Supplementary Material and Methods

Methylated DNA immunoprecipitation (MeDIP) array and data analysis

Genomic DNA samples isolated from two CRC cell lines HCT116 and LS180 and two normal colon tissues were employed for promoter methylation analysis by MeDIP array analysis (Welgene Biotech CO., LTD, Taipei, Taiwan). The Agilent Human CpG Island Array (G4492A) is a single array design including all well-characterized RefSeq promoter regions and all known CpG Islands annotated by UCSC. The array covers 5.5 kb promoter region for all 33,202 gene promoters reported in the Human RefSeq and 27,627 CGIs annotated in the UCSC genome browser. The array was scanned using the Agilent G2565BA microarray scanner and data were extracted using the Agilent G4460AA Feature Extraction software. We observed 2674 methylated CpG islands (CGIs) both of CRC cell lines and normal tissues (Supplementary table 1). Based on the ratio, we chose the first 400 genes with the highest ratio. With reference to the protein expression data in Human Protein Alta (http://www.proteinatlas.org/), we compared the ratio and the expression of corresponding candidates in normal colon tissue to further narrow to six candidates including LECT1, KHDC1, MKX, CA4, CBX8, FAM159B. mRNA expression levels of these 6 genes were measured in 5 CRC cell lines and a normal colon tissue. Only CA4 was silenced in all 5 CRC cell lines while readily expressed in normal colon tissue, which went beyond the other candidates (Supplementary figure 1A).

RNA extraction, semi-quantitative RT-PCR and real-time PCR analyses

Total RNA was extracted from cells and tissues using TRIzol Reagent (Molecular Research Center Inc, Cincinnati, OH). cDNA was synthesized from 2 μg of total RNA using Transcriptor Reverse Transcriptase (Roche, Indianapolis, IN). For semi-quantitative RT–PCR, a 137-bp

fragment of the CA4 gene was amplified using AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA). β-actin was employed as internal control. The primer sequences of CA4 were as follows: forward, 5'- CCG GCT CAG AGG ACT CTT-3' and reverse, 5'-GTT GGA GGA CTC GGC TTG AA-3'. Real-time PCR was performed using SYBR Green master mixture (Roche, Indianapolis, IN) on LightCycler® 480 Instrument. Each sample was tested in triplicate. ΔΔCT method was employed to determine the fold change in gene expression level. ΔCT method were employed to determine the relative expression levels of corresponding genes.

Bisulfite treatment of DNA, methylation-specific PCR (MSP) and combined bisulfite restriction analysis (COBRA)

Genomic DNA was extracted and the DNA was chemically modified with sodium metabisulphite. The bisulfite-modified DNA was amplified by MSP. COBRA was performed to semi-quantitate the methylated and unmethylated DNA after sodium bisulfite modification[12]. The sequences of primers were shown in Supplemental table 3.

Bisulfite genomic sequencing (BGS)

The PCR products of bisulfite-treated DNA were cloned into the pCR4-Topo vector (Life Technologies, Carlsbad, CA). Seven to eight colonies were randomly chosen and sequenced. Sequencing analysis was performed by SeqScape software (Applied Biosystems, Foster City, CA) and 40 CpG sites spanning in the promoter region of 337 bp were evaluated.

Demethylation treatment

1×10^6 cells were placed in 100-mm dishes and grown for 24 h. Cells were then treated with 2 μM of 5-aza-2'-deoxycytidine (5-Aza) (Sigma-Aldrich, St Louis, MO) for 48 hours. Culture medium containing 5-Aza was replenished every day.

Construction of CA4 expression plasmid and establishment of stable CA4-expressing cells

The full-length open reading frame sequence of CA4 was obtained by RT-PCR amplification of normal human colon cDNA. The PCR aliquots were subcloned into the mammalian expression vector pcDNA3.1 and then verified by DNA sequencing. HCT116 or SW1116 cells were transfected with pcDNA3.1 or pcDNA3.1-CA4 plasmid using lipofectamine 2000 (Life Technologies). Stable transfections were selected for 2 weeks with G418 antibiotics.

