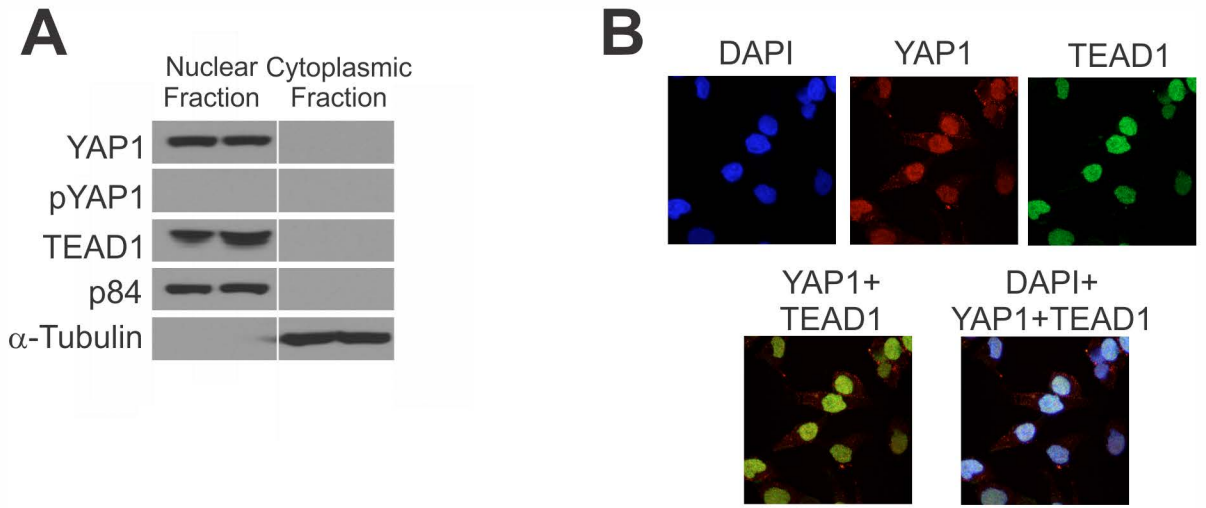
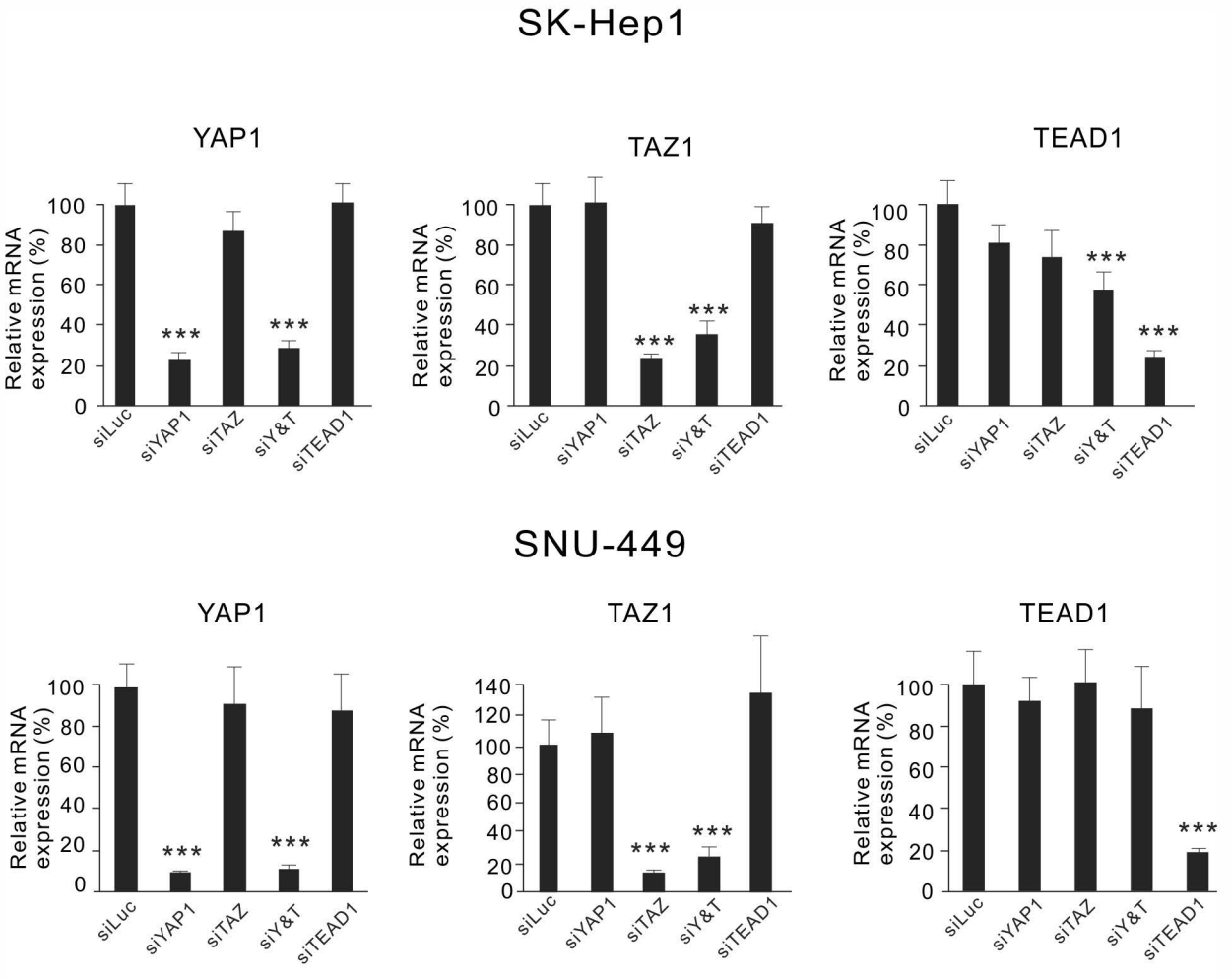


