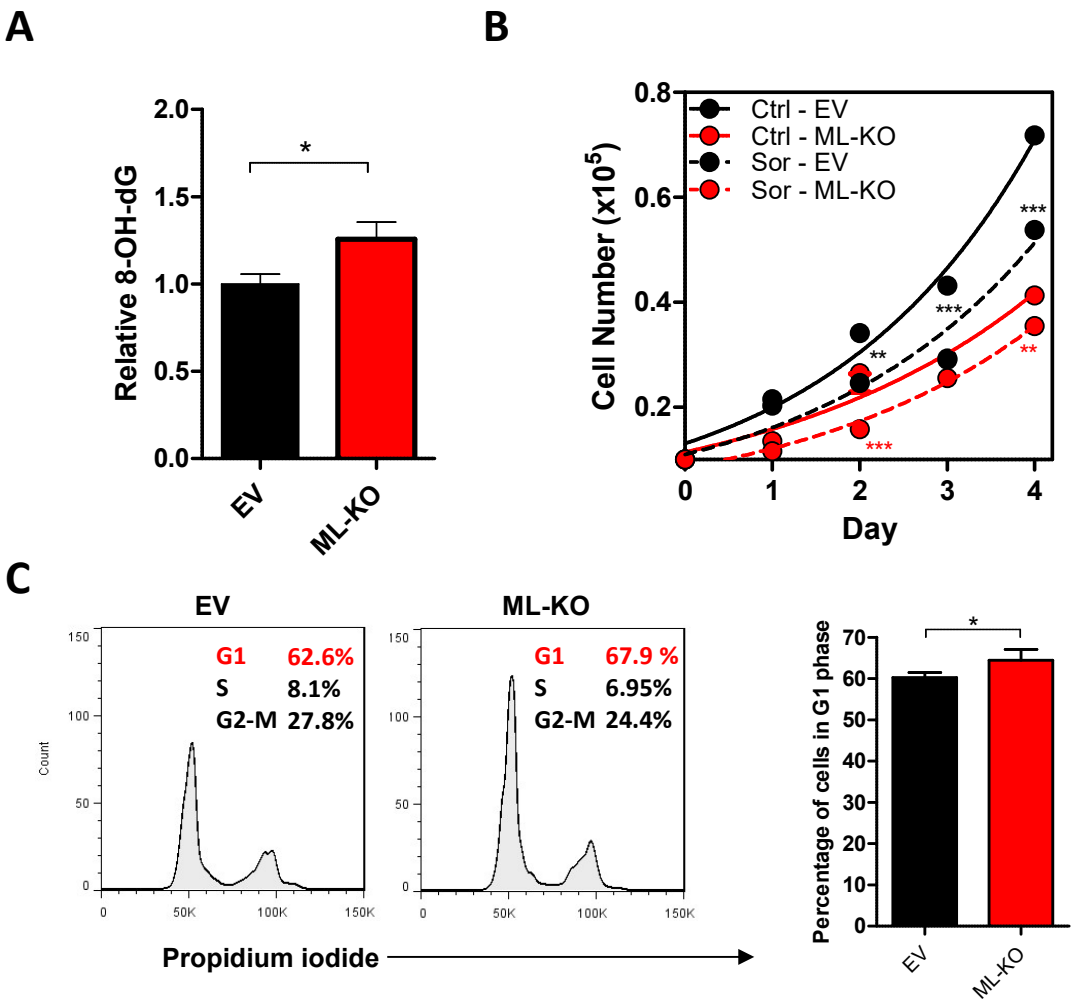


Figure S1. **Knockout of MTHFD1L increased 8-OH-dG, cell cycle delay, and sensitized HCC cells to Sorafenib treatment.** (A) Quantification of the relative amounts of 8-OH-dG in MHCC97L-EV, –ML-KO cells. (B) Cell proliferation rate of MHCC97L-EV, –ML-KO cells cultured in vehicle control (Ctrl) and Sorafenib (2.5μM) (Sor). Equal number of cells were seeded on Day 0 and cell numbers were determined by cell counting. (C) Left: representative diagrams of flow cytometry analysis of MHCC97L-EV, –ML-KO cells which were synchronized using 200 ng/mL nocodazole for 16 hours and released 15 hours before propidium iodide (PI) staining. Right: Percentage of cells in G1 phase for each group. Results are representative of 3 independent experiments. Error bars indicate mean ± SEM. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 Vs EV of the same conditions at the indicated time points. A, C: Student's *t* test. B: 1-way ANOVA.

