

Supplementary Data

Epigenetic repression of *de novo* cysteine synthetases induces intra-cellular accumulation of cysteine in hepatocarcinoma by up-regulating the cystine uptake transporter xCT

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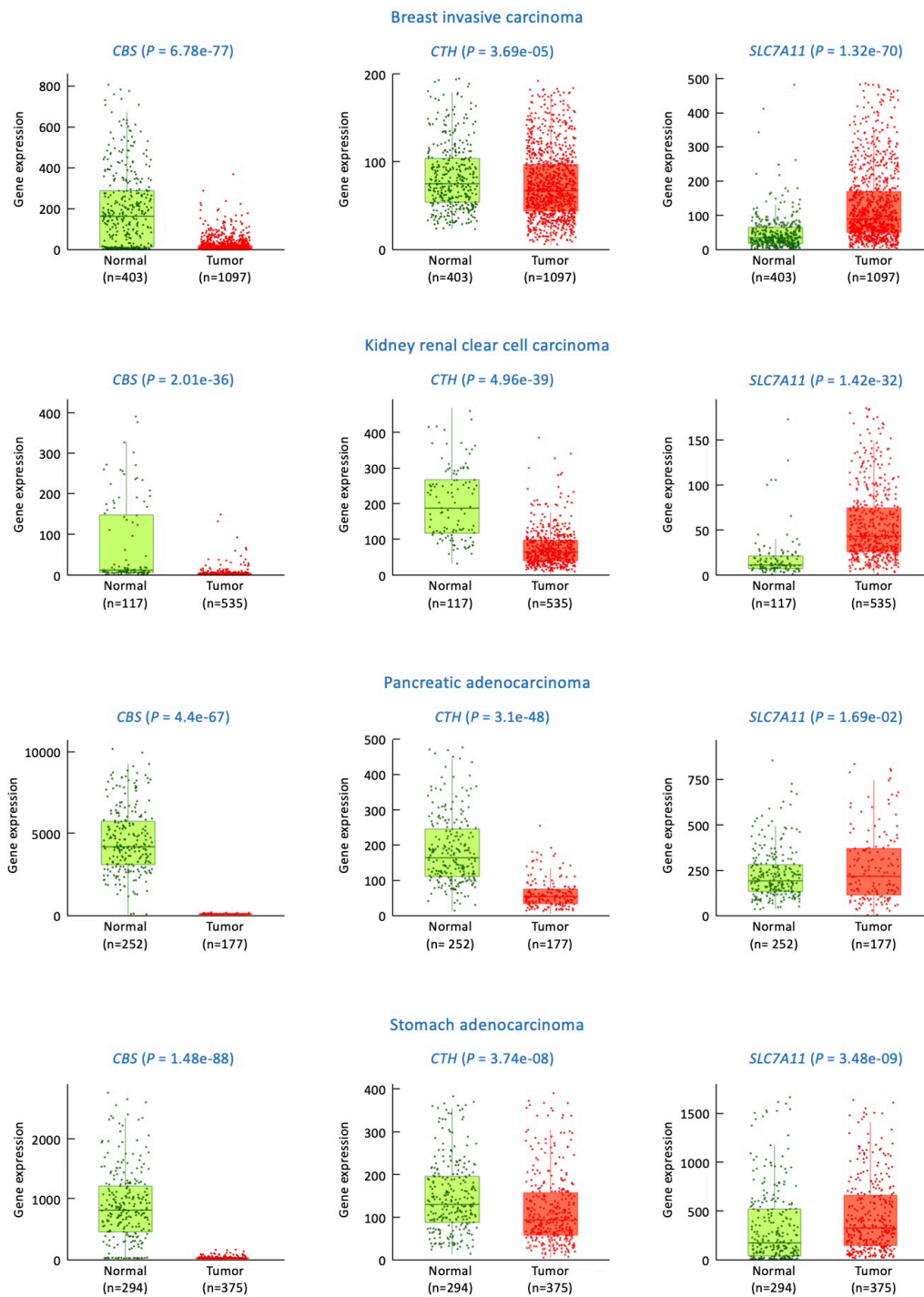
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Supplementary Table S1 Primer sets for DNA methylation analysis

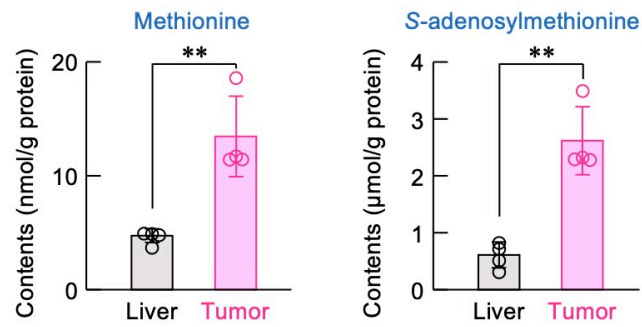
Gene	Primer sequence	
Mouse <i>Cbs</i> promoter	Forward	5'-GTTTAGGTATAGAGGAAGTTAAGTT-3'
	Reverse	5'-ACTCTCCATCCTAACACAAACC-3'
Mouse <i>Cth</i> promoter	Forward	5'-GATAAGGTGTAAAATTAGGTAAAGTTA-3'
	Reverse	5'-CCCCTTTATCTACAAAATAAAAAC-3'

