Supporting information in the Materials and Methods

Materials

Anti-PHLDA3, anti-Akt, anti-phospho-Akt (S473 or T308), anti-IRE1, anti-phospho-c-Jun (Ser73), anti-phospho-STAT3 (Tyr705), and anti-phospho-JNK antibodies were purchased from Cell Signaling Technology (Danvers, MA). Antibodies directed against Xbp1, phospho-PERK or ATF6 were provided from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phospho-IRS1 (Ser307) was purchased from Upstate Biotechnology (Lake Placid, NY). Horseradish peroxidase-conjugated goat anti-rabbit and goat anti-mouse IgGs were obtained from Zymed Laboratories (San Francisco, CA). Anti-β-actin antibody and other reagents were supplied from Sigma (St. Louis, MO).

Integrative network analysis

Gene expression data were supplied from the Gene Expression Omnibus (GSE25097) available in the public domain. Differentially expressed genes with statistical significance (P<0.01 with fold-change over 2 or under 0.5) in fibrotic patients and healthy subjects were selected, and were clustered by gene ontology using DAVID 6.7 software.[1, 2] Interactions of the genes in the apoptosis-related cluster were analyzed using STRING v9.1 database.[3] Further visualization was done with Cytoscape 3.0.0 software.[4] Filled colors indicate the genes up-regulated (red) or down-regulated (blue) in the liver of cirrhotic patients as compared to healthy subjects.

HCV-patients samples

Liver samples from hepatitis C virus (HCV)-patients without hepatitis [lower than the upper limit of normal in ALT (ULN, 40 U/L), N=3] or those with hepatitis (>4 times ULN, N=3) were used. Studies using human tissues were reviewed and approved by the Institutional Review Board.

Animal treatment and PHLDA3 knockdown *in vivo*

Animal experiments were conducted in accordance with the guidelines of the Institutional Animal Use and Care Committee at Seoul National University. Male C57BL/6 mice (6 weeks old)

were purchased from Samtako Company (Osan, Korea), and housed at 20±2°C with 12-h light/dark cycles and a relative humidity of 50±5% (Tecniplast, Varese, Italy) under filtered, pathogen-free air, with food (Purina, Korea) and water available *ad libitum*. For ER stress model, mice were intraperitoneally injected with a single dose of vehicle or tunicamycin (Tm, 2 mg/kg, for 24-72 h). Another set of mice received a single dose of vehicle or CCl_4 in corn oil (0.6 ml/kg body weight, i.p.) and were sacrificed 24 h after treatment (an acute liver injury model). Mice were also fed on either a control diet or an alcohol Lieber-DeCarli liquid diet (5%) for 5 weeks (chronic liver injury models). In a separate experiment, mice were intraperitoneally injected with 100 mg/kg PBA (or vehicle) 2 h prior to acetaminophen (500 mg/kg, i.p.) or vehicle treatment, and the liver samples were obtained 6 h later. For *in vivo* knockdown experiment, mice were injected with lentiviruses that express control shRNA or shRNA directed against PHLDA3 through tail vein ($2x10^7$ viruses in 200 μL PBS per mouse). Seven days after the injection, the mice were treated with vehicle or Tm (2 mg/kg, i.p.) and were sacrificed 72 h afterward. Blood and liver tissues were taken from mice, and the samples were then biochemically and histopathologically analyzed.

Immunohistochemistry

The paraffin-embedded tissue sections were deparaffinized with xylene, and rehydrated with alcohols series. Antigen retrieval in the sections was performed in a 37°C incubator for 10 min by proteinase K and was autoclaved for 10 min in a citric acid buffer (10 mM, pH 6.0). After cooling, the endogenous peroxidase activity in the samples was quenched by immersion in 3% H_2O_2 for 15 min. Nonspecific antibody binding to the sections was blocked using 10% normal donkey serum. The sections were interacted with anti-PHLDA3 antibody overnight at 4°C, followed by incubation with biotin-SP-conjugated affinity pure donkey anti-rabbit antibody or IgG for 2 h, and were finally mounted with Permount solution. The sections were examined using light microscope (DMRE, Leica Microsystem, Germany), and images were acquired with Fluoview-II (Soft Imaging System GmbH, Germany) attached on the microscope.

