SUPPLEMENTARY MATERIAL AND METHODS

Cell lines and cell culture

The human colon cancer cell lines Caco-2, HCT116, SW480 and SW1116 were obtained from the American Type Culture Collection (Manassas, VA, USA). HCT116 p53 knockout (KO) cell line was kindly provided by Professor Bert Vogelstein (Johns Hopkins University, Baltimore, MD). HCT116 p53 WT and HCT116 p53 KO cell lines were grown in McCoy's 5A medium (Invitrogen, Carlsbad, CA). Other cell lines were cultured in RPMI 1640 medium (Gibco BRL, Rockville, MD).

Whole genome sequencing

Genomic DNA from primary CRC tumour and peripheral blood was fragmented to an average size of 500 nucleotides. Standard Illumina protocols and Illumina paired-end adapters were then used for library preparation. DNA library sequencing was then performed on an Illumina Solexa sequencing platform as per manufacturer's instructions.

Array CGH assays

Human 244K CGH microarray (Agilent Technologies, Santa Clara, CA) was employed in a 'two-color' process to measure DNA copy number changes in the same case. Labelling reactions were performed with 1 μg genomic DNA with Agilent Genomic DNA Labeling Kit PLUS (Agilent Technologies). The microarray chip was scanned by the Agilent Microarray Scanner. Data analysis was done by the Agilent Feature Extraction 9.1 and CGH Analytics 3.4. In brief, a log2 expression ratio was

computed and normalized for forward and reverse fluor experiments using the CGH Analytics 3.4 software.

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

Total RNA was extracted from cell pellets or tissues using Quizol reagent (Qiagen, Valencia, CA) and cDNA was synthesized (Roche, Indianapolis, IN). Semi-quantitative RT-PCR was performed using Hot-star DNA polymerase (Invitrogen). Real-time PCR was performed using SYBR Green master mixture on HT7900 system (Applied Biosystems, Foster City, CA). Primer sequences are listed in supplementary Table 4.

Immunofluorescence and confocal microscopy

HCT116 cells were seeded in 12-well plates and fixed in 4% paraformaldehyde (Sigma) for 30 min. Cells were then permeabilized with PBS containing 0.1% Triton X-100 for 3 min and incubated with a 5 mg/ ml BSA blocking solution. Cells were incubated with SLC12A5 antibody (1 : 100, Abcam, Cambridge, UK), followed by incubation with a secondary antibody. Chromosomes were stained with DAPI in PBS for 5 min. The immunofluorescence images were taken with a confocal microscope (FV1000, Olympus, Tokyo, Japan).

Immunohistochemistry

Immunohistochemistry for SLC12A5 was performed on paraffin sections of paired CRC slides and TMAs using anti-SLC12A5 antibody (Millipore, Billerica, MA). The extent of *SLC12A5* 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 case with score 0, 1 or 2 was defined as low expression, while the case with score 3 was regarded as high expression. Immunohistochemistry for AIF and EndoG was also performed on paraffin slides of mouse xenograft tumours using anti-AIF and anti-EndoG antibodies (Cell Signalling Technology, Cambridge, UK).

Transfections

Cells were transfected with pCMV6-AC-GFP (PS100010, OriGene, Rockville, MD), or pCMV6-*SLC12A5* (RG223680, OriGene) using Lipofectamine 2000 (Invitrogen), respectively. RNA and proteins were harvested at 48 or 72 h after transfection.

RNA interference

The *SLC12A5* siRNA pool of three duplexes (5'-GGCUCAAUCCGGAGAAAGAdTdT-3' (sense) and 3'-dTdT CCGAGUUAGGCCUCUUUCU-5' (antisense); 5'-CAACCGCAAUGGUGAUGAAdTdT-3' (sense) and 3'-dTdT GUUGGCGUUACCACUACUU-5' (antisense) and 5'-CCUUAUGUCUUCAGUGAUAdTdT-3' (sense) and 3'-dTdT GGAAUACAGAAGUCACUAU-5' (antisense)) targeting human *SLC12A5* were synthesized by Ribo Company (RiboBio, Guangzhou, China). Scrambled siRNA was used as a negative control (siControl). 50 nM of si*SLC12A5* or siControl were transfected into cells by using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

Cell viability assay

Cell viability was monitored by the xCELLigence Real Time Cell Analyzer (Roche Applied Science, Mannheim, Germany). The cell index was derived from measured cell-electrode impedance that correlates with number of cells and cell viability. The experiment was performed in triplicate wells for three independent experiments.

