images for DAC-treated and *DNMT1* (*DNMT1*^{-/-}), *DNMT3b* (*DNMT3b*^{-/-}) and DKO cells. (B) Total number of demethylated CpG island loci detected in these knockout and DAC-treated cells relative to the HCT116 wildtype. Signal intensities of hybridized spots were calculated and normalized Cy5/Cy3 ratios of 0.5 were scored as ‘demethylated.’ (C) Scatter plot profiles. An average zero value for log₂ Cy5/Cy3 ratio indicates equal or similar methylation between test and wild-type whereas a less than zero average value (<0) suggests demethylation. As shown by the loci analyzed, there was little change in methylation profiles between the wild-type and single *DNMT* knockout cells, but markedly demethylation levels were seen in the double knockout or DAC-treated cells. (D–F) AIMS in the DKO cells. (D) AIMS of wild-type HCT-116 versus DKO cells using one set of primers. A DNA methylation fingerprint is obtained where illustrative losses of bands (indicative of DNA hypomethylation) are observed in the DKO lane. (E) Quantification of the anonymous bands obtained in each sample, showing dramatic DNA demethylation events in the DKO cells (74% of the anonymous tags are lost). (F) Schematic representation of the strategy and cloning results obtained from the AIMS study.

demonstrated again that the DKO cells had widespread DNA demethylation, exemplified by the loss of 74% of the anonymous tags (147 of 205) with respect to the wild-type HCT-116, while hypomethylation in each single knockout was minimal, 26% (53 of 205) in *DNMT1*^{-/-} and 29% (59 of 205) in *DNMT3b*^{-/-} (Fig. 2E).

As in DMH, we also selected a subset of differentially methylated sequences for further characterization. Thirty PCR bands showing hypomethylation in the DKO cells and methylation in wild-type HCT-116 cells were cloned, sequenced and blasted against the GenBank database (Fig. 2F). A total of 77% (23 of 30) of the clones matched repetitive sequences

