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Research Article

SLC6A14, a Na^+/Cl^--coupled amino acid transporter, functions as a tumor promoter in colon and is a target for Wnt signaling

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SLC6A14 is a Na^+/Cl^--coupled transporter for neutral and cationic amino acids. It is expressed at basal levels in the normal colon but is up-regulated in colon cancer. However, the relevance of this up-regulation to cancer progression and the mechanisms involved in the up-regulation remain unknown. Here, we show that SLC6A14 is essential for colon cancer and that its up-regulation involves, at least partly, Wnt signaling. The up-regulation of the transporter is evident in most human colon cancer cell lines and also in a majority of patient-derived xenografts. These findings are supported by publicly available TCGA (The Cancer Genome Atlas) database. Treatment of colon cancer cells with α-methyltryptophan (α-MT), a blocker of SLC6A14, induces amino acid deprivation, decreases mTOR activity, increases autophagy, promotes apoptosis, and suppresses cell proliferation and invasion. In xenograft and syngeneic mouse tumor models, silencing of SLC6A14 by shRNA or blocking its function by α-MT reduces tumor growth. Similarly, the deletion of *Slc6a14* in mice protects against colon cancer in two different experimental models (inflammation-associated colon cancer and genetically driven colon cancer). In colon cancer cells, expression of the transporter is reduced by Wnt antagonist or by silencing of β-catenin whereas Wnt agonist or overexpression of β-catenin shows the opposite effect. Finally, SLC6A14 as a target for β-catenin is confirmed by chromatin immunoprecipitation. These studies demonstrate that SLC6A14 plays a critical role in the promotion of colon cancer and that its up-regulation in cancer involves Wnt signaling. These findings identify SLC6A14 as a promising drug target for the treatment of colon cancer.

Introduction

Colorectal cancer is one of the major causes of cancer morbidity and mortality worldwide; it represents the third most cause of cancer death in the United States [1]. The economic burden due to colorectal cancer is huge; the total estimated direct medical cost of colorectal cancer care is more than $15 billion per year in the United States [2]. This deadly disease starts with the formation of small, benign adenomatous polyps in the colonic epithelial layer that turns into advanced adenoma with high-grade dysplasia; over time, it progresses to an invasive carcinoma (tumor–node–metastasis stages I and II) [3]. If left untreated, invasive colorectal carcinoma transforms into stage III metastatic cancer, which spreads to regional lymph nodes and finally metastasizes to distant organ sites (stage IV) [3–6]. Molecular events involved in the initiation and progression of colorectal cancer include genetic predisposition, chromosomal instability, and defect in DNA repair mechanisms. These events ultimately lead to the acquisition of multiple tumor-associated mutations, epigenetic changes (e.g. aberrant DNA

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methylation), dysregulation of miRNAs, amplification or increased activity of specific oncogenes and suppression or inactivation of specific tumor suppressors, which re-program metabolic pathways and drive cancer initiation, growth, and metastasis [7–9].

For several decades, glucose transport and glucose utilization were at the center of cancer-cell metabolism, supported by several factors such as the Warburg phenomenon (i.e. aerobic glycolysis), lactic acid production, and success of PET scans based on glucose transport for cancer detection [10,11]. In recent years, however, amino acid transport and amino acid utilization have come to the forefront in the area of cancer-cell metabolism. The reason for this shift is the recognition that amino acids play much broader biological functions than glucose that are critical for tumor growth and also that amino acid metabolism feeds into the same metabolic pathways where glucose is known to participate. The terms such as 'glutamine addiction' and 'glutaminolysis', which have become common in the vocabulary describing cancer-cell metabolism, attest to the importance of amino acids in cancer biology. Even here, the initial focus was on amino acid metabolism; the fact that there is no amino acid metabolism without the transporters that feed the amino acid substrates into these metabolic pathways did not receive much attention. This has changed in recent years [10–14]. To satisfy the increased demand for amino acids, different tumors selectively up-regulate different amino acid transporters based on their molecular signature and metabolic profile. Published reports provide convincing evidence for the up-regulation of four amino acid transporters, namely SLC7A5, SLC7A11, SLC1A5, and SLC6A14 in various cancers [15–22]. Studies in our laboratory focused on SLC6A14. We have shown that SLC6A14 is up-regulated in several cancers including colon cancer [19], estrogen receptor-positive breast cancer [20], pancreatic cancer [21], and cervical cancer [22]. We have also identified α-methyltryptophan (α-MT) as a pharmacologic blocker for this transporter [23].

These amino acid transporters that have gained attention for their role as tumor promoters differ in their substrate specificity, efficacy to concentrate amino acids inside cells, and transport mechanism [14,24,25]. SLC6A14 has the most broad substrate selectivity, transporting 18 of the 20 amino acids, including all essential amino acids, and is also of a high-capacity type. In contrast, SLC1A5 transports only alanine, serine, and glutamine, with cysteine being a transport modifier rather than a transportable substrate [26]; SLC7A5 transports all neutral amino acids plus histidine but not arginine and lysine or anionic amino acids [27]; SLC7A11 is selective for cystine and glutamate. SLC7A5, SLC7A11, and SLC1A5 are obligatory exchangers, meaning that the influx of one amino acid is coupled to efflux of another amino acid; thus, these transporters are not capable of concentrating any of their substrates inside the cells. Furthermore, SLC1A5 is coupled to a Na^+ gradient, and SLC7A5 and SLC7A11 are not dependent on any driving force. In contrast, SLC6A14 is coupled to three different driving forces: a Na^+ gradient, a Cl^- gradient, and membrane potential. As such, SLC6A14 exhibits functional features that are more ideally suited than the other three transporters to satisfy the increased demand for amino acids in tumor cells.

The present study was undertaken to investigate the role of SLC6A14 in colon cancer. Studies published thus far have only shown that SLC6A14 is up-regulated in colon cancer tissues and colon cancer cell lines [19]. The first goal of the present study was to evaluate the impact of knockdown or deletion of the transporter on colon cancer in multiple preclinical model systems and to compare the effects with pharmacologic blockade of the transporter. The second goal of the study was to determine the molecular mechanism for the up-regulation of SLC6A14 in colon cancer.

Materials and methods

Animals

Slc6a14$^{-/-}$ mice were generated in our laboratory and have been used in a previously published study on the role of this transporter in breast cancer [28]. This mouse line is on C57BL/6 background. *Apc*$^{Min/+}$ mice on C57BL/6 background were obtained from Jackson Laboratory (Bar Harbor, ME, U.S.A.). The mice were maintained in a temperature-, humidity- and light-controlled environment in the animal facility at Texas Tech University Health Sciences Center (TTUHSC). The mice had access to water and rodent diet ad libitum. Age- and gender-matched control mice were used with the experimental groups. All experimental procedures were approved by the TTUHSC Institutional Animal Care and Use Committee (protocol number, 17004). At the termination of the experiments, mice were killed by cervical dislocation under CO_2 anesthesia in accordance with the guidelines from the American Veterinary Medical Association.

Patient-derived xenografts

The patient-derived xenografts (PDXs) were obtained from TXCCR (Texas Cancer Cell Repository) at TTUHSC Cancer Center (www.TXCCR.org). This center establishes the biorepository of PDXs and PDX-derived cell lines from primary clinical samples. All PDXs samples used in this study were from human colonic adenocarcinoma patients. The protocol had approval from the Institutional Review Board.

Cell culture

Normal human colonic epithelial cell line CCD841, human colon cancer cell lines (HCT116, HT29, Colo201, Colo205, SW480, SW620, KM12C, KM12L4, Caco2, and LS174T) and the mouse colon cancer cell line MC-38 were purchased from the American Type Culture Collection (ATCC, Manassas, VA, U.S.A.). The cell lines were cultured in respective culture medium recommended by ATCC; culture media (Corning Life Sciences, Corning, NY, U.S.A.) were supplemented with 10% fetal bovine serum (Fisher Scientific, Pittsburgh, PA, U.S.A.) and 1% penicillin/streptomycin (Corning Life Sciences, Corning, NY, U.S.A.). HEK293FT cells were used for packaging lentivirus with plasmid and were maintained in DMEM, supplemented with 4.5 g/l glucose, L-glutamine, and sodium pyruvate, 10% FBS and 1% penicillin/streptomycin.

Antibodies

Anti-mTOR (#2983S), anti-P-mTOR (#5536S), anti-S6K (#9202S), anti-P-S6K (#9204S), anti-LC3A/B (#4108S) anti-β-catenin (#8814S), anti-Cyclin D1 (#2922S), anti-TCF4 (#2569S), and anti-IgG (#2729S) antibodies were purchased from Cell Signaling Technology (Danvers, MA, U.S.A.). Anti-SLC6A14 (#A10582) polyclonal antibody was obtained from Abclonal. Anti-β-actin (C4, sc-47778) monoclonal antibodies were purchased from Santa Cruz Biotechnology (Dallas, TX, U.S.A.). Horseradish peroxidase-conjugated goat anti-rabbit IgG (#1706515) and goat anti-mouse IgG (#1706516) were purchased from Bio-Rad Laboratories (Hercules, CA, U.S.A.).

Analysis of gene expression datasets

Three datasets with accession number GSE9348 [29], GSE33113 [30], and GSE34053 [31] were retrieved from publicly available gene expression omnibus database. The gene expression profiling of these datasets is based on the platform [HG-U133_Plus_2] Affymetrix Human Genome U133 plus 2.0. Additionally, Illumina HiSeq_RNASeqV2 mRNA expression data for colon adenocarcinoma (COAD) were obtained from The Cancer Genome Atlas (TCGA) data portal. Samples were grouped as tumor and normal tissue and compared for gene expression. The student's *t*-test was performed and a *p*-value of <0.05 was considered statistically significant. The interactive UALCAN data portal (http://ualcan.path.uab.edu/index.html) [32] was used to analyze SLC6A14 expression in different stages of COAD and histological subtypes of COAD compared with the normal colonic tissue samples. UALCAN uses TCGA level 3 RNA-seq transcriptome data in the form of transcripts per million (TPM) as the measure of expression.

Survival curves

SLC6A14 mRNA expression from the 'TCGA colon adenocarcinoma' dataset was used to generate overall survival (OS) and disease-free survival (DFS) curves in GEPIA: a web server for cancer and normal gene expression profiling and interactive analyses. SLC6A14 RNA fpkm values greater than the 50th percentile (median) were considered to have high SLC6A14 expression, and SLC6A14 fpkm values less than the 50th percentile were considered to have low expression. The survival curve was generated in GEPIA (http://gepia.cancer-pku.cn/) using the cox proportional hazard ratio and statistical analysis was performed using log-rank (Mantel–Cox) test.