Supplementary Table S2 Primer sets for quantitative RT-PCR analysis

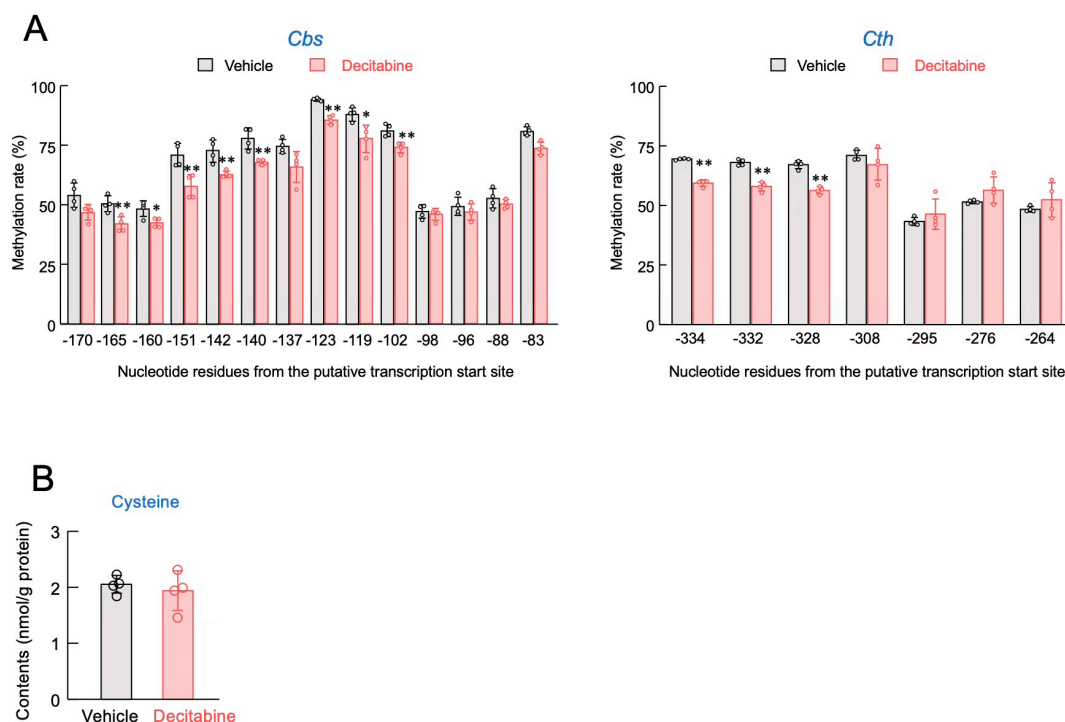
Gene	Primer sequence	
Human / Mouse <i>18s</i>	Forward	5'-CGGCTACCACATCCAAGGAA-3'
	Reverse	5'-GCTGGAATTACCGCGGCT-3'
Human <i>CBS</i>	Forward	5'-GCGGCTGAAGAACGAAATCC-3'
	Reverse	5'-TGTCCAGCTTCCCATCACAC-3'
Human <i>CTH</i>	Forward	5'-GCCAGCACTCGGGTTTTGA-3'
	Reverse	5'-TGGCTGCTAAACCTGAAGCAA-3'
Human <i>SLC7A11</i>	Forward	5'-TGGACGGTGTGTGGGGTCCT-3'
	Reverse	5'-CAGCAGTAGCTGCAGGGCGTA-3'
Mouse <i>Cbs</i>	Forward	5'-GGGACAAGGATCGAGTCTGGA-3'
	Reverse	5'-AGCACTGTGTGATAATGTGGG-3'
Mouse <i>Cth</i>	Forward	5'-TTCCTGCCTAGTTTCCAGCAT-3'
	Reverse	5'-GGAAGTCCTGCTTAAATGTGGTG-3'
Mouse <i>Slc7a11</i>	Forward	5'-GAGCCCTGTCCTATGCAGAA-3'
	Reverse	5'-CAGTAGCTCCAGGGCGTATT-3'
Mouse <i>Dnmt1</i>	Forward	5'-AGAGACCAGGATAAGAAACGCA-3'
	Reverse	5'-CTCCTTTGATTTCCGCCTCAAT-3'
Mouse <i>Dnmt3a</i>	Forward	5'-GATGAGCCTGAGTATGAGGATGG-3'
	Reverse	5'-CAAGACACAATTCGGCCTGG-3'
Mouse <i>Dnmt3b</i>	Forward	5'-CCTGTGGAGTTTCCGGCTAC-3'
	Reverse	5'-GACGCTCTTAGGTGTCACTTC-3'



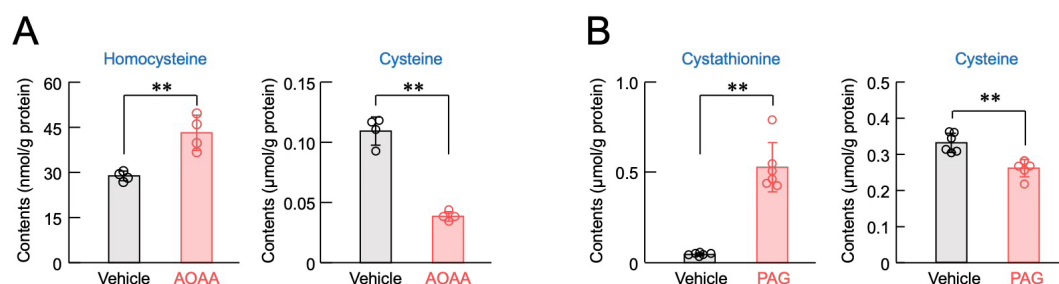
Supplementary Figure S1 Oncogenic alterations in the expression of cysteine related genes in patients with malignant cancers. The RNA-seq data obtained from patients with breast invasive carcinoma, kidney renal clear cell carcinoma, pancreatic adenocarcinoma, and stomach adenocarcinoma were analyzed using the TNMplot database. P -values were calculated using the Mann–Whitney U-test.