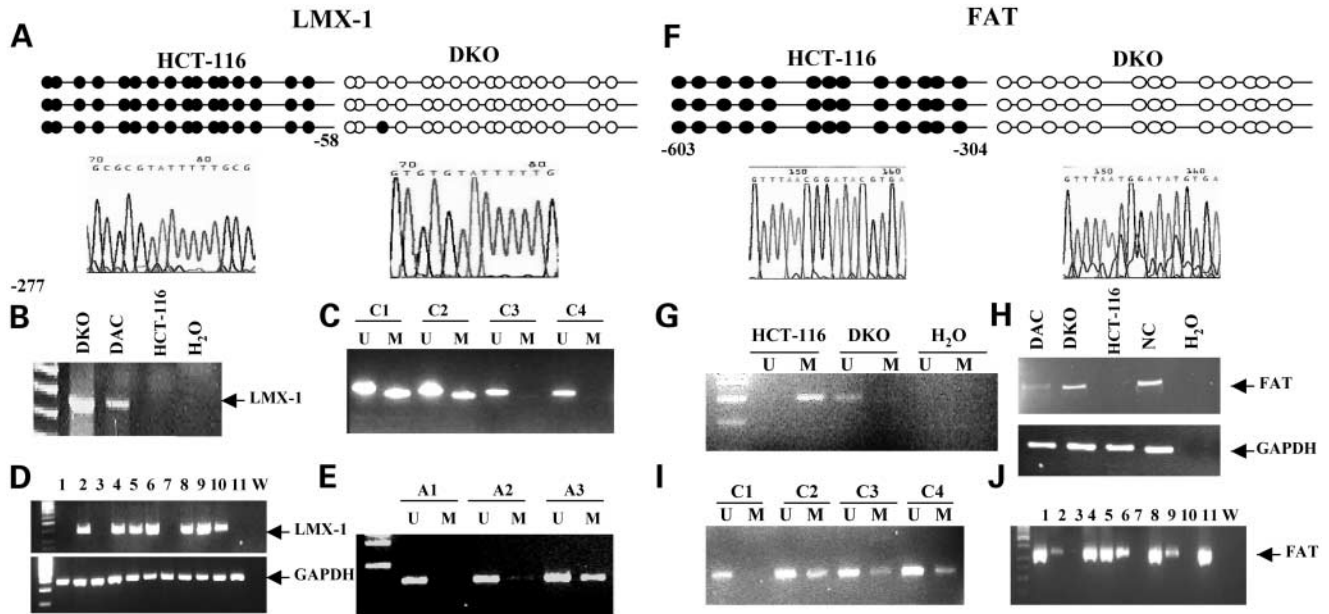


Figure 3. DNA methylation and expression analysis of the particular genes found using the DMH approach: the examples of LMX-1 and FAT. (A–E) LMX-1: (A) Bisulfite genomic sequencing of the LMX-1 CpG island. Schematic representation of the CpG sites included in the PCR fragment and three different PCR reactions, demonstrating hypermethylation in the HCT-116 wild-type cell line (retention of ‘Cs’) and demethylation in the DKO cells (transformation to ‘Ts’). Black dots indicate methylated CpGs and white dots unmethylated CpGs. (B) RT–PCR expression analysis of the LMX-1 gene demonstrates its absence in HCT-116, but its restoration in the DKO cell line or in HCT-116 cells treated with DAC. GAPDH used as internal control. (C) Analysis of the CpG island methylation status of the LMX-1 gene in colon primary tumors by MSP. C1 and C2 tumors are hypermethylated. (D) RT–PCR expression analysis of LMX-1 in colon carcinomas. The tumors 1, 3, 7 and 11 methylated at the LMX-1 promoter do not express the transcript, while it is abundant in the remaining unmethylated samples. (E) MSP analysis of LMX-1 in colon adenomas (A3 is hypermethylated). (F–J) FAT: (F) Bisulfite genomic sequencing of the FAT CpG island demonstrating hypermethylation in the HCT-116 wild-type cell line and demethylation in the DKO cells. (G) Methylation-specific PCR confirms the presence of FAT hypermethylation in HCT-116 and the demethylation in the DKO cells. (H) RT–PCR analysis shows lack of FAT transcript in HCT-116 and expression in DKO and DAC-treated cells. NC expresses FAT transcript. (I) MSP analysis of FAT in primary colon tumors. Samples C2–C4 demonstrate hypermethylation. (J) RT–PCR expression analysis of FAT in colon carcinomas. The tumors 3, 7 and 10 methylated at the FAT promoter do not express the transcript while another methylated tumor (number 2) expresses a reduced amount. The remaining unmethylated samples express abundant amounts of FAT transcript.

(12 Alus, seven MERs, two MIRs and two other endoparasitic sequences). Bisulfite genomic sequencing of two of these identified Alus demonstrated that these particular sequences were methylated in normal colon, as expected (19), and also at the wild-type HCT-116 cells and the single DNMT knock-outs, but demethylated in DKO cells (data not shown). On the other hand, 23% (seven of 30) of the clones were single-copy genes: double homeobox protein 4 (DUX-4), thromboxane A2 receptor (TBXA2R), the surfactant proteins 1 and 2 (SURF-1 and SURF-2) (involved in the biogenesis of the cytochrome c oxidase complex, that share a common CpG island but are transcribed in different directions), collagen V α 1 (COL5A), calcium channel α 1I (CALCA1I) and an unknown gene with fibronectin-homologies that we have denominated MICCC-1 (methylated in colon cancer cells-1) (Table 1). Bisulfite genomic sequencing spanning their corresponding CpG islands demonstrated hypermethylation in HCT-116 wild-type cells and demethylation in DKO cells (Fig. 4). These results were confirmed by methylation-specific PCR analysis. The single knockouts showed no demethylation events, while DAC-treated cells were hypomethylated (data not shown). Most importantly, the demethylation of the seven genes described in the DKO cells was associated with the re-expression of their transcripts, which were silenced in the wild-type HCT-116 (Fig. 4). No re-expression was observed in the single DNMT1 or

DNMT3b knockout cells. As with the DMH-obtained genes, we also analyzed a numerous set of colorectal carcinoma cell lines and primary tumors. Again, CpG island hypermethylation of these genes was not present in normal colon tissue, but aberrant DNA methylation was observed in colorectal cancer cell lines and primary tumors (Fig. 4 and Table 1). Furthermore, in 11 colon tumors where RNA was available, the methylation of CALCA1I and TBXA2R was associated with the loss of their transcripts (Fig. 4). Hypermethylation also occurred in a percentage of colorectal adenomas (Fig. 4 and Table 1).

Inhibition of colony formation by CALCA1I and TBXA2R in HCT-116 colon cancer cells

The list of new target genes of promoter hypermethylation in human cancer derived from DMH or AIMS in the DKO cells presents excellent candidates for contributing to the transformed phenotype, including the cadherin member FAT, whose mouse homolog is a bona fide tumor suppressor gene (20), or the homeobox genes LMX-1 and DUX-4 (21). To gain further understanding of the potential role of the other hypermethylated genes found, we independently introduced CALCA1I and TBXA2R expression vectors into the HCT-116 wild-type cell line, in which both genes were silenced in association with

