Supplementary Material and Methods

SubG1 Assay by Flow Cytometry

After the indicated starvation treatment, the cell cycle profiles were analyzed by FACSCalibur flow cytometer (BD Biosciences, San Jose CA), as described previously.[1] The proportion of subG1 phase was regarded as dead cells.

Western-blotting and Antibodies

Western blotting has been previously described.[1] Antibodies against ACSL3 (sc-271246), ACSL4 (sc-271800), and ACSS2 (sc-85258) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA); AceCS1 (#3658), ACSL1 (#4047), phosphor-ACC (ser79, #3661), total-ACC (#3676), phospho-AMPK (thr172, #2535), total AMPKα (#2532), C/EBPalpha (#2295 and #2843, the latter specifically detecting p42 but not p30), FASN (#3180), phospho-S6 (ser235/236, #2211), and total S6 (#2217) from Cell Signaling Technology; microtubule-associated protein 1 light chain 3 (LC3, #L7543), TMEM166 (SAB2102458) and α-tubulin (T-5168) from Sigma-Aldrich; Atg7 (#49-385) from ProScience (Poway, CA); and anti-p62 antibody (H00008878-2C11) from Abnova (Taipei, Taiwan).

siRNA Sequences for Gene Knock-down Assay

Three Stealth™ siRNA duplex sequences used for specific targeting C/EBPalpha were 1) UUG CCC AUG GCC UUG ACC AAG GAG C; 2) CCU UCA ACG ACG AGU UCC UGG CCG A; 3) AGC GCA ACA ACA UCG CGG UGC GCA A. The Stealth™ siRNA sequences used for ATG7 were 1) UUC UCU UGC UUG CAG CAA UGA CGG C; 2) AUU UCU GGA AGC UUC ACU UCG AAG A; 3) UAG AGA GGC UGC CUC ACA GCA UUG G. Two Silencer® siRNAs for TMEM166 were 1) GAU CCA UGC UUA UUA UUU ATT; and 2) AGA GGA CUU UGA ACA AGA ATT.

Nile red staining of Intracellular Lipid Droplets

Lipophilic dye Nile Red (Sigma-Aldrich) was used to detect cellular lipid droplets. Briefly, cells were fixed with 3% paraformaldehyde and then incubated with 0.1 mg/ml Nile Red for 10 mins. The images of lipid droplets with green fluorescence were captured by Eclipse TE2000U inverted microscope (Nikon, Melville, NY).

Quantitative RT-PCR

Quantitative reverse-transcription–polymerase chain reaction (qRT-PCR) was performed as previously described (2). Briefly, first-strand cDNA was synthesized with the use of ImProm II reverse transcription system (Promega, Madison, WI) and then subjected to quantitative PCR with the use of a LightCycler 480 system (Roche, Indianapolis, IN). The PCR primer sequences for C/EBPalpha are TGT ATA CCC CTG GTG GGA GA and TCA TAA CTC CGG TCC CTC TG; for TMEM166 are AGC AAC ATC CTA GCG GCC TA and TGT GTG GCA AGA GAT CCT TAT CA; for *β*-actin are GGA CTT CGA GCA AGA GAT GG and AGC ACT GTG TTG GCG TAC AG, and for SFRS4 are AAA AGT CGG AGC AGG AGT CA and CTC TTC CTG CCC TTC CTC TT.

DNA sequencing for CEBPA mutations

The DNA sequencing for CEBPA mutations in the genomic coding region was carried out from 26 pairs of primary HCCs and 5 HCC cell lines, as described previously.[1] Triplicates of DNA sequencing was conducted to ensure the robustness of the data,

Transcriptome analysis

The transcriptome analysis was conducted as described previously.[2] Briefly, total RNA was extracted from C/EBPalpha-expressing Hep3B and stable shNC, C/EBPalpha-deficient HepG2 and HCC-M, and C/EBPalpha-silenced stable sh4 and sh7 cells. The mRNAs from each cell lines were reversibly transcribed by Genechip 3'IVT Express kit (Affymetrix, Santa Cruz, CA) and biotin labeled. The final cDNAs were hybridized to human HG-U1331 and HG-U133-B oligonucleotide probe arrays (Affymetrix) according to the manufacturer's instructions.

References:

1. Lu, G.D., Leung, C.H., Yan, B., Tan, C.M., Low, S.Y., Aung, M.O., Salto-Tellez, M., Lim, S.G., and Hooi, S.C. 2010. C/EBPalpha is up-regulated in a subset of hepatocellular carcinomas and plays a role in cell growth and proliferation. *Gastroenterology* 139:632-643, 643 e631-634.
2. Ong, H.T., Tan, P.K., Wang, S.M., Hian Low, D.T., Ooi, L.L., and Hui, K.M. 2011. The tumor suppressor function of LECT2 in human hepatocellular carcinoma makes it a potential therapeutic target. *Cancer Gene Ther* 18:399-406.

Table S1: C/EBPalpha protein was over-expressed in HCC tumor. C/EBPalpha protein expression in HCC tumor and matched non-tumor tissues was determined by immunohistochemistry staining. Its expressional level was scored into 4-tiered grading system, with 0 as negative expression and 3 as the highest expression. HCC patients were ranked into four different groups according to the difference between C/EBPalpha expression in tumor (TU) and in non-tumor (NT) tissues, with 0 for no difference and 3 the highest up-regulation.