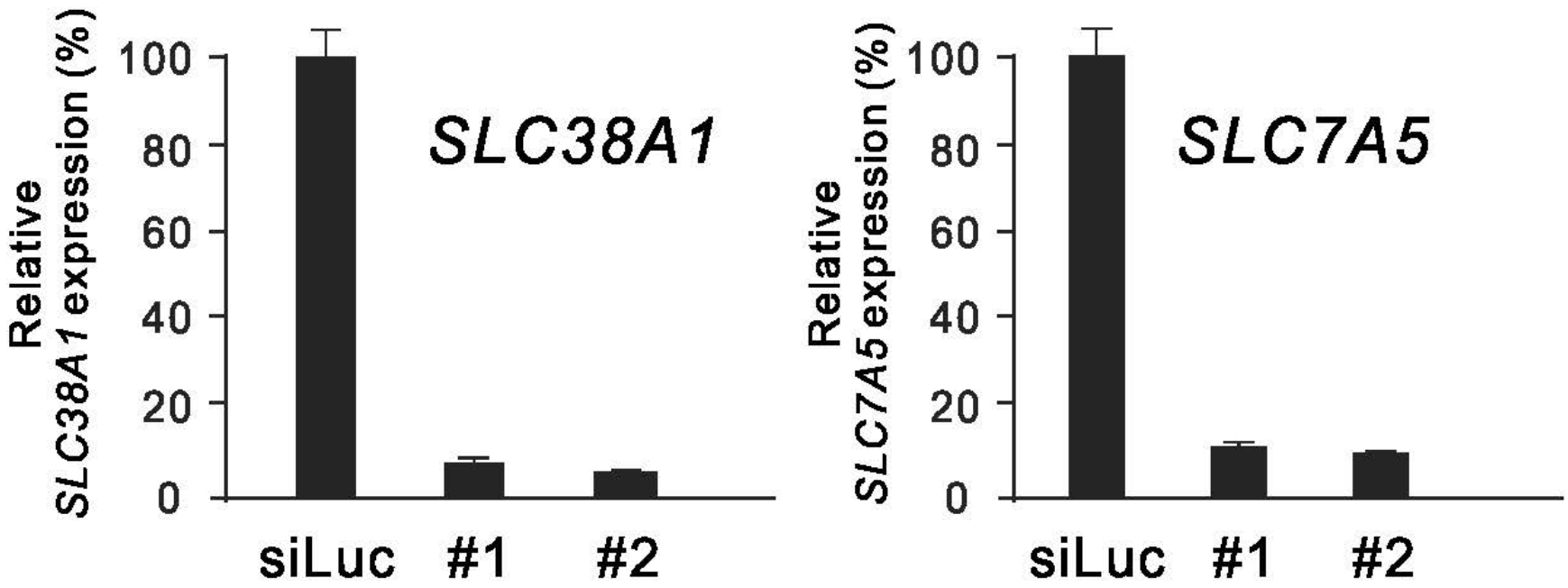
Supporting Figure 1. Cellular localization of YAP1 in HCC cells.

(A) Western blot analysis of fractionated cell lysates (nucleus and cytoplasmic parts) from SK-Hep1 with indicated antibodies.
(B) Confocal microscope images of SK-Hep1 cells stained with 4',6-diamidino-2-phenylindole (DAPI) and YAP1 or TEAD1 antibodies.



