

Supplementary Methods

1. Xenograft studies

All xenograft studies were carried out in 4-week-old female BALB/c nude mice and were approved by the Institutional Animal Care and Use Committee for Shanghai Public Health Clinical Center. For Sub-Q xenografts, the indicated cells suspended in 100 μ L Hanks buffered saline solution were injected subcutaneously into the lower flank of BALB/c nude mice. Tumor volumes were measured every 3 days starting from day 4 post-injection and calculated by multiplying length, width and depth measurements. When tumor reached $\sim$ 1 cm in diameter, mice were randomly divided into the indicated groups and the mean tumor volumes of each group were similar before the treatment started. No mice were excluded after the treatment started. To determine the effects of chemotherapy, tumor-bearing mice were treated with intraperitoneal (i.p.) sorafenib 30 mg/kg followed 2 hours later by 5-FU 50 mg/kg (all obtained from Sigma-Aldrich, Dorset, UK), twice weekly for 2 weeks. To interrupt Tyr metabolism, nitisinone (NTBC; Swedish Orphan International) was dissolved in 2 M NaOH and brought to neutral pH before administration. Nitisinone was given every other day via oral gavage at a dose of 2 mg/kg in a volume of 0.2-0.3 mL.

2. Cell proliferation assay

Cell proliferation rate was determined by XTT cell proliferation assay (Roche Diagnostics Co., Indianapolis, IN) according to the manufacturer's instructions. Absorbency was measured with a spectrophotometer (Tecan Sunrise, Tecan Trading AG, Switzerland) at 450 nm with a reference wavelength at 650 nm. Relative number of viable cells as compared to the number of cells without drug treatment was expressed as percent cell viability using the following formula: cell viability

(%)= A_{450} of treated cells/ A_{450} of untreated cells.

3. Apoptosis analysis

Cells were stained in binding buffer, propidium iodide (PI) and FITC-conjugated Annexin V as provided by the Annexin-V-FLUOS Staining Kit (Roche Diagnostics) according to manufacturer's instructions. Analysis was determined by a FACSCalibur flow cytometer and CellQuest software (BD Biosciences).

4. 3D Organoid Culture

To generate 3D organoids, each piece of the biopsies was rinsed in saline (0.9% NaCl) (S7653, Sigma-Aldrich, St. Louis, MO), and minced immediately with scissors and incubated for 45 minutes at 37°C in a 1 mL cocktail comprising 6.25 mg/mL dispase I (354235, BD Biosciences, San Jose, CA), 5 mg/ml collagenase (17018029, Thermo Fisher Scientific), 10 mM ROCK inhibitor Y27632 (1254, R&D Systems, Minneapolis, MN), and 0.5 mg/ml Fungizone (15290018, Thermo Fisher Scientific) with a periodical mixing (once every 10 minutes) via Vortex-Genie 2 (Scientific Industries, Bohemia, NY). The solution was replaced with - mL 0.25% trypsin/EDTA (R001100, Thermo Fisher Scientific) and incubated for 10 minutes at 37°C. The trypsin activity was then neutralized with 6 mL 250 mg/L soybean trypsin inhibitor (T9128, Sigma-Aldrich) in Dulbecco's phosphate-buffered saline (DPBS) (10010023, Thermo Fisher Scientific). The enzymatically dissociated tissue was forced through a 70 μ m cell strainer (22-363-548, Thermo Fisher Scientific) and cells were pelleted via centrifugation at 2,000 rpm for 5 minutes at room temperature. Cells were then resuspended in 1 mL advanced Dulbecco's modified Eagle medium (DMEM)/F12 (12491015, Thermo Fisher Scientific) containing 100 U/mL penicillin and 100 μ g/mL