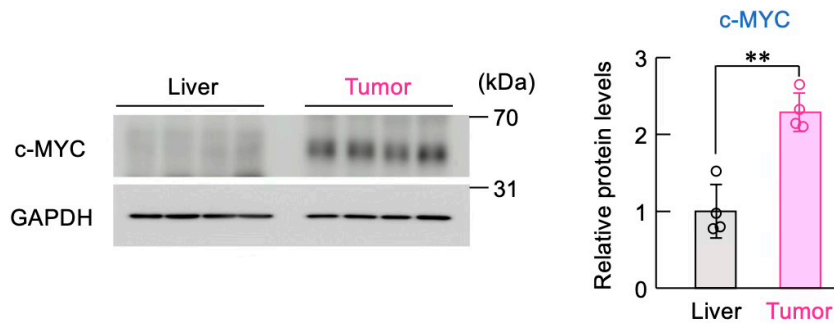
Supplementary Figure S2 Intracellular contents of methionine and S-adenosylmethionine in the liver and BNL 1ME A.7 R.1-formed tumors in mice. Each value represents the mean with S.D. ($n = 4$). ** $P < 0.01$; significant difference between the two groups ($t_6 = 4.861$, $P = 0.003$ for methionine; $t_6 = 6.283$, $P = 0.001$ for S-adenosylmethionine; unpaired t -test, two sided).



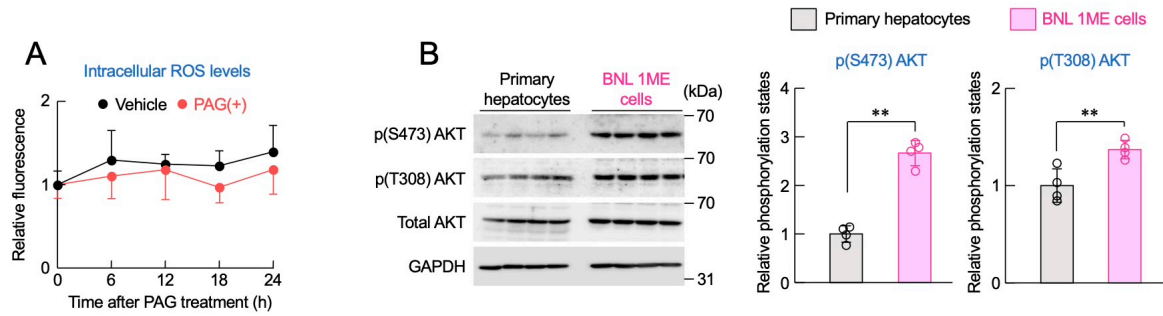
Supplementary Figure S3 Altered DNA methylation levels in the cysteine synthetic genes in hepatocarcinoma cells after decitabine treatment. **A**, Each panel represents the DNA methylation levels in the 5'-flanking region of mice *Cbs* (left) and *Cth* (right) genes. Cells were treated with methyltransferase inhibitor 500 nM decitabine for 24 h. Each value represents the mean with S.D. (n = 4). ** $P < 0.01$, * $P < 0.05$; significant difference compared with vehicle-treated cells (unpaired *t*-test, two sided). **B**, Intracellular cysteine levels in BNL 1ME A.7 R.1 cells after treatment with 500 nM decitabine for 24h. Each value represents the mean with S.D. (n = 4).



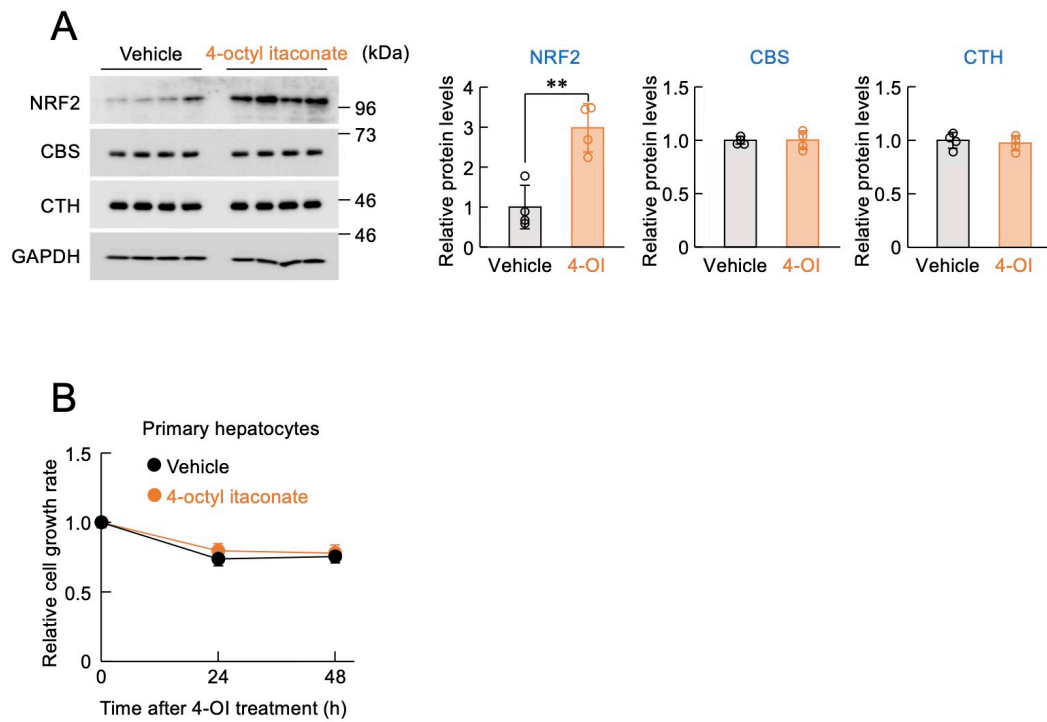
Supplementary Figure S4 Intracellular levels of cysteine-related metabolites in primary hepatocytes after treatment with inhibitors for cysteine synthases. Cells were treated with 1.5 mM AOAA (A) or 1.0 mM PAG (B) for 24 h. Each value represents the mean with S.D. ($n = 4-5$). $**P < 0.01$; significant difference between the indicated groups ($t_6 = 4.737$, $P = 0.003$ for homocysteine; $t_6 = -11.120$, $P < 0.001$ for cysteine after AOAA treatment; $t_{10} = 8.665$, $P < 0.001$ for cystathionine; $t_6 = -4.693$, $P = 0.001$ for cysteine after PAG treatment; unpaired t -test, two sided).