C/EBP*a* score	**Matched pairs**	
(Tu-NT)	**Cases**	**%**
≤0	45	23.6
1	51	26.7
2	61	31.9
3	34	17.8
Total	191	100

Table S2: Comparison of common clinicopathological parameters in human primary HCC patients with or without C/EBP*α* up-regulation. We compared the common clinicopathological parameters between HCC patients with and without C/EBPalpha up-regulation by student *t*-test or *chi*-square. The number of cases and *p*-values in two different groups were listed as indicated.

	No C/EBP*α* up-regulation (Tu-NT≤0, n=42)	**C/EBP*α* up-regulation (Tu-NT≥1, n=134)**	***p*-value**
Age (mean ± SD years)	57.4 ± 15.4	58.2 ± 13.0	0.730
Gender: Male (%)	32 (76.2%)	106 (79.1%)	0.502
Race: Chinese (%)	35 (83.3%)	103 (77.4%)	0.518
HBV carrier (% in x cases)	26 (63.4% in 41 cases)	84 (66.7% in 126 cases)	0.708
HCV carrier (% in x cases)	2 (4.9% in 41 cases)	10 (7.8% in 128 cases)	0.733
Drinker or ex-drinker (%)	12 (33.3% in 36 cases)	45 (40.2% in 112 cases)	0.556
Diabetes mellitus (%)	11 (27.5% in 40 cases)	32 (24.8% in 129 cases)	0.836
Cirrhosis (%)	23 (54.8%)	72 (53.7%)	1.000
Tumor size (%):			0.805
< 2 cm	3 (7.3%)	10 (7.5%)	
2~ 5 cm	15 (36.6%)	56 (42.1%)	
≥ 5 cm	23 (56.1%)	67 (50.4%)	
No. of tumor nodules:			0.559
Single nodule	28 (66.7%)	97 (72.4%)	
Two or more	14 (33.3%)	37 (27.6%)	
Tumor stage (%):			0.859
I	22 (53.7%)	63 (49.2%)	
II	12 (29.3%)	39 (30.5%)	
III & IV	7 (17.0%)	26 (20.3%)	
Serum AFP level (mean ± SD, ng/mL)	41.8 ± 17.8	71.5 ± 17.4	0.329
Vascular invasion (%)	11 (34.4% in 32 cases)	42 (36.5% in 115 cases)	0.823

Table S3: C/EBPalpha protein expressions in human recurrent HCC were similar to those in primary HCC. We determined the C/EBPalpha expression in 9 pairs of HCC primary tumor, neighboring non-tumor tissues and matched recurrent tumor. * $p = 0.005$, primary HCC *vs.* non-tumor (by paired-samples *t*-test) # $p = 0.008$, recurrent HCC *vs.* non-tumor (by paired-samples *t*-test) and § $p = 0.800$, primary *vs.* recurrent HCC (by Pearson correlation analysis)

Patient Code	Neighboring non-tumor liver tissue	Primary HCC tissues*	Recurrent HCC tissues #, §	Time to develop recurrence (months)
10894	0	0	0	31
10245	1	3	2	5
14778	0	2	3	5
0005	0	2	3	9
3635	1	1	1	43
8691	0	3	2	59
11421	1	1	1	14
14405	0	3	3	6
15621	1	3	3	24

Supplementary Figure Legends

Figure S1: C/EBPalpha up-regulation predicted poorer patient survival in primary HCC.

(A) HCC patients were ranked into 4 different groups according to C/EBPalpha protein level in tumor tissues, with 0 for no expression and 3 highest expressions. A Kaplan-Meier survival analysis showed that the higher the C/EBPalpha expressed in tumor HCCs the poorer overall survival was. **(B)** HCC patients were ranked into 2 different groups according to C/EBPalpha expression in adjacent normal liver tissues (no expression *vs.* overexpression). **(C and D)** Kaplan-Meier survival analyses showed that C/EBPalpha overexpression tended to predict poorer overall survival in the HCC patients with low level of serum AFP (< 300 ng/mL) but not those with high. **(E and F)** Kaplan-Meier survival analyses showed that C/EBPalpha over-expression predicted poorer overall survival in the HCC patients with microscopic vascular invasion but not those without.

Figure S2: C/EBPalpha mRNA expression was correlated with protein in primary HCC.

C/EBPalpha mRNA expression was determined by qRT-PCR and normalized by SFRS4 mRNA expression. **(A)** C/EBPalpha mRNA expression was correlated with protein expression by using Pearson correlation analysis. **(B)** C/EBPalpha mRNA expression was upregulated in HCC tumors compared with matched non-tumor tissues.

Figure S3: C/EBPalpha protected HCC cells from energy starvation induced cells death.

(A) C/EBPalpha-expressing cells (Hep3B and shNC) and C/EBPalpha–silenced cells (sh4 and sh7) were kept in full DMEM medium without changing or supplementing fresh medium for four weeks. The adherent cells and floating dead cells were collected for subG1 analysis. **(B)** C/EBPalpha-expressing shNC control and C/EBPalpha–silenced cells (sh4 and sh7) cells were cultured in the glucose and glutamine double free medium (DN) or supplemented with 1 g/L glucose (DN+Glu) or 4.0 mM L-glutamine (DN+Gln) for two days. The cell death was determined by subG1 assay. **(C)** Stable PLC/5 cells silenced of C/EBPalpha by specific shRNA (sh1 and sh2) were generated and selected as in Fig. 3. The cells were starved for

two days. **(D)** HCC cells were starved in the glucose and glutamine-free DMEM medium in a hypoxia incubator for 8 hr.

Figure S4: Lipid catabolism was essential for C/EBPalpha-mediated survival.

