Supplemental information

Hepatocellular carcinoma repression by TNFa-mediated synergistic lethal effect of mitosis defect-induced senescence and cell death sensitization

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Supplemental Figure Legends

Figure S1. Liver cancer development is comparable between *svv*$^{f/f}$ mice and *Mx-cre*; *svv*$^{f/f}$ mice before Survivin deletion

(A) Deletion efficiency of Survivin 1 week after poly(IC) treatment was determined by genotyping PCR using genomic DNA extracted from liver or bone marrow. The genomic DNA of tumor infiltrated CD3 positive and F4/80 positive cells were extracted from *Mx-cre; svv*$^{f/f}$ mice 8 months after DEN treatment and 1 week after poly(IC) treatment. Survivin deletion efficiency is characterized by genotyping. (B) Survivin mRNA levels in *Mx-cre; svv*$^{f/f}$ and *svv*$^{f/f}$ HCCs 1 week after poly(IC) treatment were determined by q-PCR. *n*=4 for each group. Data are normalized to b-actin. (C) *Mx-cre; svv*$^{f/f}$ and control *svv*$^{f/f}$ mice were treated with DEN at the age of 2 weeks. These mice were sacrificed at the age of 5 months before poly(IC)-induced Survivin deletion. H&E staining of liver sections of *Mx-cre; svv*$^{f/f}$ and control *svv*$^{f/f}$ mice were shown. Dashed lines encircled liver cancers. The number and area of liver tumors were quantified. *n*=4 for each group. Scale bar, 100 mm. (D) H&E staining of liver sections of *Mx-cre; svv*$^{f/f}$ and control *svv*$^{f/f}$ mice. These mice were treated with DEN at the age of 2 weeks and killed at the age of 8 months without poly(IC) treatment. Tumor mass was quantified on H&E stained liver sections. *n*=4 for each group. (E) Survivin was deleted in *Mx-cre; svv*$^{f/f}$ mice at the age of 5 months by poly(IC) treatment. Serum ALT, AST and total bilirubin levels were measured in these *svv*$^{\Delta li}$* mice at the age of 10 months. *n*=4 for

each group. (F) Survivin was deleted in *Mx-cre; svv*$^{f/f}$ mice at the age of 8 months by poly(IC) treatment. Serum ALT, AST and total bilirubin levels were measured in these *svv*$^{\Delta li}$* mice at the age of 10 months. *n*=4 for each group. (G) In the same experiment as (F), Kaplan-Meier survival analysis showed that *svv*$^{\Delta li}$* mice after poly(IC) treatment at the age of 8 months survived longer than the control mice. *n*=10 for *svv*$^{\Delta li}$* and *n*=8 for *svv*$^{f/f}$. (log-rank test, *P*<0.001). *: *P*<0.05, *t*-test.

Figure S2. Impaired HCC development in *Alb-cre; svv*$^{f/f}$ mice

(A) *Alb-cre; svv*$^{f/f}$ and control *svv*$^{f/f}$ mice were treated by DEN to induce liver cancers at the age of 2 weeks. HCC development was analyzed in *Alb-cre; svv*$^{f/f}$ and control *svv*$^{f/f}$ mice 10 months after DEN treatment. Liver/bodyweight ratios were measured. *n*=5 for each group. *: *P*<0.05, *t*-test. (B) H&E staining of liver sections from *Alb-cre; svv*$^{f/f}$ and control mice 10 months after DEN treatment. Liver cancers were encircled by dashed lines. Liver cancers were quantified on H&E stained sections. *n*=5 for each group. Scale bars, 100 mm. *: *P*<0.05, *t*-test. (C) Serum ALT, AST and total bilirubin levels were measured in 10-month old *Alb-cre; svv*$^{f/f}$ and control mice. *n*=5 for each group. *: *P*<0.05, *t*-test. (D) Kaplan-Meier analysis showed that *Alb-cre; svv*$^{f/f}$ mice exhibited improved survival rate after DEN treatment. *n*=28 for *Alb-cre; svv*$^{f/f}$ and *n*=25 for *svv*$^{f/f}$. *: log-rank test, *P*<0.0005. (E) HCC is induced by hydrodynamic transfection of plasmids encoding the Sleeping Beauty transposase and

transposons with oncogenes of c-Met and DN90-b-Catenin in 8-week old *Mx-cre; svv*$^{f/f}$ and control *svv*$^{f/f}$ mice. 8 weeks later, these mice were treated with poly(IC) to induce Survivin deletion. 3 weeks later, these mice were sacrificed to quantify the HCC mass. The HCC formation is markedly reduced in *svv*$^{\Delta li}$* mice. *n*=3 for each group.

Figure S3. Significant toxicity and low Survivin suppression efficiency of YM155 in DEN-induced HCCs

(A) YM155 intraperitoneal treatment at the dosage of 5 mg/Kg/day for 1 month caused about 38% body weight loss of mice with DEN-induced HCC. Animals were sick and sacrificed due to extreme body weight loss. (B) The HCC development was decreased by about 20% after 1 month YM155 treatment.

Figure S4. Survivin deficiency leads to impaired mitosis and senescence in HCCs

(A) Co-immunofluorescent staining of a-tubulin and Aurora-B kinase in liver cancer cells. The number of liver cancer cells in mitosis was quantified. Scale bars, 0.5 mm. *n*=4 for each group. *: *P*<0.05, *t*-test. (B) Gene set enrichment analysis (GSEA) of expression profiles of cell cycle and mitosis genes. These data suggested impaired mitosis and cell cycle in *svv*$^{\Delta li}$* liver cancers. (C) q-PCR analysis of senescence-associated inflammatory genes. *n*=6 for *svv*$^{f/f}$ mice and *n*=8 for *svv*$^{\Delta li}$* mice. (D) Human primary HCC cells were transfected

with a scramble siRNA or Survivin-specific siRNAs. Efficient Survivin silence was detected by q-PCR. (E) Survivin silence caused decreased cell numbers.

Figure S5. Survivin protects HCC cells from senescence-associated TNFa-induced cell death

(A) Apoptosis in $svv^{\Delta li*}$ and $svv^{f/f}$ liver cancers was determined by Western blots of Cleaved Caspase 3. (B) GSEA analysis of cell death genes in $svv^{\Delta li*}$ and $svv^{f/f}$ liver cancers. (C) The expression levels of TNFa were determined by q-PCR 1-week after PBS or Etanercept treatment. (D) Cell senescence in $svv^{\Delta li*}$ HCCs were determined by SA-b-gal staining 1 week after PBS or Etanercept treatment.

Figure S6. PLK1 inhibitor induces mitotic defects and senescence

(A) BI2536 (50nM) treatment caused decreased cell numbers. (B) SA-b-gal staining of human primary HCC cells treated with BI2536 or DMSO for 72 hours. The SA-b-gal-positive cells were quantified. *: $P<0.05$, *t*-test. (C) Senescent associated heterochromatin foci (SAHF) were characterized by DAPI-DNA staining. SAHF were indicated by arrows. (D) q-PCR analysis of senescence-associated genes in primary HCC cells treated with BI2536 for 72 hours. *: $P<0.05$, *t*-test. All results represent three independent experiments in A-D. (E) The expression levels of senescence-associated genes were measured by q-PCR in BI2536-treated HCCs. $n=7$ for DMSO group and $n=7$

for BI2536 treatment. (F) Immunofluorescent staining of F4/80 and CD3 in BI2356-treated liver cancers. Arrowheads indicate the positive cells. *n*=4 for each group. *: *P*<0.05, *t*-test. (G) Primary human HCC cells pre-treated with Smac mimetic LCL161 for 24 hours. Cells were then treated with 10ng/ml human TNFa for 4 hours. Dead cells were analyzed by propidium iodide (PI) and FITC-annexin V staining. Results represent three independent experiments. (H) The expression of Survivin were measured by q-PCR in HCCs. (I) The expression of senescence-associated genes were measured by q-PCR in HCCs. *n*=4 for DMSO group, *n*=4 for LCL group, *n*=6 for BI/LCL group.