Colony formation assay

Empty vector and different doses of *SLC12A5* plasmids (0.8 μg and 1.6 μg) were used to transfect cells in 24-well plates. After 48 h of transfection, cells were collected and seeded (2×10^3/well) in 6-well plate and selected with G418 at 0.5 mg/mL (Calbiochem, Darmstadt, Germany) for 10-14 days. Colonies (≥ 50 cells/colony) were counted after staining with 5% crystal violet. The assay was carried out in triplicate wells for three independent experiments.

Annexin V apoptosis assay

Apoptosis was assessed by flow cytometry after staining with Annexin V (FITC-conjugated) (BD Biosciences, Erembodegem, Belgium) and 7-amino-actinomycin (7-AAD) (BD Biosciences). Cell populations were counted as viable (Annexin V-negative, 7-AAD-negative), early apoptotic (Annexin V-positive, 7-AAD-negative), and late apoptotic (Annexin V-positive, 7-AAD-positive), and necrotic cells (Annexin V-negative, 7-AAD-positive). The experiments were performed in triplicate for three times independently.

In Situ DNA Nick End Labeling

Terminal deoxynucleotidyl transferase-mediated nick-end labelling (TUNEL) was performed following the Manufacturer's protocol (single-stranded DNA apoptosis

TUNEL kit, Roche, Indianapolis, IN). Nuclei with clear brown staining were regarded as apoptotic cells.

Cell cycle analysis

Cells were seeded to 6-well plates and transfected with empty vector, SLC12A5 plasmid, siSLC12A5 or siControl. Cells were starved by adding serum-free medium for G1 synchronization. After 24 hours, medium containing 10% fetal bovine serum (FBS) was added for an additional 12 hours. Cells were fixed in 75% ethanol, stained with propidium iodide (PI), and analyzed by flow cytometry (Becton Dickinson Biosciences, Bedford, MA). The results were analyzed with ModFit LT2.0 software (Coulter Electronics, Hialeah, FL).

Migration and Matrigel invasion assays

Migration and Matrigel invasion assays were performed as previously described[1]. For migration assay, cells were seeded into the upper chamber of a Transwell insert (pore size, 8 μm; Corning Falcon) and then placed into the transwell containing medium with 10% FBS in the lower chamber. For invasion assay, cells were seeded seeded in a Matrigel-coated chamber (Becton Dickinson, Waltham, MA, USA). After 48 h, cells that remained in the lower surface of the insert were stained with crystal violet. Experiments were conducted in triplicate.

Lentivirus packaging and transduction

Two short hairpin RNAs (shRNA-1, 5'-gatcgGCAGCACAACACTGTGCTTGTTTCAAGAGAACAAGCACAGTGTTGTGCTGCTTTTTTc-3' and shRNA-2, 5'-

gatcgGCGAGGTCATCACCATCTACTTTCAAGAGAAGTAGATGGTGATGACC TCGCTTTTTTc-3') targeting the SLC12A ORF (Genbank no. NM_020708) and a non-targeting RNA sequence serving as a negative control (shControl) were cloned into the pGMLV-SC1 vector (Genomeditech, Shanghai, China). Virus packaging was performed in HEK293T cells using the GM easyTM Lentiviral Packaging Kit (Genomeditech, Shanghai, China). HEK293T cells were cultured in DMEM with 10% FBS. Forty-eight hours after transfection, the supernatant was harvested and cleared by centrifugation. The HCT116 cells were transduced with the lentivirus containing sh*SLC12A5* or shControl. Forty-eight hours after infection, 1 μg/mL of puromycin was added to the media for 2 weeks to select the cells infected with the lentivirus.