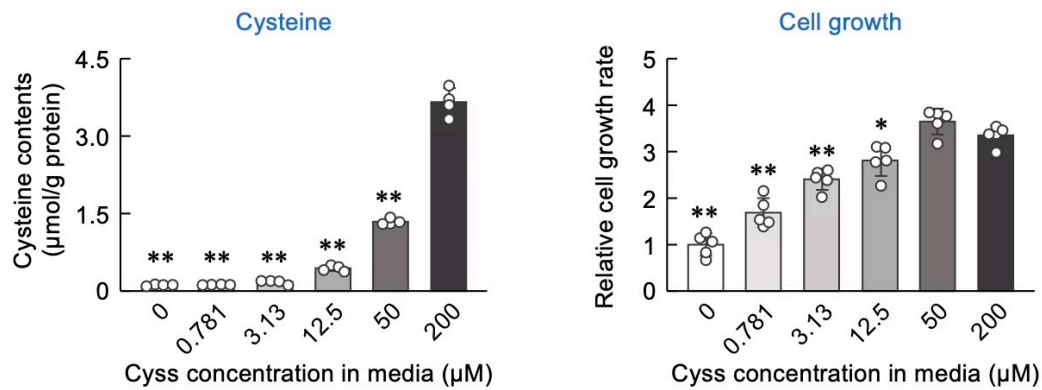
Supplementary Figure S5 The protein levels of c-MYC in the liver and hepatic tumors in mice. The values in the liver were set at 1.0. Each value represents the mean with S.D. ($n = 4$). $**P < 0.01$; significant difference between the two groups ($t_6 = 6.086$, $P < 0.001$; unpaired t -test, two sided).



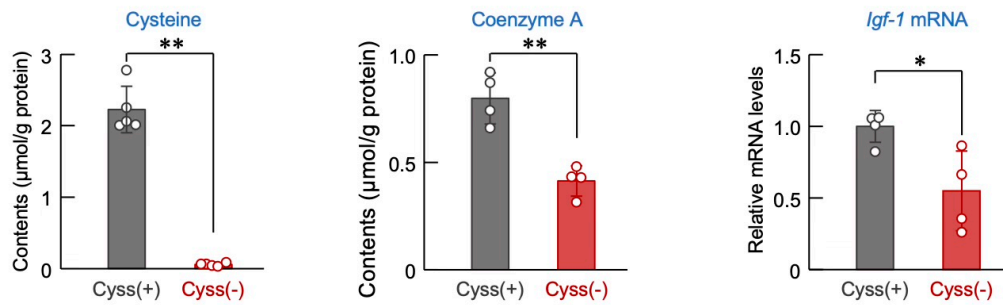
Supplementary Figure S6 Contribution of PKB/AKT signaling to the up-regulation of NRF2 in BNL 1ME A.7 R.1 cells. **A**, Time course of intracellular ROS levels in primary hepatocytes during treatment with 1.0 mM PAG for 24 h. Each value represents the mean with S.D. (n = 4). **B**, The phosphorylation states of AKT protein in primary hepatocytes and BNL 1ME A.7 R.1 cells. The protein levels were normalized to those of total AKT. The values in the primary hepatocytes were set at 1.0. Each value represents the mean with S.D. (n = 4). ** $P < 0.01$; significant difference between the two groups ($t_6 = 10.821$, $P < 0.001$ for p(S473) AKT; $t_6 = 3.736$, $P = 0.001$ for p(T308) AKT; unpaired t -test, two sided).



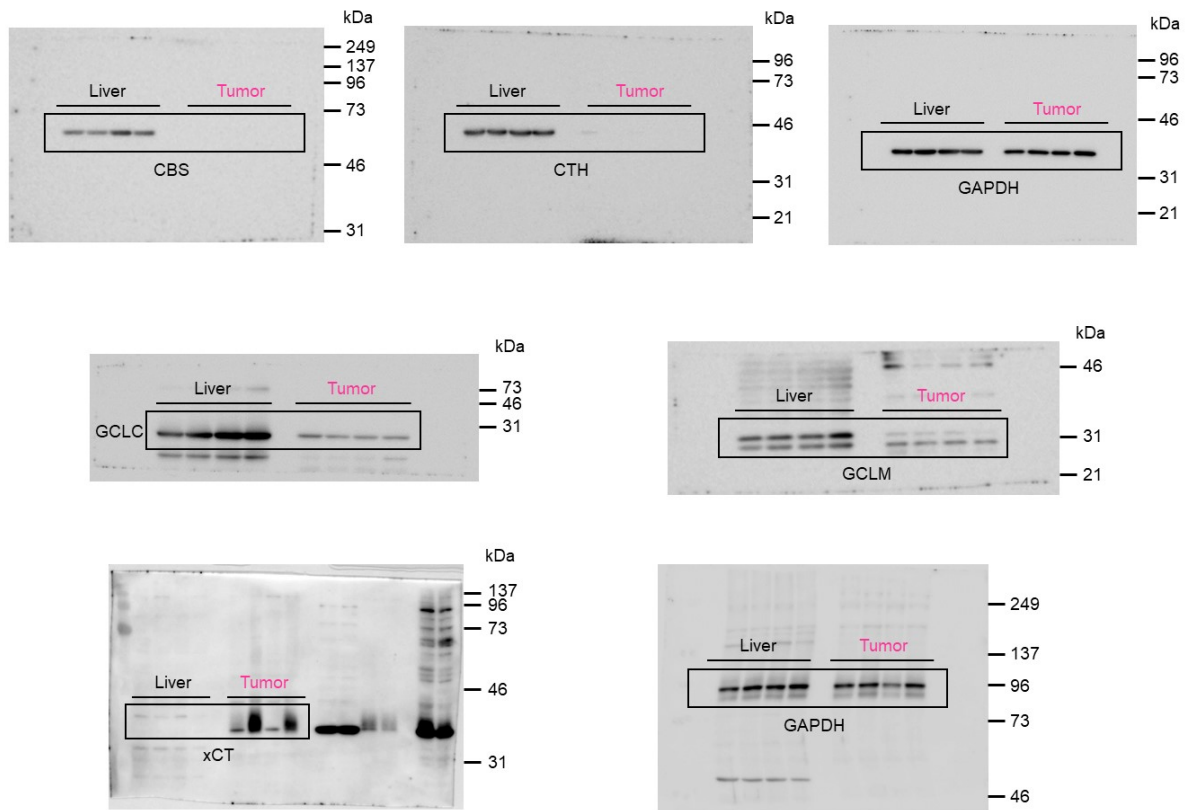
Supplementary Figure S7 Elevation of NRF2 levels has a negligible effect on the expression on de novo cysteine synthases in primary hepatocytes and their viabilities. **A**, The protein levels of NRF2, CBS, and CTH in primary hepatocytes after treatment with 100 μ M 4-octyl itaconate for 24 h. The protein levels were normalized to those of GAPDH. The values in the liver were set at 1.0. Each value represents the mean with S.D. ($n = 4$). $**P < 0.01$; significant difference between the two groups ($t_6 = 4.855$, $P = 0.003$; unpaired t -test, two sided). **B**, The viability of primary hepatocytes during incubation in media in the presence or absence of 100 μ M 4-octyl itaconate. The basal cell viability (0 h) of vehicle-treated cells was set at 1.0. Each value represents the mean with S.D. ($n = 4$).