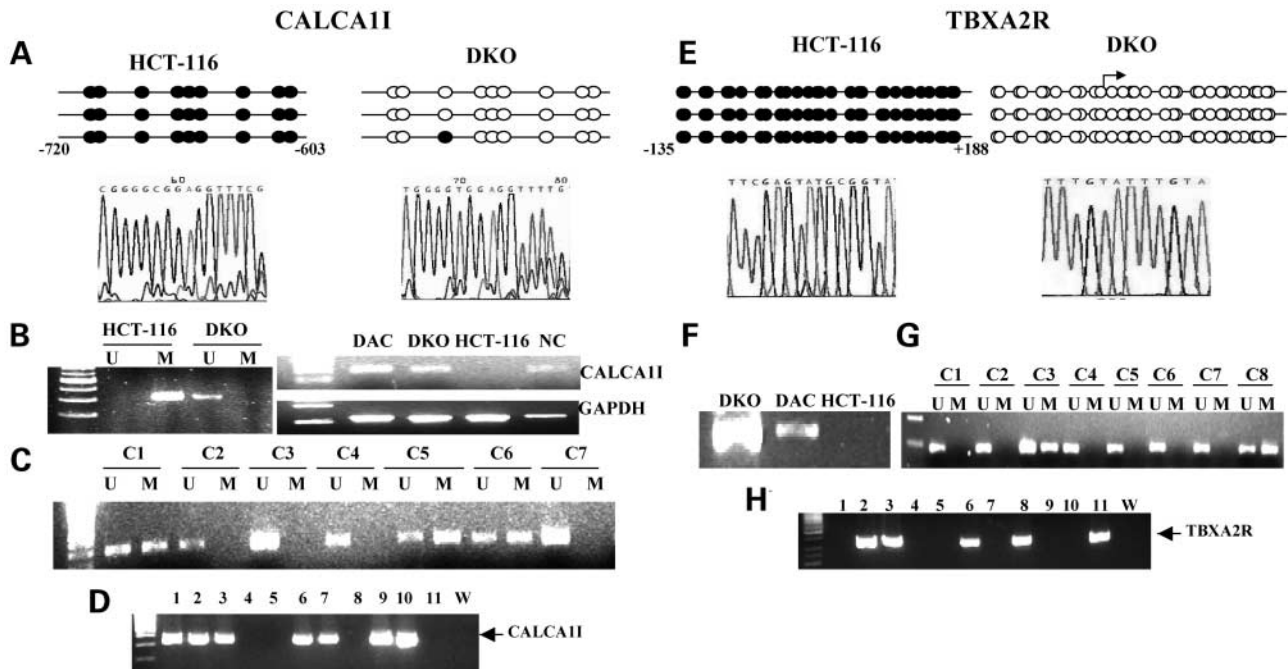


Figure 4. DNA methylation and expression analysis of the particular genes found using the AIMS approach: the examples of CALCA11 and TBXA2R. (A–D) CALCA11: (A) Bisulfite genomic sequencing of the CALCA11 CpG island demonstrating hypermethylation in the HCT-116 wild-type cell line and demethylation in the DKO cells. Schematic representation of the CpG sites included in the PCR fragment and three different PCR reactions. (B, Left) Methylation-specific PCR confirms the presence of CALCA11 hypermethylation in HCT-116 and demethylation in the DKO cells. (B, Right) RT-PCR demonstrates lack of CALCA11 transcript in HCT-116, but its expression in the DKO cell line, HCT-116 cells treated with DAC and normal colon (NC). (C) Analysis of the CpG island methylation status of the CALCA11 gene in primary colon tumors by MSP. Samples C1, C5 and C6 demonstrate hypermethylation. (D) RT-PCR expression analysis of CALCA11 in colon carcinomas. The tumors 4, 5, 8 and 11 methylated at the CALCA11 promoter do not express the transcript, while it is abundant in the remaining unmethylated samples. (E–H) TBXA2R: (E) Bisulfite genomic sequencing of the TBXA2R CpG island demonstrating hypermethylation in HCT-116 wild-type cell line and demethylation in the DKO cells. (F, Left) RT-PCR analysis shows lack of LMX-1 transcript in HCT-116 and expression in DKOs and DAC treated cells. (F, Right) MSP analysis of TBXA2R in primary colon tumors (C3 and C8 are hypermethylated). (H) RT-PCR expression analysis of TBXA2R in colon carcinomas. The tumors 1, 4, 5, 7, 9 and 10 methylated at the TBXA2R promoter do not express the transcript, while it is abundant in the remaining unmethylated samples.

methylation. Our transfection approach to measure the tumor-suppressing activity of CALCA11 and TBXA2R has been successfully applied to other methylation silenced genes, such as SEMA3B, SOCS-1 or CRIP1 (13,22,23). As internal controls, and in addition to the transfection of the empty vector, we also transfected the wild-type and mutant p16^{INK4a} tumor suppressor gene as positive and negative controls for inhibition of colony formation as described in Materials and Methods (Fig. 5). After the selection of drug-resistant colonies and the demonstration that each gene was now re-expressed, we found that the reintroduction of CALCA11 or TBXA2R was sufficient to decrease substantially the colony numbers of the transfected cells compared with those of the control (Fig. 5).