RNA interference and transfection

Knock-down CA4 expression in NCM460 cell lines was performed by a shRNA targeting CA4. Both NCM460-shRNA control and NCM460-CA4 shRNA cells were selected for 2 weeks with puromycin after transfection 48h. The cells were ready for further experiments. HCT116 and SW1116 cells were transfected with 50 nM WTAP siRNA (siWTAP: 5'-CCU UGU AAU GCG ACU AGC A-3') (Guangzhou RiboBio Co., Ltd) or control siRNA (Guangzhou RiboBio) using lipofectamine 2000. The siRNA against TBL1 (5'-GGU GUG CAU CCA UGA UCU UTT-3') was purchased (Life Technologies). 10 nM TBL1 siRNA was used to transfect HCT116 and SW1116 cells using lipofectamine 2000.

Western blot analysis

Total protein was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The proteins in SDS-PAGE were transferred onto nitrocellulose membranes (GE Healthcare, Piscataway, NJ). The membrane was incubated with primary antibodies overnight, and then with secondary antibody at room temperature for 1 hour. Proteins of interest were visualized using ECL Plus Western blotting Detection Reagents (GE Healthcare). The antibodies used and their dilutions were listed in Supplementary table 4.

Immunohistochemistry

Paired primary tumor and adjacent non-tumor samples were obtained from 12 CRC patients after surgical resection. Tissue types (tumor or normal) were assessed by histological staining. The remaining tissue specimens were fixed in 10% of formalin and embedded in paraffin. Immunohistochemistry was performed on five-micrometer paraffin sections using anti-CA4 antibodies (Novus Biologicals, Littleton, CO) with dilution of 1:200. CA4 staining was evaluated by scanning of the whole section and counting more than 1000 representative cells manually and blindly. The extent of CA4 staining was scored by assigning the percentage of positive tumour cells (0, none; 1, < 20% of positive staining cells; 2, 20-50% of positive staining cells; 3, >50% of positive staining cells). The ethics committee of the Chinese University of Hong Kong approved of this study, and written consents were obtained from all patients involved.

Cell viability assay

Cell viability of stably transfected cells was examined using the Vybrant MTT Cell Proliferation Assay Kit (Life Technologies) according to the manufacturer's instructions. All experiments were conducted three times in triplicates. Results were shown as the means ± SD.

Colony formation assay

HCT116 and SW1116 cells (2×10^5/well) were plated in a 12-well plate and transfected with pcDNA3.1-CA4 or empty vector. 48 hours post-transfection, cells were subsequently split at 1:20 ratio on six-well plates with G418 (0.5 mg/mL). After culturing for 14-21 days, cells were fixed with 70% ethanol and stained with 0.5% crystal violet solution. Colonies with more than 50 cells per colony were counted. All experiments were conducted three times in triplicates.

Cell cycle analysis

The transient transfected CRC cells (HCT116 and SW1116) were fixed in 70% ethanol-PBS for 24 hours. The cells were then labeled with 50 μg/ml of propidium iodide (BD Pharmingen, Franklin Lakes, NJ). The cells were sorted by FACSCalibur (BD Biosciences, San Diego, CA). Cell-cycle profiles were analyzed by ModFit 3.0 software (BD Biosciences). All experiments were conducted three times in triplicates.

Apoptosis assay

Cell apoptosis was determined by staining cells with Annexin V and 7-amino-actinomycin (7-AAD) (BD Biosciences) with subsequent flow cytometry analysis. Cell populations were counted as viable (Annexin V-negative, 7-AAD-negative), early apoptotic (Annexin V-positive, 7-AAD-negative), late apoptotic (Annexin V-positive, 7-AAD-positive), or necrotic (Annexin V-negative, 7-AAD-positive). The experiments were conducted three times in triplicates. In addition, terminal deoxynucleotidyl transferase-mediated nick-end labeling (TUNEL) assay was employed for apoptosis measurement of nude mice tumor biopsies. Nuclei with clear brown

staining were regarded as TUNEL-positive apoptotic cells. The apoptosis index was calculated as the percentage of TUNEL-positive nuclei after counting at least 1000 cells.