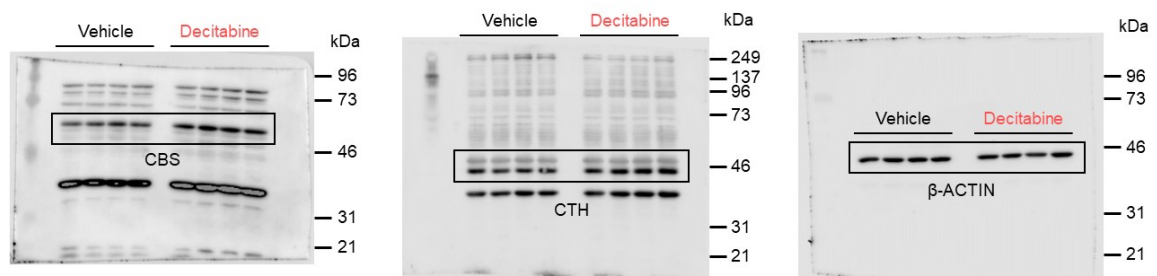
Supplementary Figure S8 The growth of hepatocarcinoma cells depends on the exogenous cysteine supply. The intracellular cysteine levels of BNL 1ME A.7 R.1 cells and their proliferation are increased in an extracellular cysteine concentration-dependent manner. Cells were incubated in conditional media containing the indicated concentrations of cystine. Intracellular contents of cysteine were normalized to protein levels. Each value represents the mean with S.D. (n = 4-6). ** $P < 0.01$; significant difference from 200 μM cystine containing media (Dunnett's test).



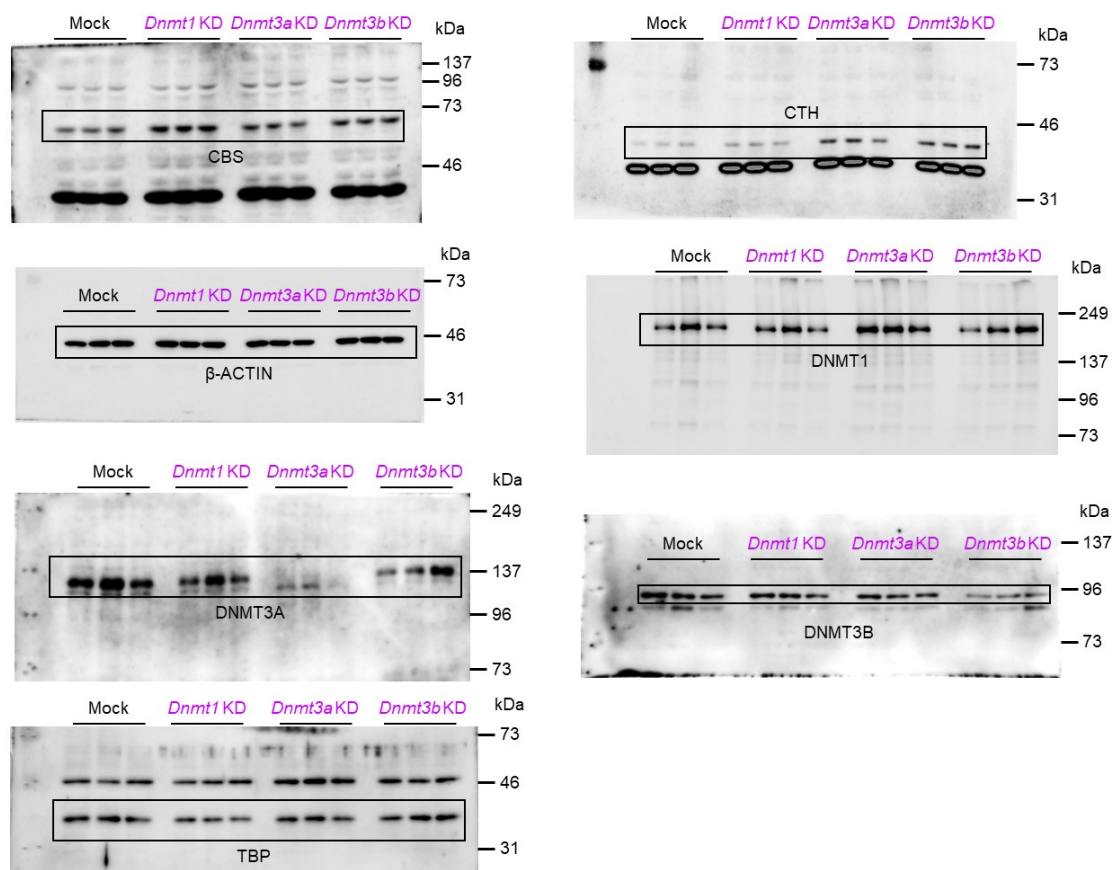
Supplementary Figure S9 Deprivation of cystine from the culture media decreased intracellular contents of coenzyme A and the expression levels of *Igf-1* in BNL 1ME A.7 R.1 cells. Cells were incubated in cystine-containing (Cyss (+)) or cystine-deficient (Cyss (-)) media for 24 h. Intracellular contents of cysteine and coenzyme A were normalized by protein levels. The *Igf-1* expression levels were normalized to those of *18s*. Each value represents the mean with S.D. (n = 4). ** $P < 0.01$, * $P < 0.05$; significant difference between the indicated groups ($t_6 = -14.924$, $P < 0.001$ for cysteine contents; $t_6 = -5.531$, $P = 0.002$ for coenzyme A contents; $t_6 = -3.010$, $P = 0.024$ for *Igf-1* mRNA; unpaired t -test, two sided).