Figure S7. Senescence and TNFa-induced cell death response in human HCCs

(A) The expression levels of Survivin were measured by q-PCR. (B) The expression levels of senescence-associated genes in PDX tumors were measured by q-PCR. *n*=5 for DMSO group, *n*=5 for BI group, *n*=3 for LCL group, *n*=5 for BI/LCL group. (C) PDX liver cancers were treated with BI2536, LCL161 or combination of BI2536 and LCL161. Infiltration of macrophages was analyzed by F4/80 immunofluorescent staining in PDX human liver cancers. *n*=3 for each group. Scale bars: 50 mm.

Figure S8. The safety of combination use of BI2536 and LCL161 in

normal or liver injured mice

(A) DEN-induced HCC-bearing mice and PDX mice treated by combination of BI2536 and LCL161 (BI/LCL) for 2 months and 1 month, respectively. Serum levels of Albumin (ALB), Total Bilirubin (TBIL), Alanine Aminotransferase (ALT) and Aspartate Aminotransferase (AST) (as indicators for liver functions) and serum levels of Creatinine (Cr) and Blood Urea Nitrogen (BUN) (as indicators for kidney functions) of these mice were measured. (B) Body weight of DEN-induced HCC-bearing mice and PDX mice were measured 2 months and 1 month after combination treatment of BI2536 and LCL161. (C) 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) diet induced mice liver injury. The serum levels of ALT and TBIL were markedly increased in mice fed with DDC diet for 2 weeks. (D) DDC diet-induced liver injured mice were treated with combined BI2536 and LCL161 at the dosage of 25 mg/Kg every other day for 2 weeks. The body weights of BI/LCL treated mice were comparable with the control mice and the serum levels of ALT and TBIL were not increased in BI/LCL treated mice compared with control mice.

Figure S9. The pharmacokinetics and pharmacodynamics of combination use of BI2536 and LCL161

(A) To evaluate the pharmacokinetics of BI2536 and LCL161, blood samples were taken at 0.5, 1, 2, 4, 8, 24, and 48 hours after intraperitoneal injection of BI2536 and LCL161 either alone or in combination, *n*=3 for each time point.

BI2536 and LCL161 were analyzed by high-performance liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). Clearance (CL), area under the concentration-time curve over the time interval from 0 extrapolated to last measured time (AUC), terminal half-life in plasma ($t_{1/2}$), maximum plasma concentration (C_{max}) were calculated and shown. (B) The pharmacological interactions between BI2536 and LCL161 were analyzed in mice with DDC-induced chronic liver injury. Pharmacokinetic studies reveal that the clearances of BI2536 or LCL161 were not much changed when used in combination or alone. (C) Immunofluorescent staining of a-tubulin showed that DEN-induced HCC cells were arrested in mitosis by BI2536 2 weeks after combination treatment of BI2536 and LCL161. a-tubulin is stained in red and DNA is stained in blue by DAPI. (D) LCL161-caused cell death of DEN-induced HCC cells was determined by TUNEL staining 2 weeks after combination treatment of BI2536 and LCL161. TUNEL positive HCC cells were quantified. Scale bars, 50 mm.

Figure S10. Higher magnification histological pictures of three HCC models were shown

Higher magnification histological pictures were shown. The DEN-induced HCCs showed moderate differentiation with prominent atypical pleomorphic and high-grade nuclei. c-Met/ΔN90-β-Catenin-induced HCC tumors showed well-differentiated carcinoma with mild atypia and low grade nuclei. PDX HCCs

showed moderate differentiation and pseudo-acinar pattern.

Supplemental Tables

Table S1 Primer pairs for q-PCR

Gene	Forward (5'-3')	Reverse (5'-3')
Actin (Mouse)	CTGGCTGGCCGGGACCTGACA	ACCGCTCGTTGCCAATAGTGATGA
p21 (Mouse)	ACCCGGGTCCTTCTTGTGTTTC	CGTTTTCGGCCCTGAGATGTTC
Survivin (Mouse)	CATCGCCACCTTCAAGAACT	AAAACACTGGGCCAAATCAG
TNFa (Mouse)	CCCTCACACTCAGATCATCTTCT	GCTACGACGTGGGCTACAG
Lamp2a (Mouse)	ATGTGCCTCTCTCCGGTTAAA	GCAAGTACCCTTTGAATCTGTCA
Oas2 (Mouse)	AAACCTCACACCCAACGAAAA	CCACCCTTAGCCACTTCCT
IL1a (Mouse)	AGTATCAGCAACGTCAAGCAA	TCCAGATCATGGGTTATGGACTG
CCL27 (Mouse)	AGGAGGATTGTCCACATGGAA	CTTGGCGTTCTAACCACCGA
CCL2 (Mouse)	TAAAAACCTGGATCGGAACCAAA	GCATTAGCTTCAGATTTACGGGT
CCL5 (Mouse)	GCTGCTTTGCCTACCTCTCC	TCGAGTGACAAACACGACTGC
Cxcl1 (Mouse)	ACTGCACCCAAACCGAAGTC	TGGGGACACCTTTTAGCATCTT
Cxcl9 (Mouse)	GGAGTTCGAGGAACCCTAGTG	GGGATTTGTAGTGGATCGTGC
TNFa (Human)	AGCCTCTTCTCCTTCCTGATCG	CCTCAGCTTGAGGGTTTGCTAC
Lamp2a (Human)	GAAAATGCCACTTGCCTTTATGC	AGGAAAAGCCAGGTCCGAAC
Oas2 (Human)	AGGTGGCTCCTATGGACGG	TTTATCGAGGATGTCACGTTGG
IL1a (Human)	AGATGCCTGAGATACCCAAAACC	CCAAGCACACCCAGTAGTCT
CCL27 (Human)	GCAGCATTCCTACTGCCAC	AGGTGAAGCACGAAAGCCTG
CCL2 (Human)	CAGCCAGATGCAATCAATGCC	TGGAATCCTGAACCCACTTCT
CCL5 (Human)	CCAGCAGTCGTCTTTGTCAC	CTCTGGGTTGGCACACACTT
Cxcl1 (Human)	ATGGCCCGCGCTGCTCTCTCC	GCAAGCACTGGCAGCGCAGTTCA
Cxcl9 (Human)	CCAGTAGTGAGAAAGGGTCGC	AGGGCTTGGGGCAAATTGTT
p21 (Human)	CCTGTCACTGTCTTGTACCCT	GCGTTTGGAGTGGTAGAAATCT

Table S2 Clinical information of 5 HCCs used in PDX assay

	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5
Age (yr)	67	32	62	44	58
Sex	Male	Male	Female	Male	Male
Therapy	Chemo-	Chemo-	No	No	No
Serum ALT (U/L)	21	59	13	44	9
Serum AST (U/L)	20	63	17	212	56
Bilirubin (mmol/L)	9.8	18.5	10	12.4	13.8
Bile acids (mmol/L)	5.1	9.8	4.3	3.9	2.4
Pre-operation AFP (mg/mL)	53.1	9452	5.2	48.7	1210
Virus infection	HBV	HBV	No	HBV	HBV
Cirrhosis	Yes	Yes	No	Yes	No
Capsule formation	Yes	Yes	Yes	Yes	N.A.
Tumor border	Clear	Clear	Clear	Clear	N.A.
Histological differentiation	Poor	Poor	Moderate	Poor	N.A.

N.A.: Not available.

Supplemental Methods

Primary cultured HCC cells and patient-derived xenograft

Human HCC samples used for primary HCC cell culture and PDX transplantation were collected from Eastern Hepatobiliary Surgery Hospital, Second Military Medical University, Shanghai, China. All procedures of human sample collection were approved by the Ethical Committee of Eastern Hepatobiliary Surgery Hospital. The clinicopathological characteristics of these samples were listed in Table S2.

For primary HCC cell culture, the HCC tissues were snipped followed by collagenase digestion. The HCC cells were cultured in RPMI 1640 medium with 10% fetal bovine serum (FBS) on collagen-coated dishes.