streptomycin (15140122, Thermo Fisher Scientific), 1× GlutaMAX (35050061, Thermo Fisher Scientific), and 1× HEPES (15630080, Thermo Fisher Scientific) (aDMEM/F12⁺). The Countess II FL Automated Cell Counter (Thermo Fisher Scientific) was used to count cells where Trypan blue exclusion test was done to determine cell viability. The cell suspension (4×10^5 /mL) was mixed further with 10× volume of ice-cold Matrigel (354234, Corning, Corning, NY) and 2×10^4 cells in 50 µL of Matrigel were seeded into each well of the flat bottom 24-well plates. After solidification, 500 µL of aDMEM/F12⁺ supplemented with 1×N2 (17502048, Thermo Fisher Scientific), 1× B27 supplements (17504044, Thermo Fisher Scientific), 0.1 mM N-acetyl-L-cysteine (A7250, Sigma-Aldrich), 50 ng/mL recombinant human EGF (236-EG, R&D Systems), 2% Noggin/R-Spondin-conditioned media, 100 ng/mL recombinant human Wnt3A (5036-WN, R&D Systems), 500 nM A83-01 (2939, R&D Systems), 10 nM SB202190 (1264, R&D Systems), 10 nM gastrin (3006, R&D Systems), 10 nM nicotinamide (N0636, Sigma-Aldrich), and 10 µM Y27632, was added and replenished every other day. 3D organoids were grown for 10-14 days at 37°C in a humidified atmosphere of 5% CO₂. Under an inverted phase-contrast microscope (Apotome, Carl Zeiss, Jena, Germany), growing organoids were observed and photomicrographed to determine their number and size. Organoid formation rate was defined as the average number of ≥ 50 µm spherical structures at day 14 that was divided by the total number of cells seeded in each well at day 0. When indicated, 3D organoids were grown in the presence or absence of 5-FU or sorafenib at indicated concentrations after organoid structure was established at day 8. Isolated organoids were dissociated by incubation with 0.25% Trypsin-EDTA, Y27632 and DNase I (1010415901, Sigma-Aldrich) into single cell suspensions and passaged.

5. Western blotting

Quantified protein lysates were resolved on sodium dodecyl sulfate polyacrylamide gel electrophoresis gel, transferred onto PVDF membrane (Millipore, Billerica, MA) and immunoblotted with the indicated primary antibodies followed by incubation with secondary horseradish peroxidase. Blots were visualized with enhanced chemiluminescence (Amersham Biosciences, Piscataway, NJ). The primary antibodies used for western blotting analysis were as follows: acetylated-lysine (#9441), CBP (#7389) from Cell Signaling Technologies; Foxd3 (AB5687) from Merck Millipore; CD13 (AB108310), FAH (AB83770), MDM2 (ab16895) and HA (ab18181) from Abcam; Flag (F1084) from Sigma-Aldrich; TAT (aa279-306) from LifeSpan BioSciences; The secondary antibodies used for immunofluorescence were Alexa Fluor 488 or 594 goat anti-mouse (#A-11001, #A-11005) or rabbit anti-mouse (#A-11034, R37117) IgG from ThermoFisher Scientific. The HRP-conjugated secondary antibodies (#7076, #7074, and #7077) used for Western blot were from Cell Signaling Technologies.

6. Immunoprecipitation

Cells were collected and lysed in lysis buffer supplemented with protease inhibitors, incubated on ice for 15 minutes, and cleared by centrifugation at $8,000 \times g$ at 4°C for 15 minutes. Total protein lysates were subjected to immunoprecipitation with the agarose-immobilized antibody (0.01 mg of anti-Foxd3, CBP, Flag, HA, MDM2, acetylated-lysine antibody, or isotype control antibody) for overnight at 4°C .

7. Plasmids construction

The Foxd3 cDNA was amplified by PCR from HeLa cell cDNA (ClonTech

Laboratories Inc.) using the primers 5'-GGCACTCAAACCCTC TTCCCCTGAGCTCCG-3' and 5'-GCAGCCTGGA GGTGCATTTGTTGCT-3', and cloned into the pCMV/hygro-Flag expression vector (Sino Biological Inc.). The QuikChange site-directed mutagenesis kit (Stratagene) was used to mutate the Foxd3 acetylation site from lysine 181 to alanine (K181A) or arginine (K181R) or glutamine (K181Q), respectively. For *in vitro* translation, Foxd3 cDNA was also subcloned into pcDNA3.1 His/myc-tagged vectors (Invitrogen).

8. Lentivirus production and gene knockdown

shRNA constructs against Foxd3, CBP and a non-effective 29-mer shRNA cassette (termed control) were cloned into the pGFP-C-shLenti plasmid (OriGene). Lentivirus was produced by transfecting HEK 293T cells with the respective pGFP-C-shLenti plasmid and packaging plasmids using the packing mix (ViraPower Lentiviral Expression System, Invitrogen) as per manufacturer's instruction. CD13⁺ cells were transduced with recombinant lentivirus at a MOI of 3 viral particles/cell, and cultured overnight in the presence of 8 µg/mL polybrene (Sigma-Aldrich). After transfection of the indicated constructs, the cells were subjected to selection with 2 µg/mL puromycin (AG Scientific). Independent clones were selected and evaluated for silencing by western blotting. The sequence of the 29mer shRNAs are listed as follows: Foxd3: GGGAGGGAAATTCT TTGTATATATTTGTA; Foxd3#: CGCTCATCAAGTCCGAGCCAAGCGCGCGG; CBP: TTGTCACATTCTCA TTGTGAATTATGTAA.