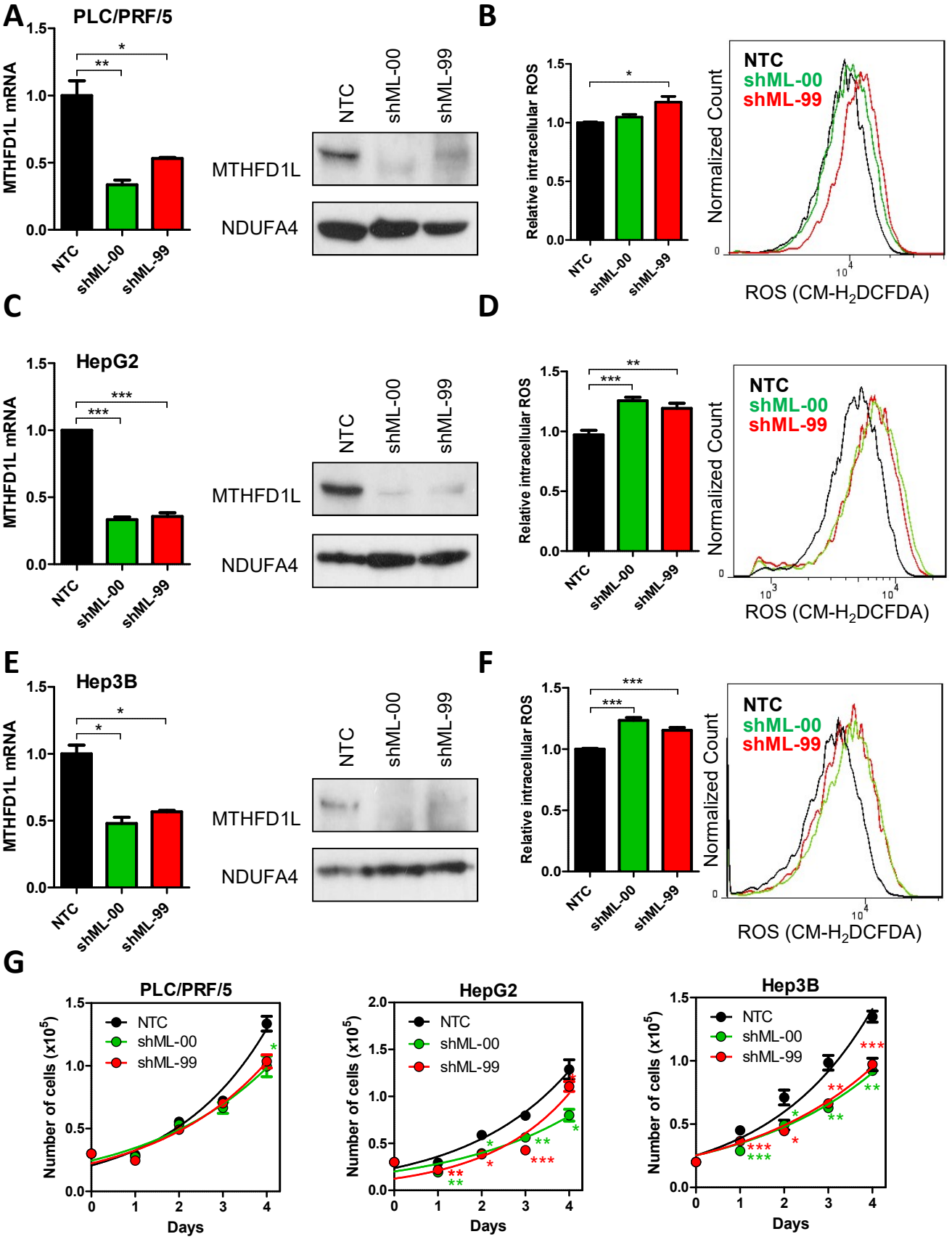


Figure S2. Knockdown of MTHFD1L in multiple HCC cell lines increased ROS. (A) MTHFD1L mRNA and protein expressions in PLC/PRF/5-NTC, -shML-00, -shML-99 stable cells. (B) Quantification (left) and representative flow cytometry analysis (right) of intracellular ROS levels in PLC/PRF/5-NTC, -shML-00, -shML-99 stable cells. (C) MTHFD1L mRNA and protein expressions in HepG2-NTC, -shML-00, -shML-99 stable cells. (D) Quantification (left) and representative flow cytometry analysis (right) of Intracellular ROS levels in HepG2-NTC, -shML-00, -shML-99 stable cells. (E) MTHFD1L mRNA and protein expressions in Hep3B-NTC, shML-00, shML-99 stable cells. (F) Quantification (left) and