Tumour xenograft mouse model

Male athymic 4-week-old Balb/c nude mice were housed under standard conditions and cared for according to the institutional guidelines for animal care. All animal experimental procedures were approved by the Animal Ethics Committee of the Chinese University of Hong Kong. To determine the tumourigenicity of *SLC12A5 in vivo*, HCT116 cells (2×10^6 cells in 0.1 mL PBS) transduced with the lentivirus containing *SLC12A5*-shRNA or control shRNA were injected subcutaneously into the dorsal left flank of 4-week-old male Balb/c nude mice (4/group). Tumour diameter was measured every 2 days until 3 weeks. Tumour volume (mm^3) was estimated by measuring the longest and shortest diameter of the tumour and calculating as previously described.[2] The mice were euthanized on the fourth week, and the tumours were excised and embedded in paraffin. Sections (5 μm) of tumours were stained with H&E to visualize the tumour structure.

Standard tail vein metastatic assay

To investigate experimental lung metastasis, male 4-week-old Balb/c nude mice were used in standard tail vein metastatic assay as previously described[3] HCT116 cells (5×10^6 cells in 0.1 mL PBS) with *SLC12A5*-shRNA or control shRNA were injected into the lateral tail veils of each nude mouse (5/group). Six weeks after injection, the mice were sacrificed and examined. The lungs and livers were dissected and paraffin embedded, and the sections were stained with hematoxylin and esosin. The metastases were counted under microscopy for occurrence of metastases in a double-blind manner.

Western blot analysis

Total protein or nuclear protein was extracted and protein concentration was measured by the DC protein assay method of Bradford (Bio-Rad, Hercules, CA). Thirty micrograms of protein from each sample were separated on 12% SDS-PAGE and transferred onto nitrocellulose membranes (GE Healthcare, Piscataway, NJ). Blots were immunostained with primary antibody and secondary antibody, respectively.

Luciferase reporter assay

To investigate the signaling pathways modulated by *SLC12A5*, five well-known signaling pathway luciferase reporters were examined in HCT116 and SW1116 cells, including p53-luc (14xp53 binding sites), p21-luc (2.1 kb p21 promoter), AP1-luc (7xAP1 binding sites), NFκB-luc (5xNFκB binding sites), and TOPFlash (4xTCF binding sites). Cells (1×10^5 cells/well) were co-transfected with luciferase report plasmid (195 ng/well), pRL-CMV vector (5 ng/well) and *SLC12A5* plasimd or control

empty vector for overexpression study, or 50nM si*SLC12A5* or Control siRNA for knockdown study using lipofectamine 2000 (Invitrogen). Cells were harvested 48 hours post-transfection and luciferase activities were analyzed by the dual-luciferase reporter assay system (Promega, Madison, WI).

Human tumour metastasis PCR array

Gene expression profiles of HCT116 cells transfected with si*SLC12A5* or siControl were analyzed by Human Tumour Metastasis RT^2 Profiler PCR array (Super Array Bioscience, Frederick, MD). This array contains 84 functionally well characterized genes involved in tumour metastasis (http://www.sabiosciences.com). Genes expression with fold-changes more than or less than 2.0 were considered to be of biological significance.

Reference

1. Zhang H, Hao Y, Yang J, *et al.* Genome-wide functional screening of miR-23b as a pleiotropic modulator suppressing cancer metastasis. *Nat Commun* 2011;2:554.
2. Xu L, Li X, Chu ES, *et al.* Epigenetic inactivation of BCL6B, a novel functional tumour suppressor for gastric cancer, is associated with poor survival. *Gut* 2012;61(7):977-85.
3. Saur D, Seidler B, Schneider G, *et al.* CXCR4 expression increases liver and lung metastasis in a mouse model of pancreatic cancer. *Gastroenterology* 2005;129(4):1237-50.