9. Small interfering RNA

For siRNA inhibition, cells were transfected with validated FAH siRNA and

MDM2 siRNA (Ambion) at a final concentration of 100 nM in the presence of an Oligofectamine reagent (12252011, Invitrogen). The siRNA sense sequences were 5'-GCCACAAAUCUGAUA GUAU-3' (for MDM2), 5'-CUUCGGAAGUGUGCAUUCA-3' (for FAH) and 5'-GGACGCAUCCUUCUAAU-3' (for scrambled siRNA).

10. *In vivo* infusions of ¹³C-labeled nutrients and isolation of CD13⁺, CD13⁻ fractions from dissected tumors

Tracer studies were performed in BALB/c nude mice bearing HepG2 xenografts. Three mice in each group were infused with ¹³C-glucose (97%-99% enrichment; Cambridge Isotope Laboratories) as follows: a bolus of 0.4 mg/g body weight (0.3 mL) was infused in ~1 minute, followed by a continuous infusion of 0.012 mg/g/min for up to 300 minutes. This protocol yielded a final plasma glucose enrichment of 62%±10% (mean±SD). Three mice in each group were infused with ¹³C-glutamine (97%-99% enrichment; Cambridge Isotope Laboratories) as a bolus of 0.28 mg/g body weight (0.3 mL) administered over ~1 minute, followed by a continuous infusion of 0.005 mg/g for up to 300 minutes. This protocol yielded a final plasma glutamine enrichment of 55%±15% (mean±SD). Three mice in each group were infused with ¹³C-tyrosine (97%-99% enrichment; Cambridge Isotope Laboratories) as a bolus of 0.25 mg/g body weight (0.3mL) administered over ~1 minute, followed by a continuous infusion of 0.005 mg/g for up to 300 minutes. This protocol yielded a final plasma tyrosine enrichment of 57%±13% (mean±SD). At the indicated time points, mice infused with the ¹³C-labeled nutrients were euthanized by decapitation with anesthesia. Tumors were rapidly dissected and slowly thawed on ice and then cut into 5mm³ pieces with tweezers and scalpel in a Petri dish on ice. Cut pieces were

loaded with 50 mL of phosphate-buffered saline (PBS) into a sterile Medicon chamber, a component of the MediMachine (BD Biosciences). Tissue was disaggregated within the chamber by microblades spinning at constant 100 rpm for 3 minutes. Minced tissue was then aspirated out of the chamber and forced into single cell suspension using a 70 μ m syringe filter (BD Biosciences). The single cell suspension was incubated with the anti-CD13-PE antibody under 4°C for 30min and centrifuged at 1500 r/min for 10 minutes. The supernatant was discarded and the cell concentration was adjusted to 1×10^7 /mL, after which CD13⁺ and CD13⁻ cells were sorted using a FACS Aria cell sorter (BD Biosciences) and collected separately in two flow tubes for subsequent mass spectrometry-based analysis of metabolite abundance.

11. Subcellular fractionation and fractionation validation

Collected cells were washed with cold PBS and pelleted by centrifugation at 200g for 7 minutes. All samples were resuspended in 300-500 μ L of STM buffer comprising 250 mM sucrose, 50 mM Tris-HCl pH 7.4, 5 mM MgCl₂, protease and phosphatase inhibitor cocktails (all chemicals were from Sigma-Aldrich) and homogenized for 1 minute on ice using a tight-fitting Teflon pestle attached to a Potter S homogeniser (Sartorius Stedium) set to 600-1,000 rpm. The homogenate was decanted into a centrifuge tube and maintained on ice for 30 minutes, vortexed at maximum speed for 15 seconds and then centrifuged at 800 $\times$ g for 15 minutes. The pellet was labelled as P0 and kept on ice, the supernatant was labelled as S0 and used for subsequent isolation of mitochondrial and cytosolic fractions. The pellet P0 (containing nuclei and debris) was resuspended in 300-500 μ L STM buffer, vortexed at maximum speed for 15 seconds and then centrifuged at 500 $\times$ g for 15 minutes. Following the above step, the nuclear pellet was labelled as P1 and kept on ice, the