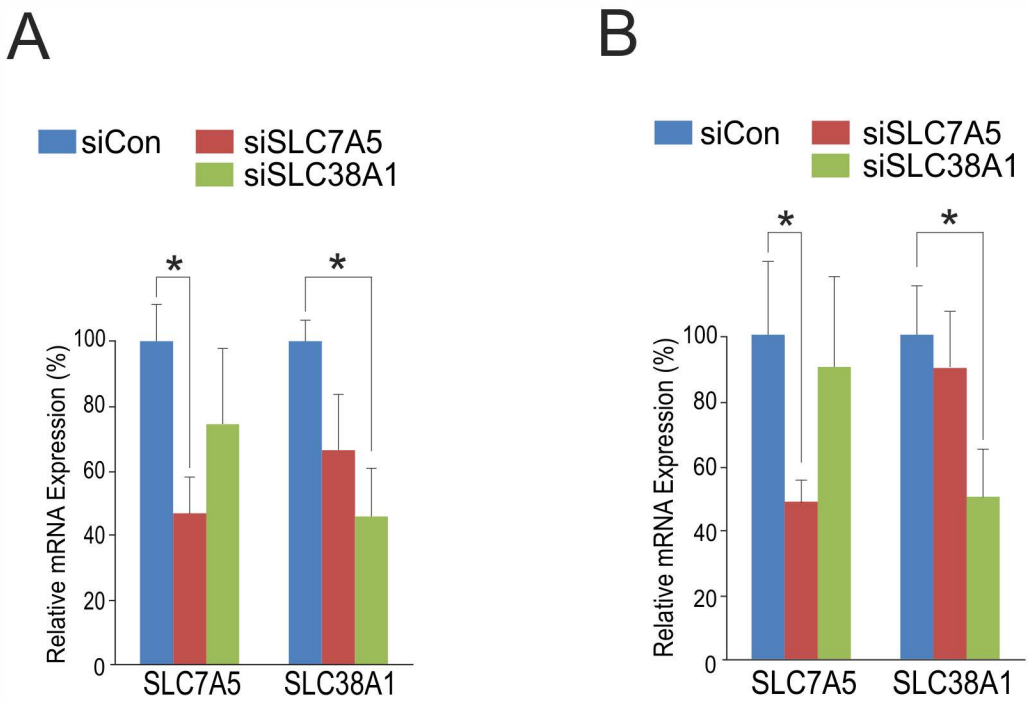
Supporting Figure 2. Knock-down efficiency of YAP, TAZ and TEAD1 genes in HCC cell lines.

SK-Hep1 and SNU-449 liver cancer cells were transiently transfected with *YAP1*, *TAZ*, and *TEAD1*-specific siRNA or control (siLuc.). Thirty nanograms of total RNA from transfected cell lines were analyzed by qRT-PCR using gene-specific primers as indicated. Student t-test (two-tailed) was applied to estimate the significance of gene expression changes ***$P < 0.001$