representative flow cytometry analysis (right) of Intracellular ROS levels in Hep3B-NTC, -shML-00, -shML-99 stable cells. (G) Cell proliferation of PLC/PRF/5, HepG2, Hep3B-NTC, -shML-00,- shML-99 stable cells. Results are representative of 3 independent experiments. Error bars indicate mean ± SEM. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 Vs NTC. 1-way ANOVA.

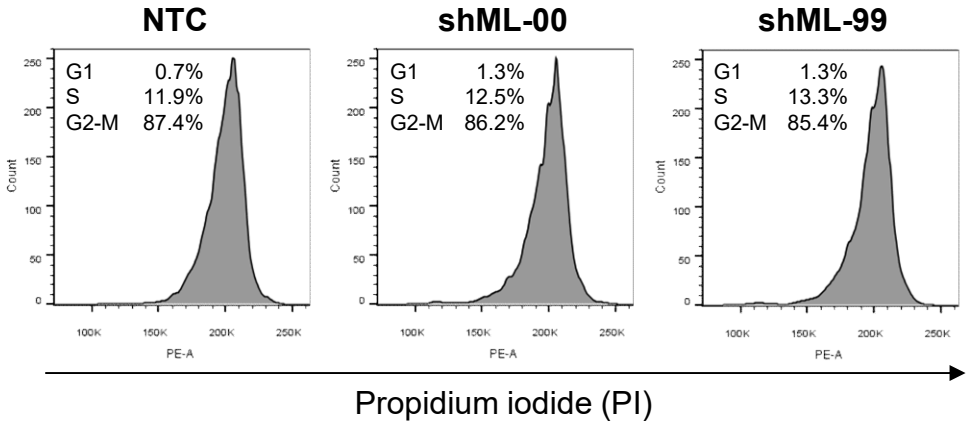


Figure S3. Synchronization of HCC cells in G2/M phase. Representative flow cytometry analysis showing the cell cycle profiles of MHCC97L-NTC, -shML-00, and -shML-99 cells right after nocodazole treatment. Cells were uniformly synchronized at the G2/M phase. Results are representative of 3 independent experiments.