(A) C/EBPalpha-expressing (Hep3B and shNC) and C/EBPalpha–silenced stable cells (sh4 and sh7) were starved in a time course. The intracellular levels of pyruvate and glycogen were determined by biochemical assays. **(B)** HCC cells were starved for 8 hr. The lipid droplets were visualized by staining with 1 μM nile red and the images captured by Nicon inverted fluorescent camera. **(C)** Hep3B cells were pretreated with liposynthesis inhibitor C75 (5μg/mL), simvastatin (statin, 10μM) and diethylum-belliferyl phosphate (DEBP, 50μM) for two weeks. The intracellular lipid droplets were determined as in panel B.

Figure S5: C/EBPalpha-expressing cells had higher expression of key enzymes involved in lipid and fatty acid metabolism.

(A) C/EBPalpha-expressing (Hep3B and shNC) and –silenced stable cells (sh4 and sh7) were starved for 4 and 8 hours. The alterations of autophagic upstream AMPK and mTOR pathways were analyzed by Western blotting. **(B)** The key enzymes involved in lipid and fatty acid metabolism in the stable cells were determined by Western blotting. **(C)** Different HCC cell lines were starved for 6 hr and the proteins were collected for Western blotting.

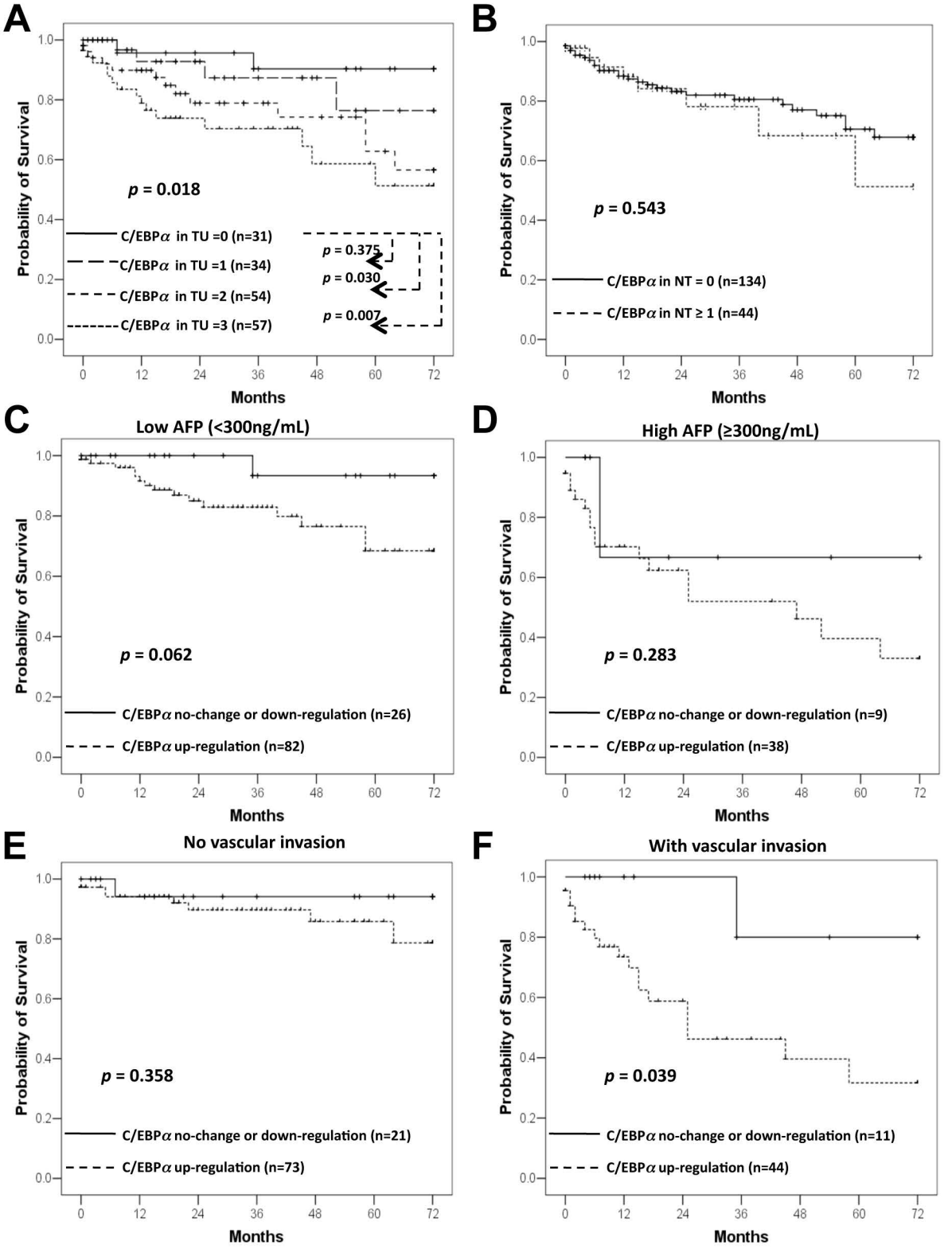
Figure S6: TMEM166 was required for autophagy-involved lipid metabolism.