RT-PCR

Total RNA was isolated either from cells or tissue samples using TRIzol™ (Invitrogen, #15596018) according to the manufacturer's instructions. RNA samples for human total colon were obtained from a commercial source (Takara Bio USA Inc; #636553). Two micrograms of total RNA was used for cDNA synthesis using SuperScript™ II cDNA synthesis kit (Invitrogen, #18064022). After reverse transcription, the relative expression of mRNA was analyzed either by regular RT-PCR using Takara PCR master mix or by real-time RT-PCR using SYBR_Green PCR Master Mix (Bio-Red, #1725121). Appropriate primer pairs were used for the study (Supplementary Table S1) with GAPDH or 18S (for PDXs analysis) as an internal control. Expression levels of ASNS mRNA and CHOP mRNA were analyzed by regular RT-PCR, followed by separation of the PCR

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products in 1.5% agarose gel, staining with ethidium bromide, and imaging with the AlphaImager system. Relative mRNA expression of SLC6A14, Cyclin D1, and β-catenin were obtained by on the StepOnePlus real-time PCR system (Applied Biosystems, Foster City, CA, U.S.A.). All the samples were analyzed in duplicates and the experiments were repeated three times; the relative expression of genes studied was calculated by normalizing cycle threshold (Ct) value of each gene with that of the housekeeping gene 18S.

Western blot

Whole-cell protein lysates were prepared from cells grown at 80–90% confluency by using RIPA lysis buffer with protease-phosphatase inhibitor cocktail. The total protein contents of the samples were quantified using BCA Protein Assay kit. Lysates were prepared at a final protein concentration of 1 μg/μl, which also contained 100 μl/ml β-mercaptoethanol, 250 μl/ml 4× Laemmli buffer. Lysates were heated at 95°C for 3 min, and then separated by electrophoresis on 8% SDS–PAGE or 6% SDS–PAGE and transferred to Polyvinylidene difluoride membrane at 70 V for 2 h at 4°C. After transfer, the membranes were blocked with 5% milk or 5% bovine serum albumin (for phospho-protein) for 1 h, and incubated with specific primary antibodies overnight at 4°C with gentle shaking. After incubation, membranes were washed three times with TBST, 10 min each time, then incubated with HPR-conjugated secondary antibodies for another hour, washed three times with TBST as before and bands were visualized using Pierce ECL Western blotting substrate (Thermo Scientific, # 32106).

Cell proliferation assay

Colony formation assay was performed as described previously [33]. Cells were seeded at a very low density (5000 cells per well) in six-well plates and allowed to attach overnight. After attachment, cells were cultured in either normal media or media containing 2.5 mM α-MT. The media were changed every other day for 10 days. At the end of the experiment, cells were washed with phosphate-buffered saline, fixed with methanol, and stained with KaryoMax Giemsa stain; the colonies were then visualized following which the stain was extracted and quantified.

Cell migration and invasion assay

The effect of α-MT on cell migration and invasion was monitored by using Corning® BioCoat™ Matrigel® Invasion assay kit according to the manufacturer's instructions. Briefly, cells were serum-starved and their invasion and migration to the other side of the membrane were analyzed in the presence or absence of α-MT for 48 h. At the end of this treatment, non-invaded cells were removed by scrubbing. Invaded and migrated cells were fixed with 100% methanol, stained with crystal violet, and counted under an inverted microscope; images were also captured.

Immunofluorescence analysis of autophagy

Cells were seeded in chamber slides (10^4 cells/chamber) and allowed to grow overnight. After attachment, cells were treated with or without α-MT for 48 h. At the end of treatment, cells were fixed with 4% formaldehyde, permeabilized with 0.1% Triton-X for 5 min, blocked by 10% goat serum for an hour, stained with rabbit anti-LC3 A/B antibody overnight at 4°C. After that, cells were washed and stained with Alexa Fluor 488 conjugated secondary antibody (goat anti-rabbit IgG). Punctate localization of LC3 A/B was examined by fluorescence microscopy.

Apoptosis assay

CCD841 or LS174T cells (50 000cells/well) were seeded in six-well culture plates and allowed for the attachment with overnight incubation. After attachment, cells were treated with or without α-MT in regular medium for another 48 h. Apoptosis assay was performed using Annexin V Apoptosis Detection Kit FITC (Invitrogen, # 88-8005) according to the manufacturer's instructions. Briefly, cells were harvested by trypsinization, washed one time with phosphate-buffered saline and resuspended in 1× binding buffer at a concentration of 10^6 cells/ml. The cell suspensions were treated with Annexin V-FITC and propidium iodide, followed by analysis for apoptotic cell death by flow cytometry (BD Accuri™ C6 cytometer).

Inhibition or activation of Wnt signaling

LS174T or HT29 cells (0.3×10^6 cells/well) were seeded in six-well culture plates and allowed for the attachment with overnight incubation. After attachment, LS174T cells were treated with the Wnt antagonist

Calphostin C (Cayman Chemicals) at concentrations of 0, 0.5, and 5 μM for 6 h; HT29 cells were treated with the Wnt agonist N^4-(1,3-Benzodioxol-5-ylmethyl)-6-(3-methoxyphenyl)-2,4-pyrimidinediamine hydrochloride (AMBMP; Tocris Bioscience) at concentrations of 0, 5, and 10 μM for 24 h. After incubation, RNA and protein were isolated. Expression levels mRNA and protein for SLC6A14 and Cyclin D1 (positive control for Wnt target) were measured by RT-qPCR and Western blot.

shRNA-mediated knockdown of SLC6A14 and β-catenin

shRNA-mediated knockdown of SLC6A14 and β-catenin was carried out using a Lentivirus-based transduction system. A set of shRNA-lentiviral vectors for SLC6A14 was purchased from Open Biosystems (RHS4533-NM_007231); shRNA-lentiviral vectors for β-catenin were obtained from Addgene (shRNA1, #42543; shRNA2, #43544; pLKO.1, #84530). Lentiviral particles were generated in HEK293FT cell line by transfecting the plasmids along with the packaging plasmids pLP-1, pLP-2, and pVSVG (Invitrogen). Lipofectamine-2000 was used as the transfection reagent. After 72 h of transfection, the lentiviral supernatant was harvested and filtered through 0.45 μm filter. LS174T cells were infected with lentiviral particles carrying shRNA or empty vector for 24 h in a media containing 8 μg/ml Polybrene (Hexadimethrine bromide; Sigma) and cultured for an additional 24 h. Positive cells for transfection were selected by resistance to treatment with 3 μg/ml puromycin for 72 h. The resistant cells were maintained under the selective pressure of 0.5 μg/ml puromycin. Expression levels of mRNA and protein for SLC6A14 and β-catenin were measured by RT-qPCR and Western blot.

Overexpression of β-catenin

Overexpression of β-catenin was carried out in HT29 cells by lipofectamine-2000 based transfection system. pcDNA3.1 vector (#52535) and β-catenin vector (#16828) were obtained from Addgene. HT29 cells were transiently transfected with either pcDNA3.1 empty vector or β-catenin plasmid using lipofectamine-2000 according to the manufacturer's protocol. Expression levels of mRNA and protein for SLC6A14 and β-catenin were measured by RT-qPCR and Western blot.

Chromatin immunoprecipitation (ChIP) assay

Chromatin immunoprecipitation (ChIP) was performed using commercially available Magna ChIP™ A/G Chromatin Immunoprecipitation Kit (# 17-10085) according to the manufacturer's protocol. Briefly, LS174T cells at 80–90% confluency were treated with formaldehyde (1% final concentration) to cross-link the proteins–DNA complexes; the reaction was then stopped by the addition of glycine. Cells were lysed and sonication was performed to shear the chromatin into ∼200 to 1000 bp long. Immunoprecipitation of the chromatin–protein complex was performed by incubating overnight with 3 μg of anti-β-catenin, anti-TCF4, and anti-IgG antibodies followed by addition of protein A/G-conjugated magnetic beads to precipitate the antibody–chromatin complex. Immunoprecipitated DNA was analyzed using qPCR using appropriate primers for *SLC6A14* promoter (Supplementary Table S1).

Xenograft of human colon cancer cells in immunosuppressed nude mice

Male athymic BALB/c nude mice (8-weeks-old) were obtained from the Jackson laboratory and acclimatized with the environment before initiating the experiment. Mice were dived into two groups (control and treatment) with 5 mice in each group. The control group was provided with sucrose-water and treatment group with α-MT (2 mg/ml) in sucrose-water 7 days prior to cancer cell injection. α-MT was used as the D/L enantiomeric mixture. At day 0, both groups of mice were subcutaneously injected with SLC6A14-positive human colon cancer cell line LS174 T (1×10^6 cells/mouse). Mice in the treatment group continued to receive α-MT in sucrose-water and the control group sucrose-water throughout the experiment. To analyze the effect of SLC6A14 knockdown on tumor growth, LS174T/pLKO.1 (empty vector) and LS174T/SLC6A14 shRNA (knockdown) cells were generated by lentiviral mediated transduction and injected subcutaneously as described above. The size of the tumors was measured periodically by slide-caliper once it reached a palpable size, and tumor volume was calculated using the formula (width^2 × length)/2.

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Syngeneic transplantation of mouse colon cancer cells in immunocompetent mice

C57BL/6 mice were separated into two groups (5 mice/group; 3 males and 2 females). The control group received sucrose-water and the treatment group received α-MT (2 mg/ml) in sucrose-water. After 7 days, MC-38 cells were injected subcutaneously (1×10^6 cells/mouse). The control group continued to receive sucrose-water and treatment group α-MT throughout the experiment. In a separate experiment, we also evaluated the efficacy of α-MT with that of two other chemically related compounds: 1-methyltryptophan (1-MT; 2 mg/ml) and tryptophan (2 mg/ml) throughout the experiment. This second study had its own control group. α-MT, 1-MT, and Trp were all used as the D/L enantiomeric mixtures. The size of the tumors was measured periodically by slide-caliper once it reached a palpable size, and tumor volume was calculated using the formula (width^2 × length)/2.