For PDX transplantation, healthy purebred BALB/C nude mice, male, 6 weeks old, were purchased from the Shanghai SLAC Laboratory Animal Center of Chinese Academy of Sciences (Shanghai, China). The fresh HCC tissues from 5 HCC patients were placed in DMEM (Dulbecco's Modified Eagle Medium) containing 10% fetal bovine serum (FBS), and quickly cut to a depth of 2 mm in each piece using a sharp blade, which was subcutaneously buried in the right axilla of the nude mice using a trocar puncture. The tumor tissue from each patient was transplanted to 8 nude mice, and the 8 mice were assigned to 4 experimental groups (DMSO, BI2536, LCL161, BI2536/LCL161), that is, the 10 tumor xenografts of the 10 nude mice in each experimental group represented the 5 patients. The mice were continuously fed, and the growth of

the tumors was regularly observed. Three weeks after the transplantation, the tumor xenografts had grown in size to a diameter of approximately 8 mm. For the treatment, the mice were received 25mg/kg body weight BI2536 or 25mg/kg body weight LCL161 (Which were dissolved in 0.9% NaCl saline containing 25% DMSO) or both through intraperitoneal injection every other day. The tumor size was measured regularly, and the tumor volume was calculated with the formula of "$a \times b^2 \times 0.5$" (a: maximum diameter, b: minimum diameter), resulting in the tumor growth curve. At the end of the study, the mice were sacrificed with body and tumor weights recorded and the tumors harvested for analysis. All mice were maintained according to the Guide for the Care and Use of Laboratory Animals published by the Institutional Animal Care and Use Committee (IACUC) of the Institute of Biochemistry and Cell Biology.

Mice and liver tumorigenesis protocol

All mice were maintained in a mixed genetic background (C57Bl/6; 129Sv). *Alb-cre*; *Survivin*$^{f/f}$ (*svv*$^{\Delta li}$) and *Mx-cre*; *Survivin*$^{f/f}$ (*svv*$^{\Delta li}$*) were generated by crossing *svv*$^{f/f}$ mice to *Alb-cre* mice, with the Cre recombinase gene under the control of the *albumin* promoter and enhancer (Postic and Magnuson, 2000) and *Mx-cre* mice, with the Cre recombinase gene under the control of the *Mx1* promoter (Kuhn et al., 1995). Genotyping was performed by polymerase chain reaction (PCR) of tail genomic DNA.

To specifically delete the Survivin gene in the liver of adult mice, 6-8 weeks old

Mx-cre; *Survivin*$^{f/f}$ mice were treated with 7.5 Gy γ-irradiation and received syngeneic wild type bone marrow transplantation. 2 months after transplantation, the mice were treated with 3 intraperitoneal injections of poly(IC) 13 mg/Kg body weight at 2-day intervals as described previously (Leung et al., 2007). To induce liver tumors in mice, a single dose of DEN (Sigma, 25 mg per kilogram of body weight) was injected into 2-week-old male mice intraperitoneally and euthanized at the age of 10 months.

Genotyping primers are listed in the Supplemental Experimental Procedures. Mice were kept under specific pathogen-free conditions at the Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences. All mouse experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of the Institute of Biochemistry and Cell Biology and performed in accordance with this committee's guidelines.

Dosage and route of administration of BI2536, LCL161 and Etanercept

BI2536 (MedCam) and LCL161 (MedCam) are dissolved in DMSO for storage. For *in vivo* treatment, these compounds are diluted with 20% DMSO and 5% Tween-80 and intraperitoneally injected into mice every other day. Etanercept (Amgen) is dissolved in sterile 0.9% saline solution and intraperitoneally injected into mice twice a week.

Serum contents measurement

Blood was collected and centrifuged at 3000 g for 10 min. Freshly isolated supernatant plasma was used for measuring alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activity, total bilirubin and total bile acid levels according to the manufacturer's instructions (Shanghai Shensuo UNF Medical Diagnostic Articles Co., Ltd.). Albumin, blood urea nitrogen and creatinine were measured by 7600-020 clinical analyser (Hitachi).

Human macrophage culture and treatment

The human monocytic leukemia cells THP-1 were maintained in complete RPMI-1640 supplemented with 10% FBS, penicillin and streptomycin (100U/ml). THP-1-derived macrophages were obtained by stimulating THP-1 monocytes (7 X 10^5/mL) with 150ng/ml phorbol 12-myristate 13-acetate for 12 hours, and then followed with PMA-free PRMI-1640 culture for another 48 hours. The medium was changed with conditioned medium of senescent HCC cells for another 24 hours. mRNA levels of TNFa were analyzed by q-PCR.

Histology, Immunohistochemistry and Immunofluorescence

Liver tissue samples were fixed overnight in 4% neutral buffered paraformaldehyde (Solarbio), embedded in paraffin, cut into 5 micrometer thick sections, and placed on adhesion microscope slides. Sections were subjected to hematoxylin-eosin (H&E), TdT-mediated dUTP nick end labelling (TUNEL), immunohistochemical (IHC) or immunofluorescence (IF) staining.

IHC staining was performed using the Elite ABC kit (Vector Laboratories). For immunohistochemistry, deparaffinized and rehydrated slides were subjected to autoclave antigen retrieval in a 10 mmol/L citric acid buffer (pH 6.0) and allowed to cool to room temperature. Slides were blocked with 3% H_2O_2 for 30 minutes, washed in phosphate-buffered saline, then blocked with 5% normal goat serum in PBS. Slides were incubated with diluted primary antibodies overnight at 4°C. The following primary antibodies were used: rabbit anti-phospho-Histone H3Ser10 (1:1000 dilution, Upstate biotechnology); mouse anti-BrdU (1:1000 dilution, Sigma); rabbit anti-CD3 (1:1000 dilution, Dako). Biotinylated anti-rabbit (Vector Laboratories) and anti-mouse (Vector Laboratories) antibodies were used as secondary antibodies according to the manufacturer's protocol. Biphenyl-3,3',4,4'-tetrayltetraammonium tetrachloride (Dako) was used as a chromogen according to the manufacturer's protocol. TUNEL assays were performed using the Fluorescein cell death detection kit (Clontech). Images were observed using a Leica BX51 microscope.

For immunofluorescence staining, frozen sections were air-dried before washed in PBS for 10 min at room temperature. Blocking was performed in 1% BSA and 0.1% Triton X-100 for 1 hour and then incubated with primary antibodies at room temperature for 2 hours or at 4°C overnight. The following primary antibodies were used: mouse anti-a-Tubulin (1:1000 dilution, Sigma), rabbit anti-Aurora-B (1:1000 dilution, Abcam), rat anti-p19Arf (1:100 dilution, Novus, NB200-174), mouse anti-p16 antibody (1:1000 dilution, Santa Cruz,

sc-1661), rat anti-F4/80 (1:100 dilution, eBioscience), rabbit anti-cleaved caspase 3 antibody (1:50 dilution, Cell Signaling Technology, 9661). After incubation with FITC-conjugated goat anti-rabbit IgG (1:1000 dilution, Molecular Probes), Cy3 conjugated goat anti-mouse IgG (1:1000 dilution; Jackson Labs), FITC-conjugated donkey anti-rat IgG (1:1000 dilution, Molecular Probes) secondary antibodies for 1 hour at room temperature and counterstaining with DAPI, fluorescence images were observed using a Leica BX51 microscope.

Microarray Analysis

Total RNAs were hybridized to whole-mouse gene expression microarray (Agilent) in accordance with the manufacturer's instructions. Data were normalized using Gene-Spring (Agilent). Original data are available in the NCBI Gene Expression Omnibus (GSE64804).

Pharmacokinetics of BI2536 and LCL161

Blood samples for the evaluation of pharmacokinetics (PK) of BI2536 and LCL161 were taken from mice after the treatment course at 0, 0.5, 1, 2, 4, 8, 24, and 48 hours after intraperitoneal injection of BI2536 and LCL161 either alone or in combination. For each time point, 3 wild-type C57/B6 mice were sacrificed for blood collection. The plasma concentration of BI2536 and LCL161 were determined by LC-MS/MS using a Shimadzu 20A HPLC system

coupled to an 8030 mass spectrometer (Shimadzu, Kyoto, Japan) equipped with an API electro spray ionization (ESI) source. The pharmacokinetic parameters were determined by WinNonlin Professional (version 6.3, Pharsight Corp., St. Louis, MO).