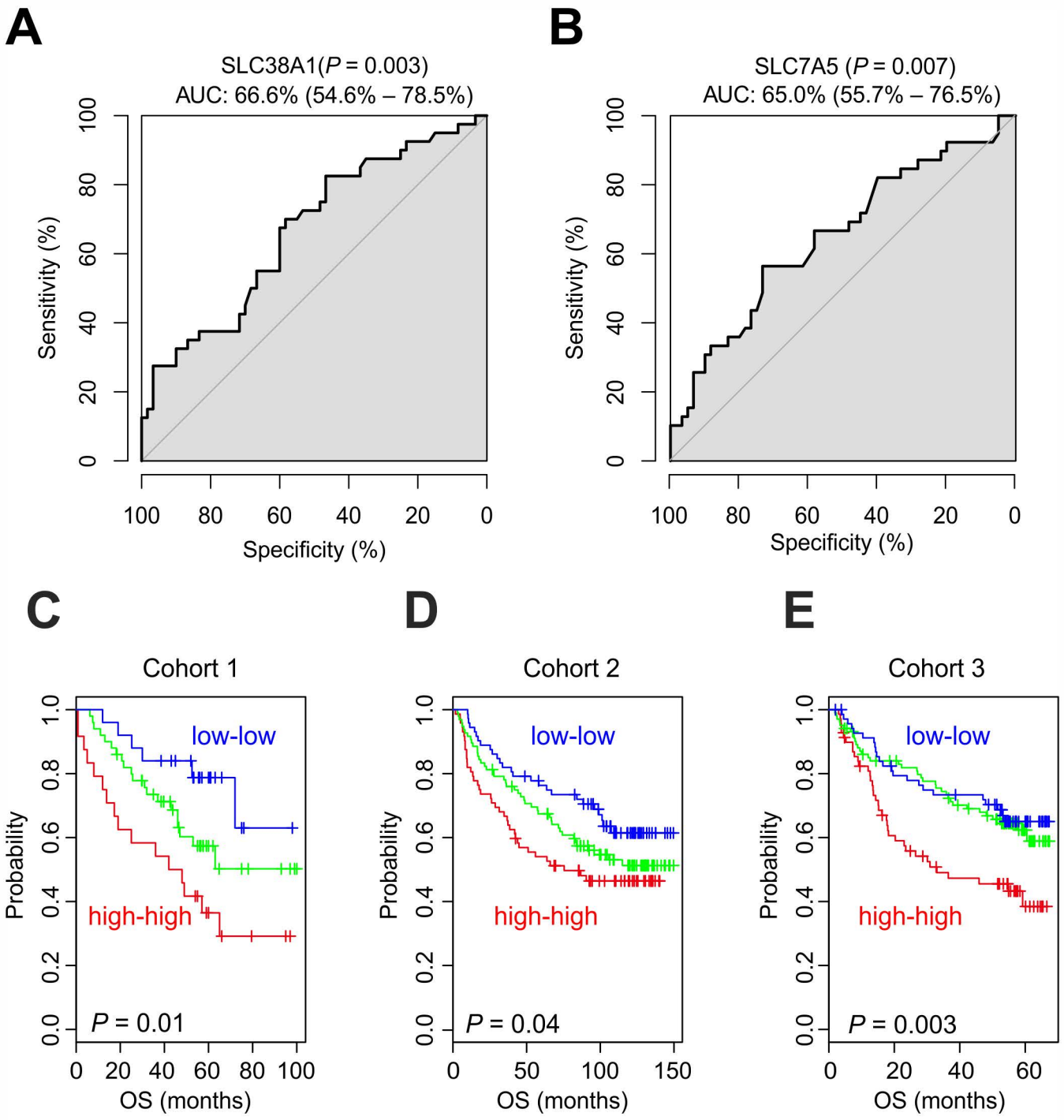
Supporting Figure 3. Silencing of *SLC38A1* and *SLC7A5* in HCC cells.

SK-Hep1 cells were transiently transfected with *SLC38A1*-specific or *SLC7A5*-specific siRNA (Mission RNA; Sigma-Aldrich) or control siLuciferase-specific siRNA (siLuc.). Thirty nanograms of total RNA from transfected cell lines was analyzed by qRT-PCR using gene-specific primers as indicated.



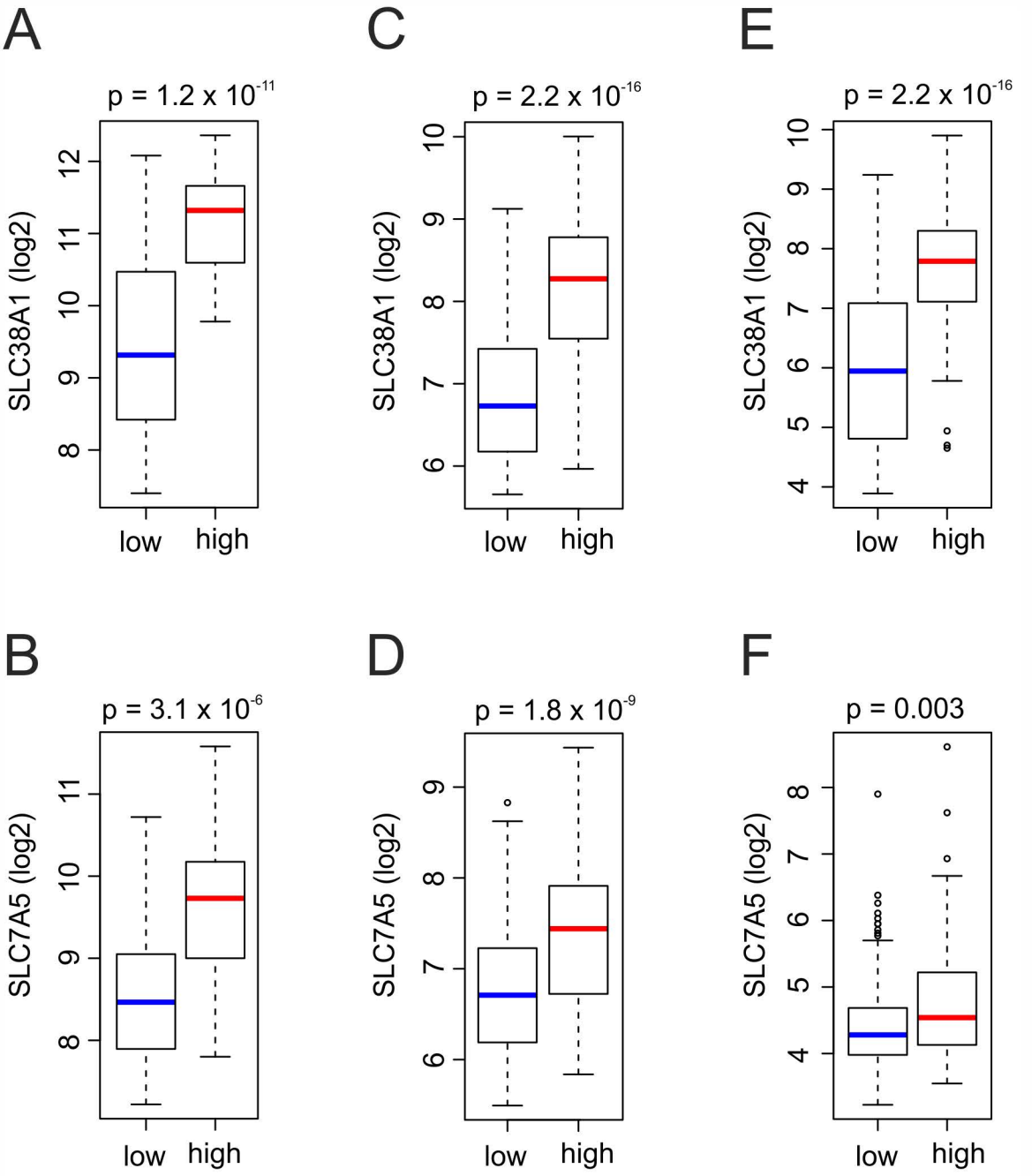
Supporting Figure 4. Silencing of amino acid transporters in mouse xenograft model