Competitive genomic hybridization reveals the chromosomal distribution of DNA hypomethylation events in DKO cells

Finally, we determined the 'geographical' disturbance of DNA methylation present in the DKO cells. We competitively hybridized the AIMS PCR products to metaphases to determine DNA demethylation throughout chromosomal regions (16,24). The AIMS products from each of the four HCT-116 cell lines: wild-type, DNMT1^{-/-}, DNMT3b^{-/-} and DKO, was competitively hybridized with whole genomic DNA from normal

lymphocytes. Our results show that the AIMS-amplified sequences are unevenly distributed along the chromosomes, discriminating regions that are richer than others in DNA-methylated sites (Fig. 6). Telomeric and centromeric regions showed the highest density of AIMS products, irrespective of their DNMT genotype. Concerning gene-rich areas of the genome, specific regions of chromosomes 1 (at band 1p36), 3 (3p21), 7 (7q21-22), 9 (9q34), 17 (17q24-25), 19q, 20q and 22q were recurrently enriched in AIMS products. These regions include hot-spots of imprinted-methylated genes (NOEY2 and ARHI genes in 1p31 and PEG10, MEG1 and MEST in 7q21) and genes hypermethylated in cancer (i.e. RASSF1 in 3p21). In all these loci, while the wild-type HCT-116 cell line retained DNA methylation at these sites, discrete losses of DNA methylation were observed in the DNMT1 and 3b single knockouts (Fig. 6). Most importantly, in the DKO cells, synergistic DNA hypomethylation events were observed, with demethylated bands spanning larger regions than those expected by the mere addition of each single knockout (Fig. 6). Finally, we analyzed 10 genes with known promoter hypermethylation in human cancer (2,3) that are located in the specific chromosomal loci pointed-out by the AIMS hybridization approach: COL1A2, TFPI-2 and PIK3CG at 7q22, MYO18B and COMT at 22q11-12, DAPK1 and TGFBR1 at 9q34, SEMA3B and BLU at 3p21, and CACNA1G at 17q22.

