Supporting Information

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SI Materials and Methods

Cell Lines, Culture Conditions, and Viability Assays. HepG3 and QGY-7703 human HCC cell were cultured as described (1). For serum starvation assays, cells were cultured in serum-free medium. Generation of Hep-AEG-1–14 and Hep-AEG-1–8 clones, HepG3 cells stably expressing AEG-1, and Hep-pc-4, HepG3 cells stably transduced with empty vector, has been described previously (1). Cell viability was determined by standard MTT assays as described (1). Thymidine, 5-FU, and 5′dFUrd were used at a dose of 20 μM, 50 μM, and 10 μM, respectively.

Plasmids, siRNAs, and Lentiviruses. LSFwt and LSFdn constructs have been described before (2). All siRNAs were obtained from Santa Cruz Biotechnology. The 19-bp AEG-1 sequence used to generate AEG-1 shRNA is 5′ CAGAAGAAGAAGAACCGGA 3′. Detailed description of lentivirus vector production is described previously (3).

Transient Transfection and Luciferase Assay. Transfection was carried out using lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. For luciferase assays, cells were plated into 24-well plates and the next day transfected with empty vector (pGL3-basic), pGL3B-WT4-E1b (luciferase reporter plasmid containing 4 tandem LSF-binding sites), or pgL3B-MT4-E1b (luciferase reporter plasmid containing 4 tandem mutated LSF-binding sites) and renilla luciferase expression plasmid for transfection control. Luciferase assays were measured using a Dual Luciferase Reporter Assay kit (Promega) according to the manufacturer's protocol and firefly luciferase activity was normalized by renilla luciferase activity. To knock down AEG-1 or DPYD, cells were cultured for 2 days after transfection of 20 pmol of siRNA for AEG-1 or DPYD, respectively.

Preparation of Whole Cell Lysates and Western Blot Analyses. Preparation of whole cell lysates and Western blot analyses was performed as described (1). The primary antibodies used were anti-AEG-1 (1:500; chicken polyclonal), anti-LSF (1:2,000; mouse monoclonal; BD Biosciences), anti-actin (1:1,000; rabbit polyclonal; Santa Cruz), anti-laminB (1:1,000; goat polyclonal; Santa Cruz), anti-TS (1:1,000; mouse monoclonal; Abcam) and anti-DPYD (1:1,000; mouse monoclonal; Abcam). Blots were stripped and normalized by re-probing with anti-β-tubulin (1:2,000; mouse monoclonal; Sigma).

Extraction of Total RNA and Real-time PCR. Total RNA was extracted using a Qiagen mRNAeasy mini kit (Qiagen). Real-time PCR was performed using ABI 7900 fast real-time PCR system and Taqman gene expression assays for LSF, DPYD, TS, and α-globin according to the manufacturer's protocol (Applied Biosystems).

Immunofluorescence and Immunohistochemical Analyses. Immunofluorescence studies in cells and tumor sections and immunohistochemical studies in tumor sections were performed as described (1). For immunofluorescence the primary antibodies used were: anti-LSF (1:100; mouse monoclonal; BD Biosciences), anti-AEG-1 (1:400; rabbit polyclonal; Invitrogen), and anti-Ki67 (1:200; mouse monoclonal; BD Biosciences). The secondary antibody was Alexa 488-conjugated anti-mouse IgG or anti-rabbit IgG (Molecular Probes). The samples were mounted in VectaShield fluorescence mounting medium containing 4,6 -diamidino-2- phenylindole (DAPI; Vector Laboratories). Images were analyzed by a Zeiss confocal laser scanning microscope. For immunohistochemistry anti-AEG-1 antibody was used at 1:200 dilution. The sections were developed by avidin-biotin-peroxidase complexes with DAB substrate solution (Vector Laboratories). The slides were co-stained with 10% hematocylin solution. The images were taken by an Olympus microscope.

Preparation of Cytosolic and Nuclear Extracts and Electrophoretic Mobility Shift Assay. Fractionation of cytosolic and nuclear extracts was performed using the nuclear extract kit (ActiveMotif), according to the manufacturer's protocol. EMSA was performed using Gel Shift Assay System (Promega) with minor modifications. ^{32}P-label oligonucleotides containing LSF-binding site was incubated with purified nuclear extracts from the cells at 4°C for 30 min. The reactions were subjected to electrophoresis and autoradiography. For competition experiments, wild-type and mutated oligonucleotides corresponding to LSF-binding sites were added, using identical conditions as described. For supershift experiments, anti-LSF antibody was preincubated for 30 min at 4°C before adding the oligonucleotides probe. The sequences of the wild-type probe are, sense: 5′-ANA ACT GGG TNG AGC CNG C- 3′ and antisense: 5″- G CNG GCT CNA CCC AGT TNT-3′ and that of the mutated probe are, sense: 5′-TAT GGG TNG AGA CNG C-3′ and antisense: 5′- G CNG TCT CNA CCC ATA TNT-3′.

Nude Mice Xenograft Studies. QGY-7703 cells were transduced with either a lentivirus expressing control (scrambled) shRNA or a lentivirus expressing AEG-1 shRNA at a concentration of 2 moi per cell for 48 h. One million cells were s.c. implanted in the flanks of athymic nude mice. 5-FU was injected 3 times/week for 2 weeks at a dose of 30 mg/kg. Tumor diameter was measured with calipers at 2 weeks later after injection, and the tumor volume in mm^3 was calculated by the formula: (width)^2 $\times$ length/2.

TUNEL Assay. Apoptotic cell death was detected by the deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) method using ApoAlert DNA fragmentation assay kit (Clontech) according to the manufacturer's protocol. Images were analyzed using an Olympus immunofluorescence microscope.

Statistical Analysis. Data were represented as the mean $\pm$ SEM and analyzed for statistical significance using 1-way analysis of variance (ANOVA) followed by Newman-Keuls test as a post-hoc test. A P value of <0.05 was considered significant.

1. Yoo BK, *et al.* (2009) Astrocyte elevated gene 1 regulates hepatocellular carcinoma development and progression. *J Clin Invest* 119:465–477.
2. Shirra MK, Zhu Q, Huang HC, Pallas D, Hansen U (1994) One exon of the human LSF gene includes conserved regions involved in novel DNA-binding and dimerization motifs. *Mol Cell Biol* 14:5076–5087.
3. Kock N, Kasmieh R, Weissleder R, Shah K (2007) Tumor therapy mediated by lentiviral expression of shBcl-2 and S-TRAIL. *Neoplasia* 9(5):435–442.

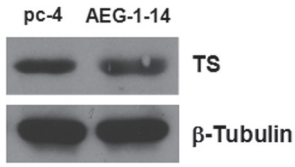

Fig. S1. Steady-state expression of thymidylate synthase (TS) protein is unchanged between Hep-pc-4 (pc-4) and Hep-AEG-1-14 (AEG-1-14) clones. Lysates from exponentially growing indicated cells were subjected to Western blot for TS and β-tubulin.