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'-dTdTCCGAGUUAGGCCUCUUUCU-5' (antisense); 5'-CAACCGCAAUUGUGAUGAAdTdT-3' (sense) and 3'-dTdTGUUGGCGUUACCACUACUU-5' (antisense); 5'-CCUUAUGUCUUCAGUGAUAdTdT-3' (sense) and 3'-dTDGGAUAACAGAAGUCACUAU-5' (antisense)) targeting human SLC12A5 were synthesized by Ribo Company (RiboBio, Guangzhou, China). Scrumbled siRNA was used as a negative control (siControl). 50 nM of siSLC12A5 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 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'-gatcgGCAGCACAACACTGTGCTTTTCAAGAGAACAAGCACAAGCAGTGGTGTG and shRNA-2, 5'-CTGCTTTTTTc-3') were used for the experiments.
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 shSLC12A5 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 (2x10⁶ 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 (mm³) 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. HCT116 cells (5×10^6 cells in 0.1 mL PBS) with SLC12A5-shRNA or control shRNA were injected into the lateral tail veins 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 eosin. 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 plasmid or control
empty vector for overexpression study, or 50nM siSLC12A5 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 siSLC12A5 or siControl were analyzed by Human Tumour Metastasis RT² 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          | Log₂ 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   | CCATGCAGGTGAGCACA   |
| β-actin-F   | GTCTTCCCCTCCATCGTG  |
| β-actin-R   | AGGGTGAGGATGCTCTCTT |
| FN1-F       | ACAACACCGAGGTGACTGAGAC |
| FN1-R       | GGACACAACGATGCTCTCT |
| HGF-F       | GAGAGTTGGGTCTTACTGCACG |
| HGF-R       | CTCACTCCTCCTTCGTGGACA |
| MMP2-F      | AGCGAGTGGATGCCGCCTTTAA |
| MMP2-R      | CATTCCAGGCATCTGGATGAG |
| MMP13-F     | CCTTGATGCCATTACCAGTCTCC |
| MMP13-R     | AAACAGCTCCGCATCAACCTGC |
| MTSS1-F     | TTCAGTGCTCCAGCGGCTACAG |
| MTSS1-R     | GGAATGGTGAGGACTTGTGCA |