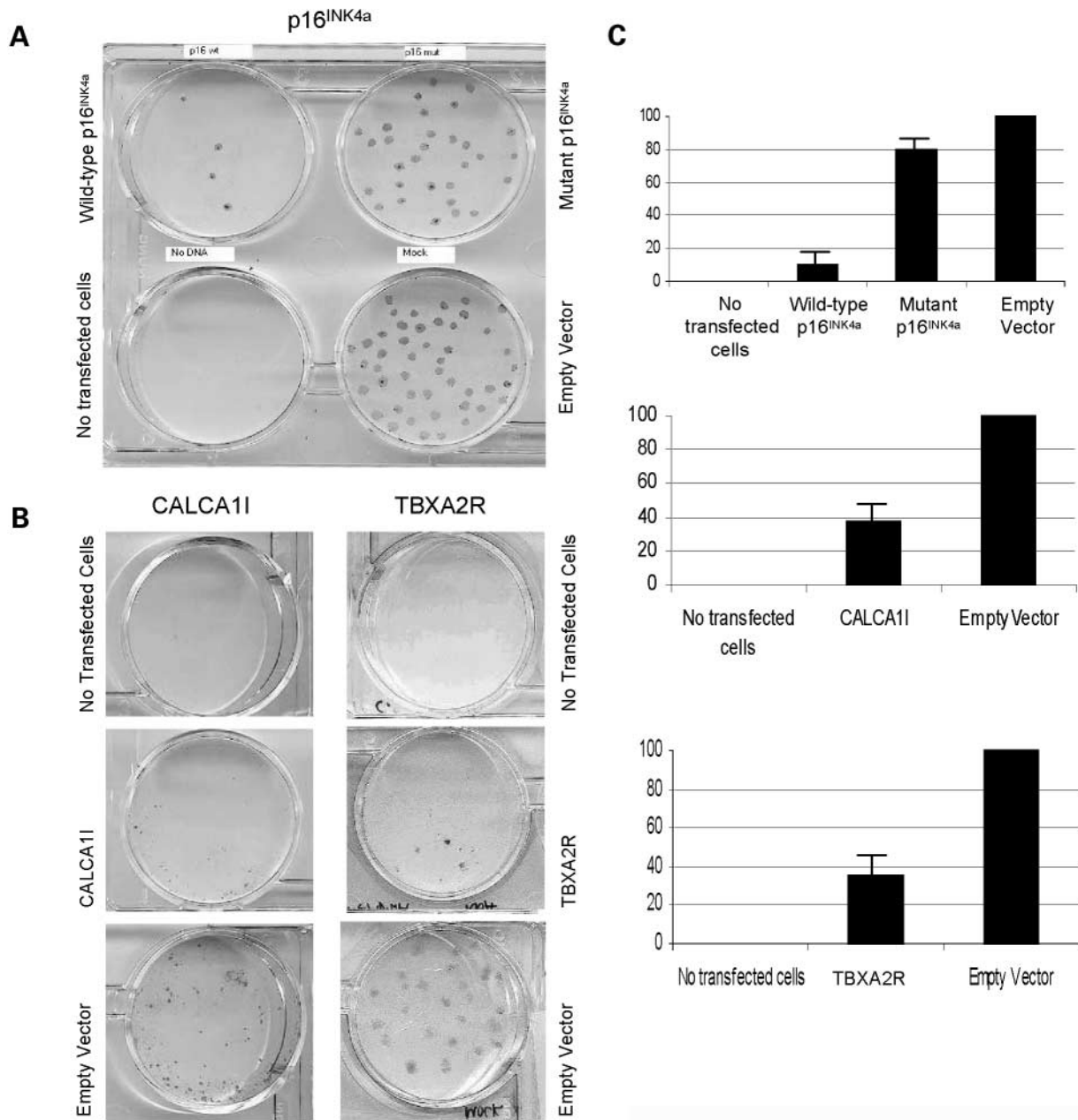


Figure 5. Colony focus assay in wild-type HCT-116 colon cancer cells. All results are an average of three independent experiments. (A) Colony formation after 2 week selection of G418 stained with methylene blue using empty vector, wild-type p16^{INK4a} and mutant p16^{INK4a}. (B) The effect of exogenous expression of CALCA11 and TBXA2R on the colony formation of HCT-116 colon cancer cells. (C) Quantitation of the number of G418-selected HCT-116 colonies. The vector control was set at 100%. The degree of suppression of colony formation was 90% for wild-type p16, 62% for CALCA11 and 65% for TBXA2R.

We found that all of them had hypermethylated CpG islands in the wild-type HCT-116 cells that were demethylated in DKO cells, supporting the versatility of the AIMS competitive genomic hybridization approach.

DISCUSSION

The generation of a HCT-116 colon cancer cell line, where both the DNA methyltransferase 1 (DNMT1) and DNA methyltransferase 3b (DNMT3b) are disrupted through gene

targeting, has proven to be extremely useful in elucidating the epigenetic mechanisms underlying malignant transformation (7,8). These DKO cells show a dramatic reduction in the DNA methyltransferase activity and demethylation of repeated sequences (8). Furthermore, the finding that two genes, p16^{INK4a} and TIMP3 are demethylated and re-expressed in DKO cells (8), suggests that both DNA methyltransferases tightly cooperate to silence tumor suppressor genes. The erasure of the methylation at the p16^{INK4a} gene in DKO cells also leads to histone modifications (25). We have taken these previous results one step further using the DKO cells to ‘catch’

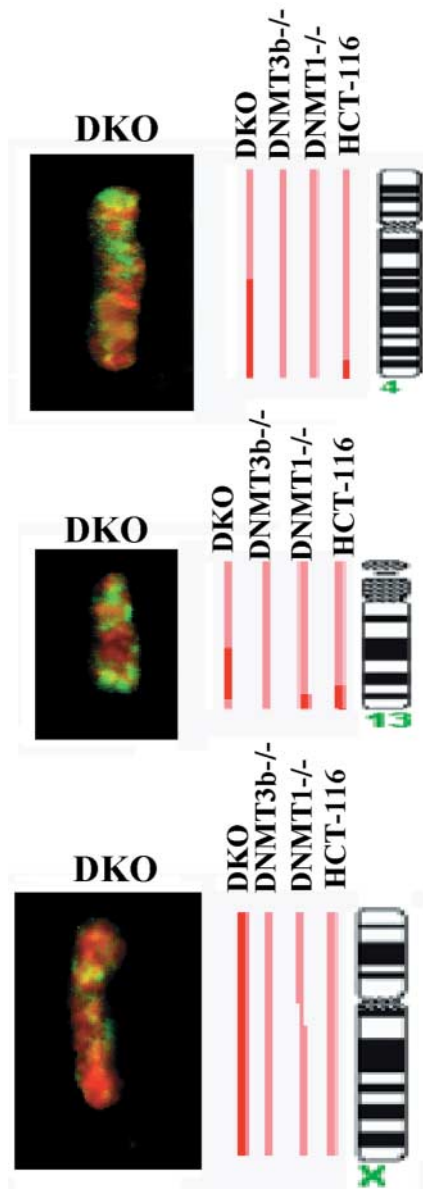


Figure 6. Comparative genomic hybridization for methylated DNA. Competitive hybridization to metaphase chromosomes of the AIMS products generated from the wild-type HCT-116 cell line, the single DNMT1 knockout, the single DNMT3b knockout and the DKO cells are shown from the closer line to the chromosome to the farthest, respectively. Examples of the hybridization of chromosomes 4, 13 and X are displayed. Normal DNA was labeled in red and it stains the whole chromosome. AIMS products were labeled in green and they are visualized as green signals that hybridize only to specific chromosome regions and stain partially the chromosomes. The DKO cell line presents the most comprehensive demethylation events displayed as thick red blocks.

new genes undergoing methylation associated silencing in human cancer.