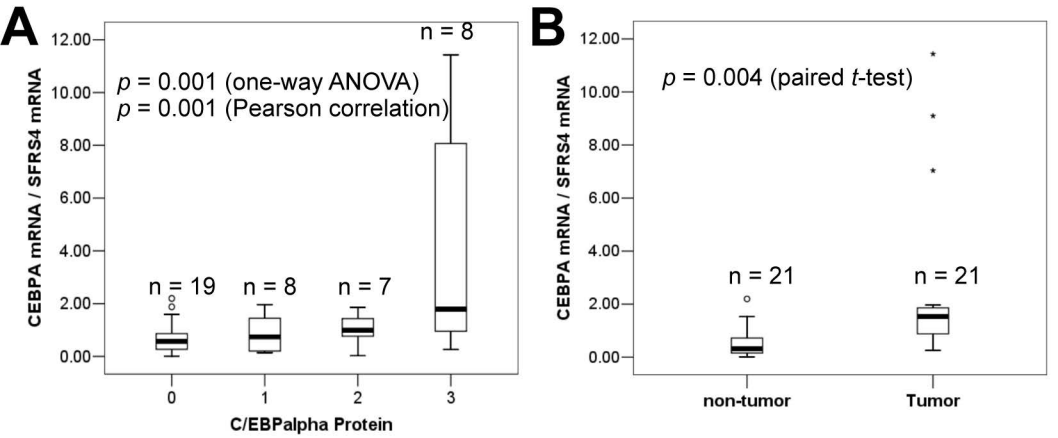
(A) To identify autophagic executive genes that may be regulated by C/EBPalpha, we compared the transcriptomes from cells expressing C/EBPalpha with those from cells without. The first comparison was between C/EBPalpha-expressing Hep3B and C/EBPalpha-deficient HepG2 and HCC-M cells. The second comparison was between C/EBPalpha-expressing shNC control cells and C/EBPalpha–silenced cells (sh4 and sh7). 84 genes were found to be differentially expressed at least tenfold in both sets of comparisons. **(B)** The mRNA level of C/EBPalpha and TMEM166 were determined by qRT-PCR and normalized with those in Hep3B cells respectively. **(C)** TMEM166 was silenced by specific siRNA (si1 and si2) before energy starvation. The intracellular lipid droplets were detected by nile red staining as in Fig.

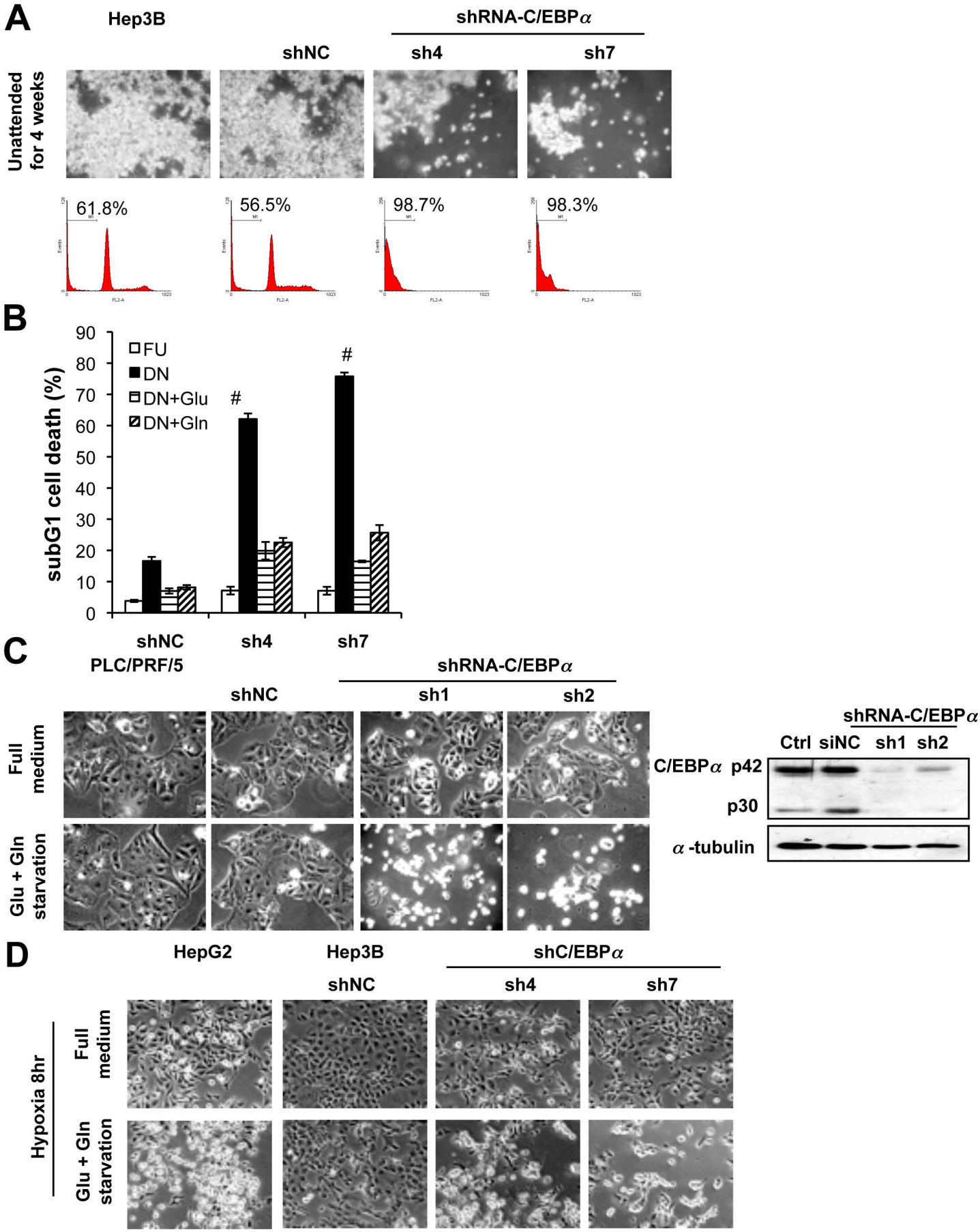
S4B. **(D)** TMEM166 was overexpressed in the C/EBPalpha-deficient HCC-M cells. The fatty acid beta-oxidation rates were determined as in Fig. 4C.

