Supporting Information

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SI Materials and Methods

Genotyping of Conditional Cyclin E Knockout Mice. Genotyping of conditional E1 knockout mice was performed using primers E11a: 5′-CGCCATGGTTATCCGGGAGATGG-3′, 11–2: 5′-CGCATACTGAGACACAGACT-3′, and E4in6: 5′-GAAGAGGGCATCAGATCCTATTAC-3′. PCR conditions were as follows: denaturation step at 94 °C for 3 min, followed by 30 cycles of amplification: 94 °C for 1 min, 64 °C for 1 min, 72 °C for 1 min, and a final extension step at 72 °C for 5 min. For semiquantitative PCR, only 25 cycles of amplification were performed. Wild-type cyclin E1, $E1^{F}$, and $E1^{\Delta}$ alleles yielded, respectively, 229 bp, 328 bp, and 257 bp products. The genotype at cyclin E2 locus was determined as described (1).

Acute Ablation of Cyclin E. To ubiquitously delete cyclin E, cyclin $E1^{F/F}E2^{+/-}$ female mice were bred with cyclin $E1^{+/F}E2^{+/-}$/Esr1-Cre male mice. At day 17.5 of gestation, pregnant $E1^{F/F}E2^{+/-}$ females were intraperitoneally (IP) injected with tamoxifen (Sigma) 75 mg per kg of body weight. Mice born to these mothers, carrying cyclin $E1^{F/+}E2^{+/-}$/Esr1-Cre (control) or $E1^{F/F}E2^{-/-}$/Esr-1-Cre (experimental) genotype were IP injected with tamoxifen 150–225 mg per kg of body weight starting at postnatal day 19. Tamoxifen was injected every 3 d, five times in total. Mice were observed for 10–13 mo before being killed and subjected to histopathological analyses. Peripheral blood was collected for blood chemistry analyses at 10–13 mo of age.

For deletion of cyclin E in liver and in liver tumors, cyclin $E1^{F/F}E2^{-/-}$ mice were bred with Mx1-Cre mice. Cyclin $E1^{+/F}E2^{+/-}$/Mx1-Cre (control) and $E1^{F/F}E2^{-/-}$/Mx1-Cre mice were IP injected three to five times with polyinosinic–polycytidylic acid (pI–pC, Amersham), 2.5 mg per gram of body weight every other day.

Injections of DEN. To induce liver tumors, diethylnitrosamine (DEN) (Sigma) was injected once to 2-wk-old male mice at the dose of 25 mg/kg of body weight.

Histological Analyses of Tissues and Tumors. Tissues or tumors were fixed in 10% neutral buffered formalin for 36 h and transferred to 70% ethanol. The Rodent Histopathology Core at Harvard Medical School carried out paraffin embedding, sectioning, and staining with hematoxylin and eosin. Tumors and normal organs from a minimum of five mice were analyzed for each experimental and control group.

Hematological Analyses. Mice were killed and blood was collected by cardiac puncture for complete blood count analysis (using BD Microtainer tubes with K2-EDTA) or for blood serum analysis (using BD Microtainer serum separator tubes). Analysis was performed by the Department of Laboratory Medicine of Boston Children's Hospital.

Apoptosis Analyses. Annexin staining was performed using a kit from BD Biosciences, following manufacturer's instructions. The TUNEL staining was performed by DF/HCC Pathology Core at Brigham and Women's Hospital.

Tumor Burden Estimation. Volumes of liver tumors were calculated according to the formula $V = a \times b^2/2$ (a is tumor length and b is tumor width) (2). Tumor sizes (mm^2 area) were measured on hematoxylin and eosin stained sections of mouse livers using ImageJ software. Each mouse liver sample was step sectioned with 500 μm apart, and tumor sizes were measured on three sections.