Inflammation-associated and genetically driven colon cancer mouse models

We used two different mouse models of colon cancer. For inflammation-associated colon cancer, intraperitoneal injection of the carcinogen azoxymethane coupled with induction of inflammation with three cyclic administrations of dextran sulfate sodium (7 days/cycle with 7 days interval) in C57BL/6 mice. For genetically driven colon cancer, $Apc^{Min/+}$ mice (C57BL/6 background) were used. In the first model, wild type and *Slc6a14*-null mice, both on C57BL/6 background, were injected i.p. with azoxymethane at a dose of 10 mg/kg body weight in phosphate-buffered saline. After 5 days of this injection, mice received 2.5% dextran sulfate sodium in drinking water for a week, followed by 7 days of regular water. After two additional cycles of dextran sulfate sodium, mice were killed at day 70 of azoxymethane injection and colonic tissues were evaluated for the number and size of polyps. In the second model, $Apc^{Min/+}$ mice were crossed with *Slc6a14*-null mice to generate $Apc^{Min/+}/Slc6a14^{+/+}$ and $Apc^{Min/+}/Slc6a14^{-/-}$ mice. At the age of 6 months, many polyps in colon and small intestine was counted. In all experiments, each group contained 6 mice (3 males and 3 females).

Statistical analysis

Experiments were repeated at least twice. The data shown are means ± SEM (*in vivo* studies) or means ± SD (*in vitro* studies). Statistical analyses and graphing were performed using Microsoft Excel or OriginPro. Statistical differences between control and experimental groups were analyzed by a two-tailed, paired Student's *t*-test for single comparison. Differences were judged statistically significant when the p-value < 0.05.

Results

SLC6A14 is up regulated in colon cancer

Several years ago we reported that SLC6A14 is up-regulated at mRNA and protein levels in primary and metastatic colon cancer [19]. Beyond this simple observation, nothing else is known on the relevance of this transporter to colon cancer. In the present study, we first expanded this original observation using human colon cancer cell lines and PDXs (Figure 1). We compared the levels of SLC6A14 mRNA in a panel of human colon cancer cell lines with that in the normal colonic epithelial cell CCD841. A majority of the cancer cell lines showed significantly higher levels of SLC6A14 mRNA and protein than CCD841 cells (Figure 1A,B). The increased level of SLC6A14 mRNA was also true with PDXs (Figure 1C). We then analyzed publicly available TCGA and GSE colon cancer datasets to examine the relative expression and fold changes for SLC6A14 mRNA compared with mRNAs for the other three amino acid transporters SLC1A5, SLC7A5, and SLC7A11. Among these four amino acid transporters, SLC6A14 mRNA showed the most significant increase with the highest fold changes in tumor tissue compared with normal colon tissue (Supplementary Figure S1 and Supplementary Table S2). In the TCGA dataset, which has the largest sample size, the up-regulation in colon cancer was significant only for SLC6A14. Analysis of the TCGA-COAD data by UALCAN web portal also showed significant up-regulation of SLC6A14 mRNA in all four stages (stages I–IV) of colon cancer as well as in different histological subtypes (Supplementary Figure S2). Furthermore, the analysis of the GSE34053 dataset showed higher levels of SLC6A14 mRNA in CD133-positive colon cancer than in CD133-negative colon cancer (Supplementary Figure S3). CD133 is a marker for cancer stem cells, and CD133-positive cancer cells are more aggressive and metastatic [34]. However, analysis of OS or DFS in colon cancer patients showed no significant difference between those with low levels of SLC6A14 mRNA and those with high levels of SLC6A14 mRNA.

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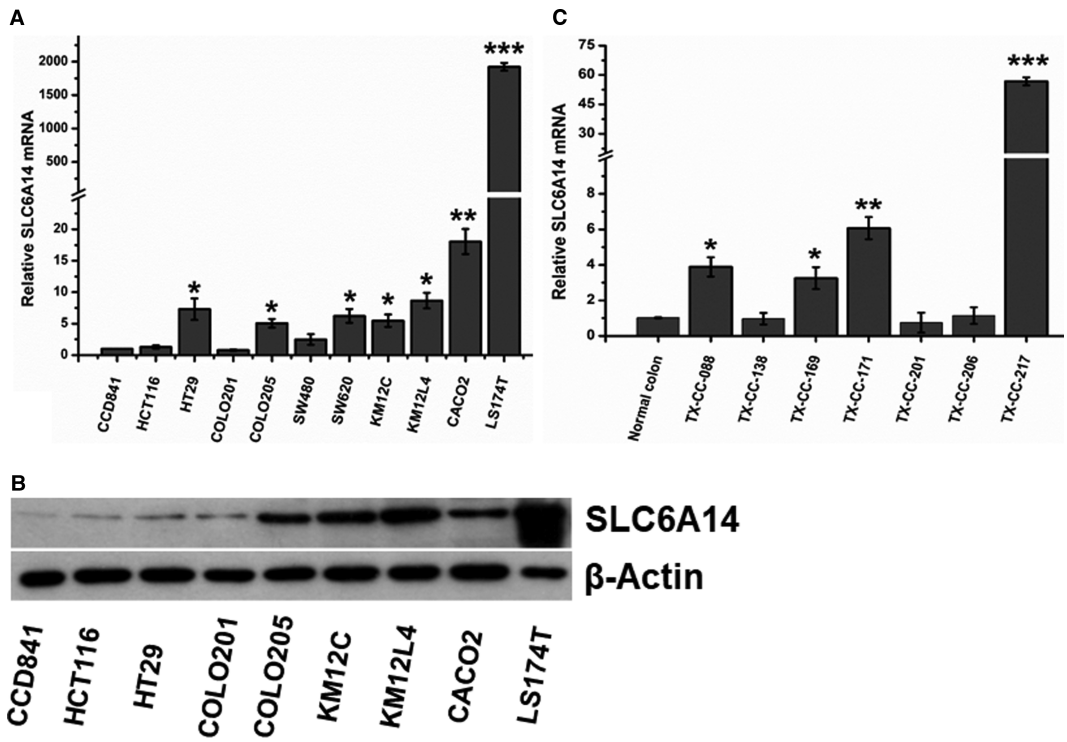

Figure 1. Up-regulation of SLC6A14 at mRNA level in colon cancer.

(**A**) Quantitative RT-PCR analysis of SLC6A14 mRNA in the normal human colonic epithelial cell line CCD841 and in several human colon cancer cell lines. (**B**) Western blot analysis of SLC6A14 protein levels in CCD841 cells and in colon cancer cells. (**C**) Quantitative RT-PCR analysis of SLC6A14 mRNA in normal human colon tissue (commercially available pooled RNA from five different normal human tissues) and in patient-derived xenograft (PDXs) tissues. Data represent means ± SD from three independent RT-PCR runs. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

Pharmacologic blockade of SLC6A14 with α-MT induces amino acid starvation, inhibits mTOR signaling and promotes autophagy selectively in colon cancer cells

α-MT is a non-transportable blocker of SLC6A14 [23] and thus can be used to achieve pharmacologic blockade of the transporter function. We have used this strategy successfully in two other SLC6A14-positive cancers, namely ER-positive breast cancer [20] and pancreatic cancer [21]. Here, we used α-MT to evaluate the consequences of blocking SLC6A14 function in the colon cancer cell line LS174T, which showed the highest expression of the transporter. For comparison, we used the normal colonic epithelial cell line CCD841, which showed the lowest expression of the transporter. The cells were treated with α-MT (2.5 mM) for 72 h and RNA and protein lysates were prepared. RNA was used to monitor the expression levels of asparagine synthetase and C/EBP-homologous protein as a readout of amino acid starvation [35,36]. The rationale was that if SLC6A14 is essential for the maintenance of amino acid nutrition in a given cell, blockade of its transport function should cause amino acid starvation and hence should increase the expression of these two markers. This was what we observed in LS174T cells (Figure 2A). While α-MT increased the expression of the markers in this cell line, it had no effect in CCD841 cells, suggesting that SLC6A14 does not contribute significantly to amino acid nutrition in the latter cell line. Cellular levels of amino acids constitute an important signal for the activation of mTOR signaling to co-ordinate amino acid availability with protein synthesis. To determine whether amino acid starvation caused by α-MT in LS174T cells translates into mTOR inhibition, we monitored the phosphorylation status of mTOR and its downstream mediator S6 kinase. α-MT treatment significantly reduced phosphorylation of mTOR and S6 kinase, but again only in LS174T cells and not in CCD841 cells (Figure 2B), thus mirroring the differential effects of α-MT on amino acid nutrition in these two cell lines. Induction of autophagy is another hallmark of cells undergoing amino acid starvation, and mTOR suppresses autophagy [37]. As

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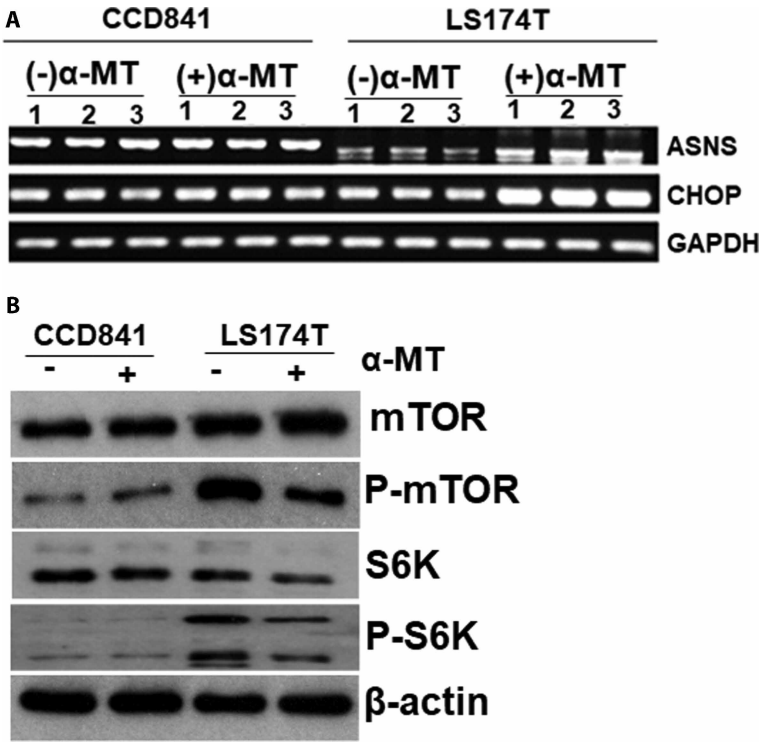

Figure 2. Pharmacologic blockade of SLC6A14 by α-MT induces amino acid starvation and suppresses mTOR signaling in colon cancer cell lines.