Small interfering RNA (siRNA) knockdown

siRNAs against Survivin and scrambled siRNA (negative control) were synthetized by RiboBio Co., Ltd., China according to the sequence, Survivin (human): sense: 5' ACCGCAUCUCUACAUUCAA dTdT 3', anti-sense: 3' dTdT UGGCGUAGAGAUGUAAGUU 5'; scramble control: sense: 5' CCACCUACAUGGCCAAGAA dTdT 3'; anti-sense: 3' dTdT GGUGGAUGUACCGGUUCUU 5'. The primary HCC cells were transfected with siRNA using Lipofectamine RNAimax (Invitrogen, CA, USA). The cells were lysed 24, 48 or 72 hours later, gene expression levels were measured by q-PCR. Each treatment was repeated three times.

Quantitative Real-Time PCR (q-PCR)

Total RNA was prepared from liver tissue samples using Trizol (Invitrogen) according to the manufacturer's protocol. Reverse transcriptase PCR was performed using a M-MLV reverse transcriptase (Promega). Real-time PCR reactions were performed using SYBR® Premix Ex Taq™ (Takara) and 300 nmol/L of each primer. Amplification was performed according to the

manufacturer's protocol of the 7500 Fast Real-Time PCR Systems (Applied Biosystems, CA, USA). Primer sequences used are listed in table S1.

ELISA

Enzyme linked immunosorbent assay was performed following the user manual. Tissues were lysed in PBS buffer containing protease inhibitor cocktail and 0.1% TritonX-100 and equal amount of total protein of tissue lysates from each condition were resolved by Sample Diluent followed by ELISA. Mouse TNF-a, DY410, R&D Systems.

Western Blot

Western blot was performed following the routine protocol. Tissues were lysed in HNTG buffer (20mM HEPES, 150mM Nacl, 0.1% TritonX-100, 10% glcerol) and equal amount of total protein of tissue lysates from each condition were resolved by 8%-15% SDS-PAGE followed by immunoblotting.

Senescence-Associated b-Galactosidase Analysis

For senescence-associated b-galactosidase (SA-b-gal) staining, 10 micrometer sections were cut from OCT-embedded frozen tissue and allowed to adhere to coated slides at 25°C for 1min before fixation for 15min in PBS containing 0.5% glutaraldehyde (Sigma). Sections were rinsed with PBS, pH5.5, containing 1mM MgCl2 and incubated at 37°C for 16h in prewarmed

and filtered 5-bromo-4-chloro-3-indolyl-b-D-galactoside (X-gal) solution (0.1% X-gal (Sigma) dissolved in PBS, pH5.5, containing 1mM MgCl2, 5mM potassium ferrocyanide and 5mM potassium ferricyanide). Sections were rinsed with PBS, post-fixed in 95% ethanol, rehydrated, counterstained with nuclear fast red, dehydrated and mounted(Dimri et al., 1995), or subsequently subjected to IHC detection of cleaved caspase 3.

Assessment of apoptosis activation in vitro

Primary human HCC cells were cultured in RPMI 1640 medium with 10% fetal calf serum, when cell confluence reached 80%, the medium was changed to serum free RPMI 1640, containing 10 ng/ml of recombinant human TNFa (R&D Systems, Indianapolis, IN, USA). After three hours incubation, cells were processed for Annexin V levels using a commercially available kit (BD Biosciences, San Diego, CA, USA). Cells were further treated with propidium iodide and analyzed by fluorescence-activated cell sorting (FACS, Calibur, BD Instruments, San Diego, CA, USA). Annexin V-positive / propidium iodide-negative cells were assigned to be early apoptotic cells, while double-positive cells were assigned as late-apoptotic/dead cells.

Statistics

For most statistic analyses, the unpaired Student's t test was applied for calculating statistical probability. For survival analyses, the Kaplan-Meier log rank test was applied. Statistic calculations were performed using the

Statistical Program for Social Sciences software (SPSS, IBM). For all statistics, data from at least three independent samples or repeated experiments were used.