Cell Growth and Cell Cycle Analyses. For analyzing cell growth, equal numbers of control and experimental cells were plated in triplicate. Cells were fixed 1 d after plating (day 0), and at the indicated time points. The increase in cell number was measured as described (1). For analyzing cell cycle profile, cells were incubated in a medium containing 75 μM 5-bromo-2′-deoxyuridine (BrdU) for 1 h, collected, and fixed overnight in 90% ethanol. Cells were then treated with 2 M HCl containing 0.5% Triton X-100, neutralized in borate buffer, pH 8.5, incubated with an anti-BrdU antibody (556028, BD Biosciences), 5 μg/mL propidium iodide and 200 μg/mL RNase A for 30 min, and analyzed by FACS. Inhibitor 3MM-PP1 (EMD Millipore) was used at 10 μM concentration.

Immunohistochemistry. Formalin-fixed paraffin-embedded tissue sections were deparaffinized, boiled with 10 mM sodium citrate (pH 6.0) for 10 min, and treated with 3% H_2O_2 for 10 min. For BrdU staining, sections were incubated in 1 M HCl for 10 min on ice, in 2 M HCl for 10 min at room temperature, in 2 M HCl for 20 min at 37 °C, and neutralized in 0.1 M sodium tetraborate (pH 9.0) for 10 min at room temperature. Blocking was performed with 5% goat serum (in TBS-T with 1% BSA), followed by incubation with the primary anti-BrdU antibody (1:100, diluted in TBS-T with 5% goat serum and 1% BSA) for 1 h followed by incubation with biotinylated secondary antibody (diluted in TBS-T with 1% BSA) for 30 min at room temperature. Subsequently, antibody binding was visualized using the VECTASTAIN ABC Kit and the ImmPACT DAB substrate, and counterstained using Vector Hematoxylin QS (all according to the manufacturer's instructions).

Viral Transduction and Transfection. Retroviral and lentiviral plasmids were packaged in 293T cells by cotransfecting expression plasmids together with the packaging plasmid (pCMV-Gag-Pol for retroviral infections, pCMV-delta8.9 for lentiviral infections) and the envelope plasmid (pCMV-VSV-G), using the calcium phosphate precipitation method. Virus-containing medium was collected 2 d after transfection and filtered and used for infection in the presence of 10 μg/mL polybrene. For double infections, the viruses were concentrated on Amicon Ultra-15 100-kDa centrifugal columns. Twenty-four hours after the infection, virus-containing medium was replaced by a fresh medium. Cells were selected for 3–5 d with puromycin (1–2 μg/mL), or 7 d with neomycin (0.2–1 mg/mL).

Immunoprecipitation and Western Blotting. For immunoprecipitation, 100 μg to 1 mg of proteins were immunoprecipitated overnight at 4 °C with antibodies against cyclin E1 (sc-248AC, Santa Cruz), CDK2 (sc-163AC, Santa Cruz), CDK1 (sc-54AC, Santa Cruz), hemagglutinin (HA) (MMS-101P, Covance) or Flag (M2, Sigma). Immunoprecipitated complexes were resolved on SDS/PAGE gels. The proteins were detected using specific antibodies with enhanced chemiluminescence reagents (GE Healthcare).

In Vitro Kinase Assays. Kinase assays were performed in a final volume of 30 μL of a kinase buffer: 50 mM Hepes pH 7.5, 10 mM $MgCl_2$, 1 mM DTT, 1 mM EGTA, 0.1 mM NaF, containing 10 μM ATP, and 4 μCi [^{32}P]γATP (Perkin-Elmer). Endogenous cyclin E1, CDK2, or HA-tagged CDK2 was immunoprecipitated from whole cell lysates. Histone H1 (EMD Millipore, 14-155) was used as kinase substrate, 2 μg per reaction.

Plasmids. Plasmids expressing HA-CDK2-WT and HA-CDK2-DN (D145 → N) were kind gifts from David Livingston, Dana-Farber Cancer Institute. Mouse analog-sensitive CDK2 was constructed

by subcloning mouse CDK2 cDNAs carrying F80 → G mutation into p3xFlag-CMV-10 vector (Sigma). All CDK2 mutants were generated with the QuikChange XL Site-Directed Mutagenesis kit (Stratagene). Plasmid expressing cyclin $E1^{188\text{-}192A}$ mutant was obtained from Bruce Clurman and Fred Hutchinson Cancer Research Center.