(**A**) CCD841(a normal human colon cell line) and LS174T (a human colon cancer cell line) were treated with or without 2.5 mM α-MT for 72 h followed by RT-PCR analysis of mRNA for ASNS and CHOP, the widely used markers for amino acid starvation. (**B**) CCD841 and LS174T cells were treated with or without 2.5 mM α-MT for 72 h and then cell lysates were prepared and used for Western blot analysis for total mTOR, phospho-mTOR (P-mTOR), total S6 kinase (S6K), phospho-S6 kinase (P-S6K) and β-actin (internal control).

expected, α-MT treatment induced autophagy in LS174T cells as evident from the increased appearance of punctate localization of autophagy-associated markers LC3A/B in autophagosomes (Supplementary Figure S4). Again, there was no change in autophagy in CCD841 cells.

Relative expression of various amino acid transporters in CCD841 cells and LS174T cells

We compared the steady-state mRNA levels for 16 different amino acid transporters between CCD841 cells and LS174T cells (primar sequences for these transporters are given in Supplementary Table S1). These transporters are: SLC1A1, SLC1A5, SLC6A14, SLC6A15, SLC6A19, SLC6A20, SLC7A5, SLC7A8, SLC7A9, SLC7A11, SLC36A1, SLC36A2, SLC38A1, SLC38A2, SLC38A3, and SLC38A5. Only two transporters were expressed in CCD841 cells at a greater magnitude than in LS174T cells; these were SLC6A15 and SLC38A3. All remaining transporters were expressed at higher levels in LS174T cells than in CCD841 cells; however, the fold-increase was the highest for SLC6A14.

Pharmacologic blockade of SLC6A14 with α-MT inhibits proliferation and invasion/migration, and induces apoptosis in colon cancer cells

Next, we used the colony formation assay to investigate the effects of SLC6A14 blockade on cancer cell proliferation. Treatment with α-MT significantly decreased the proliferation of LS174T cells but had no effect on CCD841 cells (Figure 3A). The treatment also induced apoptotic cell death, again only in LS174T cells (Figure 3B). We also evaluated the impact of α-MT on invasion and migration of LS174T cells. α-MT showed a remarkable inhibitory effect on these cells in their ability to invade and migrate Matrigel-coated trans-well chambers (Supplementary Figure S5).

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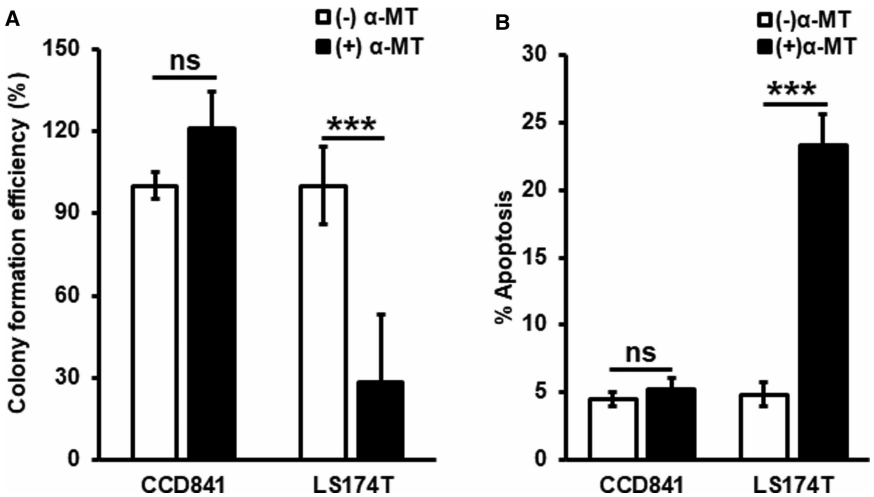

Figure 3. Pharmacologic blockade of SLC6A14 by α-MT decreases cell proliferation and induces apoptosis in colon cancer cells.

(**A**) Colony formation assay for CCD41 and LS174T cells treated with or without 2.5 mM α-MT for 10 days. Colonies were visualized by staining with KaryoMax Giemsa reagent and the dye was eluted and quantified by measuring absorbance at 630 nm. (**B**) CCD841 and LS174T cells were treated with or without α-MT for 48 h. The cells were then labeled with annexin-V and propidium iodide and subjected to flow cytometry to detect apoptotic cells positive for both labels. Data represent means ± SD. ***$P < 0.001$; ns, not significant.

Pharmacologic blockade or shRNA-mediated knockdown of SLC6A14 suppresses *in vivo* growth of human colon cancer cells in a xenograft model with immunocompromised mice

To evaluate the efficacy of SLC6A14 as a drug target for colon cancer, we studied the effect of interfering with the transport function or expression of the transporter on the growth of LS174T cells xenografted into immunocompromised athymic BALB/c mice. First, we tested whether pharmacologic blockade of SLC6A14 with α-MT suppressed the tumor growth. A marked reduction of tumor growth was observed in mice treated with α-MT (D/L enantiomeric mixture; 2 mg/ml in drinking water) compared with the untreated group (Figure 4A). Similar results were obtained with shRNA-mediated knockdown of SLC6A14 (Figure 4B).

Pharmacologic blockade of SLC6A14 suppresses *in vivo* growth of mouse colon cancer cells in a syngeneic transplantation model with immunocompetent mice

We then evaluated the role of SLC6A14 in colon cancer using a syngeneic transplantation model in mice with intact immune system. Here, we used the mouse colon cancer cell line MC-38, which is syngeneic with C57BL/6 mice; when transplanted in this mouse line, MC-38 cells will proliferate and grow into a tumor without immune rejection. These cells were injected subcutaneously into C57BL/6 mice and the effect of α-MT on tumor growth was evaluated. Mice treated with α-MT (D/L enantiomeric mixture; 2 mg/ml in drinking water) showed markedly reduced tumor growth compared with the control group (Figure 5A). There was also a reduction in final tumor weight at the termination of the experiment in response to α-MT (Figure 5B). We also investigated the specificity of α-MT in the observed anti-cancer effect by comparing its efficacy with that of two structurally and pharmacologically related compounds, 1-MT and tryptophan. 1-MT is a transportable substrate for SLC6A14 [23]; it is a competitive inhibitor of amino acid transport via SLC6A14 but not a blocker like α-MT. Tryptophan is a substrate for SLC6A14 and the parent molecule for α-MT and 1-MT. In all three cases, we used the same dose (2 mg/ml in drinking water) in the form of a D/L enantiomeric mixture. Neither 1-MT nor Trp showed significant effect on tumor progression (Figure 5C). There was a small inhibitory effect on the final tumor weight for 1-MT (Figure 5D), but this effect was significantly less than that of α-MT.

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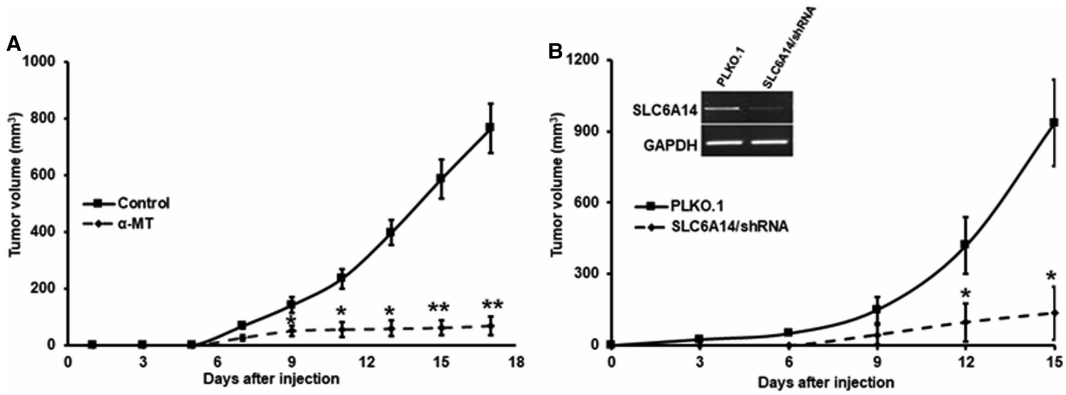

Figure 4. shRNA-mediated knockdown or pharmacologic blockade of SLC6A14 by α-MT decreases tumor growth in an immunocompromised mouse xenograft model.

(**A**) Immunocompromised nude mice were injected subcutaneously with LS174T cells (10^6 cells/mouse) and then divided into two groups (5 mice/group). One group was given drinking water with sucrose while the second group was given α-MT (2 mg/ml) in drinking water with sucrose. Tumor volume was measured periodically. Data are means ± SE. *$P < 0.05$; **$P < 0.01$.
(**B**) Immunocompromised nude mice were injected subcutaneously with control LS174T cells transfected with the empty vector (pLKO.1) or LS174T cells transfected with shRNA vector (SLC6A14/shRNA) (10^6 cells/mouse). Tumor volume was measured periodically. Data are means ± SE. *$P < 0.05$; 5 mice in each group.

Slc6a14-null mice show protection against colon cancer

We compared the susceptibility of wild type mice and *Slc6a14*-null mice to colon cancer in two different models of colon cancer. First, we used the inflammation-associated colon cancer (azoxymethane plus dextran sulfate sodium, AOM + DSS). This also represents an immunocompetent model. In this model, we observed significant protection against colon cancer in *Slc6a14*-null mice (Figure 6A,B). The number of colonic polyps decreased significantly in *Slc6a14*-null mice and the sizes of the polyps were also reduced in *Slc6a14*-null mice. We then used a genetically driven colon cancer model in mice ($Apc^{Min/+}$). APC is the gene that is mutated in most cases of familial colon cancer. However, the $Apc^{Min/+}$ mice differ from humans in that the Apc mutation in mice induces polyps in the small intestine as well as in colon with more polyps in the former than in the latter. In contrast, mutations in APC cause cancer only in colon. Nonetheless, this mouse model is used widely in investigations relating to colon cancer. We found significantly reduced number of polyps in the small intestine as well as in colon in $Apc^{Min/+}$ mice on *Slc6a14*-null background than in $Apc^{Min/+}$ mice on $Slc6a14^{+/+}$ background (Figure 6C,D).