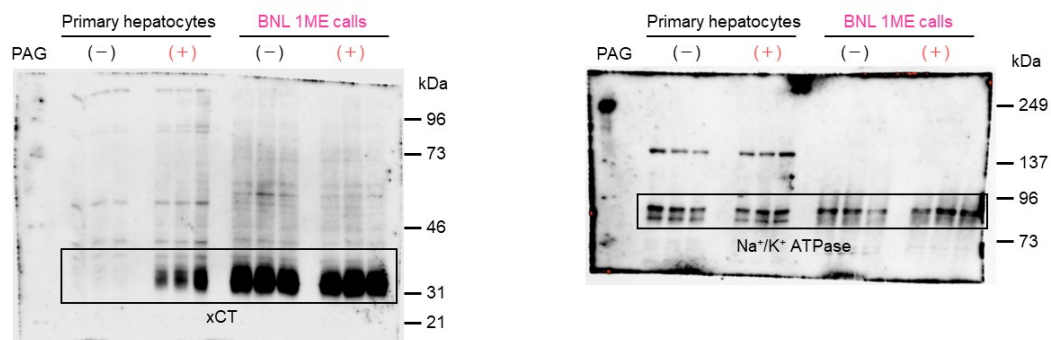
Supplementary Figure S10 Unedited full blots of Figure 2A



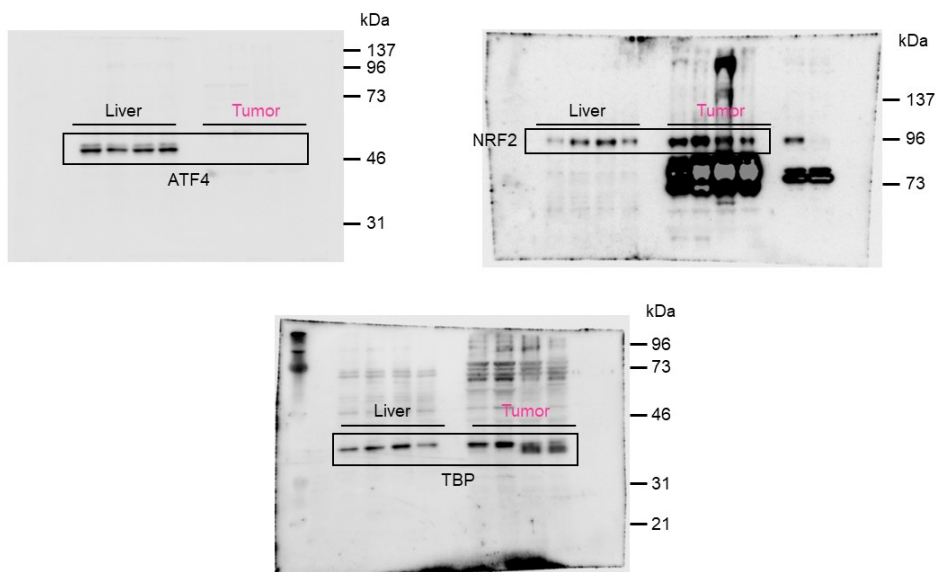
Supplementary Figure S11 Unedited full blots of Figure 4B