1. Geng Y, et al. (2003) Cyclin E ablation in the mouse. *Cell* 114:431–443.
2. Carlsson G, Gullberg B, Hafström L (1983) Estimation of liver tumor volume using different formulas: An experimental study in rats. *J Cancer Res Clin Oncol* 105:20–23.

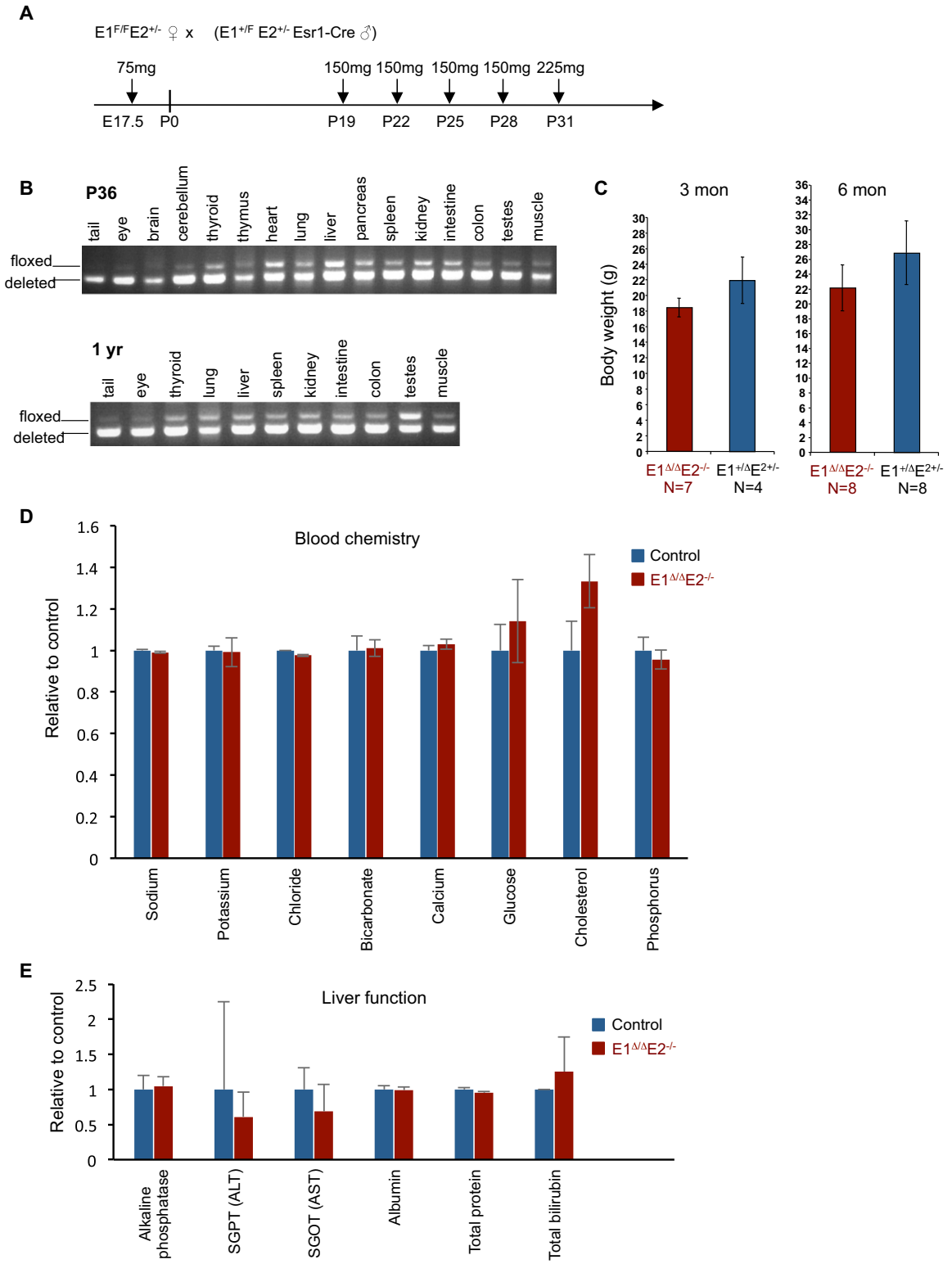

Fig. S1. E-type cyclins are not essential in postnatal mice. (*A*) Experimental approach to ablate E cyclins in vivo. Cyclin $E1^{F/F}E2^{+/-}$ females were mated with $E1^{+/F}E2^{+/-}$/Esr1-Cre males. The ratio of 1:16 of the resulting embryos was expected to be of the cyclin $E1^{F/F}E2^{-/-}$/Esr1-Cre genotype (experimental) and 1:8 cyclin $E1^{+/F}E2^{+/-}$/Esr1-Cre (control). Pregnant females were injected with tamoxifen, to activate Cre, at day E17.5 of gestation. Neonates were again injected with tamoxifen at the indicated postnatal (P) days. The dose administered at each time point is indicated as milligrams (mg) per kilogram of body weight. (*B*) Semiquantitative PCR analysis of the deletion efficiency of the genomic cyclin E1 locus in the indicated organs of cyclin $E1^{\Delta/\Delta}E2^{-/-}$/Esr1-Cre mice. *Top* shows analyses of mouse organs collected 5 d after the last tamoxifen dose (P36), and *Bottom* shows organs collected 1 y after the last injection of tamoxifen. (*C*) Mean body weights of cyclin E deleted ($E1^{\Delta/\Delta}E2^{-/-}$/Esr1-Cre) and control ($E1^{+/\Delta}E2^{+/-}$/Esr1-Cre) mice at 3 mo (*Left*) and 6 mo (*Right*) of age. (*D*) Peripheral blood chemistry analyses of cyclin E deleted (four samples pooled from 11 mice) and control mice (four samples pooled from 12 mice). (*E*) Analysis of markers of liver function in the peripheral blood of cyclin E-deleted (four samples pooled from 11 mice) and control mice (four samples pooled from 12 mice). In *C*–*E*, mean values are shown; error bars indicate SD.