Cell culture

Hepatocytes were isolated from Sprague–Dawley rats weighing ~400 g, as previously described.[5] Briefly, under anesthesia with Zoletil, livers were perfused with Ca^{2+}-free Hank's buffered salt solution (Invitrogen) for 10 min, followed by continuous perfusion with a 0.1% w/v collagenase (Sigma, Type IV). The whole liver was removed, and minced in the phosphate-buffered saline. Cell suspension was filtered through the gauze, and purified with Percoll. Primary hepatocytes were harvested into collagen-coated plates (5×10^5 cells/well) in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 50 units/ml penicillin, and 50 μg/ml streptomycin. HepG2 cells (a human hepatocyte-derived cell line) and AML12 cells (a mouse hepatocyte-derived cell line) were purchased from American Type Culture Collection (Rockville, MD). HepG2 cells were maintained in the DMEM containing 10% FBS, 50 units/ml penicillin, and 50 μg/ml streptomycin. AML12 cells were cultured in the DMEM/F-12 containing 10% FBS, insulin-transferrin-selenium X (ITSX), dexamethasone (40 ng/ml; Sigma), and the antibiotics. The cells with less than 20 passage numbers were used.

RNA isolation and real-time PCR assays

Total RNA was extracted using Trizol (Invitrogen, Carlsbad, CA), and was reverse-transcribed using oligo-$(dT)_{16}$ primers to obtain cDNA. The cDNA was amplified by polymerase chain reaction (PCR). Quantitative real-time PCR (qRT-PCR) was carried out according to the manufacturer's instructions using a Light CyclerDNA master SYBR green-I kit (Light-Cycler 1.5, Roche, Mannheim, Germany). The following primer sequences were used: mouse PHLDA3 (sense: 5'-CAGTAGGGGCTGAGCATGAA-3', antisense: 5'-GCAGTCTGCAGAACCCAGAA-3'), mouse Xbp1s (sense: 5'-GGTCTGCTGAGTCCGCAGCAGG-3', antisense: 5'-AGGCTTGGTGTATACATGG-3'), mouse GRP78 (sense: 5'-TGGTATTCTCCGAGTGACAGC-3', antisense: 5'-AGTCTTCAATGTCCGCATCC-3'), mouse Erdj4 (sense: 5'-CCCCAGTGTCAAACTGTACCAG-3', antisense: 5'-AGCGTTTCCAATTTTCCATAAATT-3'), mouse CHOP (sense: 5'-CATACACCACCACACCTGAAAG-3', antisense: 5'-

CCGTTTCCTAGTTCTTCCTTGC-3'), human PHLDA3 (sense: 5'-CATGCTACCCACCACCTCAG-3', antisense: 5'-AGAGTCTGGGACCATCCAGG-3'), and rat PHLDA3 (sense: 5'-TTCCTGGTGTGCTAAGGCAG-3', antisense: 5'-AGGTGAGGGAGGAAGTGTGC-3'). The levels of target mRNAs were normalized to those of glyceraldehyde-3-phosphate dehydrogenase. A melting curve of each amplicon was determined to verify its accuracy.

Small interfering RNA (siRNA) knockdown

Scrambled siRNA (control) and siRNAs of Xbp1, IRE1 or PHLDA3 were supplied from Dharmacon (Dharmacon, Chicago, IL). ATF6 siRNA was provided from Santa Cruz Biotechnology (Santa Cruz, CA). Cells were transfected with each siRNA (100 pmol) using FuGENE HD (Promega, Madison, WI) according to the manufacturer's instructions.