Figure S7: C/EBPalpha up-regulation predicted poorer patient survival in recurrent HCC.

Kaplan-Meier survival analyses showed that neither C/EBPalpha upregulation **(A)** nor its expression levels in tumor tissues **(B)** were associated with tumor recurrence. **(C)** The HCC patients with recurrent tumors and who had elevated C/EBPalpha expression in the primary tumor had poorer prognosis.


A
Probability of Survival
p = 0.018
C/EBPα in TU =0 (n=31)
C/EBPα in TU =1 (n=34)
C/EBPα in TU =2 (n=54)
C/EBPα in TU =3 (n=57)
p = 0.375
p = 0.030
p = 0.007
Months
B
Probability of Survival
p = 0.543
C/EBPα in NT = 0 (n=134)
C/EBPα in NT ≥ 1 (n=44)
Months
C
Low AFP (<300ng/mL)
Probability of Survival
p = 0.062
C/EBPα no-change or down-regulation (n=26)
C/EBPα up-regulation (n=82)
Months
D
High AFP (≥300ng/mL)
Probability of Survival
p = 0.283
C/EBPα no-change or down-regulation (n=9)
C/EBPα up-regulation (n=38)
Months
E
No vascular invasion
Probability of Survival
p = 0.358
C/EBPα no-change or down-regulation (n=21)
C/EBPα up-regulation (n=73)
Months
F
With vascular invasion
Probability of Survival
p = 0.039
C/EBPα no-change or down-regulation (n=11)
C/EBPα up-regulation (n=44)
Months


A
CEBPA mRNA / SFRS4 mRNA
12.00
10.00
8.00
6.00
4.00
2.00
0.00
n = 8
p = 0.001 (one-way ANOVA)
p = 0.001 (Pearson correlation)
n = 19
n = 8
n = 7
0
1
2
3
C/EBPalpha Protein
B
CEBPA mRNA / SFRS4 mRNA
12.00
10.00
8.00
6.00
4.00
2.00
0.00
p = 0.004 (paired t-test)
n = 21
n = 21
non-tumor
Tumor


A
Hep3B
shRNA-C/EBPα
shNC
sh4
sh7
Unattended for 4 weeks
61.8%
56.5%
98.7%
98.3%
B
subG1 cell death (%)
FU
DN
DN+Glu
DN+Gln
#
#
shNC
sh4
sh7
C
PLC/PRF/5
shRNA-C/EBPα
shNC
sh1
sh2
Full medium
Glu + Gln starvation
shRNA-C/EBPα
Ctrl
siNC
sh1
sh2
C/EBPα p42
p30
α-tubulin
D
HepG2
Hep3B
shC/EBPα
shNC
sh4
sh7
Hypoxia 8hr
Full medium
Glu + Gln starvation