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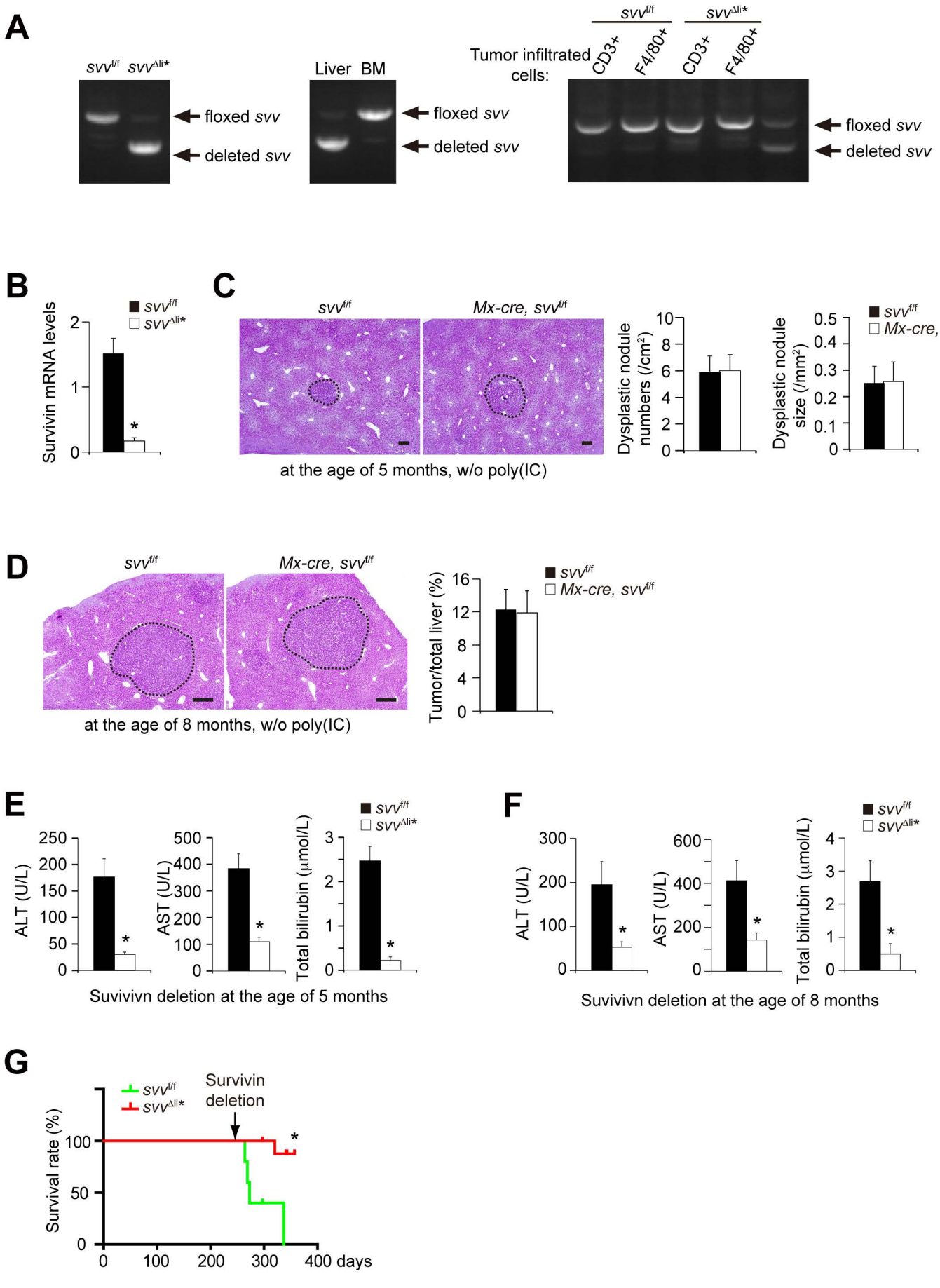
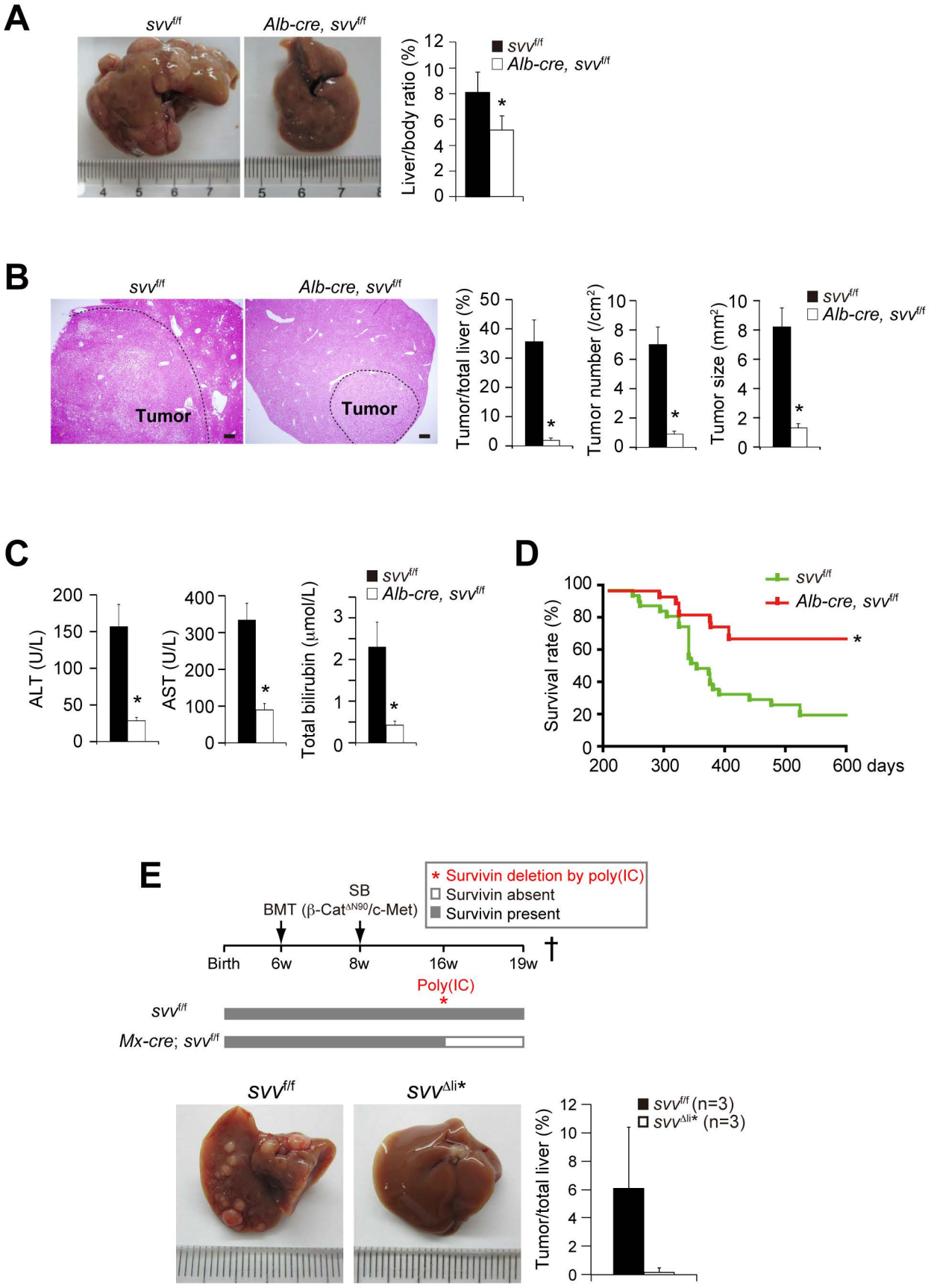
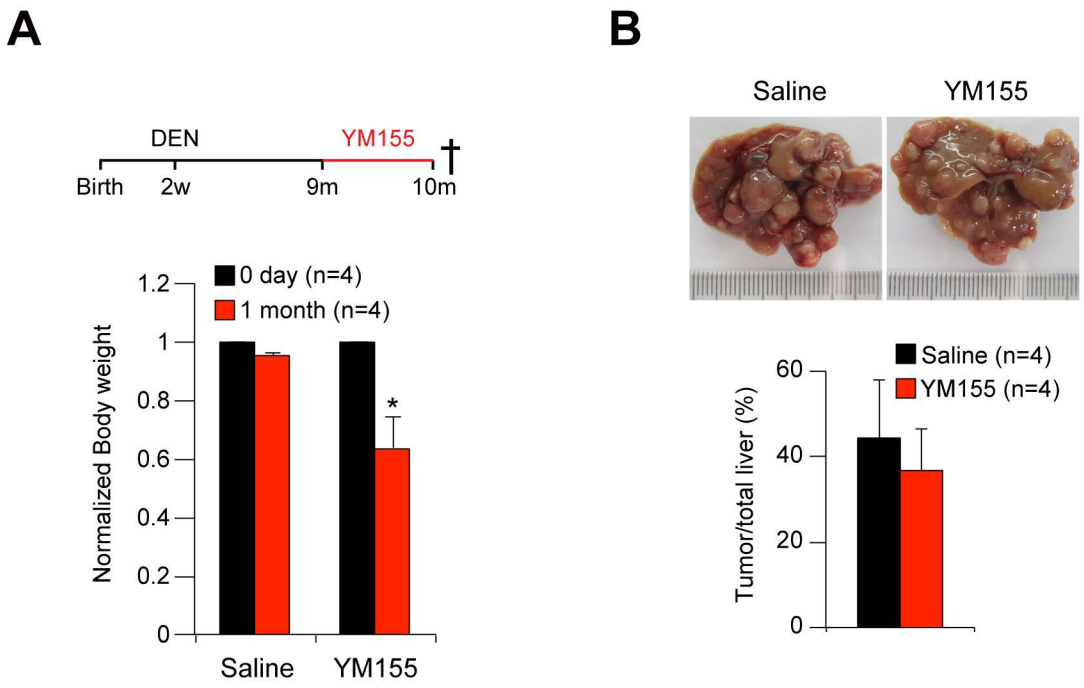