Chromatin immunoprecipitation (ChIP) assays

HepG2 cells were treated with Tm for 12 h, and then formaldehyde was added to the cells to a final concentration of 1% for cross-linking of chromatin. The chromatin immunoprecipitation assay was performed according to the ChIP assay kit protocol (Upstate Biotechnology, Lake Placid, NY). PCR was done using the primers flanking the Xbp1 regions located in the promoter region of human *PHLDA3* gene (Xbp1-RE1: sense, 5'-AGGGCAAAACTCCGTCTCAA-3' and antisense, 5'-AGGGACCCACAAGGTCTCAG-3', 214 bp; Xbp1-RE2: sense, 5'-GGCCCCCATCATTCTCTAAA-3' and antisense, 5'-TCTCCCTTGTTGGCTGTGTC-3', 234 bp; and Xbp1-RE3: sense, 5'-GACTGCAGACAGTGGCATCC-3' and antisense, 5'-ACCCAAGAGAGGCTGTCCAT-3', 202 bp), or irrelevant regions (irrelevant region 1: sense, 5'-GCCTGGGTGACAAAGTGAGA-3' and antisense, 5'-GAACTCAAAAGCCAGGGGAG-3', 216 bp; irrelevant region 2: sense, 5'-GTTGAGTAAGCAGCCCCCTC-3' and antisense, 5'-GCATTCCTACCCTGGCCG-3', 153 bp). One tenth of cross-linked lysates served as the input control.

Transient transfection and reporter gene assays

The plasmids encoding Xbp1s, DN-PERK and PHLDA3 were supplied from Addgene (Cambridge, MA) or GeneCopoeia (Rockville, MD). Cells were plated in six-well plates overnight, serum-starved for 3 h, and transiently transfected with the plasmids in the presence of FuGENE HD Reagent (Promega, Madison, WI). The transfected cells were then incubated in Eagle's minimum essential medium containing 1% fetal bovine serum for 18 h. For reporter gene assays, the region containing –2500 bp to +385 bp of the human *PHLDA3* gene was cloned into the pGL3 luciferase vector. A mutation of Xbp1-RE1 was done by replacing the sequence of Xbp1 binding element from 5'-GATCATGGCA-3' to 5'-GAT**A**A**AAA**CA-3' (bolds indicate mutation). Cells were transfected with pGL3-PHLDA3 for 12 h in the presence of FuGENE HD reagent, and luciferase activity was measured by adding luciferase assay reagent (Promega, Madison, WI).

Immunoblot analysis

Immunoblot analysis was performed according to the previously published procedures.[5] Cells were centrifuged at 3,000g for 3 min and allowed to swell after the addition of lysis buffer in the ice for 30 min. The lysates were centrifuged at 10,000g for 10 min to obtain supernatants. Proteins of interest in lysates were resolved using polyacrylamide gels and transferred to nitrocellulose membrane. The bands were developed using ECL chemiluminescence system (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK). Equal loading of proteins was verified by immunoblotting for β-actin. At least three samples were used for each experiment.

MTT assays

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay was carried out according to the previously published method.[6] AML12 cells were plated at a density of 1×10^5 cells per well in a 48-well plate to measure the degree of cell survival. After treatment, viable cells were stained with 0.25 μg/ml MTT for 2 h. The media were then removed, and formazan crystals produced in the wells were dissolved by the addition of 300 μl dimethylsulfoxide. Absorbance was measured at

540 nm using an ELISA microplate reader (Tecan, Research Triangle Park, NC). Cell viability was defined relative to untreated control [i.e. viability (% control) = 100 × (absorbance of treated sample)/(absorbance of control)].