Tumor-associated up-regulation of SLC6A14 expression in colon is controlled by TCF4/β-catenin and is mediated by Wnt signaling

In almost all cases of colon cancer, TCF4/β-catenin-mediated transcriptional activity mediated by the canonical Wnt signaling is enhanced. This is true not only for the sporadic form of colon cancer but also for APC-associated colon cancer. Therefore, we hypothesized that SLC6A14 is a transcriptional target for TCF4/β-catenin and hence is controlled by the canonical Wnt signaling pathway in colorectal cancer. To test this hypothesis, we first analyzed the sequence of *SLC6A14* gene promoter for the consensus binding motif of TCF4. We found four consensus motifs: −1904 to −1898, −1342 to −1335, −832 to −826, and −228 to −222 in the promoter (Figure 7). We then evaluated experimentally the involvement of Wnt signaling in the control of SLC6A14 expression in colon cancer cells. We treated LS174T cells, which showed abundant expression of the transporter, with calphostin C, an inhibitor of TCF4/β-catenin-mediated transcription. Treatment of the cells with this inhibitor showed significant inhibitory effect on SLC6A14 mRNA and protein (Figure 8A,B). Cyclin D1, a known Wnt target and hence a positive control, also showed significant reduction in expression in response to calphostin C. We then performed the reciprocal experiment in which the effect of a Wnt agonist was evaluated in HT29 cells, a colon cancer cell line that showed relatively low basal expression of SLC6A14 in comparison with LS174T cells. Activation of Wnt signaling by treatment of HT29 cells with AMBMP, an

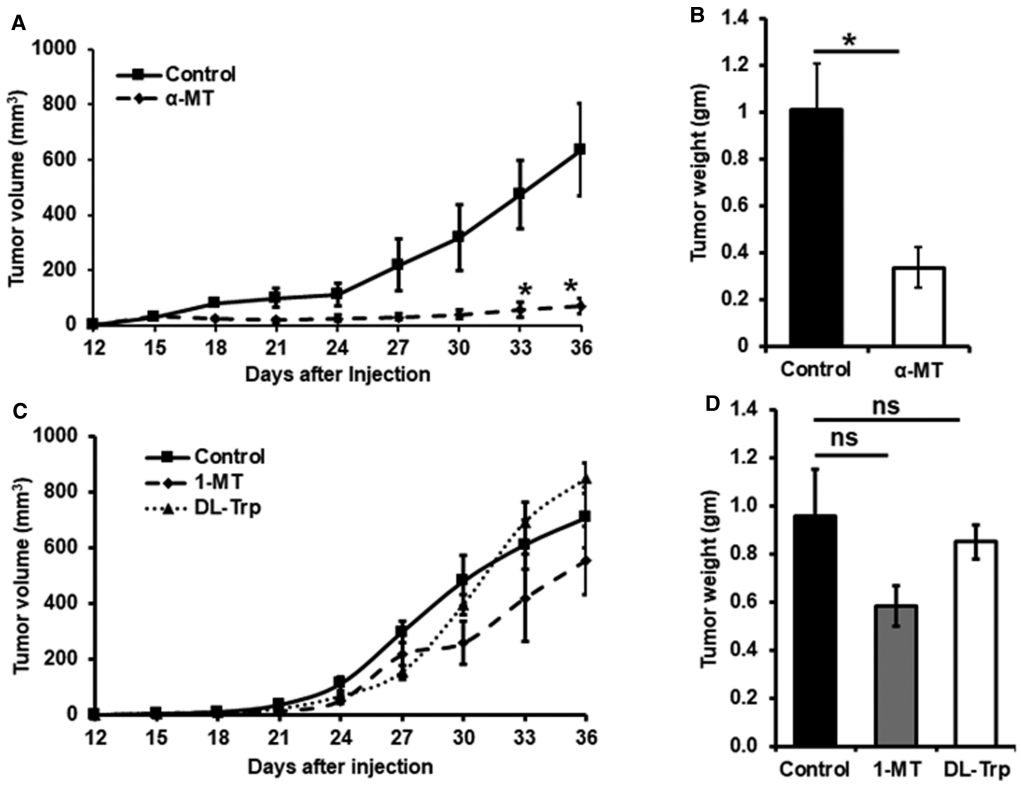

Figure 5. Pharmacologic blockade of SLC6A14 by α-MT prevents tumor growth in an immunocompetent syngeneic mouse model.

(A,**B**) C57BL/6 mice were separated into two groups (5 mice each). Mice in both groups were injected subcutaneously with the mouse colon cancer cell line MC-38 (10^6 cells/mouse). One group of mice received drinking water with sucrose while the other group received α-MT (2 mg/ml) in drinking water with sucrose. Tumor volume was monitored periodically (**A**). At the end of the study, mice were killed and the tumors collected and weighed (B). Data are means ± SE. *$P < 0.05$. (**C**,**D**) C57BL/6 mice were separated into three groups (5 mice each). Mice in all groups were injected subcutaneously with MC-38 cells (10^6 cells/mouse). Group 1 received drinking water with sucrose; the other two groups received either 1-MT or Trp at a concentration of 2 mg/ml in drinking water with sucrose. Data are means ± SE. ns, not significant.

agonist for Wnt signaling, increased the expression of SLC6A14 mRNA and protein (Figure 8C,D). Again, cyclin D1 was used as a positive control for Wnt target. These studies were then complemented with an experimental strategy involving the alteration of β-catenin levels in colon cancer cells. We knocked down β-catenin in LS174T cells with shRNA and found the expression of SLC6A14 mRNA and protein to be decreased (Figure 9A,B). Then, we increased the cellular levels of β-catenin in HT29 cells by the ectopic expression, and found the expression of SLC6A14 mRNA and protein to be increased (Figure 9C,D). Finally, we examined the binding of TCF4/β-catenin to *SLC6A14* gene promoter by ChIP assay using LS174T cells. There was significant enrichment of immunocomplexes of TCF4 and β-catenin on *SLC6A14* gene promoter (Figure 10). Anti-IgG antibodies were used as a negative control. These experiments provide convincing evidence for the control of SLC6A14 expression by canonical Wnt signaling and also for its involvement in tumor-associated up-regulation of SLC6A14 in colon cancer.

Discussion

In the present study, we provide strong evidence to support our notion that SLC6A14 has great potential as an effective drug target for the treatment of colon cancer. The evidence can be summarized as follows. (a) SLC6A14 is up-regulated in a majority of colon cancer cell lines and PDXs; (b) *in vitro* data show that pharmacologic blockade of SLC6A14 leads to amino acid starvation, suppresses mTOR signaling, induces

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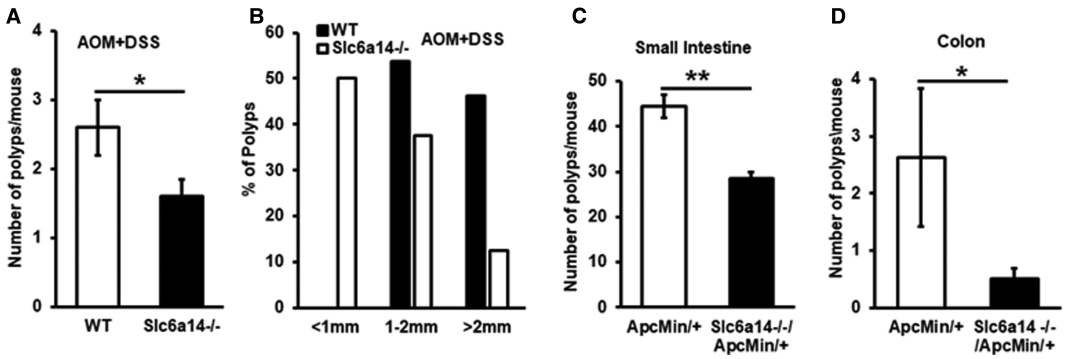

Figure 6. Deletion of *Slc6a14* in mice provides protection against colon cancer.

(**A**,**B**) Wildtype and *Slc6a14*$^{-/-}$ mice (males; 5 mice/genotype) were subjected to an experimental model of inflammation-associated colon cancer, which involves one-time injection of the carcinogen azoxymethane followed by three cycles of dextran sulfate sodium (AOM + DSS). At the end of the experiments, mice were killed and colon was monitored for the number (**A**) and size (**B**) of polyps. Data are means ± SE. *$P < 0.05$. (**C**,**D**) *Apc*$^{Min/+}$ mice were crossed with *Slc6a14*$^{-/-}$ mice to generate *Apc*$^{Min/+}$ mice on *Slc6a14*-null background. At 6 months of age, mice were killed and polyps in small intestine (**C**) and colon (**D**) were counted. Data are means ± SE. 8 mice/group; *$P < 0.05$; **$P < 0.01$.

apoptosis despite the initial induction of autophagy, inhibits cell proliferation, and also interferes with cell migration and invasion; (c) pharmacologic blockade or genetic knockdown of the transporter markedly reduces cancer cell proliferation and growth into tumors in immunocompromised mice as well as in immunocompetent mice; (d) *Slc6a14*-null mice show significant protection not only against inflammation-associated colon cancer but also against genetically driven colon cancer; (e) SLC6A14 is a direct target for the transcription factor complex TCF4/β-catenin; (f) the tumor-associated up-regulation of the transporter is likely to be driven by Wnt signaling with TCF4/β-catenin transcriptional activity as the downstream mediator, a signaling pathway highly active in colon cancer.

There are more than three dozen amino acid transporters in mammalian cells and many of them have considerable overlap in substrate specificity. They differ in the transport mechanism and driving forces. As such, there has to be a rational biological basis for tumor cells to up-regulate a given specific amino acid transporter to meet their amino acid demands. In addition to essential amino acids, the 'non-essential' amino acid glutamine has attracted special attention in cancer cell nutrition because this amino acid serves multiple functions (e.g. protein synthesis, nucleotide synthesis, mTOR activation), all of which are critical for cell proliferation. SLC6A14 is an excellent transporter for glutamine. There are two other transporters that also transport glutamine, and both of them have been shown to be up-regulated in specific cancers; these transporters are SLC1A5

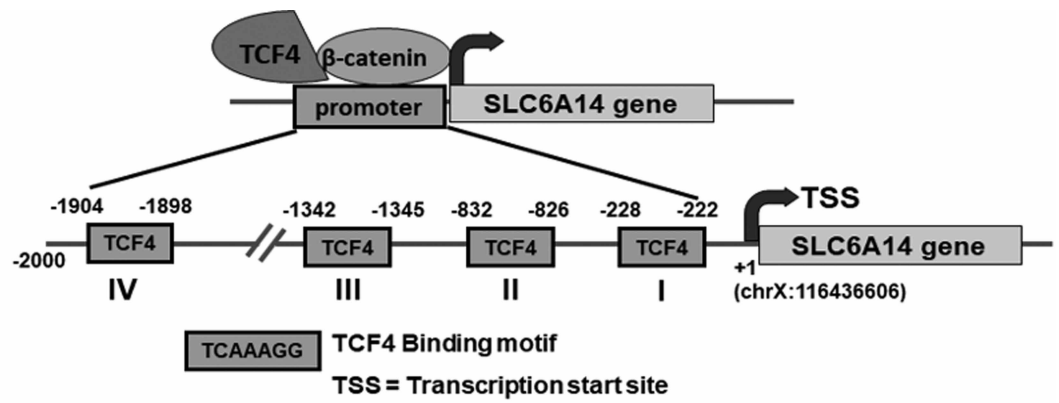

Figure 7. Model for SLC6A14 transcriptional regulation by TCF4/β-catenin complex.