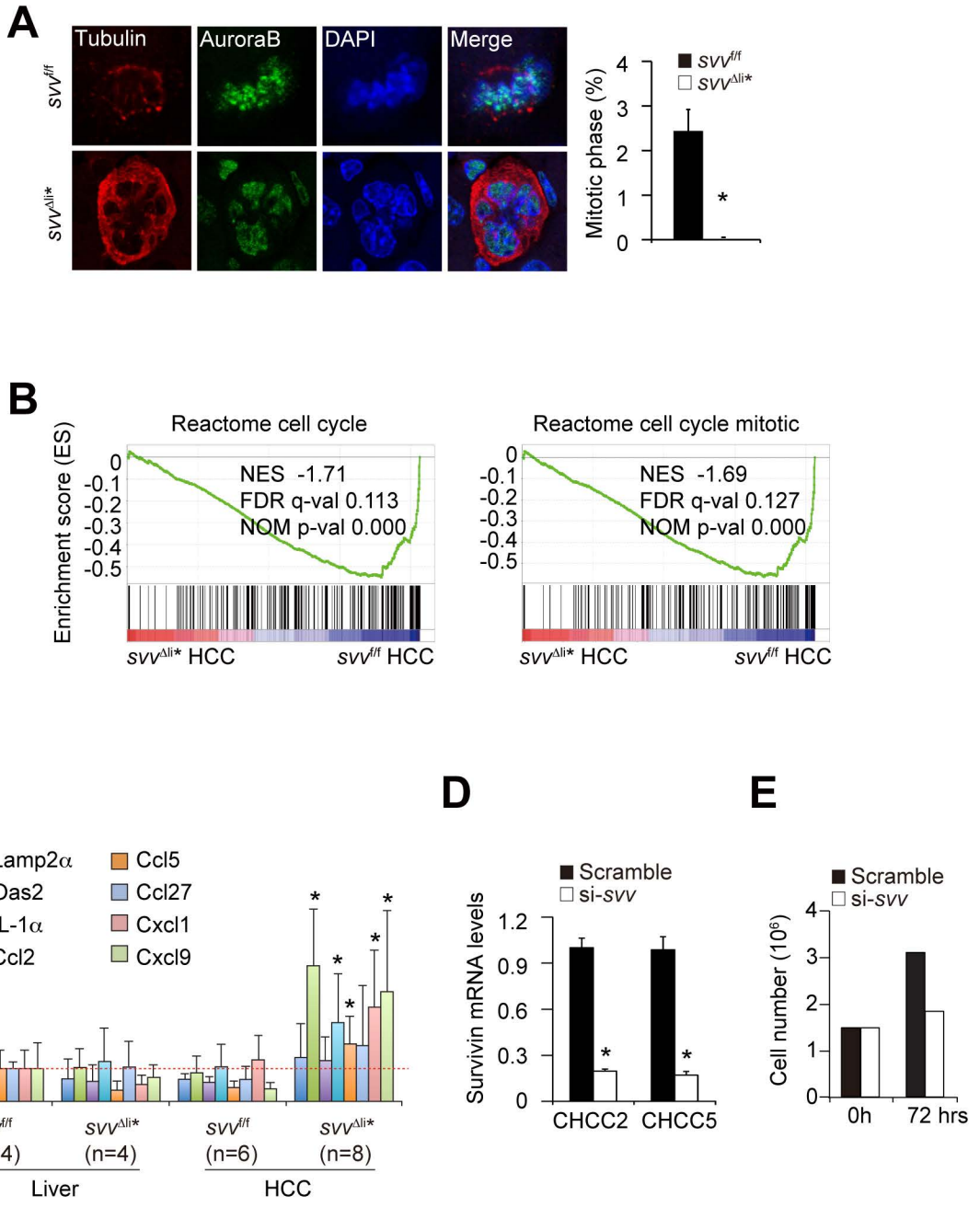
Supplementary Fig. 1



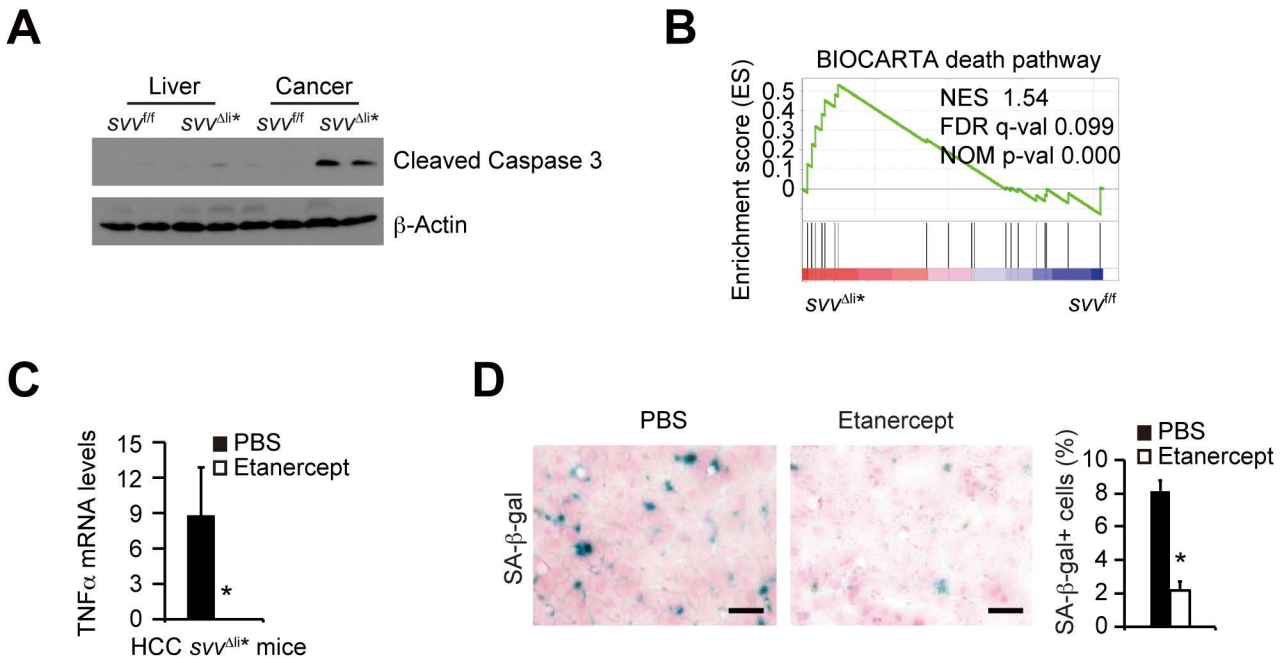
Supplementary Fig. 2



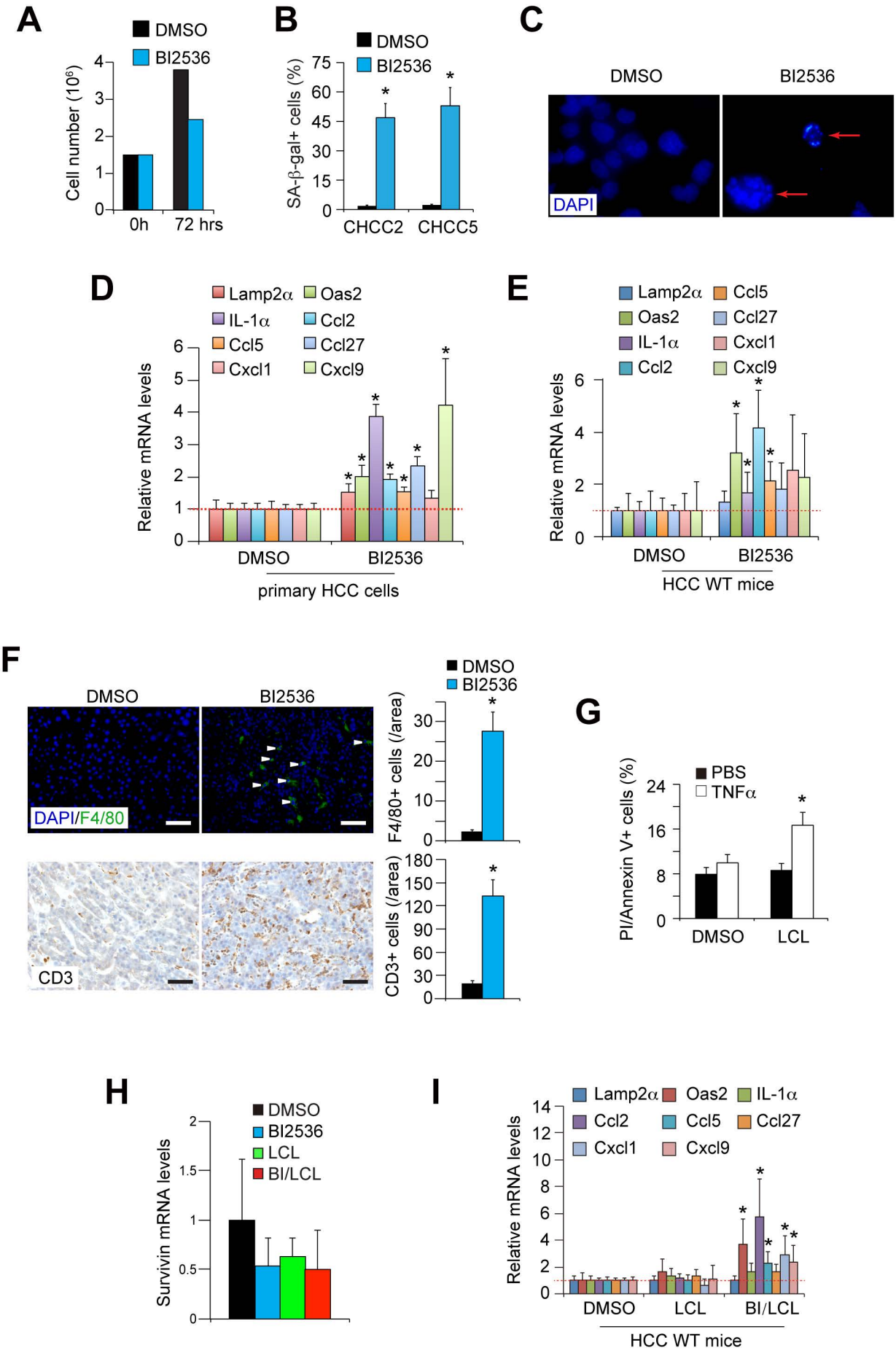