SK-Hep1 cells were xenografted subcutaneously or orthotopically to immuno-compromised mice. At 7 days after xenografting, mice were treated with indicated siRNA-DOPC mixtures until sacrifice. Expression of SLC38A1 and SLC7A5 in siRNA-treated tumors from subcutaneous model (A) and orthotopic model (B). Expression of mRNAs was quantified by qRT-PCR. (* $P<0.05$).



Supporting Figure 5. Clinical Significance of SLC38A1 and SLC7A5 in HCC cohort1.

(A,B) Prognostic significance of SLC38A1 and SLC7A5 estimated by AUC from receiver operating characteristics analysis for 5-year overall survival rate. AUC: area under curve, CI: confident internal of AUC.
(C) Kaplan-Meier plots of overall survival of HCC patients in Cohort 1 when patients were stratified into three groups according to expression levels of SLC38A1 and SLC7A5: high expression of both transporters (red), high expression of one of the transporters (green), and low expression of both transporters (blue). P-values were calculated with the log-rank test.
(D,E) Kaplan-Meier plots of overall survival of HCC patients in Cohort 2 (D) and Cohort 3 (E) when patients were stratified into three groups according to expression levels of SLC38A1 and SLC7A5. P-values were calculated with the log-rank test.



Supporting Figure 6. SLC38A1 and SLC7A5 are highly expressed in poor prognosis subgroups in HCC.

Box plots of SLC38A1 and SLC7A5 expression in two prognostic subtypes stratified by 65-gene risk score in cohort 1 (A,B), cohort 2 (D, D), and cohort 3 (E, F). low: favorable prognostic subgroup with low risk scores, high: poor prognostic subgroup with high risk scores.

Supporting Information

YAP1 and TAZ Activates mTORC1 Pathway by Regulating Amino Acid Transporters in hepatocellular carcinoma

Western Blot Analysis

Cells were maintained and Western blot analysis performed as described previously 1. Liver tissues from 4-week-old Mst1/2$^{-/-}$ mutant and wild-type mice were used for Western blots. Antibodies used were against YAP1 (sc-500; Santa Cruz Biotechnology), pYAP1 (#4911; Cell Signaling Technology [CST]), TAZ (560235; BD Biosciences), TEAD1 (610922; BD Biosciences), p70 S6K (#9202; CST), p-p70 S6K (Thr389) (#9205; CST), b-actin (ab3280; Abcam), S6 (#2217; CST), pS6 (Ser240/244) (#2215; CST), ERK1/2 (#4695; CST), pERK1/2(Thr202/Tyr204) (#4284; CST), AKT (#4691; CST), pAKT (Ser473) (#9271; CST), and a-tubulin (#3873; CST). All antibodies were diluted with bovine serum albumin (BSA) as 1:1000 ratio and membrane was blocked with BSA.

siRNA

siYAP1, siTAZ, and siTEAD1 were purchased from Dharmacon (SMARTPool). Cells were transfected with indicated siRNA using oligofectamine (Invitrogen) for the indicated times.