CF4 consensus sequences in SLC6A14 promoter as assessed with JASPAR software and also by NCBI nucleotide blast.

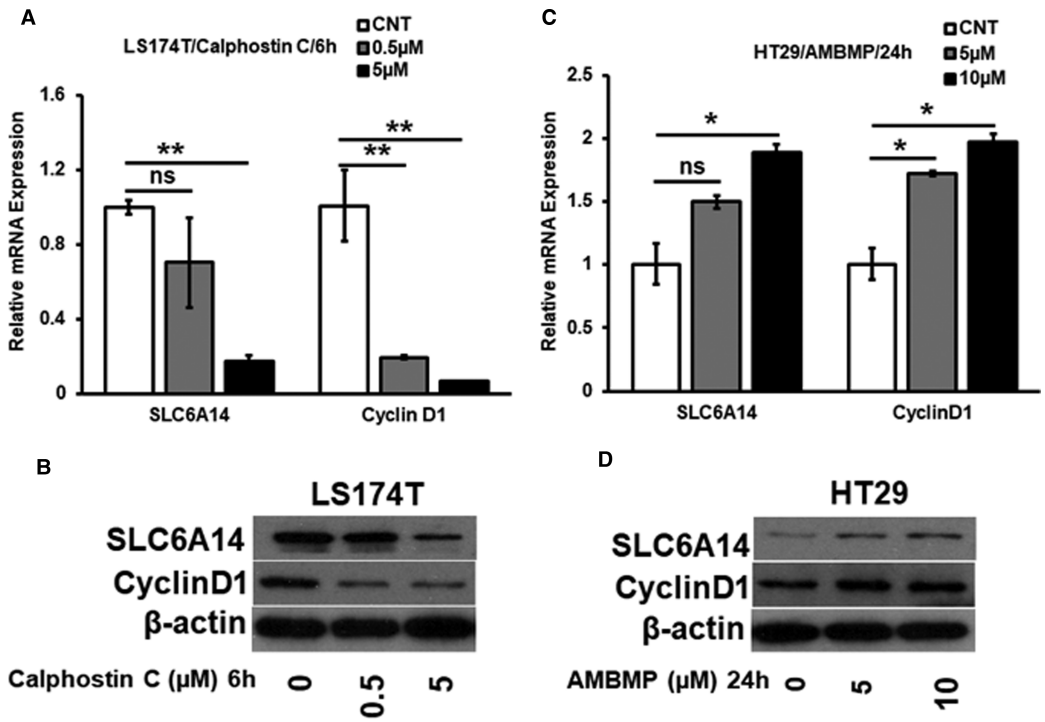

Figure 8. Effects of Wnt antagonist (Calphostin C) and agonist (AMBMP) on SLC6A14 expression in human colon cancer cells.

(**A**,**B**) As LS174T cells express robust levels of SLC6A14, these were used to monitor the effect of Wnt antagonist. Cells were treated without or with two different concentrations of Calphostin C for 6 h. At the end of the experiment, RNA and protein lysates were prepared from the control and treated cells and used for quantitative RT-PCR (**A**) or Western blot (**B**) to determine SLC6A14 mRNA and protein levels. Cyclin D1 was used as a positive control as a Wnt target. (**C**,**D**) As HT29 cells express low levels of SLC6A14, these cells were used to monitor the effect of Wnt agonist. Cells were treated without or with two different concentrations of AMBMP for 24 h. At the end of the experiment, RNA and protein lysates were prepared from the control and treated cells and used for quantitative RT-PCR (**C**) or Western blot (**D**) to determine SLC6A14 mRNA and protein levels. Cyclin D1 was used as a positive control as a Wnt target. Data are means ± SD. *$P < 0.05$; **$P < 0.01$; ns, not significant.

and SLC7A5 [13]. Both are obligatory exchangers. Nicklin et al. [37] proposed that these two transporters are functionally coupled in tumor cells with a net result of mTOR activation. It appears that tumor promotion by this functional coupling between SLC1A5 and SLC7A5 is all related to mTOR activation but little to do with amino acid nutrition. It has to be noted, however, that a more recent study has shown that SLC1A5 is able to promote tumor growth even when it is not functionally coupled to SLC7A5 [15]. Another amino acid transporter that has received increasing attention with regard to tumor growth is SLC7A11, also known as xCT or cystine/glutamate exchanger [38,39]. The focus on this transporter as a tumor promoter is even more interesting because it has nothing to do with amino acid nutrition. This transporter is an exchanger and mediates the entry of cystine into cells coupled to the efflux of glutamate out of the cells [40]. As cysteine is the limiting amino acid for the synthesis of the antioxidant glutathione, SLC7A11 plays an essential role in providing this limiting amino acid in the form of cystine and thus promotes the activity of the antioxidant machinery in cancer cells. With this mode of action, SLC7A11 also provides protection against iron-induced cell death pathway ferroptosis [41]. As tumor cells accumulate iron to support their growth, these cells are at risk for ferroptosis-mediated cell death; up-regulation of SLC7A11 prevents ferroptosis even in the presence of increased levels of iron within the cells. In fact, one of the unique features of SLC7A11 is its up-regulation in cells when subjected to oxidative stress [42] and nitrosative stress [43].

SLC6A14 is a high-capacity, concentrative, transporter. Leucine, the most effective activator of mTOR, is an excellent substrate for SLC6A14. As such, SLC6A14 is able to accumulate leucine in cancer cells for mTOR activation. There is also the potential for SLC6A14 to be involved in cellular glutathione synthesis due to its ability

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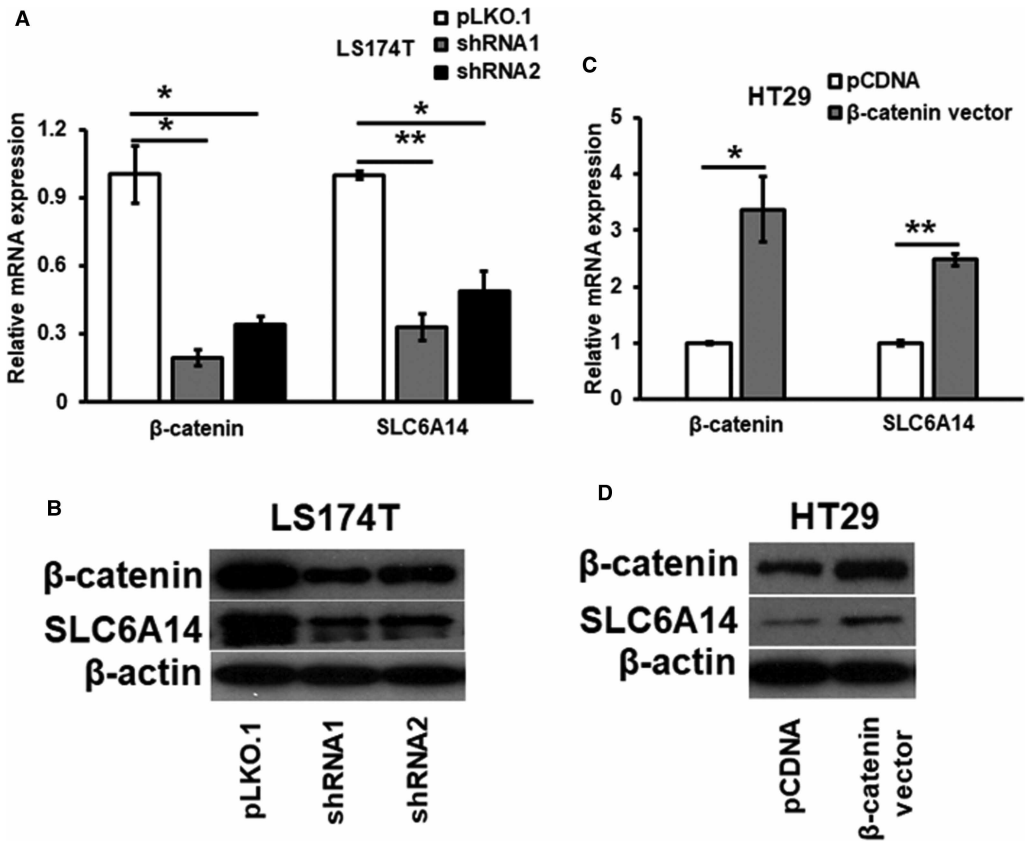

Figure 9. Effect of β-catenin knockdown and overexpression on SLC6A14 expression in human colon cancer cells.
(**A**,**B**) LS174T cells were transfected with empty vector (pLKO.1), β-catenin/shRNA1 vector, or β-catenin/shRNA2 vector. After selection with puromycin, cells were processed for the preparation of RNA and protein lysates. Quantitative RT-PCR was used to determine the levels of mRNA for β-catenin and SLC6A14 (**A**) and Western blot was used to determine protein levels for β-catenin and SLC6A14 (**B**). (**C**,**D**) HT29 cells were transfected with either empty vector (pCDNA) or pCDNA/β-catenin vector. Seventy-two hours following transfection, cells were processed for preparation of RNA and protein lysates. Quantitative RT-PCR was used to determine the levels of mRNA for β-catenin and SLC6A14 (C) and Western blot was used to determine protein levels for β-catenin and SLC6A14 (D). Data are means ± SD. $^{*}P < 0.05$; $^{**}P < 0.01$.

to transport glycine, a constituent of glutathione. Taken collectively, the functional features of SLC6A14 ensure optimal amino acid nutrition, mTOR activation, and protection against oxidative stress to support tumor cell growth and proliferation.