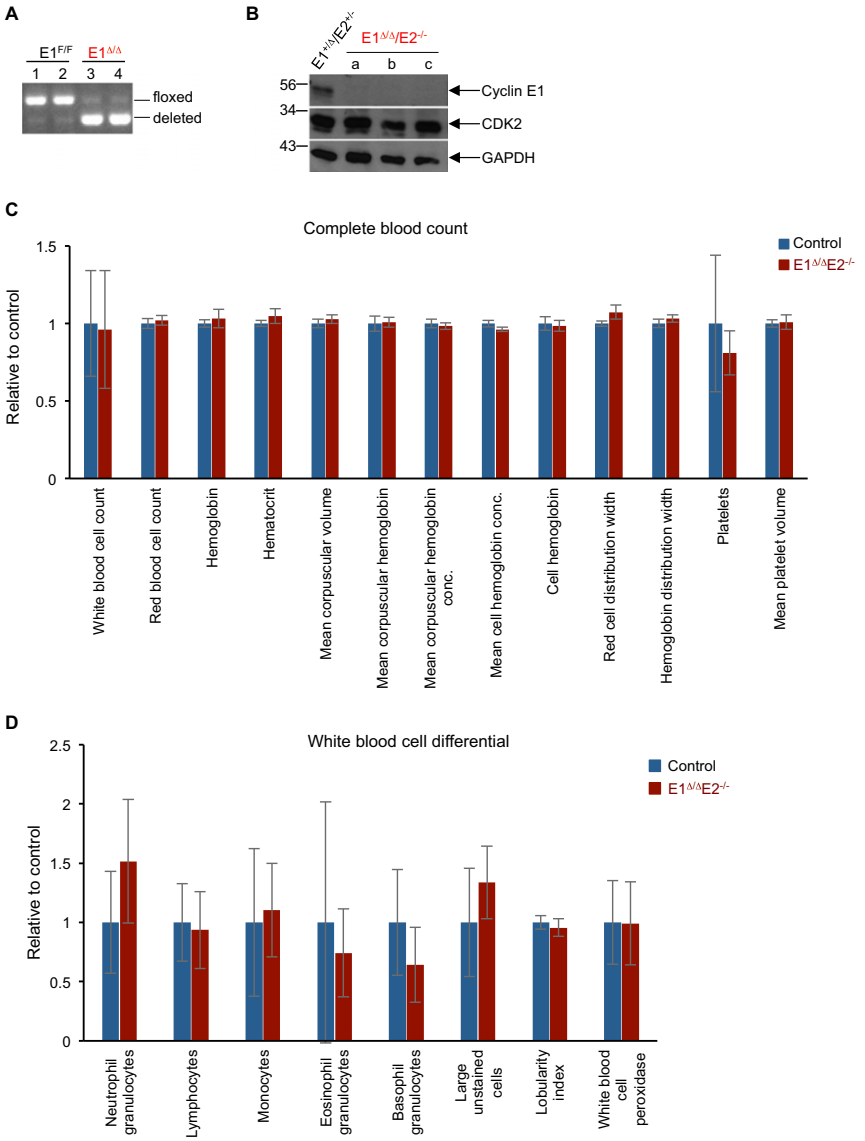

Fig. S2. Analyses of mice that sustained ablation of cyclin E. (*A*) Semiquantitative PCR analysis of cyclin E1 locus deletion efficiency in the livers of two cyclin $E1^{F/F}E2^{-/-}$/Mx1-Cre mice before injection of pI–pC ($E1^{F/F}$, lanes 1 and 2), and two mice 1 wk after the last pI–pC dose ($E1^{\Delta/\Delta}$, lanes 3 and 4). Bands corresponding to cyclin $E1^{F}$ (floxed) and $E1^{\Delta}$ (deleted) alleles are indicated on the *Right*. (*B*) Three cyclin $E1^{F/F}E2^{-/-}$/Mx1-Cre (*a*–*c*) and one control $E1^{+/F}E2^{+/-}$/Mx1-Cre mouse were treated with pI–pC as in *A*. Livers ($E1^{\Delta/\Delta}E2^{-/-}$ and $E1^{+/\Delta}E2^{+/-}$, respectively) were analyzed by immunoblotting with the indicated antibodies. GAPDH was used as a loading control. Note that cyclin E1 expression is quite low in control mouse liver. (*C*) Complete blood counts and (*D*) white blood cell differential, determined 3 mo after induction of cyclin E deletion (by pI–pC injection) in cyclin $E1^{\Delta/\Delta}E2^{-/-}$/Mx1-Cre mice ($n = 7$) and in control $E1^{+/\Delta}E2^{+/-}$/Mx1-Cre mice ($n = 6$). In *C* and *D*, mean values are shown; error bars indicate SD.