TUNEL assays

The terminal transferase-mediated dUTP nick-end labeling (TUNEL) assay was done as previously described.[6] The assay was performed using the DeadEnd Colorimetric TUNEL System, according to the manufacturer's instruction. Liver tissues or AML12 cells were fixed with 10% buffered formalin in PBS at room temperature for 30 min, and permeabilized with 0.2% Triton X-100 for 5 min. After washing, each sample was incubated with biotinylated nucleotide and terminal deoxynucleotidyltransferase in an equilibration buffer at 37°C for 1 h. The reaction was stopped by immersing the samples in 2× saline sodium citrate buffer for 15 min. Endogenous peroxidases were blocked by immersing the samples in 0.3% H_2O_2 for 5 min, treated with horseradish peroxidase-labeled streptavidin solution (1:500), and further incubated for 30 min. Finally, the samples were developed using the chromogen, H_2O_2 and diaminobenzidine for 10 min, and were examined under a light microscope (200×). The counting of TUNEL-positive cells was repeated three times, and the percentage from each counting was calculated.

Data analysis

Statistically significant differences were assessed by the Student's *t*-test or one-way analysis of variance tests. For each statistically significant effect of treatment, the Bonferroni's method was used for comparisons between multiple group means. The data were expressed as the mean ± S.E. The criterion for statistical significance was set at $P<0.05$ or $P<0.01$.

REFERENCES

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Supplementary Figure Legends

Supplementary Figure S1. Activation of Xbp1 by ER stress and Xbp1-RE1-dependent *PHLDA3* transactivation

(A) qRT-PCR assays for spliced Xbp1 (Xbp1s) or Erdj4 were done on the liver of mice injected with a single dose of Tm (2 mg/kg, at day 1-3)(N=4 each).

(B-C) qRT-PCR assays or immunoblottings for Xbp1s in AML12 cells treated with Tm (2 μg/ml).

(D) Xbp1-RE1-dependent PHLDA3 promoter assays using primary hepatocytes. Luciferase assays were done in mouse primary hepatocytes transfected with PHLDA3 promoter luciferase construct or its Xbp1-RE1 mutant and continuously treated with vehicle or Tm (2 μg/ml) for 24 h.

Data represent the mean±S.E of at least 3 samples. Statistical significance of differences between each treatment group and the control (*P<0.05, **P<0.01) was determined.

Supplementary Figure S2. PHLDA3 induction by serum starvation

(A) The effect of serum starvation on Xbp1s or PHLDA3 transcript levels and cell viability. qRT-PCR and MTT assays were done on AML12 cells incubated in a medium without FBS for the indicated times.