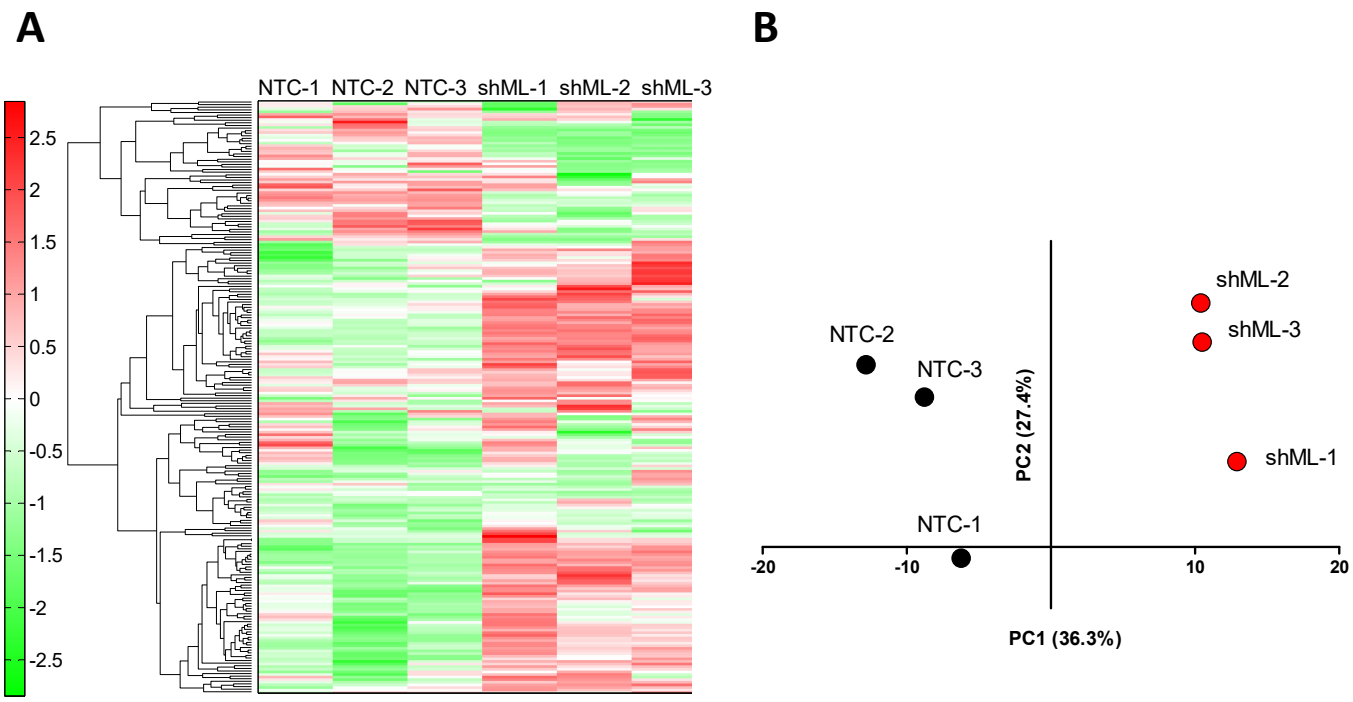


Figure S4. Metabolite profiles of MTHFD1L knockdown HCC cells. (A) Hierarchical clustering analysis of 228 metabolites in MHCC97L-NTC (NTC-1, NTC-2, NTC-3) and –shMTHFD1L (shML-1, shML-2, shML-3) cells in triplicates is plotted as a heat map diagram. One row represents a single intracellular metabolite. Increasing levels of metabolites are illustrated as increasing intensity of red while decreasing levels of metabolites are illustrated as increasing intensity of green. Levels of metabolites are calculated relative to internal control in the CE-TOF-MS. (B) Principal component (PC) analysis demonstrates that NTC triplicates cluster together as a group while MTHFD1L knockdown triplicates cluster together as another group, confirming the metabolite extraction was consistent.