MTT Assay

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to assess cell proliferation after treatment with specific siRNA. Cells were plated into 12-well plates, transfected with specific siRNA, and incubated for the indicated time periods. After 20 μl of MTT was added to each well, the wells were incubated for 3 hours, and 100 μl of dimethyl sulfoxide was then added. The absorbance values of individual wells were read at 570 nm.

ChIP Assay

Chromatin immunoprecipitation was performed according to the protocol from Upstate Biotechnology with minor modifications[1]. 10^7 SK-Hep1 and SNU449 cells were used for each reaction. Cells were treated with 1% formaldehyde for cross-linking and harvested. After sonication, 1% of soluble chromatin fraction was de-cross-linked by heating at 65°C overnight and used as an input. The rest of the chromatin fraction was immunoprecipitated with YAP1, TEAD1 and TAZ antibody and de-cross-linked by heat. DNA was purified with the QiaQuick PCR purification kit (Qiagen) and analyzed by quantitative real time PCR (qPCR). Quantitative real time PCR was performed using

SYBR Green master mix (Applied Biosystems). Antibodies used were against YAP1 (A302-309A; Bethyl Laboratories), TEAD1 (610922; BD Biosciences), TAZ (560235; BD Biosciences), and normal rabbit immunoglobulin G (SC-2027; Santa Cruz Biotechnology).

Primer sequences:
SLC7A5 promoter region primer I (–2951 to –2801 bp)
Forward 5¢TGCTGAGATGAAAACCACAATC-3¢
Reverse 5¢AGGCCACCCTCTCTCACAAG-3¢
SLC7A5 promoter region primer II (–1401 to –1201 bp)
Forward 5¢GTTTTATCACCGCAGAGGAATG-3¢
Reverse 5¢AATTCTGAACAATAGCCGTGGT-3¢
SLC38A1 promoter region primer I (–2951 to –2701 bp)
Forward 5¢ACCAGTGCAGGAAAACACATATT-3¢
Reverse 5¢TAGCTTGTTTTTGTATGGTTCCAG 3¢
SLC38A1 promoter region primer II (–2301 to –2001 bp)
Forward 5¢CAAGATTTGGATGTGCCACTTAG-3¢
Reverse 5¢TGATTCCTCTATTCACTGTGTGCT-3¢

Quantitative RT-PCR
Total RNA was extracted from the indicated cell lines using a mirVana RNA isolation kit (Ambion) according to the manufacturer's instructions, and RNA was assayed by using real-time quantitative RT-PCR with TaqMan primers specific to each gene (Applied Biosystems). Real-time RT**-**PCR was performed using the 7700HT real-time RT**-**PCR system with a 96-well block module (Applied Biosystems). Cycling conditions were 45°C for 30 minutes and 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 60 seconds. Relative amounts of mRNA were calculated from the threshold cycle number using expression of cyclophilin A as an endogenous control. All experiments were performed in triplicate and the values averaged. Primers were purchased from Applied Biosystems (YAP1; Hs00902712-g1, TAZ; Hs00210007_m1, TEAD1; Hs00173359_m1, SLC38A1; Hs01562175_m1, SLC7A5; Hs01001183_m1. PPIA; Hs04194521_s1)

Glutamine Uptake Assay
Glutamine uptake was measured using Multiparameter Bioanalytical System (YSI-2900; YSI Life Sciences) following the manufacturer's protocol. After SK-Hep1 cells were seeded on 12-well plates, the cells were treated with siRNAs for 72 hours. Glutamine levels were measured in the medium from each well of cultured cells and were normalized to cell numbers.

Xenograft Experiments

Female athymic nude mice were purchased from the National Cancer Institute's Frederick National Laboratory for Cancer Research and maintained according to guidelines set forth by the Association for Assessment and Accreditation of Laboratory Animal Care International and the United States Public Health Service policy on Humane Care and Use of Laboratory Animals. All mouse studies were approved and supervised by The University of Texas MD Anderson Cancer Center Institutional Animal Care and Use Committee. All mice used in the xenograft experiments were between 8 and 12 weeks of age at the time of injection.

For both intrasplenic delivery to the liver and subcutaneous injection, SK-Hep1 cells were trypsinized, washed, and resuspended in Hank's balanced salt solution (Gibco) before injection. To establish the tumors, cells were injected either intrasplenically (2.5×10^6 cells in 50 µl of Hank's balanced salt solution) or subcutaneously over the posterior flank (1×10^6 cells in 200 µl of Hank's balanced salt solution). For the intrasplenic injections, mice were anesthetized with ketamine. Following skin cleaning with an alcohol swab, an incision was made to visualize the spleen. A cell suspension was injected directly into the spleen using a 30-gauge needle. After injection, the incision was closed using surgery clips.

