Combined Bisulfite Restriction Analysis

To confirm the Pyrosequencing and genomic sequencing results, we performed combined bisulfite restriction analysis with PCR-amplified products used in the Pyrosequencing reaction. The bisulfite-converted PCR products were subjected to two independent enzymatic digestions: AciI and CfoI. Restriction digestions were performed using 5–10 U AciI and CfoI (New England Biolabs, Ipswich, MA, USA) in a total volume of 10 µL at 37°C overnight. The digestion reaction was separated in a 2% agarose gel, visualized by ethidium bromide staining, and quantitated with a densitometer. To evaluate complete digestion, the pCR2.1 plasmid, that has both CfoI and AciI recognition sites, was subjected to the restriction reaction.

AciI recognizes three CpG sites: one is the CpG site interrogated in the Pyrosequencing reaction (Figure 2), the second is a CpG site within the CTCF binding site (Supplementary Figure S1, delimited in a box), and the third is adjacent to the CTCF binding site. We calculated the average methylation of the CpG site interrogated in the Pyrosequencing (Supplementary Figure S1, open circles marked by the asterisk) by the ratio of the density of the 98-bp band versus the 226-bp band. The combined bisulfite restriction analysis (COBRA) assay quantitated average methylation of both alleles to be 44%, which is in agreement with the 45% average of combining both G and T alleles from our Pyrosequencing analyses (Supplementary Figure S1).

Bisulfite Sequencing

The H19 differentially methylated region (DMR) was amplified using bisulfite PCR ([5′-A A A A A A T A T T C C C C A A T T C C A-3′ and 5′-G T T T T T T A T G A G T G T T T A T T T T T T A T G A T G-3′]), and the PCR product was cloned using the TA cloning kit (Invitrogen, Carlsbad, CA, USA). Plasmid DNA was purified from 17 clones using the Wizard® Plus kit (Promega, Madison, IL, USA). The nucleotide sequences of each clone representing a single allele was verified by cycle sequencing at the Laragen Sequencing Facility (Los Angeles, CA, USA) using the M13 F (5′-G T A A A A C G A C G C G C A T T A G T-3′) or M13 R (5′-C A G G A A A C A G C T A T G A C-3′) primers (Supplementary Figure S2).
Supplementary Figure S1. Average methylation by combined bisulfite restriction analysis (COBRA). Diagram shows the H19 differentially methylated region (DMR) and the methylation sites (open circles) within the Pyrosequencing PCR product. The CTCF binding site is marked with the box. The box indicates the location of the five CpG sites in the CTCF binding site (CB6, sixth of seven CTCF-binding sites in the H19 imprinting center). The restriction map of the PCR product for AcI and CfoI are shown. The COBRA gel: lane M, 100-bp ladder (New England Biolabs); lane 1, Colo205 bisulfite-converted PCR product untreated; lane 2, Colo205 AcI digestion; lane 3, Colo205 CfoI digestion.

Supplementary Figure S2. Genomic bisulfite sequencing of 14 CpG sites within and flanking the H19 imprinting center in the colorectal cancer cell line, Colo205. Each row represents a single sequenced molecule/allele. The single nucleotide polymorphism (SNP) used to differentiate between the parental alleles in the allele-specific methylation assay is indicated by the notation G/T, and the sites interrogated by our Pyrosequencing assay are the two CpG sites to the right of the G/T SNP (rs2071094; GenBank dbSNP accession no. build 124). Filled circles, methylated CpGs; empty circles, unmethylated CpGs.