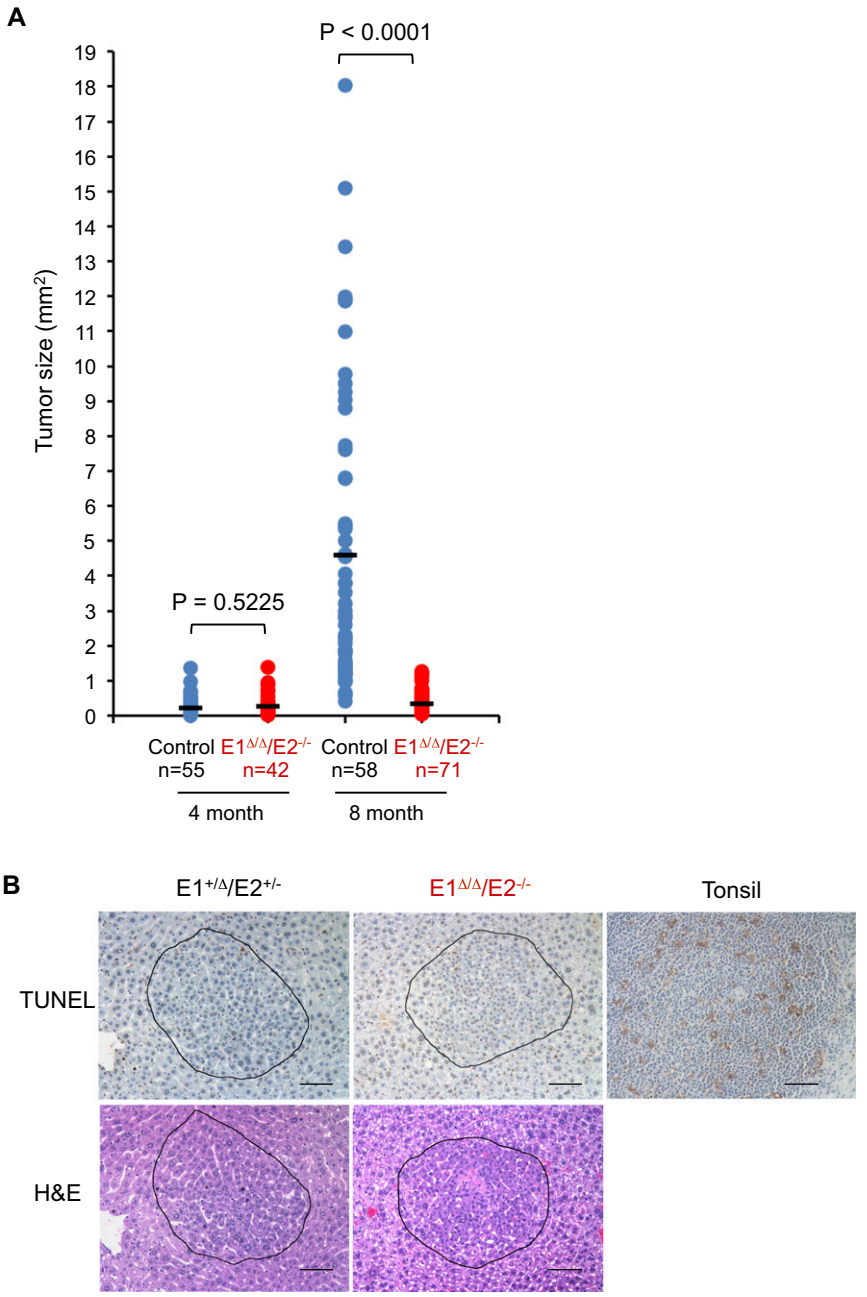

Fig. S3. Analyses of mouse tumors. (*A*) Liver tumor size (mm^2 area) was measured on hematoxylin and eosin-stained liver sections from control (cyclin $E1^{+/\Delta}E2^{+/-}$) and from cyclin E-deleted ($E1^{\Delta/\Delta}E2^{-/-}$) mice at 4 mo, i.e., 1–2 wk after the last polyI–polyC injection (55 tumors in seven control and 42 tumors in six cyclin E-deleted mice), and at 8–9 mo after DEN injection (58 tumors in five control and 71 tumors in six cyclin E-deleted mice). Each dot represents a different tumor; black lines denote mean values. *P* values were calculated using two-tailed *t* test. (*B*) Sections of livers with tumors from mice at 4 mo of age (1 d after the last polyI–polyC injection) stained for TUNEL (*Upper*) or with hematoxylin and eosin (H&E, *Lower*). Solid lines indicate boundaries of small tumors. A section of a mouse tonsil was stained as positive control for TUNEL. (Scale bar, 25 μm.)