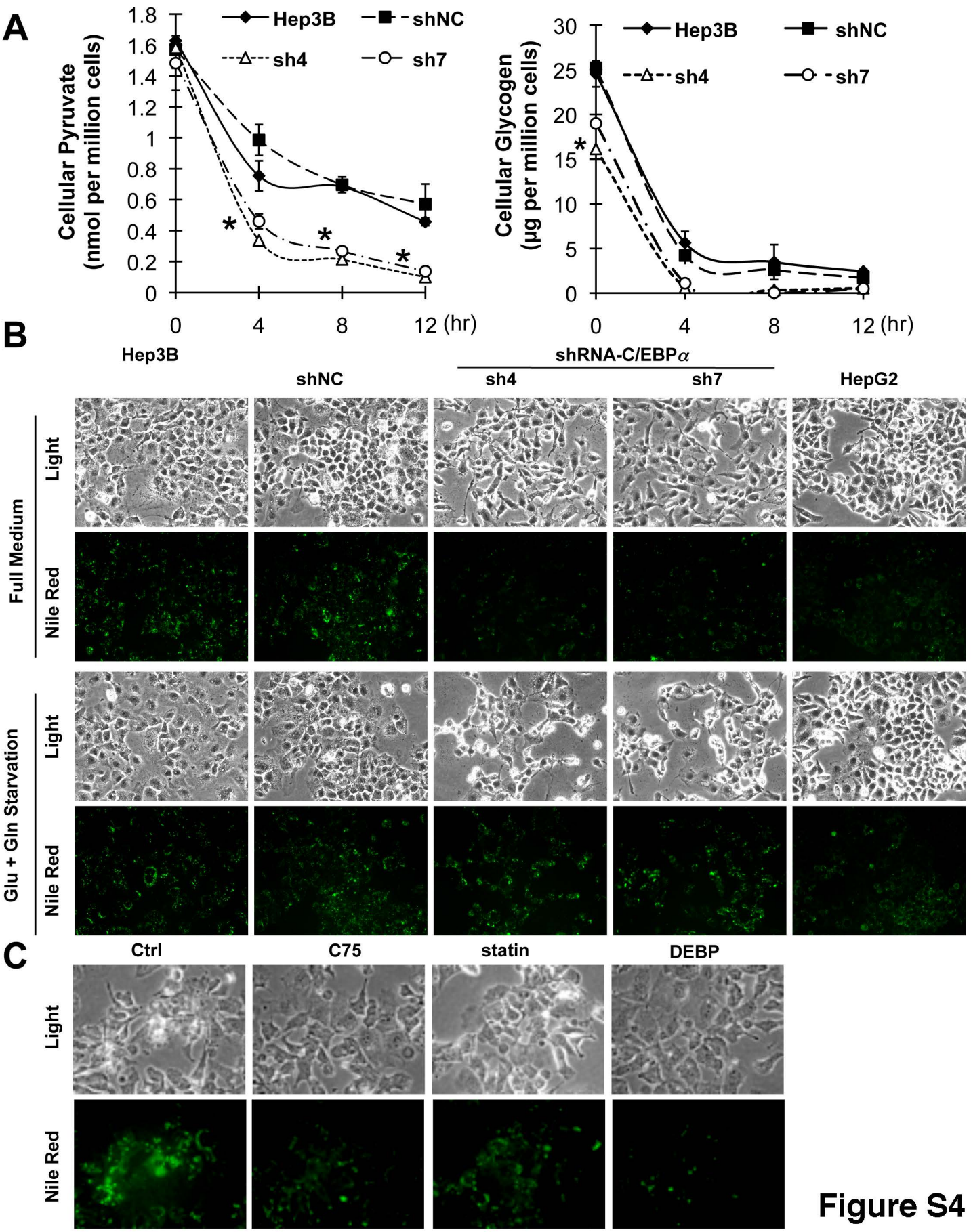

A
Cellular Pyruvate (nmol per million cells)
Hep3B
shNC
sh4
sh7
0
0.2
0.4
0.6
0.8
1
1.2
1.4
1.6
1.8
0
4
8
12 (hr)
*
*
*
Cellular Glycogen (μg per million cells)
Hep3B
shNC
sh4
sh7
0
5
10
15
20
25
30
0
4
8
12 (hr)
*
B
Hep3B
shRNA-C/EBPα
shNC
sh4
sh7
HepG2
Full Medium
Light
Nile Red
Glu + Gln Starvation
Light
Nile Red
C
Ctrl
C75
statin
DEBP
Light
Nile Red