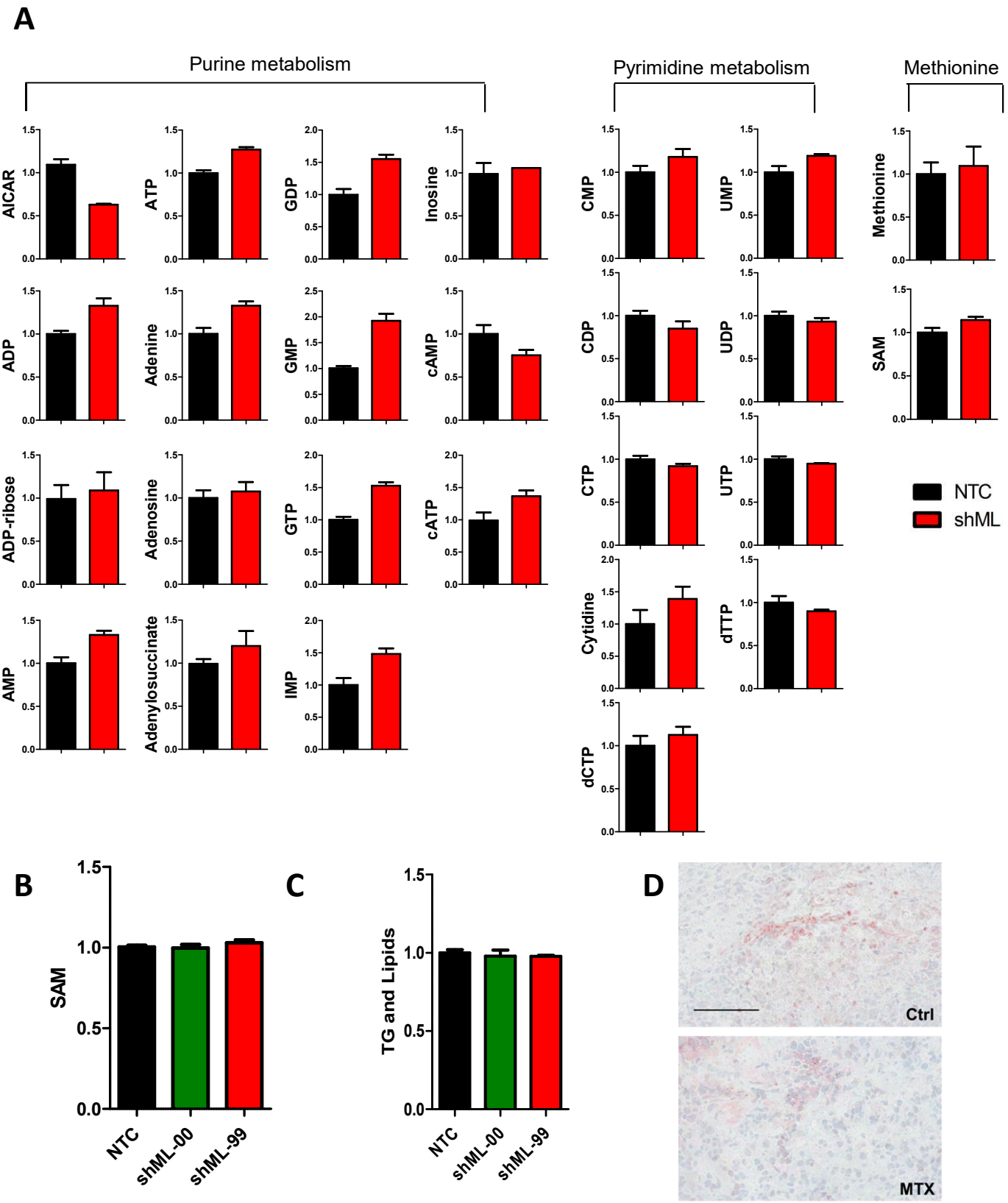


Figure S5. Knockdown of MTHFD1L did not reduce nucleotides, methionine, SAM, and lipids. (A) Quantification of metabolites by CE-TOFMS. AICAR, ADP, ADP-ribose, AMP, ATP, adenine, adenosine, adenylsuccinate, GDP, GMP, GTP, IMP, inosine, cAMP, cATP, CMP, CDP, CTP, cytidine, dCTP, UMP, UDP, UTP, dTTP, methionine, SAM in MHCC97L-NTC and -shML-99 cells. (B) Levels of SAM were further measured in MHCC97L-NTC, shML-00, shML-99 cells by ELISA-based SAM quantification assay (Cloud-Clone Corp). Levels of metabolites are calculated relative to NTC. (C) Levels of triglyceride (TG) and lipids in MHCC97L-NTC, -shML-00, shML-99 cells were measured by Oil Red O staining assay. (D) Oil Red O staining on mouse HCC tumors (Hepa1-6) from C57BL/6N immune competent mice which were treated with vehicle control (Ctrl) and methotrexate (6mg/kg/day) (MTX) for 7 days. A, B, C: Results are representative of 3 independent experiments. Error bars indicate mean ± SEM. D: scale bar = 100 μm.