Supplementary Fig. 3



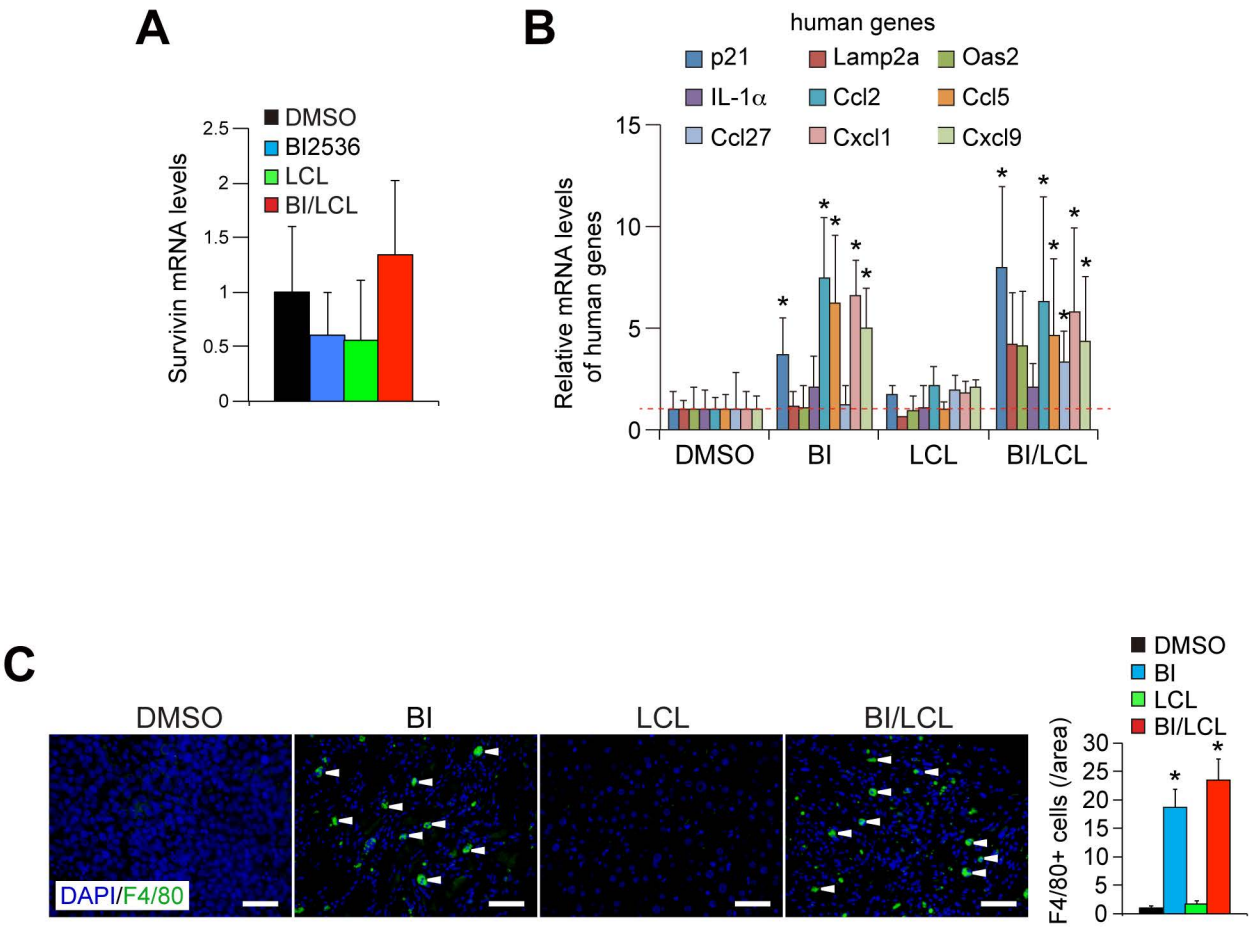
Supplementary Fig. 4



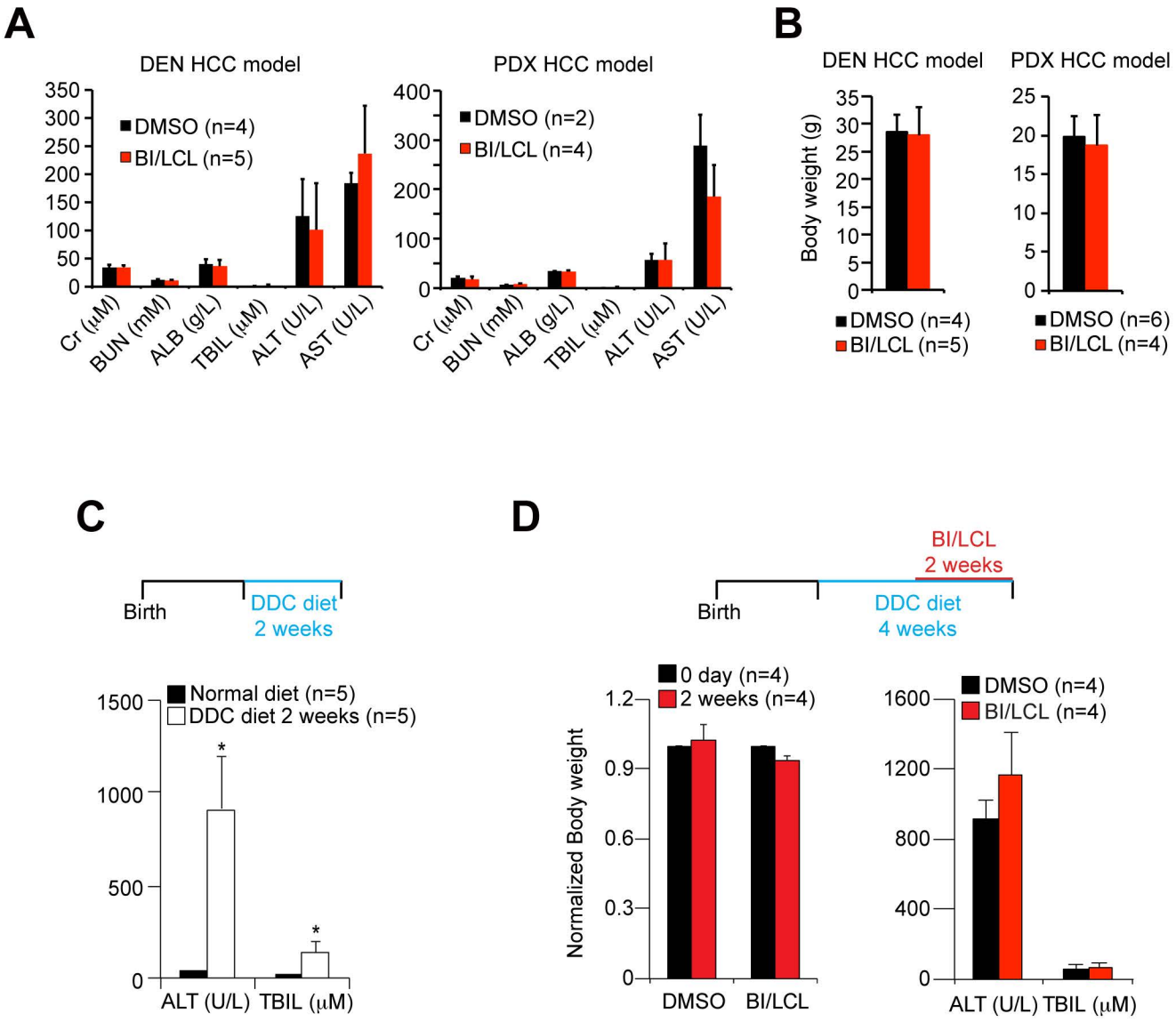
Supplementary Fig. 5



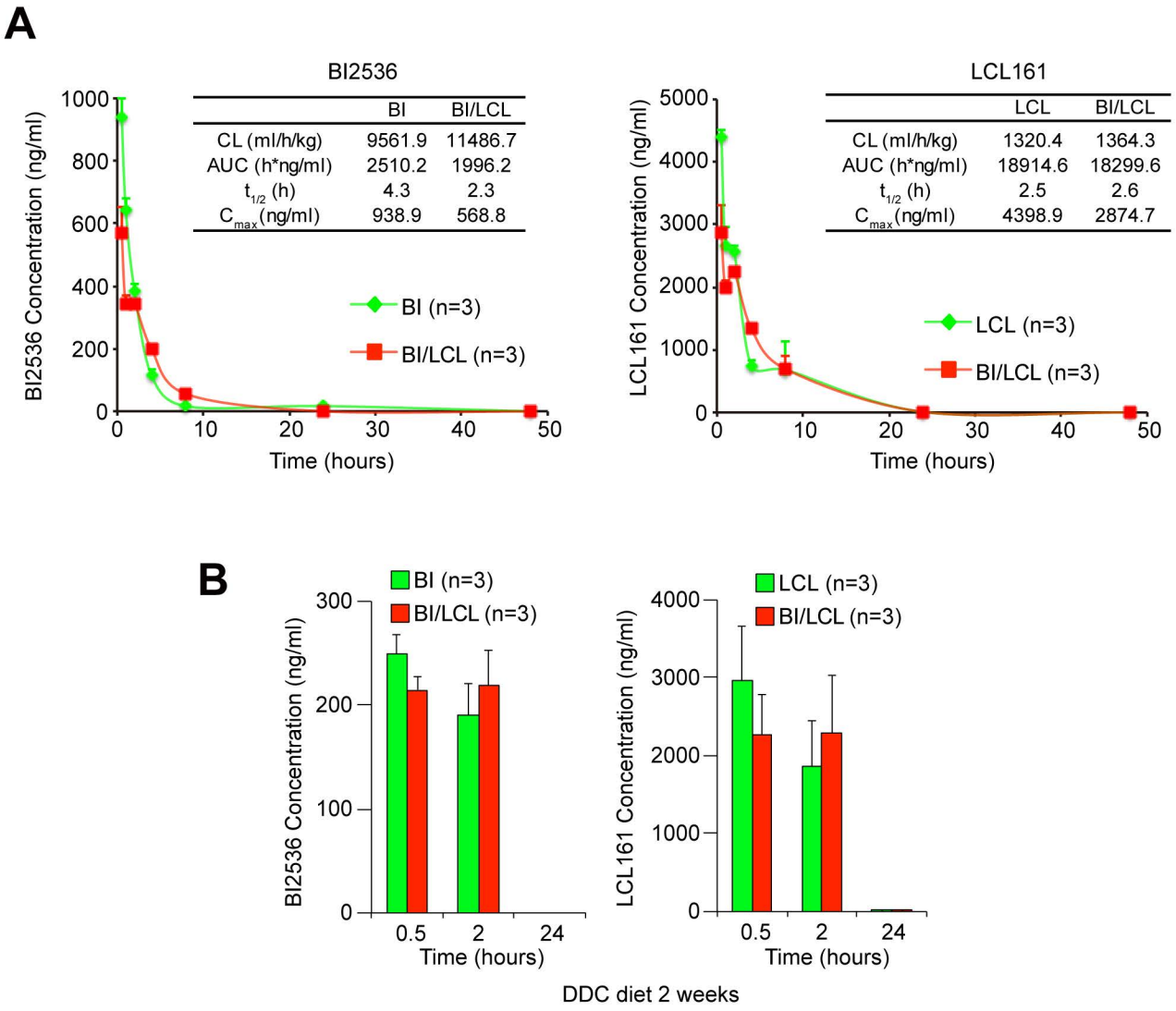