The search for new targets of promoter hypermethylation in tumoral cells has so far adopted two main approaches. One has been the candidate gene approach, whereby a gene having putative tumor suppressor-like capabilities and a history of loss of expression in cancer cells, in the absence or low frequency of somatic mutations, has been catalogued as 'suspicious' to be

hypermethylated and, thus, analyzed in depth for this epigenetic lesion. In using this strategy, we and others have found the methylation associated silencing of many tumor suppressor genes in human cancer, including hMLH1, BRCA1, p16^{INK4a}, p14^{ARF}, p15^{INK4b} and VHL (3). A second approach uses global genomic strategies: pharmacological screening of cancer cell lines with DNA demethylating drugs, alone or in combination with inhibitors of histone deacetylases, followed by hybridization of the RNA to expression microarrays (12,13), or alternatively by comparing the DNA from normal and tumoral tissue of the same patient by global methylation techniques, such as restriction landmark genomic scanning (10). These genomic approaches have provided the scientific community with a long list of epigenetically silenced genes, such as the cystein rich protein with LIM gene (CRIP1) (13) or the secreted frizzled-related protein 1 (SFRP1) (12), with potential tumor suppressor functions that are currently under study. These approaches are useful but have their shortcomings. The candidate gene approach is time-consuming, the number of genes studied is limited and due to the fact that only those genes with special features are studied, it may be biased towards certain type of genes. Several drawbacks are also present in the genomic approaches. The DNA demethylating drugs used in the pharmacological screenings have pleiotropic effects in addition to their hypomethylating action, and, for instance, 5-aza-2-deoxycytidine is able to induce the re-expression of genes with unmethylated CpG islands, such as CDKN2D (26). Finally, the mere comparison of normal versus tumoral DNAs in primary neoplasms can be compromised for the omnipresent contamination of normal tissue, lymphocytes and stroma that can mask many hypermethylation lesions, although studying the DNA methylation patterns among numerous samples (11,17,18) has shown to be of excellent value. In this same setting, DMH and AIMS approaches have been used previously to compare normal breast and colon tissue versus primary breast and colon carcinomas (15,16), respectively.

The complementary spectrum of DNA methylation changes picked up by each technique, more CpG island single copy gene changes for DMH (14,15) and more repeat sequences for AIMS (16), makes them promising tools to learn more on the extent and quality of genomic methylation in different biological and experimental situations. Future suitable sources of DNA, where this technology can be applied, include patients with immunodeficiency, centromeric instability and facial anomalies (ICF) syndrome due to germline mutations in DNMT3b (4,5) or the mice models where DNMTs have been disrupted (27).

The generation of cancer cells disrupted at the two major DNA methyltransferases have proven to be a useful avenue to characterize the DNA methylation patterns observed in malignant transformation. This is a pure population of cancer cells that can be compared to the original cell line because it only differs in its DNA methylation pattern. By using such DKO cells we have revealed a constellation of genes with methylation associated silencing in human tumors. Although cancer lines have a tendency to be more hypermethylated than primary tumors (28–30), we have shown that starting from the particular cell line HCT-116, the CpG island hypermethylation of those genes is not a unique feature of that single cell line but

is also common among colorectal tumorigenesis, being found in other colon cancer cell lines, primary colon tumors and colon adenomas. The list of epigenetically silenced genes unveiled covers most of the cellular pathways: from cell adherence (the cadherin family member FAT) to development (the homeobox genes LMX-1 and DUX-4), and from tissue structure (the collagens XIV and V) to cell metabolism (the SURF-1 and SURF-2 genes). For some of these genes a contributing role in malignant transformation has previously been defined. This is the case of the FAT tumor suppressor gene which defect in *Drosophila* causes tumor-like overgrowth of larval imaginal discs (20), and it becomes the third cadherin gene hypermethylated in cancer following E-cadherin and H-cadherin (3). For others, such as CALCA11 and TBXA2R, we have found that their transfection in the wild-type HCT-116 provokes a strong reduction of colony formation. Further studies are now required to carefully determine the tumor suppressor action of some of these candidate genes, though, that said, the observation that many of them lie in regions of frequent loss of heterozygosity in many human cancer cell lines and primary tumors, such as the chromosomal region 9q32-34 for ZPF37, SURF-1, SURF-2 and COL5A (31,32) and 19p13 for TBXA2R (33,34), supports their putative role as tumor suppressor-like genes.

Overall, we have demonstrated that cancer cells disrupted simultaneously at the DNMT1 and DNMT3b genes, but not each single DNMT knockout, have an epigenetic 'awakening' demonstrated by the reactivation of those 'dormant' tumor suppressor genes that were transcriptionally silenced by CpG island hypermethylation in the wild-type cells. This type of genetic disruption strategy may yield a complete picture of the genes undergoing epigenetic silencing in all tumor and cell types.

MATERIALS AND METHODS

Cancer cell lines and tumor samples

HCT-116 colon cancer cells were cultured in McCoy's 5A modified medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. HCT-116 DNMT1^{-/-}, HCT-116 DNMT3b^{-/-} and HCT-116 double DNMT1^{-/-}DNMT3b^{-/-} (DKO) cells were grown supplemented with 0.05 mg/ml hygromycin. HCT-116 cells were treated with 5-aza-2-deoxycytidine (1 μM) for 48 h, with drug and medium replaced 24 h after the beginning of the treatment. Human colorectal cancer cell lines SW48, SW480, SW837, DLD-1, HCT-15, LoVo, Colo205 and Caco2 were cultured in RPMI 1640 or Minimal Essential Medium (Gibco BRL) supplemented with 10% fetal bovine serum. Tissue samples of colorectal cancer, normal colon and normal lymphocytes were all from specimens obtained at the time of clinically indicated surgical procedures.