Figure S4

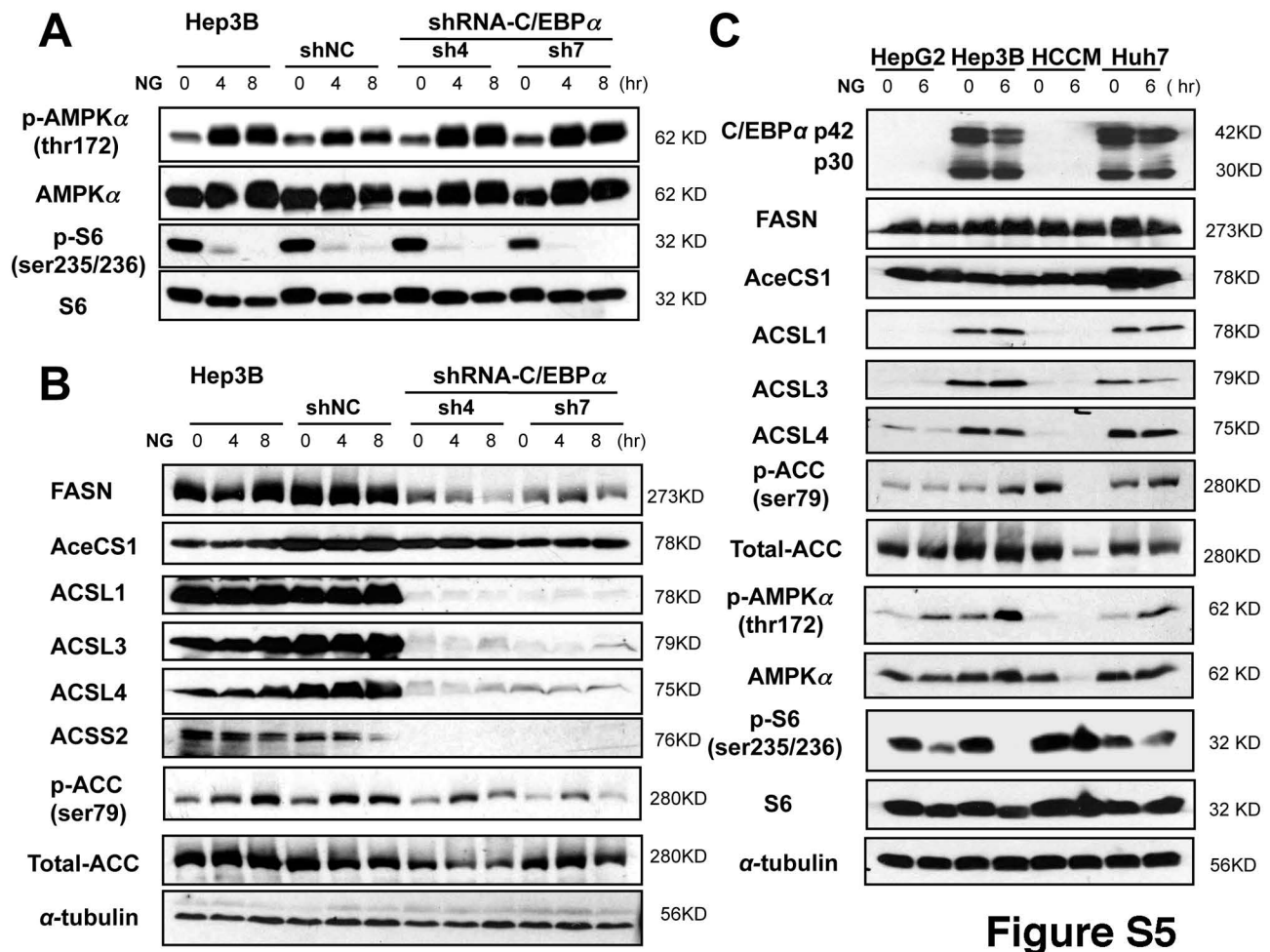
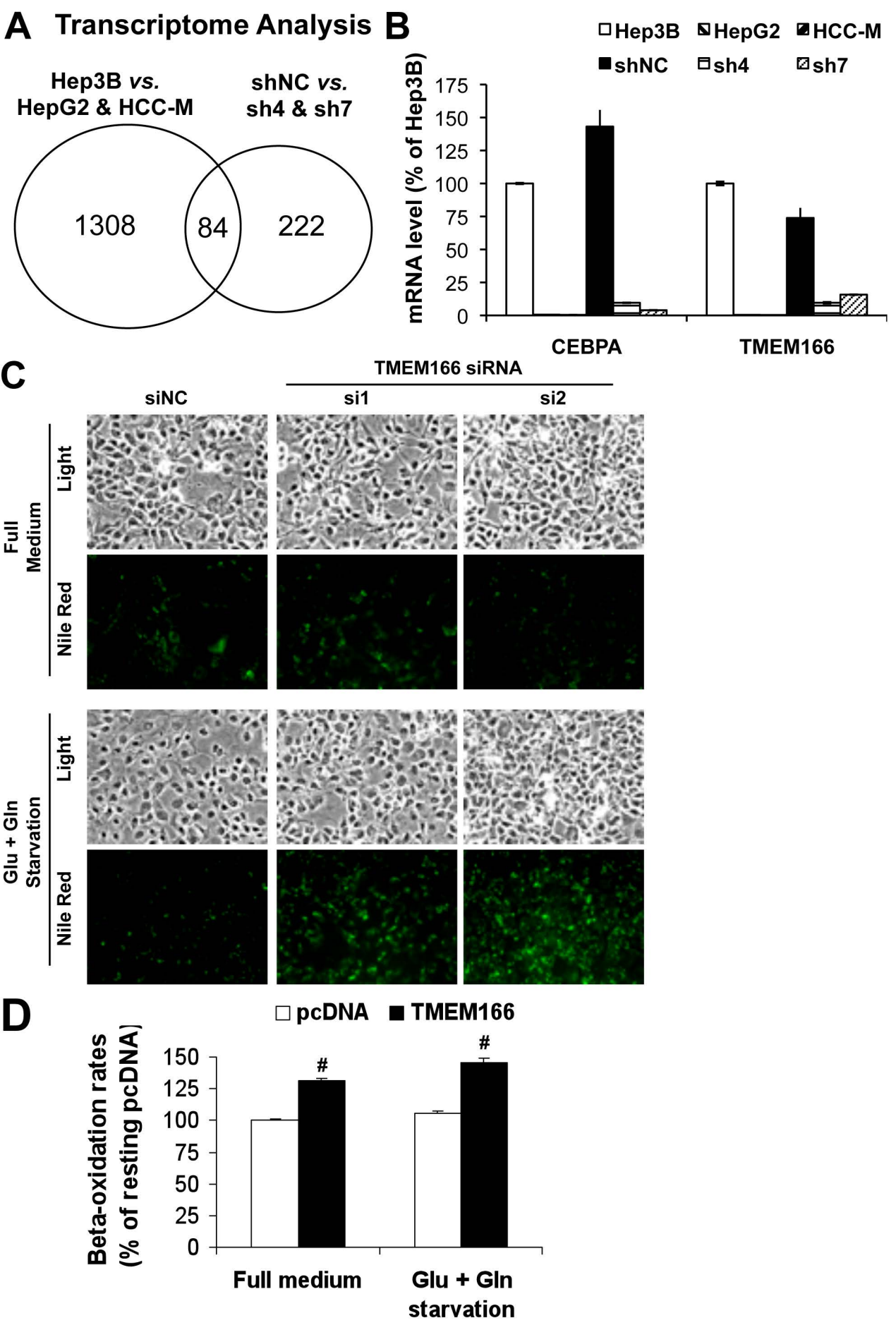

A
Hep3B
shNC
shRNA-C/EBPα
sh4
sh7
NG 0 4 8 0 4 8 0 4 8 0 4 8 (hr)
p-AMPKα (thr172)
AMPKα
p-S6 (ser235/236)
S6
62 KD
62 KD
32 KD
32 KD
B
Hep3B
shNC
shRNA-C/EBPα
sh4
sh7
NG 0 4 8 0 4 8 0 4 8 0 4 8 (hr)
FASN
AceCS1
ACSL1
ACSL3
ACSL4
ACSS2
p-ACC (ser79)
Total-ACC
α-tubulin
273KD
78KD
78KD
79KD
75KD
76KD
280KD
280KD
56KD
C
HepG2 Hep3B HCCM Huh7
NG 0 6 0 6 0 6 0 6 (hr)
C/EBPα p42
p30
FASN
AceCS1
ACSL1
ACSL3
ACSL4
p-ACC (ser79)
Total-ACC
p-AMPKα (thr172)
AMPKα
p-S6 (ser235/236)
S6
α-tubulin
42KD
30KD
273KD
78KD
78KD
79KD
75KD
280KD
280KD
62 KD
62 KD
32 KD
32 KD
56KD