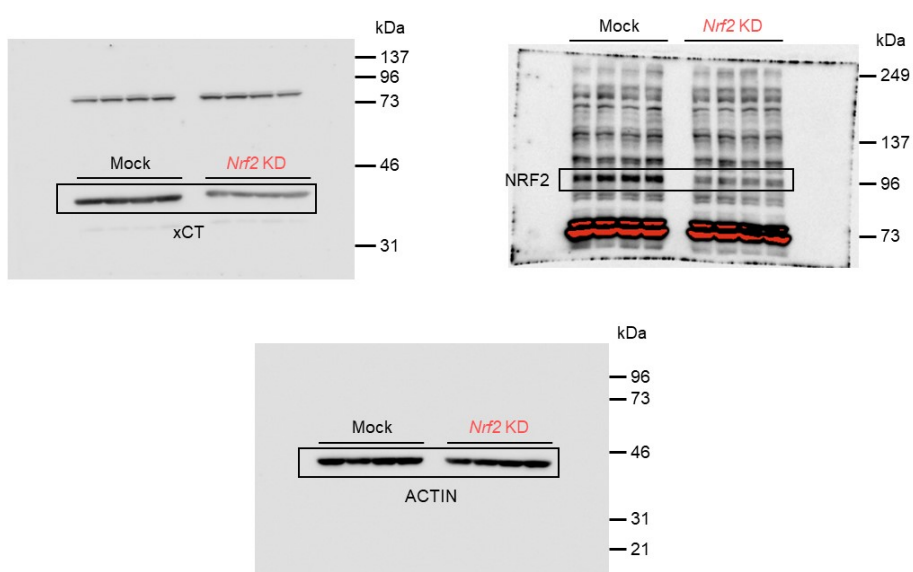
Supplementary Figure S12 Unedited full blots of Figure 4D



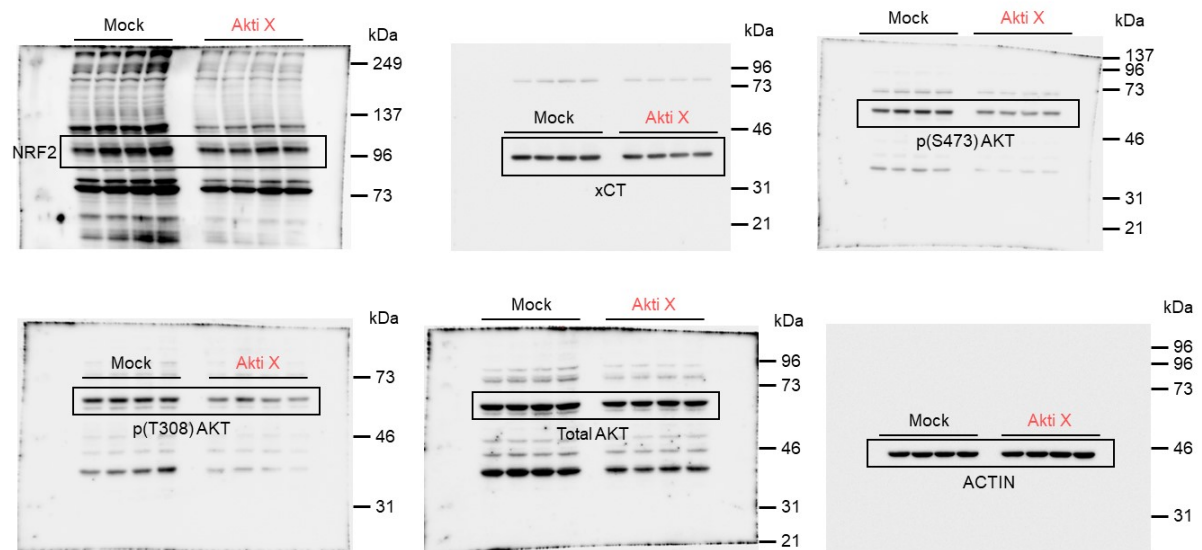
Supplementary Figure S13 Unedited full blots of Figure 5D



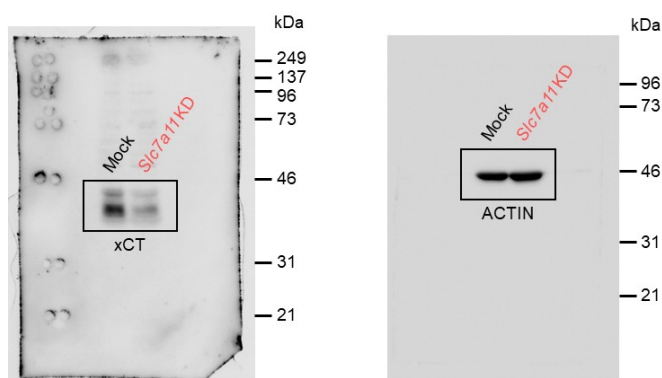
Supplementary Figure S14 Unedited full blots of Figure 5E



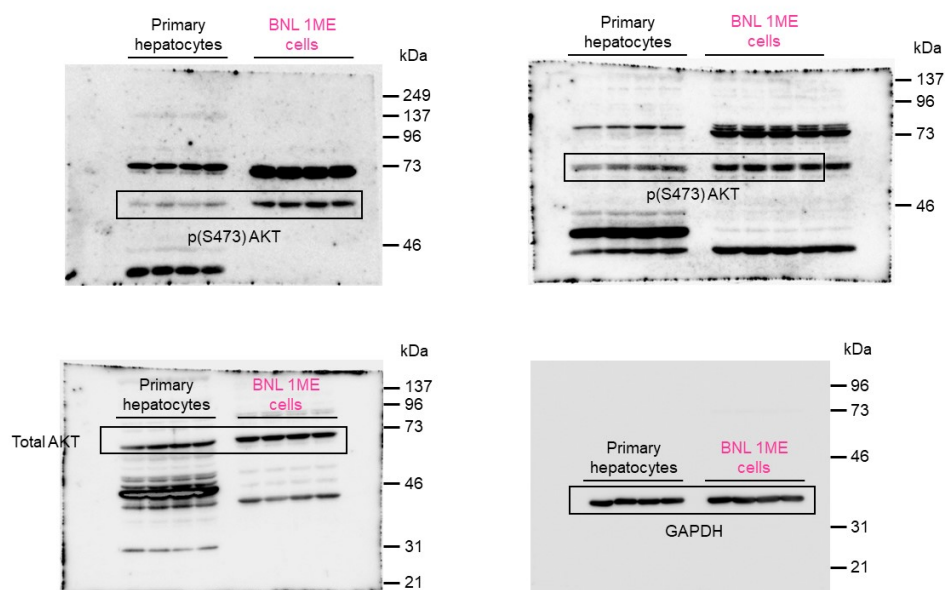
Supplementary Figure S15 Unedited full blots of Figure 5F