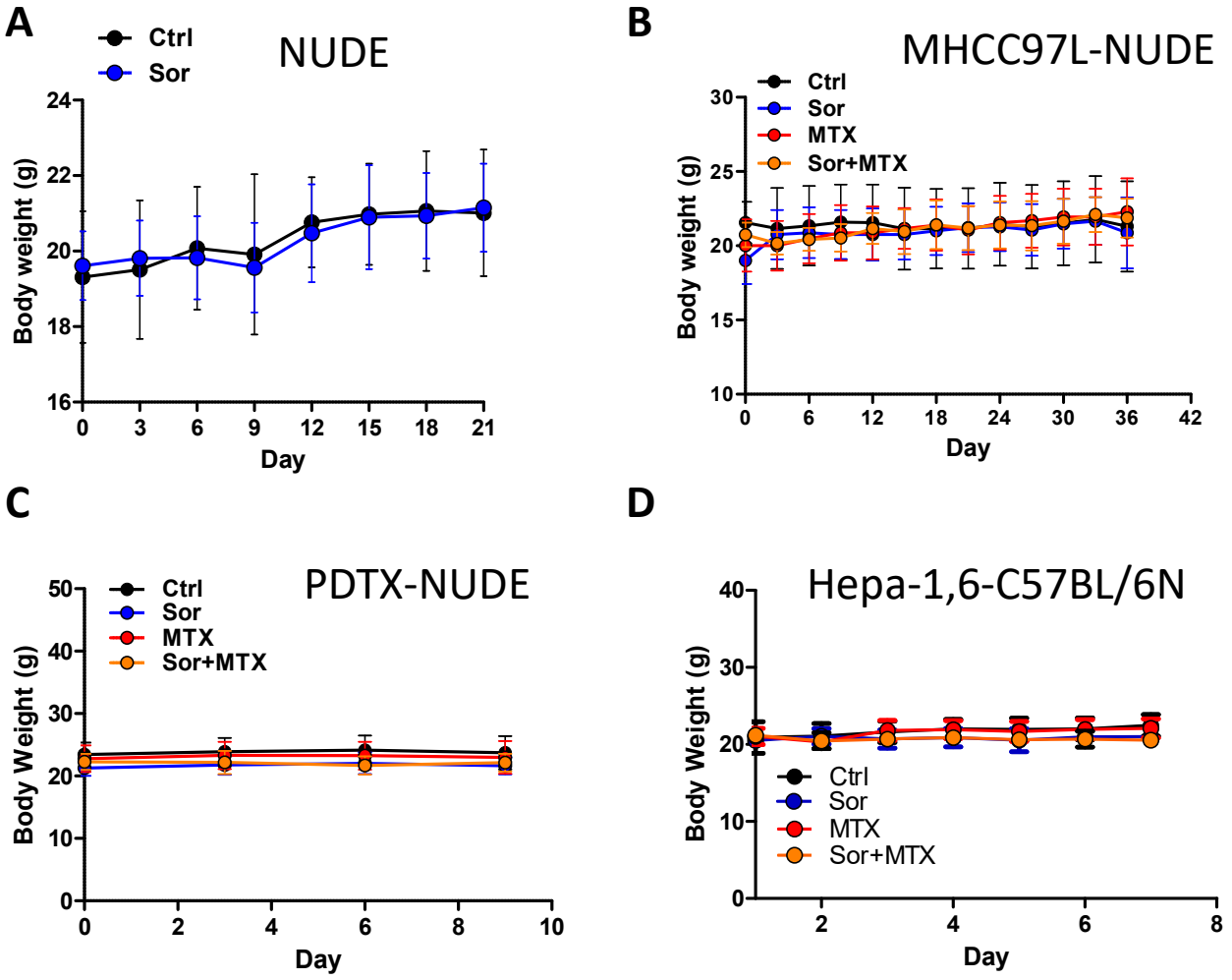
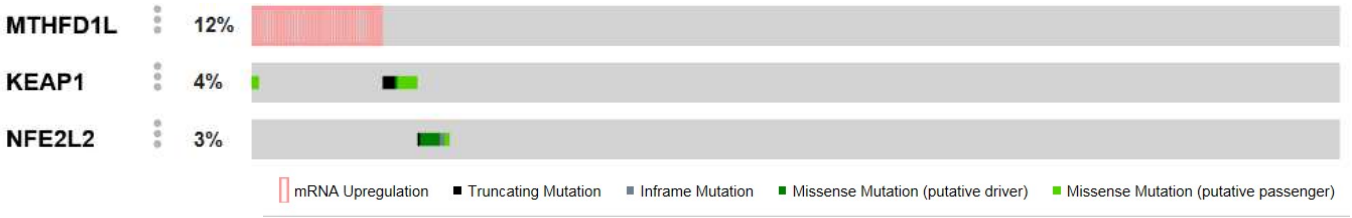


Figure S6. Body weights of mice after drug administration. (A) Nude mice were subcutaneously injected with MHCC97L-NTC, and –shML cells on the left and right flanks, respectively. After 1 week of injection, mice were administered with vehicle control (Ctrl) or Sorafenib (Sor) daily and body weights were recorded every other two days (*n* = 6 mice per experimental group). (B) Nude mice were subcutaneously injected with parental MHCC97L cells on the left and right flanks. After 1 week of injection, mice were administered with vehicle control (Ctrl), Sorafenib (Sor), methotrexate (MTX), or both (Sor+MTX). Body weights were recorded every other two days (*n* = 10 mice per experimental group). (C) Nude mice were subcutaneously injected with PDTX on the right flanks. After 11days of inoculation, mice were administered with vehicle control (Ctrl), Sorafenib (Sor), methotrexate (MTX), or both (Sor+MTX). Body weights were recorded every other two days (*n* = 4 mice per experimental group). (D) C57BL/6N immune competent mice (syngeneic) were orthotopically injected with Hepa1-6 cells. 4 days after implantation, mice were administered either with vehicle control (Ctrl), Sorafenib (Sor), methotrexate (MTX), or combination of both (Sor+MTX) for 7 days. Body weight of the mice throughout the treatment (*n* = 6 mice per experimental group).