Figure S5


A
Transcriptome Analysis
Hep3B vs. HepG2 & HCC-M
shNC vs. sh4 & sh7
1308
84
222
B
Hep3B
HepG2
HCC-M
shNC
sh4
sh7
mRNA level (% of Hep3B)
175
150
125
100
75
50
25
0
CEBPA
TMEM166
C
TMEM166 siRNA
siNC
si1
si2
Full Medium
Light
Nile Red
Glu + Gln Starvation
Light
Nile Red
D
pcDNA
TMEM166
Beta-oxidation rates (% of resting pcDNA)
150
125
100
75
50
25
0
#
#
Full medium
Glu + Gln starvation

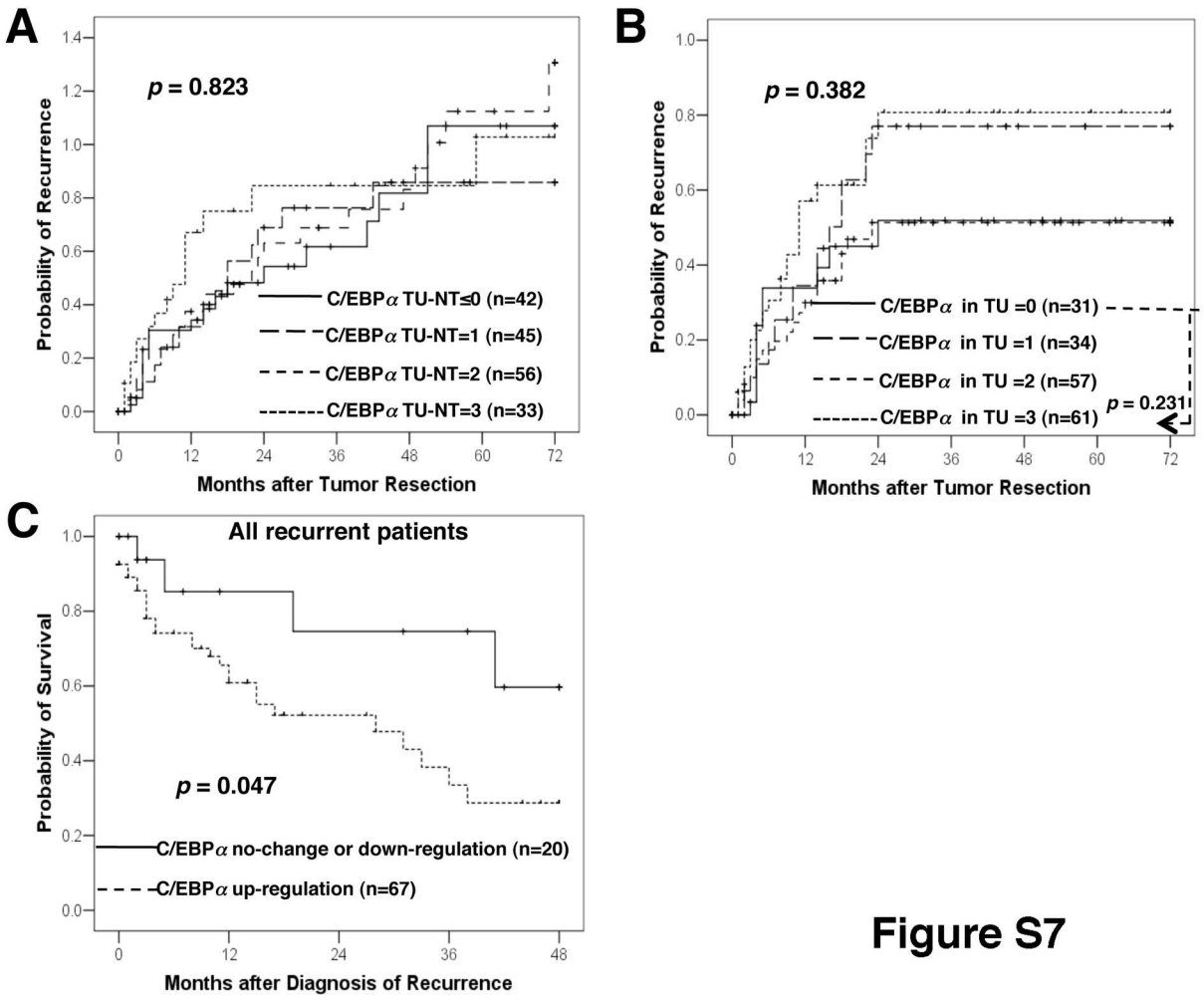

A
p = 0.823
Probability of Recurrence
C/EBPα TU-NT≤0 (n=42)
C/EBPα TU-NT=1 (n=45)
C/EBPα TU-NT=2 (n=56)
C/EBPα TU-NT=3 (n=33)
Months after Tumor Resection
B
p = 0.382
Probability of Recurrence
C/EBPα in TU =0 (n=31)
C/EBPα in TU =1 (n=34)
C/EBPα in TU =2 (n=57)
C/EBPα in TU =3 (n=61)
p = 0.231
Months after Tumor Resection
C
All recurrent patients
Probability of Survival
p = 0.047
C/EBPα no-change or down-regulation (n=20)
C/EBPα up-regulation (n=67)
Months after Diagnosis of Recurrence

Figure S7