Differential methylation hybridization analysis (DMH)

We analyzed DNA methylation in HCT116 colorectal carcinoma cells with disrupted DNMT1, DNMT3b or DNMT1/DNMT3b genes and in cells treated with DAC using the DMH approach (14,15). Microarray slides containing the 7776 CpG

island loci and the DNA samples were processed essentially as described previously (14,15). Briefly, we digested the DNA with *MseI*, ligated it to end linker, and interrogated DNA methylation by digesting the ligated samples with a methylation-sensitive endonuclease, *BstUI*. We then amplified the DNA from the end linkers. DNA derived from the wild type HCT116 cell line was similarly prepared as a control. Genomic fragments containing methylated sites were protected from the digestion and could be amplified, while other fragments containing the unmethylated sites were restricted away and not present in the amplified samples. Each test amplicon was labeled with Cy5 (red) and the control amplicon was labeled Cy3 (green). Both samples were co-hybridized onto the slides, which were processed according to the method of De Risi *et al.* (www.microarrays.org). We then scanned the slides using GenePix 400A (Axon) and analyzed the images with the GenePix Pro3.0 program. Sequence characterization of the microarray clones indicates that about 80% of the clones have CpG island-like sequences, 10% are ribosomal DNA sequences and the remainder are residual bulk genomic or mitochondrial DNAs (35).

The data were analyzed by measuring the methylation differential between the test and wild-type genomes on the basis of the microarray spot Cy5: Cy3 ratios. For a CpG island locus, this was determined from the net pixel intensity, subtracting its local background, for each of the dyes. We normalized the Cy5: Cy3 ratios for hybridization variations using internal control and systematically applied a Cy5: Cy3 ratio cut-off of ~0.5 for demethylation, a threshold verifiable for the presence of green spots on the slide. Yellow spots are indicative of the absence of any methylation differential between the test and wild-type cells whereas red spots, in principle, imply hypermethylation in the test cells.

Amplification of inter-methylated sites (AIMS)

AIMS was developed as described (16). The non-methylated sites were cut in an initial digestion using the methylation-sensitive endonuclease *SmaI*, which leaves blunt ends. A second digestion was performed using the isoschizomer *PspAI*, which leaves a CCGG overhang. Adaptors are ligated to the sticky ends. DNA fragments flanked by two ligated adaptors were amplified by PCR using specific primers that hybridize to the adaptor sequence and the restriction site and one or more additional arbitrarily chosen nucleotides.

Briefly, 1 μg of genomic DNA was digested with 20 U of the methylation-sensitive restriction endonuclease *SmaI* (Amersham Pharmacia Biotech, Buckinghamshire, UK) for 16 h at 30°C, which cleaves to leave blunt ends (CCC/GGG). Subsequently, the DNA was digested with 4 U of *PspAI* (Stratagene, La Jolla, CA) for 6 h at 37°C, which leaves sticky ends (C/CCGGG). Adaptors are ligated to the sticky ends. The adaptors were prepared by incubation of the oligonucleotides MCF (5'-CCGGTCAGAGCTTTGCGAAT-3') and Blue (5'-ATTCGCAAAGCTCTGA-3') at 65°C for 2 min, followed by cooling to room temperature for 30–60 min. An aliquot of 1 μg of digested DNA was ligated to 2 nmol of adaptor using T4 DNA ligase (New England Biolabs, Boston, MA). The ligation product was purified using the GFX Kit (Amersham Pharmacia Biotech) and eluted into 250 μl of water. In a second

step, PCR amplification of sequences flanked by adaptors was performed using different primers or sets of primers corresponding to the Blue primer extended with the CCGGG (overhanging) end and 2–4 arbitrarily chosen nucleotides. This allows the amplification of a limited number of sequences that typically range from ~200 to ~2000 bp. In our previous study, we found that ~60% of the sequences identified by AIMS are repeat sequences and 40% are non repetitive (16). The following primer sets were used (3'-arbitrarily chosen nucleotides are shown underlined): Blue-CCGGG-CTA + Blue-CCGGG-TGG (set A), Blue-CCGGG-CTG + Blue-CCGGG-TGG (set B), Blue-CCGGG-CGCG + Blue-CCGGG-CAAC (set C), Blue-CCGGG-TG (set D). The obtained PCR products were diluted 1:4 in formamide dye buffer, denatured for 3 min at 95°C and 3 µl were run on a 6% polyacrylamide 8 M urea sequencing gel at 55 W for 5 h. The gels were dried under vacuum at 85°C and exposed to an X-ray film at room temperature without an intensifier screen for 3–6 days.