To assess the effects of siRNA therapy on tumor growth, treatment with siRNA (150 mg/kg intraperitoneally twice weekly) was initiated 1 week after injection of tumor cells. Mice were randomized into three groups (n = 10 mice per group) and treated with siRNA incorporated in neutral nanoliposomes (intraperitoneal administration): (a) control siRNA-1,2-dioleoyl-sn-glycero-3-phosphatidylcholine (DOPC), (b) SLC7A5 siRNA-DOPC, or (c) SLC38A1 siRNA-DOPC. Treatment was continued until mice in any group became moribund (typically 4-6 weeks following tumor cell injection), at which point they were killed. The mouse weight, tumor weights, and tumor locations were then recorded. Tumor tissue was either fixed in formalin for paraffin embedding, frozen in optimal cutting temperature medium to prepare frozen slides, or snap-frozen for lysate preparation. The individuals who performed the necropsies, tumor collections, and tissue processing were blinded to the treatment group assignments.

siRNA sequences for xenograft experiments:

control siRNA (siCon): UUCUCCGAACGUGUCACGU [dT][dT]

siSLC7A5: CUCUUUGCCUAUGGAGGAU[dT][dT]

siSLC38A1: CAAUUUACAGUGAGCUUAA[dT][dT]

Gene Expression Data from SK-Hep1 After Silencing of YAP1 and TAZ

YAP1 and TAZ were transiently silenced in SK-Hep1 cells using siRNA. Three days after transfection, cells were harvested for RNA isolation. Total RNA was extracted using a mirVana RNA isolation labeling kit (Ambion). Five hundred nanograms of total RNA were used for labeling and hybridization (Human BeadChip v3 microarray;

Illumina) according to the manufacturer's protocol. After the bead chips were scanned with an Illumina BeadArray Reader, the microarray data were normalized using the quantile normalization method in the Linear Models for Microarray Data package in the R language environment.[2] The expression level of each gene was transformed into a log2 base before further analysis. Data are available in the Gene Expression Omnibus public database from the National Center for Biotechnology Information (accession number GSE54617).

Gene Expression Data from Patients' Tissues

To examine the clinical relevance and significance of SLC38A1 and SLC7A5 in HCC, we generated gene expression data from 100 patients with HCC (Cohort 1). Tumor specimens and clinical data were obtained from patients undergoing hepatectomy as primary treatment for HCC at Seoul National University Hospital, Seoul, Korea, and Chonbuk National University Hospital, Jeonju, Korea. Samples were frozen in liquid nitrogen and stored at –80°C until RNA extraction. The study protocols were approved by the Institutional Review Boards at both institutions and at The University of Texas MD Anderson Cancer Center, and all participants provided written informed consent. Gene expression data from Cohort 1 were generated using the Illumina microarray platform human-6 v.2. Patients were monitored prospectively at least once every 3 months after surgery. Primary microarray data from Cohort 1 are available in the Gene Expression Omnibus public database (accession number GSE16757).

To validate the clinical significance of SLC38A1 and SLC7A5, we analyzed additional gene expression data that are publicly available from the Gene Expression Omnibus database. The gene expression data in Cohort 2 were generated from frozen tumor tissues of 240 patients who underwent hepatectomy as primary treatment for HCC at Samsung Medical Center, Seoul, Korea (accession number GSE36376).[3] Data in Cohort 3 were collected from frozen tumor tissues of 242 patients who underwent hepatectomy as primary treatment for HCC at the Fudan University Liver Cancer Institute, Shanghai, China (accession number GSE14520).[4]

Immunohistochemical Staining of Human HCC Tissues

To assess correlation between YAP1 and mTORC1 activation in HCC tissues, 106 HCC tissue samples from 106 patients treated at Yonsei University Severance Hospital, Seoul, Korea, were used.

CTGF was stained using the Ventana automated immunostainer Discovery XT (Ventana Medical Systems, Tucson, AZ, USA). Briefly, the slides were deparaffinized using EZ Prep (Ventana Medical Systems) at 75°C for 4 minutes. Cell conditioning was performed using CC1 solution (Ventana Medical Systems) at 100°C for 32 minutes. HCC tissue arrays were incubated at room 37℃ for 36 min with a 1:300 dilution of CTGF rabbit monoclonal concentrated primary antibody (#ab5097; Abcam). Signals

were detected using the Discovery DAB IHC Detection Kit (Ventana Medical Systems). Counterstaining was performed with Hematoxylin I (Ventana Medical Systems) for 4 minutes at room temperature.