supernatant (cell debris) was discarded. To increase the P1 fraction purity further it was washed in STM buffer (300-500 μ L), vortexed at maximum speed for 15 seconds and then centrifuged at 1,000 $\times$ g for 15 minutes. The washed pellet was labelled as P2 and resuspended in 200-500 μ L NET buffer (comprising: 20mM HEPES pH 7.9, 1.5 mM $MgCl_2$, 0.5 M NaCl, 0.2 mM EDTA, 20% glycerol, 1% Triton-X-100, protease and phosphatase inhibitors) using a pipette to triturate until homogeneous. Pellet P2 was vortexed at maximum speed for 15sec and incubated on ice for 30 minutes, this fraction contained the nuclei. The nuclei were lysed with 10-20 passages through an 18-gauge needle at high setting for 10-15 seconds with 30 seconds pauses whilst being kept on ice throughout. The lysate was centrifuged at 9,000 $\times$ g for 30 minutes (at 4°C), the resultant supernatant was the final “nuclear fraction.” Cytosolic and mitochondrial fractions were extracted from S0 by centrifugation at 800 $\times$ g for 10 minutes. The supernatant S2 was then centrifuged at 11,000 $\times$ g for 10 minutes to produce the supernatant S3 (containing cytosol and microsomal fraction) and the pellet P3 (containing mitochondrial fraction). The supernatant S3 was precipitated in cold 100% acetone at -20°C for at least 1 hour followed by centrifugation at 12,000 $\times$ g for 5 minutes, the pellet (P4) was then resuspended in 100-300 μ L STM buffer and labelled as “cytosolic fraction” that likely included some microsomal content. Meanwhile, the pellet P3 was resuspended in 100-200 μ L STM buffer and centrifuged at 11,000 $\times$ g for 10 minutes. Once centrifuged, supernatant was discarded, the mitochondrial pellet (P4) was resuspended in 50-100 μ L SOL buffer (comprising: 50 mM Tris-HCl pH 6.8, 1 mM EDTA, 0.5% Triton-X-100, protease and phosphatase inhibitors) by sonication on ice at high setting for 5-10 seconds with 30 seconds pauses and labelled as “mitochondrial fraction.” Validation of the purity of the subcellular fractions derived from the same starting sample was determined by

examining “house-keeper” (HK) protein markers by standard sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis. Protein samples (20 µg/fraction) were separated by a 12% SDS-PAGE and transferred onto a nitrocellulose membrane (Sigma) and probed using monoclonal antibodies for Histone H3 (Cell Signaling, #9715), GAPDH (Abcam, ab8245), and Cox IV (Abcam, ab16056) for nuclei, cytosolic and mitochondrial HK fractions respectively.

12. Mass spectrometry for intermediates in Tyr metabolism.

For metabolite collection, media from biological triplicates was fully aspirated and 4 mL of 80% (v/v) methanol was added at dry ice temperatures. Cells and the metabolite-containing supernatants were collected into conical tubes. Insoluble material in lysates was centrifuged at 2,000 ×g for 15 minutes, and the resulting supernatant was evaporated using a refrigerated SpeedVac. Samples were resuspended using 20 mL HPLC grade water for mass spectrometry. Ten microliters were injected and analyzed using a 5500 QTRAP hybrid triple quadrupole mass spectrometer (AB/SCIEX) coupled to a Prominence UFLC HPLC system (Shimadzu) via selected reaction monitoring (SRM) of a total of 254 endogenous water soluble metabolites for steady-state analyses of samples. Some metabolites were targeted in both positive and negative ion mode for a total of 285 SRM transitions using positive/negative switching. ESI voltage was +4,900 V in positive-ion mode and –4,500 V in negative ion mode. The dwell time was 4ms per SRM transition and the total cycle time was 1.89 seconds. Approximately 9-12 data points were acquired per detected metabolite. Samples were delivered to the MS via normal phase chromatography using a 4.6 mm i.d×10 cm Amide Xbridge HILIC column (Waters Corp.) at 300 mL/min. Gradients were run starting from 85% buffer B (HPLC grade acetonitrile) to 42% B from 0-5

minutes; 42% B to 0% B from 5-16 minutes; 0% B was held from 16-24 minutes; 0% B to 85% B from 24-25 minutes; 85% B was held for 7 minutes to re-equilibrate the column. Buffer A was comprised of 20 mM ammonium hydroxide/20mM ammonium acetate (pH 9.0) in water: acetonitrile (95:5). Peak areas from the total ion current for each metabolite SRM transition were integrated using MultiQuant v2.0 software (AB/SCIEX). For ^{13}C labeled experiments, SRMs were created for expected ^{13}C incorporation in various forms for targeted LC-MS/MS. Data analysis was performed in Cluster3.0 and TreeViewer.