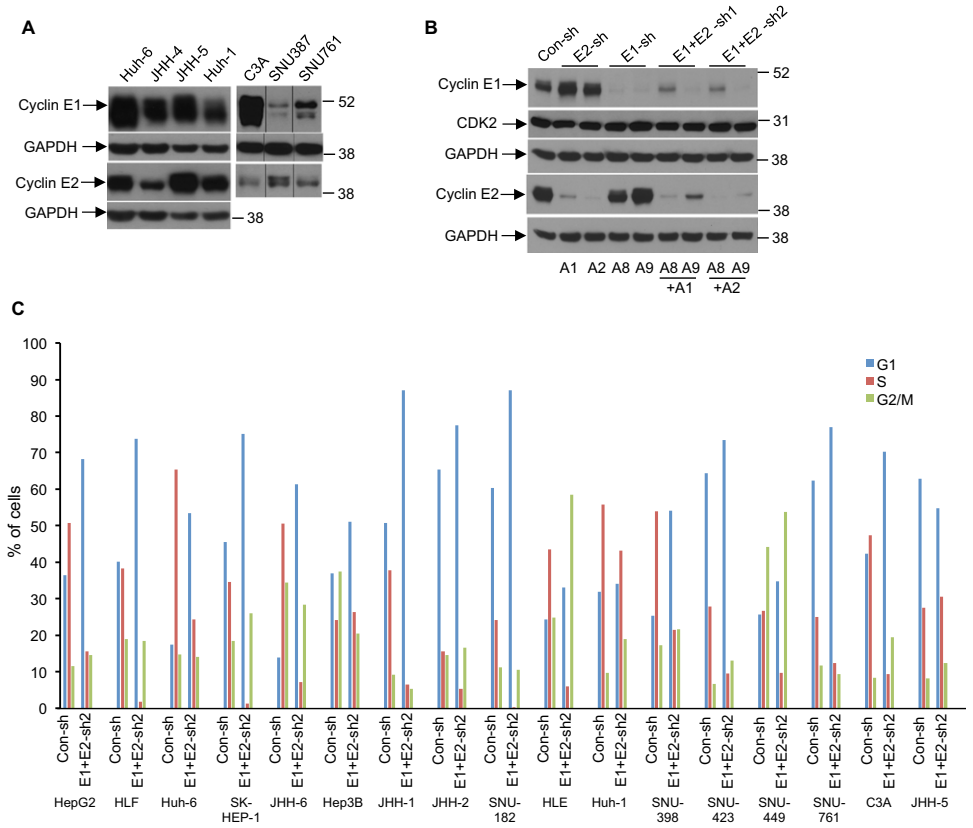

Fig. S4. Analyses of human HCC cell lines. (*A*) Western blot analysis of cyclin E1 and E2 levels in seven human HCC cell lines. The middle portions of the *Right* were spliced out (indicated by lines). GAPDH was used as a loading control. Please see Fig. 2*A* for analyses of the remaining cell lines. (*B*) Western blot analysis of cyclin E1 and E2 levels in SK-HEP-1 cells to assess the efficiency of knockdown. Two independent shRNAs against cyclin E1 (E1-sh: A8, A9) or two independent shRNAs against cyclin E2 (E2-sh: A1, A2), or a combination of shRNAs against cyclins E1 and E2 (E1+E2-sh1, E1+E2-sh2) were used to knock down cyclins E1 and E2. Con-sh, control shRNA. The blots were probed with the indicated antibodies. GAPDH was used as a loading control. Note that the levels of CDK2 protein remained unchanged after depletion of cyclins E1 and E2. (*C*) Cell cycle profiles of 17 human HCC lines following depletion of cyclins E1 and E2 (E1+E2-sh2). Con-sh, cells expressing control shRNA. Cells were pulsed with BrdU, stained with an anti-BrdU antibody and propidium iodide, and analyzed by flow cytometry. The percentages of cells in each cell cycle phase are indicated.