While it has been shown convincingly that SLC1A5, SLC7A5, SLC7A11, and SLC6A14 are all up-regulated in specific cancer types, molecular mechanisms underlying this up-regulation remain to be explored in greater detail. With regard to SLC1A5 and SLC7A5, the oncogene MYC appears to be at least one of the drivers of up-regulation [44,45]. In the present study, we investigated the molecular mechanism associated with the up-regulation of SLC6A14 in colon cancer. An accumulating body of evidence suggests that TCF4/β-catenin-mediated canonical Wnt signaling pathway is predominantly altered in colon cancer [46–49]. We thought that *SLC6A14* could be one of the genes up-regulated at the transcriptional level by TCF4/β-catenin in colon cancer. Our present studies have shown that it indeed is the case.

In the present study, we used a small molecule blocker of SLC6A14 to assess the impact on colon cancer using three different mouse models: xenograft of human colon cancer cells in immunocompromised nude mice, syngeneic transplantation of mouse colon cancer cells in immunocompetent mice, and experimentally induced spontaneous colon cancer in mice. In all three approaches, blockade of SLC6A14 by α-MT suppressed colon cancer. With 2 mg/ml in drinking water, we have shown in a previously published study that the plasma levels of α-MT is ∼10 μM [20]. As the blocker was used in the form of D/L enantiomeric mixture, the dose in

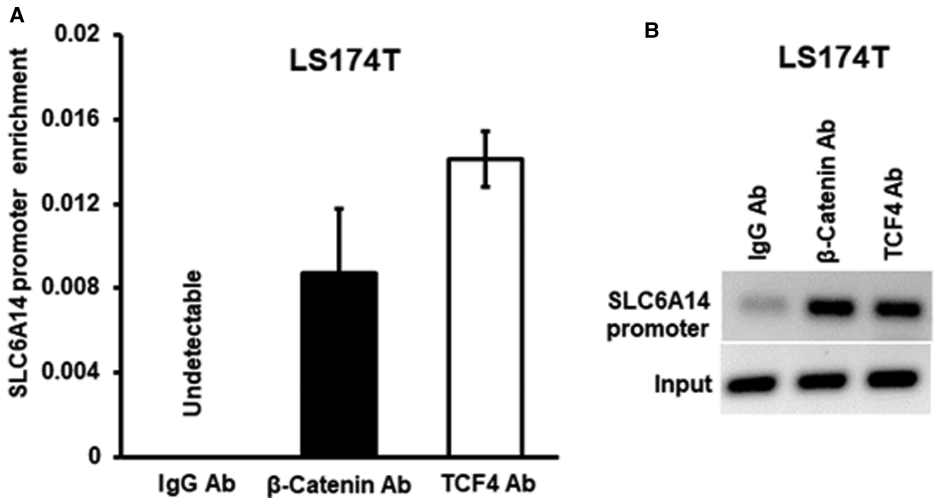

Figure 10. Binding of β-catenin and TCF4 to human *SLC6A14* promoter.

Quantitative RT-PCR (**A**) and regular RT-PCR (**B**) for the region of *SLC6A14* promoter with the binding motifs for β-catenin and TCF4 using the ChIP assay. Chromatin complexes from LS174T cells were immunoprecipitated using IgG (negative control) or antibodies specific for β-catenin and TCF4. Enrichment of *SLC6A14* promoter was normalized with the starting amount of DNA (input).

drinking water was actually 1 mg/ml for the L-enantiomer, the likely active form. The dose of 1 mg/ml in drinking water in mice (25 g body weight) translates approximately to a human dose of 600 mg per day for a person with 60 kg body weight, assuming an intake of 3 ml drinking water per day in mice and 100% oral bioavailability of the drug [50]; this calculation takes into account the marked difference between mice and humans in body surface area per given body weight. These data identify α-MT as a potential drug for the treatment of colon cancer; it could also be effective for other SLC6A14-positive cancers.

Summary Statement

The amino acid transporter SLC6A14 is up-regulated in colon cancer in humans, at least partly via Wnt signaling, and deletion or pharmacologic inhibition of the transporter in mice protects against colon cancer; these studies identify SLC6A14 as a potential drug target for colon cancer treatment.

Competing Interests

The authors declare that there are no competing interests associated with the manuscript.

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Author Contribution

M.O.F.S. and S.S. performed most of the experiments; T.P.B. contributed to the Western blot experiments; M.T. and Y.D.B. performed the xenograft experiments with human colon cancer cell lines; M.O.F.S., S.S., and V.G. designed the study and interpreted the data; M.O.F.S. and V.G. wrote the manuscript.

Abbreviations

1-MT, 1-methyl-DL-tryptophan; APC, adenomatous polyposis coli; ASNS, asparagine synthetase; ChIP, chromatin immunoprecipitation; CHOP, CCAAT/enhancer-binding protein (C/EBP) homologous protein;

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CRC, colorectal cancer; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; SLC, solute-linked carrier; SLC6A14, 14th member of the *SLC6A* gene family; TCF4, T-cell factor 4; α-MT, α-methyl-DL-tryptophan.

References

1 Siegel, R.L., Miller, K.D. and Jemal, A. (2019) Cancer statistics, 2019. *CA Cancer J Clin.* **69**, 7–34 https://doi.org/10.3322/caac.21551
2 Mariotto, A.B., Yabroff, K.R., Shao, Y., Feuer, E.J. and Brown, M.L. (2011) Projections of the cost of cancer care in the United States: 2010–2020. *J. Natl. Cancer Inst.* **103**, 117–128 https://doi.org/10.1093/jnci/djq495
3 Markowitz, S.D. and Bertagnolli, M.M. (2009) Molecular origins of cancer: molecular basis of colorectal cancer. *N. Engl. J. Med.* **361**, 2449–2460 https://doi.org/10.1056/NEJMra0804588
4 Arena, E.A. and Bilchik, A.J. (2013) What is the optimal means of staging colon cancer? *Adv. Surg.* **47**, 199–211 https://doi.org/10.1016/j.yasu.2013.03.001
5 Markowitz, S.D., Dawson, D.M., Willis, J. and Willson, J.K. (2002) Focus on colon cancer. *Cancer Cell* **1**, 233–236 https://doi.org/10.1016/S1535-6108(02)00053-3
6 Liu, Q., Luo, D., Cai, S., Li, Q. and Li, X. (2018) P-TNM staging system for colon cancer: combination of P-stage and AJCC TNM staging system for improving prognostic prediction and clinical management. *Cancer Manag. Res.* **10**, 2303–2314 https://doi.org/10.2147/CMAR.S165188
7 Tariq, K. and Ghias, K. (2016) Colorectal cancer carcinogenesis: a review of mechanisms. *Cancer Biol. Med.* **13**, 120–135 https://doi.org/10.20892/j.issn.2095-3941.2015.0103
8 Baltruskeviciene, E., Schveigert, D., Stankevicius, V., Mickys, U., Zvirblis, T., Bublevic, J. et al. (2017) Down-regulation of miRNA-148a and miRNA-625-3p in colorectal cancer is associated with tumor budding. *BMC Cancer* **17**, 607 https://doi.org/10.1186/s12885-017-3575-z
9 Bai, J.W., Xue, H.Z. and Zhang, C. (2016) Down-regulation of microRNA-143 is associated with colorectal cancer progression. *Eur. Rev. Med. Pharmacol. Sci.* **20**, 4682–4687 PMID: 27906435
10 Ganapathy, V., Thangaraju, M. and Prasad, P.D. (2009) Nutrient transporters in cancer: relevance to Warburg hypothesis and beyond. *Pharmacol. Ther.* **121**, 29–40 https://doi.org/10.1016/j.pharmthera.2008.09.005
11 Brown, T.P. and Ganapathy, V. (2020) Lactate/GPR81 signaling and proton motive force in cancer: role in angiogenesis, immune escape, nutrition, and Warburg phenomenon. *Pharmacol. Ther.* **206**, 107451 https://doi.org/10.1016/j.pharmthera.2019.107451
12 Sikder, M.O.F., Yang, S., Ganapathy, V. and Bhutia, Y.D. (2017) The Na^+/Cl^- coupled, broad-specific, amino acid transporter SLC6A14 ($ATB^{0,+}$): emerging roles in multiple diseases and therapeutic potential for treatment and diagnosis. *AAPS J.* **20**, 12 https://doi.org/10.1208/s12248-017-0164-7
13 Fuchs, B.C. and Bode, B.P. (2005) Amino acid transporters ASCT2 and LAT1 in cancer: partners in crime? *Semin. Cancer Biol.* **15**, 254–266 https://doi.org/10.1016/j.semcancer.2005.04.005
14 Bhutia, Y.D., Babu, E., Ramachandran, S. and Ganapathy, V. (2015) Amino acid transporters in cancer and their relevance to "glutamine addiction": novel targets for the design of a new class of anticancer drugs. *Cancer Res.* **75**, 1782–1788 https://doi.org/10.1158/0008-5472.CAN-14-3745
15 Cormerais, Y., Massard, P.A., Vucetic, M., Giuliano, S., Tambutte, E., Durivault, J. et al. (2018) The glutamine transporter ASCT2 (SLC1A5) promotes tumor growth independently of the amino acid transporter LAT1 (SLC7A5). *J. Biol. Chem.* **293**, 2877–2887 https://doi.org/10.1074/jbc.RA117.001342
16 Ansari R, E., Craze, M.L., Miligy, I., Diez-Rodriguez, M., Nolan, C.C., Ellis, I.O. et al. (2018) The amino acid transporter SLC7A5 confers a poor prognosis in the highly proliferative breast cancer subtypes and is a key therapeutic target in luminal B tumours. *Breast Cancer Res.* **20**, 21 https://doi.org/10.1186/s13058-018-0946-6
17 Koppula, P., Zhang, Y., Shi, J., Li, W. and Gan, B. (2017) The glutamate/cystine antiporter SLC7A11/xCT enhances cancer cell dependency on glucose by exporting glutamate. *J. Biol. Chem.* **292**, 14240–9 https://doi.org/10.1074/jbc.M117.798405
18 van Geldermalsen, M., Wang, Q., Nagarajah, R., Marshall, A.D., Thoeng, A., Gao, D. et al. (2016) ASCT2/SLC1A5 controls glutamine uptake and tumour growth in triple-negative basal-like breast cancer. *Oncogene* **35**, 3201–3208 https://doi.org/10.1038/onc.2015.381
19 Gupta, N., Miyauchi, S., Martindale, R.G., Herdman, A.V., Podolsky, R., Miyake, K. et al. (2005) Upregulation of the amino acid transporter $ATB^{0,+}$ (SLC6A14) in colorectal cancer and metastasis in humans. *Biochim. Biophys. Acta* **1741**, 215–223 https://doi.org/10.1016/j.bbadis.2005.04.002
20 Karunakaran, S., Ramachandran, S., Coothankandaswamy, V., Elangovan, S., Babu, E., Periyasamy-Thandavan, S. et al. (2011) SLC6A14 ($ATB^{0,+}$) protein, a highly concentrative and broad specific amino acid transporter, is a novel and effective drug target for treatment of estrogen receptor-positive breast cancer. *J. Biol. Chem.* **286**, 31830–8 https://doi.org/10.1074/jbc.M111.229518
21 Coothankandaswamy, V., Cao, S., Xu, Y., Prasad, P.D., Singh, P.K., Reynolds, C.P. et al. (2016) Amino acid transporter SLC6A14 is a novel and effective drug target for pancreatic cancer. *Br. J. Pharmacol.* **173**, 3292–3306 https://doi.org/10.1111/bph.13616
22 Gupta, N., Prasad, P.D., Ghamande, S., Moore-Martin, P., Herdman, A.V., Martindale, R.G. et al. (2006) Up-regulation of the amino acid transporter $ATB^{0,+}$ (SLC6A14) in carcinoma of the cervix. *Gynecol. Oncol.* **100**, 8–13 https://doi.org/10.1016/j.ygyno.2005.08.016
23 Karunakaran, S., Umapathy, N.S., Thangaraju, M., Hatanaka, T., Itagaki, S., Munn, D.H. et al. (2008) Interaction of tryptophan derivatives with SLC6A14 ($ATB^{0,+}$) reveals the potential of the transporter as a drug target for cancer chemotherapy. *Biochem. J.* **414**, 343–355 https://doi.org/10.1042/BJ20080622
24 Bhutia, Y.D. and Ganapathy, V. (2016) Glutamine transporters in mammalian cells and their functions in physiology and cancer. *Biochim. Biophys. Acta* **1863**, 2531–2539 https://doi.org/10.1016/j.bbamcr.2015.12.017
25 Bhutia, Y.D., Babu, E., Prasad, P.D. and Ganapathy, V. (2014) The amino acid transporter SLC6A14 in cancer and its potential use in chemotherapy. *Asian J. Pharm. Sci.* **9**, 293–303 https://doi.org/10.1016/j.ajps.2014.04.004
26 Scalise, M., Pochini, L., Sonsole, L., Losso, M.A. and Indiveri, C. (2018) The human SLC1A5 (ASCT2) amino acid transporter: from function to structure and role in cell biology. *Front. Cell Dev. Biol.* **6**, 96 https://doi.org/10.3389/fcell.2018.00096
27 Scalise, M., Galluccio, M., Console, L., Pochini, L. and Indiveri, C. (2018) The human SLC7A5 (LAT1): the intriguing histidine/large neutral amino acid transporter and its relevance to human health. *Front. Chem.* **6**, 243 https://doi.org/10.3389/fchem.2018.00243
28 Babu, E., Bhutia, Y.D., Ramachandran, S., Gnanaprakasam, J.P., Prasad, P.D., Thangaraju, M. et al. (2015) Deletion of the amino acid transporter Slc6a14 suppresses tumour growth in spontaneous mouse models of breast cancer. *Biochem. J.* **469**, 17–23 https://doi.org/10.1042/BJ20150437