Differences in the intensity of bands between the tumor and its paired normal tissue were ascertained by naked eye inspection of the film. Densitometric analysis was considered unnecessary in normal-tumor comparisons since only clear and reproducible changes were taken into account. Relative decreases in the intensity of a band in the tumor with respect to its paired normal tissue fingerprint were considered a symptom of hypomethylation. Bands appearing differentially methylated were excised from dried polyacrylamide gels, and DNA was re-eluted in 50 µl of sterile H₂O (80°C, 10 min). PCR with the same primers and conditions as used in the AIMS experiment was performed to amplify the fragment. PCR products were cloned into plasmid vectors using the TA cloning kit (Invitrogen, Groningen, The Netherlands). Auto-matic sequencing of multiple colonies was performed to ascertain the unique identity of the isolated band. Sequence homologies were searched using the BLAST engine available at www.ncbi.nlm.nih.gov.

Competitive hybridization of AIMS products to metaphase chromosomes

To study the distribution of the AIMS amplified DNA along the chromosomes, we hybridized equimolecular quantities of human normal DNA against the unrestrained AIMS product. The whole genomic DNA was labeled with Spectrum Red dUTP by CGH nick-translation kit (Vysis Inc., Downer Grove, IL, USA) and the AIMS products of the cell lines were labeled with Spectrum Green. The mixture was then hybridized onto normal metaphase chromosome spreads, following the Comparative Genomic Hybridization protocol described previously (24). Images of metaphases were captured under a fluorescent microscope (Olympus BX60) with a CCD camera (Photometrics Sensys), then analyzed using the chromofluor image analysis system (Cytovision, Applied Imaging Ltd, Newcastle, UK). A wide range of chromosomes (13–25) was analyzed for each hybridization. Two different kinds of control experiment were performed. Negative controls were done by hybridizing normal male human DNA versus normal female human DNA, labeled in red and green, respectively. Positive controls were done by hybridizing normal male DNA versus whole DNA from HCT-116 cells, labeled in red and green,

respectively. Both types of controls yielded the expected values. In addition, all experiments with the AIMS products were carried out twice in order to assess their reproducibility.

DNA methylation analysis of particular genes

We carried out bisulfite modification of genomic DNA as described previously (36). We established methylation status by PCR analysis of bisulfite-modified genomic DNA using two procedures. First, all genes investigated were analyzed by bisulfite genomic sequencing of their corresponding CpG islands. Both strands were sequenced. The second analysis used methylation-specific PCR for all genes analyzed in several cancer cell lines and tissue samples, as previously described (36). We designed all of the bisulfite genomic sequencing and methylation-specific PCR primers according to genomic sequences around presumed transcription start sites of investigated genes. Primer sequences and PCR conditions for methylation analysis are available upon request.

Semiquantitative RT-PCR expression analysis

We reverse-transcribed total RNA (2 µg) treated with Dnase I (Ambion) using oligo (dT)_{12–18} primer with Superscript II reverse transcriptase (Gibco BRL). We carried out PCR reactions in a 50 µl volume containing 1× PCR buffer (Gibco BRL), 1.5 mM of MgCl₂, 0.3 mM of dNTP, 0.25 µM of each primer and 2 U of Taq polymerase (Gibco BRL). We used 100 ng of cDNA for PCR amplification, and we amplified all of the genes with multiple cycle numbers (20–35 cycles) to determine the appropriate conditions for obtaining semi-quantitative differences in their expression levels. RT-PCR primers were designed between different exons to avoid any amplification of DNA. We also carried out PCR with GAPDH (25 and 28 cycles) to ensure cDNA quality and loading accuracy. Primer sequences are available upon request.

Transfection experiments and colony formation assays

pSV-β-galactosidase control vector and the expression vectors for the calcium channel α1I (pDNA-3 Ca_v 3.3, provided by Dr Edward Perez-Reyes) and the thromboxane A₂ receptors (pCMV5-TxR, provided by Dr Colin D. Funk), also containing neomycin resistance genes, were transfected into the DKO HCT116 cells by the lipofection method. Briefly, cells were seeded in a 6-well plate, 1 day before transfection, at a density of 2 × 10⁵ cells/well. An aliquot of 2 µg of purified plasmid DNA were transfected with LipofectAmine Plus reagent, according to the manufacturer's recommendations. The experiment was repeated three times. Twenty-four hours after transfection, βgal activity was measured in the cells carrying the control vector using a βgal activity kit. Clones expressing the transfected proteins were selected in complete medium supplemented with 1 mg/ml G418, 48 h posttransfection. Stable clones were maintained in complete medium with G418 (800 µg/ml). Total RNA from individual clones was extracted and RT-PCR was performed to confirm that the clones were expressing the transfected genes. HCT116 DKO cells were also transfected with the vector containing no inserts and stable clones isolated. As a positive control, we transfected wild-type

p16^{INK4a} (pLPC-hp16 wt-HA-puro provided by Dr Manuel Serrano), a well-known tumor suppressor gene that is lost in HCT-116 cells, and as a negative control, we transfected a mutant p16^{INK4a}, a form without tumor suppressor growth activity (pLPC-hp16 mut-HA-M531-puro also provided by Dr Manuel Serrano). After ~16 days of selection, stable G418 resistant colonies were fixed, stained with 2% methylene blue in 60% methanol and the average number of colonies present in each well was counted.

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