Wound-healing assay

Cell migration was assessed using the wound-healing assay. Briefly, HCT116 and SW1116 cells stably transfected with pcDNA3.1 or pcDNA3.1-CA4 were cultured in six-well plates. When the cells reached 80% confluence, three scratch wounds in each well were made using a P-200 pipette tip. Fresh medium supplemented with reduced (5%) fetal bovine serum was then added, and the wound-closure was observed for 48 hours. Photographs were taken at 0, 24 and 48 hours, respectively.

Invasion assay

Invasion assay was performed using BD BioCoat MATRIGEL Invasion Chamber (BD Biosciences) according to the manufacturer's instructions. Complete culture medium (supplemented with 10% fetal bovine serum) was used as the chemoattractant. Insert membranes were stained with 0.5% crystal violet. Invaded cells were counted under an inverted microscope and photographed. Three independent experiments were performed and the data were expressed as the means ± SD.

***In vivo* tumorigenicity assay**

For *in vivo* tumorigenicity assay, 1×10^7 empty vector- or CA4-transfected cells were injected subcutaneously into dorsal right flank of 4-week-old male Balb/c nude mice (five mice per group). Tumor volume was measured every 3 days over a 3-week period. Tumor volume (mm^3)

was estimated by measuring the longest and shortest diameter of the tumor (Formula: Volume = 0.5 × Length × 2 × Width). Mice were sacrificed at 3 weeks after injection. Tumors were excised and weighed. The excised tissues were either fixed in 10% neutral-buffered formalin or snap frozen in liquid nitrogen. Tumor sections from paraffin-embedded blocks were used for histologic examination. All animal studies were performed in accordance with guidelines approved by the Animal Experimentation Ethics Committee of The Chinese University of Hong Kong.

Ki-67 staining

Ki-67 was detected in paraffin-embedded colon sections of mice using an avidin-biotin complex immunoperoxidase method (Abcam, Chambridge, MA). The proliferation index was determined by counting the numbers of positive staining cells for Ki-67 as a percentage of the total number of cells. At least 1000 cells were counted each time. The experiments were conducted in 5 mice per group.

Dual-luciferase reporter activity assay

HCT116, SW1116 and HEK293 cells were seeded at cell density of 1×10^5 cell/well in 24-well plate. Cells were transiently transfected with pcDNA3.1 empty vector, pcDNA3.1-CA4, pCS2+/Wnt-1 + pcDNA3.1 empty vector , pCS2+/Wnt-1 + pcDNA3.1-CA4 and co-transfected with TOPflash plasmid (0.2 μg/well; TK-luciferase reporter) or FOPflash plasmid (0.2 μg/well; TK-luciferase reporter) using lipofectamine 2000 (Life Technologies). Cells were harvested at 24 hours post-transfection. Luciferase activity was measured by the Dual Luciferase Assay System (Promega, Madison, WI). The experiments were conducted three times in triplicates.

Co-immunoprecipitation

HCT116 and SW1116 cells were transfected with pcDNA3.1-CA4 expressing vector or pcDNA3.1 empty vector. After 48 hours post-transfection, total proteins were extracted by CytoBuster Protein Extraction Reagent (Novagen, Darmstadt, Germany). For each immunoprecipitation, 300 ug of precleared cell lysate and 30 μl of protein G PLUS-Agarose (Santa Cruz Biotechnology, Santa Cruz, CA) were used for overnight incubation at 4°C. The immunoprecipitated proteins were mixed with 2 x SDS-PAGE loading buffer and boiled at 95°C for 10 minutes. The proteins were separated by SDS-PAGE and analyzed by mass spectrometry. The identity of proteins was validated by Western blot.