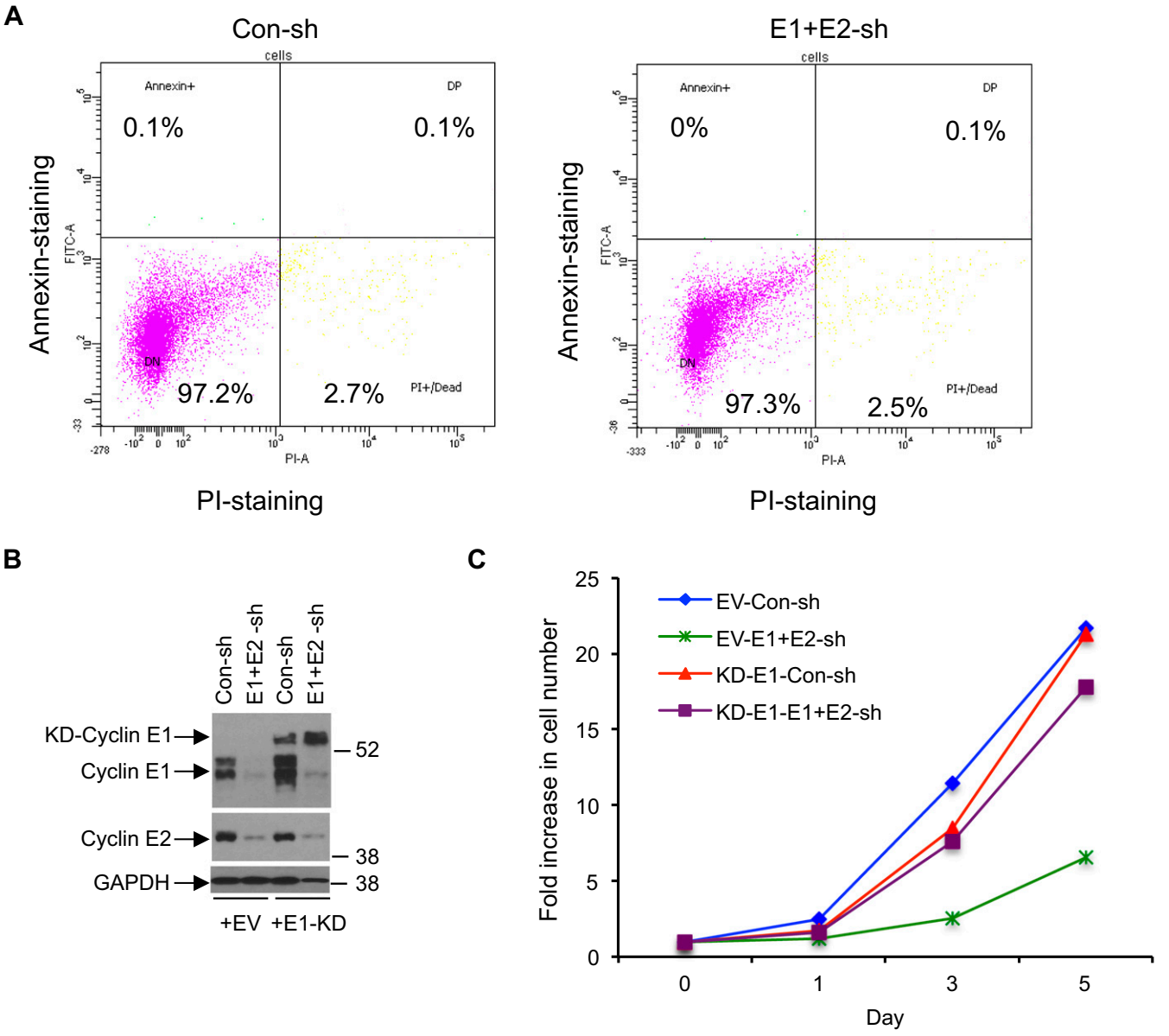

Fig. S5. (*A*) Apoptosis analysis of HepG2 cells expressing control shRNA (Con-sh), or shRNAs against cyclins E1 and E2 (E1+E2-sh). The cells were stained with annexin and propidium iodide (PI), and analyzed by flow cytometry. The percentage of cells in each quadrant is indicated. (*B*) Western blot analysis of cyclin E1 and E2 protein levels in HLF cells expressing control shRNA (Con-sh) or shRNAs against cyclins E1 and E2 (E1+E2-sh). Cells were engineered to express shRNA-resistant Myc-tagged cyclin E1$^{188-192A}$ kinase-dead mutant (+E1-KD) or an empty vector (+EV). GAPDH was used as a loading control. (*C*) Growth curves of control HLF cells expressing empty vector and control shRNA (EV-Con-sh), or empty vector and anti-cyclin E1 and E2 shRNA (EV-E1+E2-sh, cyclin E-depleted cells), or cyclin E1 kinase-dead mutant and control shRNA (KD-E1-Con-sh), or cyclin E1 kinase-dead mutant and anti-cyclin E1 and E2 shRNAs (KD-E1-E1+E2-sh).