Supplementary Table 1. Clinicopathological features of CRC patients

Variable	No.	%
Mean age, y ±SD	64.3±12.8	
Gender		
M	104	55.5%
F	87	45.5%
Location		
Colon	40	24.5%
Rectum	123	75.5%
Grade		
High	5	2.6%
Moderate	177	92.7%
Low	9	4.7%
TNM		
I	28	14.7%
II	81	42.4%
III	56	29.3%
IV	26	13.6%

Supplementary Table 2. Amplification of *SLC12A5* was verified by comparative genomic hybridization (array CGH)

Gene Name	Probe Name	Log_2 Ratio	Copy Ratio
SLC12A5	A_16_P03524624	0.355	1.279
SLC12A5	A_16_P03524631	0.739	1.669
SLC12A5	A_16_P21143897	0.778	1.715
SLC12A5	A_16_P03524640	0.714	1.640
SLC12A5	A_18_P13807773	0.695	1.619
SLC12A5	A_16_P21143916	0.841	1.791
SLC12A5	A_16_P41338750	0.443	1.359
SLC12A5	A_16_P34722776	0.572	1.487
SLC12A5	A_16_P03524656	0.142	1.103
SLC12A5	A_16_P03524659	0.632	1.550
SLC12A5	A_16_P21143939	0.874	1.833
SLC12A5	A_16_P03524668	0.523	1.437
SLC12A5	A_16_P03524673	0.702	1.627
SLC12A5	A_16_P03524678	0.328	1.255
SLC12A5	A_16_P03524681	0.889	1.852
SLC12A5	A_16_P34723117	0.216	1.162
SLC12A5	A_14_P138449	0.669	1.590
Average		**0.595**	**1.528**

Supplementary Table 3. Changes in gene expression after knockdown of *SLC12A5* in HCT116 cells

Gene name	Symbol	Fold-change	Gene location	Gene function
Metastasis Suppressor	MTSS1	12.69	8p22	Inhibits metastasis and proliferation
Tumor Protein P53	p53	10.7	17p13.1	Induces apoptosis, inhibits proliferation and metastasis
NME/NM23 Nucleoside Diphosphate Kinase	NME1	8.07	17q21.3	Negatively regulates proliferation and participates in cell adhesion
Fibronectin 1	FN1	7.22	2q34	Inhibits tumor growth, angiogenesis and metastasis
Neurofibromin 2	NF2	6.88	22q12.2	Suppresses cell proliferation and tumorigenesis
FAT Tumor Suppressor Homolog 1	FAT1	5	4q35	Inhibits cell migration
Breast Cancer Metastasis Suppressor 1	BRMS1	3.59	11q13	Inhibits metastasis and promotes apoptosis
Tissue Inhibitor Of Metalloproteinase 2	TIMP2	2.69	17q25	Inhibits metastasis
Cathepsin K	CTSK	2.58	1q21	Participates in tumor progression and metastasis
Matrix Metallopeptidase 10	MMP10	-2.02	11q22.3	Protein hydrolysate, promotes metastasis
Matrix Metallopeptidase 13	MMP13	-2.06	11q22.3	Decomposes, protein hydrolysate and promotes metastasis
Hepatocyte Growth Factor	HGF	-12.67	7q21.1	Participates in proteolysis,
Matrix Metallopeptidase 2	MMP2	-14.19	16q13-q21	Decomposes, protein hydrolysate and promotes metastasis

Supplementary Table 4. DNA sequences of primers used in this study

Primer name	Sequence (5'-3')
RT-PCR	
SLC12A5-F	GCAGGAGCCATGTACATCCT
SLC12A5-R	CCATGCAGGTGAGCACACA
β-actin-F	GTCTTCCCCTCCATCGTG
β-actin-R	AGGGTGAGGATGCCTCTCTT
FN1-F	ACAACACCGAGGTGACTGAGAC
FN1-R	GGACACAACGATGCTTCCTGAG
HGF-F	GAGAGTTGGGTTCTTACTGCACG
HGF-R	CTCATCTCCTCTTCCGTGGACA
MMP2-F	AGCGAGTGGATGCCGCCTTTAA
MMP2-R	CATTCCAGGCATCTGCGATGAG
MMP13-F	CCTTGATGCCATTACCAGTCTCC
MMP13-R	AAACAGCTCCGCATCAACCTGC
MTSS1-F	TTCAGTGCTCCAGCGGCTACAG
MTSS1-R	GGAATGGTGGAGGACTTGTCGA