Supplementary Fig. 6



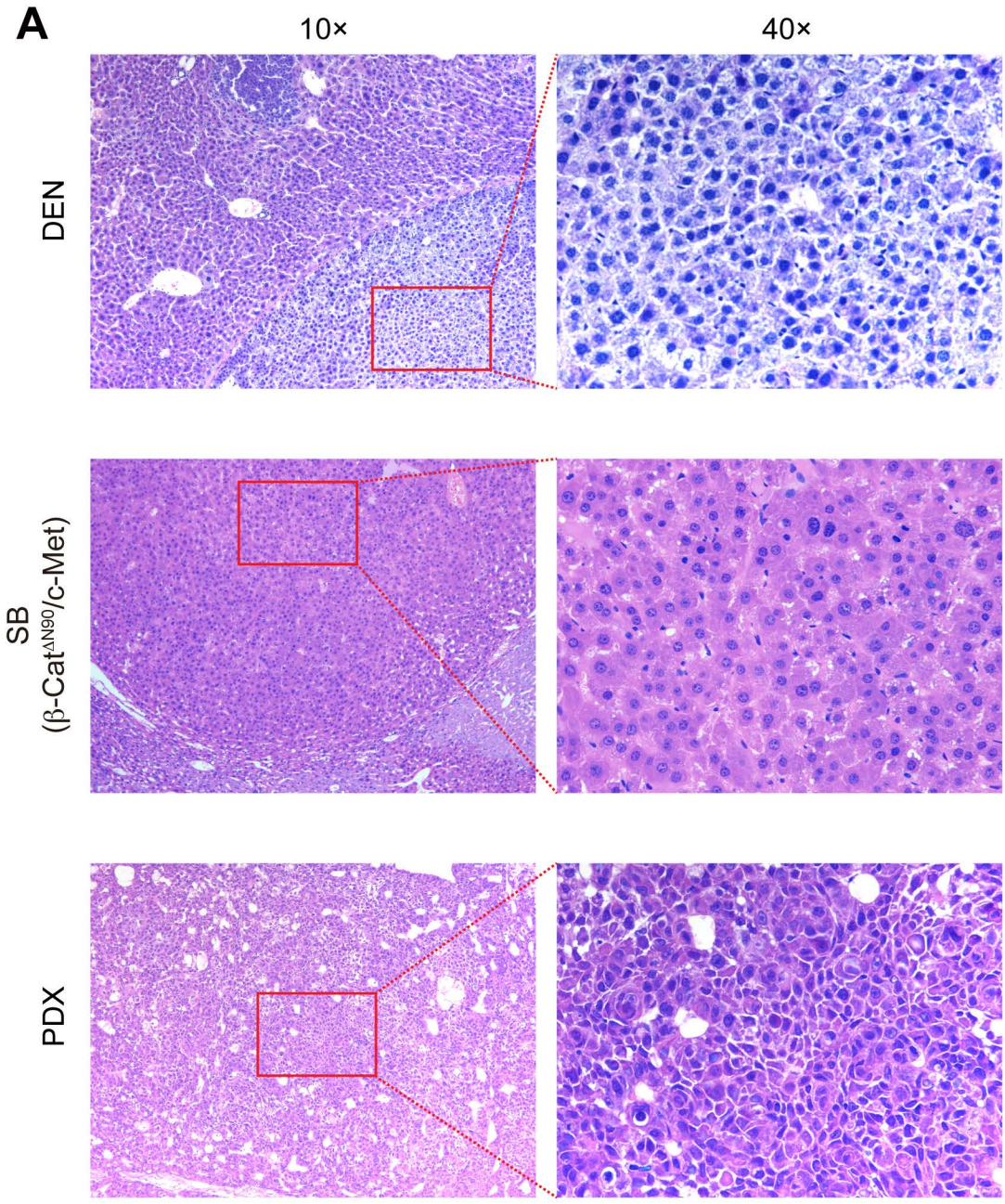
Supplementary Fig. 7



Supplementary Fig. 8



Supplementary Fig. 9



Supplementary Fig. 10