29 Hong, Y., Downey, T., Eu, K.W., Koh, P.K. and Cheah, P.Y. (2010) A 'metastasis-prone' signature for early-stage mismatch-repair proficient sporadic colorectal cancer patients and its implications for possible therapeutics. *Clin. Exp. Metastasis* **27**, 83–90 https://doi.org/10.1007/s10585-010-9305-4
30 de Sousa, E.M.F., Colak, S., Buikhuisen, J., Koster, J., Cameron, K., de Jong, J.H. et al. (2011) Methylation of cancer-stem-cell-associated Wnt target genes predicts poor prognosis in colorectal cancer patients. *Cell Stem Cell* **9**, 476–485 https://doi.org/10.1016/j.stem.2011.10.008
31 Chao, C., Carmical, J.R., Ives, K.L., Wood, T.G., Aronson, J.F., Gomez, G.A. et al. (2012) CD133+ colon cancer cells are more interactive with the tumor microenvironment than CD133- cells. *Lab. Invest.* **92**, 420–436 https://doi.org/10.1038/labinvest.2011.185
32 Chandrashekar, D.S., Bashel, B., Balasubramanya, S.A.H., Creighton, C.J., Ponce-Rodriguez, I., Chakravarthi, B. et al. (2017) UALCAN: a portal for facilitating tumor subgroup gene expression and survival analyses. *Neoplasia* **19**, 649–658 https://doi.org/10.1016/j.neo.2017.05.002
33 Coothankandaswamy, V., Elangovan, S., Singh, N., Prasad, P.D., Thangaraju, M. and Ganapathy, V. (2013) The plasma membrane transporter SLC5A8 suppresses tumour progression through depletion of survivin without involving its transport function. *Biochem. J.* **450**, 169–178 https://doi.org/10.1042/BJ20121248
34 Behrooz A, B., Syahir, A. and Ahmad, S. (2019) CD133: beyond a cancer stem cell biomarker. *J. Drug. Target* **27**, 257–269 https://doi.org/10.1080/1061186X.2018.1479756
35 Chen, H., Pan, Y.X., Dudenhausen, E.E. and Kilberg, M.S. (2004) Amino acid deprivation induces the transcription rate of the human asparagine synthetase gene through a timed program of expression and promoter binding of nutrient-responsive basic region/leucine zipper transcription factors as well as localized histone acetylation. *J. Biol. Chem.* **279**, 50829–50839 https://doi.org/10.1074/jbc.M409173200
36 Averous, J., Bruhat, A., Jousse, C., Carraro, V., Thiel, G. and Fafournoux, P. (2004) Induction of CHOP expression by amino acid limitation requires both ATF4 expression and ATF2 phosphorylation. *J. Biol. Chem.* **279**, 5288–5297 https://doi.org/10.1074/jbc.M311862200
37 Nicklin, P., Bergman, P., Zhang, B., Triantafellow, E., Wang, H., Nyfeler, B. et al. (2009) Bidirectional transport of amino acids regulates mTOR and autophagy. *Cell* **136**, 521–534 https://doi.org/10.1016/j.cell.2008.11.044
38 Ishimoto, T., Nagano, O., Yae, T., Tamada, M., Motohara, T., Oshima, H. et al. (2011) CD44 variant regulates redox status in cancer cells by stabilizing the xCT subunit of system x_c^- and thereby promotes tumor growth. *Cancer Cell* **19**, 387–400 https://doi.org/10.1016/j.ccr.2011.01.038
39 Timmerman, L.A., Holton, T., Yuneva, M., Louie, R.J., Padro, M., Daemen, A. et al. (2013) Glutamine sensitivity analysis identifies the xCT antiporter as a common triple-negative breast tumor therapeutic target. *Cancer Cell* **24**, 450–465 https://doi.org/10.1016/j.ccr.2013.08.020
40 Bridges, C.C., Kekuda, R., Wang, H., Prasad, P.D., Mehta, P., Huang, W. et al. (2001) Structure, function, and regulation of human cystine/glutamate transporter in retinal pigment epithelial cells. *Invest. Ophthal. Vis. Sci.* **42**, 47–54 PMID: 11133847
41 Jiang, L., Kon, N., Li, T., Wang, S.J., Su, T., Hibshoosh, H. et al. (2015) Ferroptosis as a p53-mediated activity during tumour suppression. *Nature* **520**, 57–62 https://doi.org/10.1038/nature14344
42 Lewerenz, J., Hewett, S.J., Huang, Y., Lambros, M., Gout, P.W., Kalivas, P.W. et al. (2013) The cystine/glutamate antiporter system x_c^- in health and disease: from molecular mechanisms to novel therapeutic opportunities. *Antioxid. Redox Signal.* **18**, 522–555 https://doi.org/10.1089/ars.2011.4391
43 Dun, Y., Mysona, B., Van Ells, T., Amarnath, L., Ola, M.S., Ganapathy, V. et al. (2006) Expression of the cystine-glutamate exchanger (x_c^-) in retinal ganglion cells and regulation by nitric oxide and oxidative stress. *Cell Tissue Res.* **324**, 189–202 https://doi.org/10.1007/s00441-005-0116-x
44 Zhao, X., Petrashen, A.P., Sanders, J.A., Peterson, A.L. and Sedivy, J.M. (2019) SLC1A5 glutamine transporter is a target of MYC and mediates reduced mTORC1 signaling and increased fatty acid oxidation in long-lived Myc hypomorphic mice. *Aging Cell* **18**, e12947 https://doi.org/10.1111/acel.12947
45 Venkateswaran, N., Latifa-Navarro, M.C., Hao, Y.H., Kilgore, J.A., Perez-Castro, L., Braveman, J. et al. (2019) MYC promotes tryptophan uptake and meabolism by the kunurenine pathway in colon cancer. *Genes Dev.* **33**, 1236–1251 https://doi.org/10.1101/gad.327056.119
46 Zhan, T., Rindtorff, N. and Boutros, M. (2017) Wnt signaling in cancer. *Oncogene* **36**, 1461–1473 https://doi.org/10.1038/onc.2016.304
47 Christie, M., Jorissen, R.N., Mouradov, D., Sakthianandeswaren, A., Li, S., Day, F. et al. (2013) Different APC genotypes in proximal and distal sporadic colorectal cancers suggest distinct WNT/beta-catenin signalling thresholds for tumourigenesis. *Oncogene* **32**, 4675–4682 https://doi.org/10.1038/onc.2012.486
48 Anastas, J.N. and Moon, R.T. (2013) WNT signalling pathways as therapeutic targets in cancer. *Nat. Rev. Cancer* **13**, 11–26 https://doi.org/10.1038/nrc3419
49 Schneikert, J. and Behrens, J. (2007) The canonical Wnt signalling pathway and its APC partner in colon cancer development. *Gut* **56**, 417–425 https://doi.org/10.1136/gut.2006.093310
50 Reagan-Shaw, S., Nihal, M. and Ahmad, N. (2008) Dose translation from animal to human studies revisited. *FASEB J.* **22**, 659–661 https://doi.org/10.1096/fj.07-9574LSF