13. Isotopomer data analysis

The extent of isotopic labeling for each metabolite was determined by dividing the summed peak height ion intensities of all labeled species by the ion intensity of both labeled and unlabeled species, expressed in percent. Label-specific ion counts were corrected for naturally occurring ^{13}C species (i.e., [M+1] and [M+2]). The relative abundance of each isotopically labeled species was determined by dividing the peak height ion intensity of each isotopic form (corrected for naturally occurring ^{13}C species as above) by the summed peak height ion intensity of all labeled species.

14. Glucose uptake assay

Glucose uptake assay was performed using Wako Glucose kit according to manufacturer's protocol.

15. Lactate secretion

Lactate secretion was determined using Trinity Lactate Kit according to manufacturer's protocol.

16. Histone preparation for mass spectrometry

Histone extraction was performed using the commercially available EpiQuik Global Histone Acetylation Assay kit (Epigentek). Briefly, isolated nuclei and whole cells were lysed and proteins were precipitated with 25% trichloroacetic acid. Extracted histone pellets were dissolved in HPLC grade distilled water.

17. Mass spectrometry for protein acetylation

Protein was resolved by SDS-PAGE, and the band to be analyzed was excised from the gel by the use of a razor blade and divided into $\sim 1 \text{ mm}^3$ pieces. Gel pieces were destained using 100 mM ammonium bicarbonate (pH 8.9) in 50% acetonitrile and then treated with 100 μL of 50mM ammonium bicarbonate (pH 8.0) and 10 μL of 10 mM TCEP [Tris(2-carboxyethyl)phosphine HCl] at 37°C for 30 minutes. Protein digestion was carried out using 1:50 sequencing-grade trypsin or chymotrypsin in 50mM ammonium bicarbonate (pH 7.5). Digested peptide samples were desalted with a C8 OptiPak column (Optimize Technologies) and analyzed by liquid chromatography-electrospray tandem mass spectrometry (LC-ESI/MS/MS) on a Thermo LTQ Orbitrap Hybrid FT mass spectrometer. Positive-ion mass spectra were acquired in the reflectron mode. Ions selected for MS/MS were subsequently placed on an exclusion list using an isolation width of 1.6Da, a low-mass exclusion of 0.8Da, and a high-mass exclusion of 0.8Da. Tandem mass spectra were extracted by Readw.exe ver. 3.0. All MS/MS samples were analyzed using Mascot (Matrix Science, London, UK) data explorer software, and Scaffold (version Scaffold_2.1.03; Proteome Software, Inc., Portland, OR) was used to validate MS/MS-based peptide and protein identifications.

18. Sphere formation assays

Cells were resuspended in serum-free medium in DMEM/F12 medium (Invitrogen) containing 20ng/mL EGF (Sigma-Aldrich), 10 ng/mL FGF2 (Sigma-Aldrich), B27 (GIBCO), and ITSS (Roche). Cells were plated onto ultralow attachment plates (Corning). Every three days, 200 μ L DMEM/F12 medium containing EGF, FGF2 was added. Seven days after seeding, the number of spheroids was measured under a microscope. For serial passage of spheres, tumor spheres were dissociated into single-cell suspension and passaged at a ratio of 1:3 and cultured under conditions described above, after which new tumor spheres formed within 1 week were counted.

19. Limiting dilution analysis

For *in vivo* limiting dilution assays, transduced CD13⁺ cells were dissociated into single cells or diluted serially to the desired cell doses. Cells were injected subcutaneously in the axillary region of the right chest in BALB/c nude mice, and the number of tumors formed out of the number of sites injected was scored to determine the frequency of CSCs calculated using the ELDA software.

20. Cell cycle analysis

FACS-sorted CD13⁺ CSCs were incubated for 45 minutes at 37°C with 20 μ g/mL Hoechst 33342 (Invitrogen) in DMEM/F12 medium containing 20 ng/mL EGF (Sigma-Aldrich), 10 ng/mL FGF2 (Sigma-Aldrich), B27 (GIBCO), and ITSS (Roche). Pyronin Y (Sigma-Aldrich) was then added at 1 μ g/mL, and the cells were incubated for another 15 minutes at 37°C, washed, and immediately analyzed using a MoFlow

cytometer (Cytomation) and FlowJo program (Cytomation).

21. 3D Matrigel differentiation assay

For 3D Matrigel cultures, trypsin-dissociated spheroid cultures were dissolved in ice-cold, liquid Matrigel and allowed to solidify at 37°C before addition of CSC culture medium. Treatment were added to the 3D Matrigel cultures 2 days thereafter and continued for 4 days. Quantification of differentiated spheres was performed by three independent observers that were blinded for the treatment.