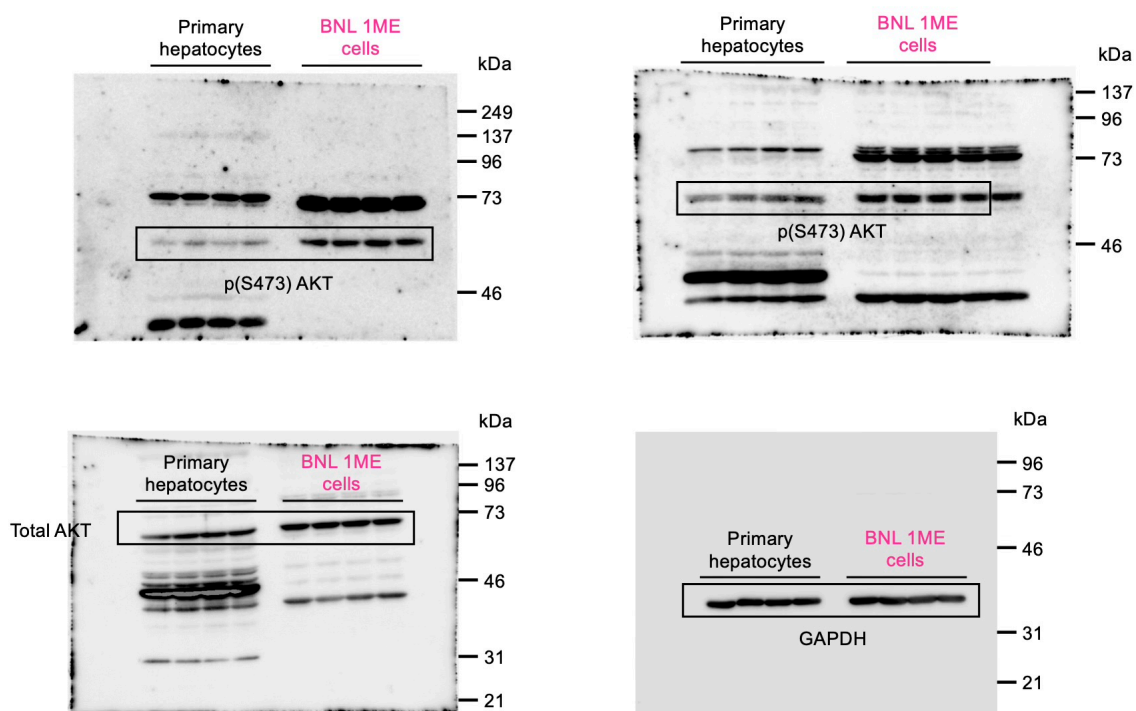
Supplementary Figure S16 Unedited full blots of Supplementary Figure 5G



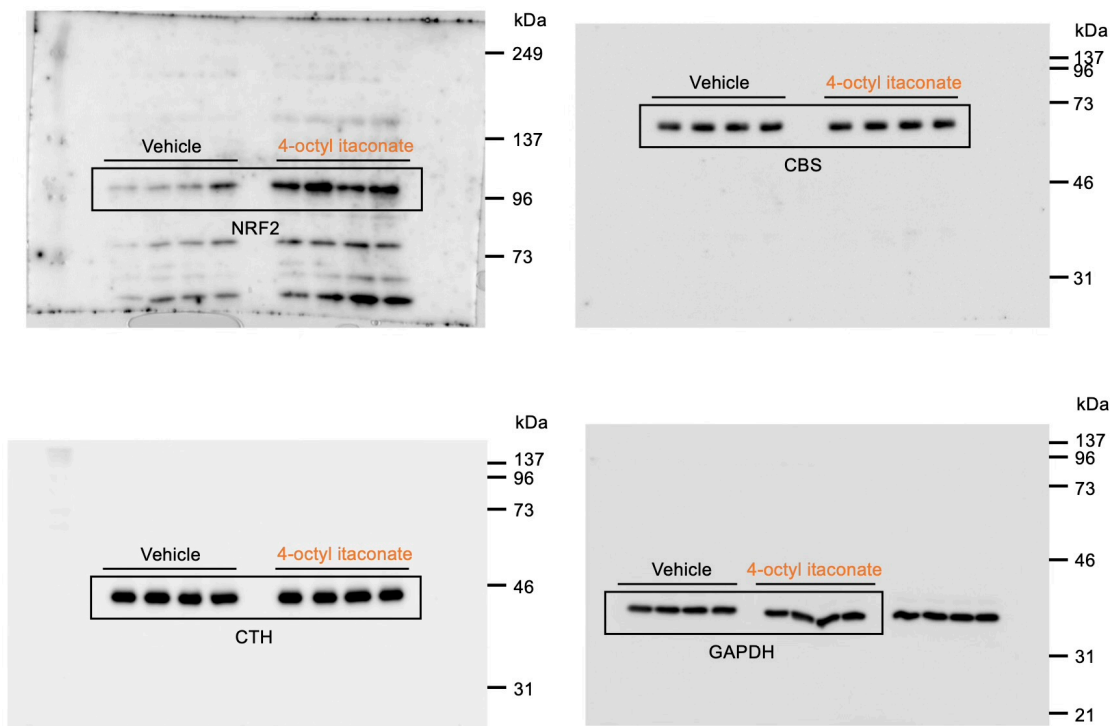
Supplementary Figure S17 Unedited full blots of Figure 6A



Supplementary Figure S18 Unedited full blots of Supplementary Figure S5B



Supplementary Figure S19 Unedited full blots of Supplementary Figure S7



Supplementary Figure S20 Unedited full blots of Supplementary Figure S9