For detection of phophorylated S6 ribosomal protein, the sections were deparaffinized in xylene and rehydrated in graded ethanol. Heat inducible antigen retrieval was performed using citrate buffer (pH 6.0) in microwave oven for 15min and cool down in the running water for 20min. After immersing the slide in 3% hydrogen peroxide solution for 20min, the slides were incubated at room temperature for 1 hour with a 1:2,000 dilution of phospho-S6 ribosomal protein (S240/244) rabbit monoclonal concentrated primary antibody (#2215; CST), After rinsing, a secondary antibody (EnVision Rabbit/Mouse kit, Dako) was applied and then developed with 3,3'-diaminobenzidine (DAB). Slides were later counterstained with Mayer's hematoxylin.

Mst1/2-Deficient Mouse Models and Rapamycin Treatment

Generation and breeding of *Mst1*$^{fl/fl}$ and *Mst2*$^{fl/fl}$ mice were done as described in a previous report.[5] The mice were subsequently bred to albumin-Cre mice and then backcrossed to homozygous floxed mice. All mice were housed in a conventional facility with a 12-hour/12-hour light/dark schedule and access to food and water *ad libitum*. All procedures were approved by The University of Texas MD Anderson Cancer Center Animal Care and Use Committee. Rapamycin (LC Laboratories) was dissolved in ethanol at 50 mg/ml and aliquots stored at –80°C. A working solution was made by further dilution into an aqueous solution of 5% Tween 80 and 5% polyethylene glycol 400 to a concentration of 1 mg/ml immediately before use. Rapamycin or an equal volume of vehicle was injected intraperitoneally into *Mst1/2*$^{-/-}$ or *Mst1/2*$^{fl/fl}$ mice at a dose of 6 mg/kg of body weight. The treatment was started when the mice were 3 months of age and continued on a schedule of one injection per mouse every other day for 62 days.

Statistical Analysis

BRB-ArrayTools was primarily used for statistical analysis.[6] We identified genes that were differentially expressed between the two subtypes using t-test. Differences in gene expression were considered statistically significant if $p < 0.001$. This stringent significance threshold was chosen to minimize the number of false-positive findings. We also performed a global test of whether expression profiles differed between the subtypes by permuting the labels of which arrays corresponded to which subtype. For each permutation, the p-values were recomputed and the number of genes whose expression differed significantly between subtypes noted. A heat map of gene expression was generated using Cluster and TreeView software.[7]

Receiver operating characteristic curve analyses were carried out to estimate the discriminatory power of the two amino acid transporters. We calculated the area under

the curve, which ranges from 0.5 (for a noninformative predictive marker) to 1 (for a perfect predictive marker), and a bootstrap method (1000 resampling) was used to calculate the 95% CI for the area under the curve. We used multivariate Cox proportional hazards regression analysis to evaluate independent prognostic factors associated with overall survival time, using tumor stage, vasculature invasion, tumor grade, and number of liver tumors as covariates.[8] A p-value of <0.05 indicated statistical significance, and all statistical tests in this analysis were two-tailed.

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Supporting Table 1. Univariate and Multivariate Cox Regression Analyses of Overall Survival in HCC Patients in Cohort 1

Variable	Univariate Analysis		Multivariate Analysis for SLC38A1		Multivariate Analysis for SLC7A5	
	Hazard Ratio (95% CI)	P value	Hazard Ratio (95% CI)	P value	Hazard Ratio (95% CI)	P value
Patient Sex (Male or Female)	0.74 (0.34-1.62)	0.46				
Age (>60 years)	0.7 (0.35-1.47)	0.35				
AFP (>300 ng/ml)	1.6 (0.86-2.99)	0.13				
Cirrhosis (Present or not)	0.77 (0.4-1.47)	0.43				
T stage (2/3 or 1)	1.9 (1.0-3.5)	0.04	1.22 (0.53-2.8)	0.63	1.26 (0.52-3.0)	0.6
Tumor number (>1)	2.3 (0.72-7.6)	0.1	4.6 (1.2-17.1)	0.01	3.6 (1.0-12.7)	0.04
Grade (3/4 or ½)	1.6 (0.88-3.0)	0.1	1.1 (0.57-1.1)	0.79	1.4 (0.7-2.6)	0.33
Vasculature Invasion (Present or not)	2.2 (1.2-4.2)	0.01	2.1 (0.9-4.0)	0.08	2.3 (0.95-5.3)	0.06
SLC38A1 (High or Low)	1.98 (1.06-3.6)	0.02	1.9 (1.0-3.8)	0.04		
SLC7A5 (High or Low)	1.92 (1.02-3.6)	0.04			1.3 (1.2-4.3)	0.01

AFP, alpha fetoprotein.