	MTHFD1L high	MTHFD1L low			
KEAP1 WT	50	373		P = 0.431	q = 0.861
KEAP1 Mut	3	14			
NFE2L2 WT	53	374		P = 0.137	q = 0.861
NFE2L2 Mut	0	13			

MTHFD1L High: z-score > 1
MTHFD1L Low: z-score < 1

Figure S7. Mutation status of KEAP1 and NFE2L2 (NRF2) genes in HCC patients with MTHFD1L up-regulation. Data of MTHFD1L expression and mutation status of KEAP1 and NFE2L2 genes in 440 HCC samples were obtained from TCGA database . MTHDF1L up-regulation (z-score > 1) was detected in 12% of HCC samples. Mutation of KEAP1 and NFE2L2 were detected in 4% and 3% of HCC samples, respectively. We found no significant correlation between MTHFD1L up-regulation and KEAP1 or NFE2L2 mutations. Chi-square test.

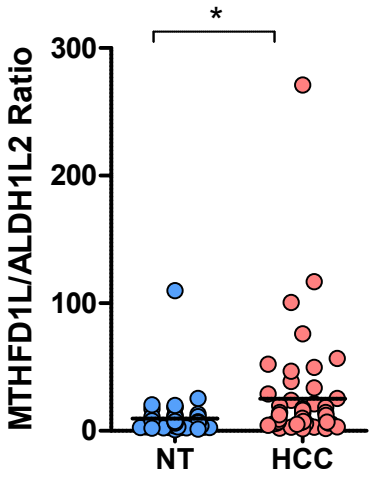


Figure S8. MTHFD1L/ALDH1L2 ratio in HCC. MTHFD1L and ALDH1L2 mRNA expressions in NT and HCC tissues from TCGA database were used to determine MTHFD1L/ALDH1L2 ratio. *P < 0.05, Student's t test.

Table S1 Demographic data of HCC patients at Queen Mary Hospital

Ethnic group	Chinese	85 (100%)
Age	Mean ± SD	53.98 ± 11.76
Gender	Male	64 (76%)
	Female	20 (24%)
HBV infection	Positive	67 (81%)
	Negative	16 (19%)
Cirrhosis	Present	43 (53%)
	Absent	38 (47%)
Tumor size	< 5 cm	31 (39%)
	≥ 5 cm	49 (61%)
pTNM stage	I/II	32 (39%)
	III/IV	50 (61%)

Table S2 Primer sequence

Primer	Sequence
MTHFD1L-Forward	CAACATCAAGTGCCGAGCTT
MTHFD1L-Reverse	AAGAGGAACACCAGCCGTTA
NRF2-Forward	GGTTGCCCACATTCCCAAAT
NRF2-Reverse	AGCAATGAAGACTGGGCTCT
ChIP-MTHFD1L-Site 1- Forward	GGAAGCTCACAAGGCCATTT
ChIP-MTHFD1L--Site 1- Reverse	TTGGGGTTACTCATTCCTACAG
ChIP-MTHFD1L-Site 2- Forward	ACATTTCTGGGCTCCACTGA
ChIP-MTHFD1L--Site 2- Reverse	GGTGAAACCCCATCTCTAGT
ChIP-MTHFD1L-Site 3- Forward	CCTGAGTAGCTAGGACTGCA
ChIP-MTHFD1L--Site 3- Reverse	TTGAGCCCAGGAGTTCGAG
18S-Forward	GAGGATGAGGTGGAACGTGT
18S-Reverse	AGAAGTGACGCAGCCCTCTA

Table S3 shRNA and sgRNA target sequences

shRNA/sgRNA	Target Sequence
shMTHFD1L-00	GGCCAAAGCTGTAATTGAACTTCT
shMTHFD1L-99	GCTCTGTATAATCGGCTGGTTC
shNRF2-1	GCTCCTACTGTGATGTGAAAT
shNRF2-2	AGTTTGGGAGGAGCTATTATCC
sgNRF2-dCas9	GTCGCCTTCGGGCTGGGACC
sgMTHFD1L-dCas9	ACCTCGGCCTTTCAGGCTGA
sgMTHFD1L-Cas9	CCGCCGCTGCTAGCGCGACA