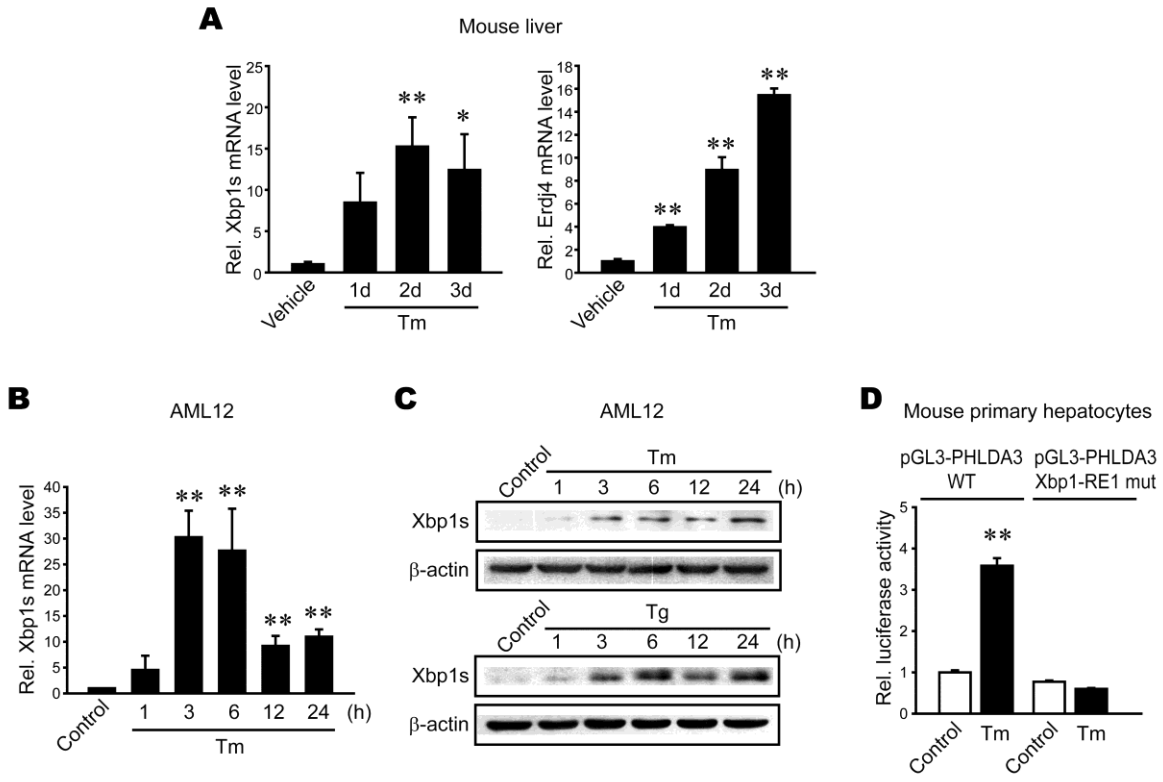
(B) The effects of chemical chaperones on cell death elicited by serum starvation. The cells were incubated in a medium deprived of FBS with or without 100 μM TUDCA or 1 mM 4-PBA for 48 h.

Data represent the mean±S.E of at least 3 samples. Statistical significance of differences between each treatment group and the control (*P<0.05, **P<0.01) or serum starvation ($^{\#}P$<0.05) was determined.

Supplementary Figure S3. Inhibition of cell growth by PHLDA3 overexpression

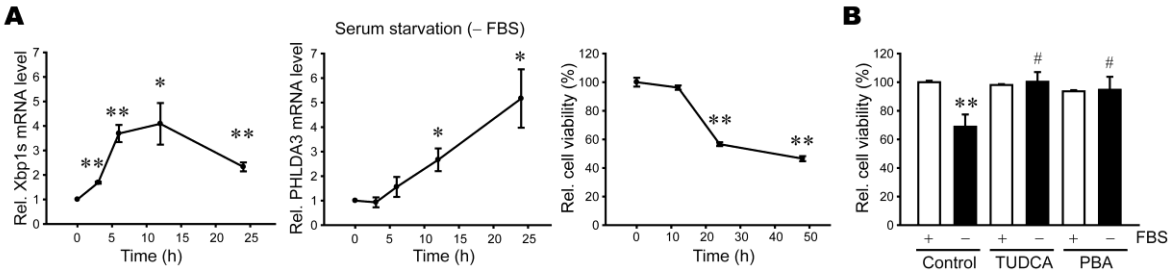
AML12 cells were transfected with Mock or PHLDA3 construct for 12 h, and continuously incubated in a growth medium containing 5% FBS for 0, 12, or 24 h. Cell growth was assessed using MTT assays. Data represent the mean±S.E of at least 3 samples.

Supplementary Figure S1



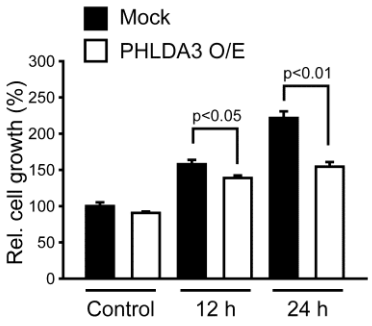
Supplementary Figure S1

Supplementary Figure S2



Supplementary Figure S2

Supplementary Figure S3



Supplementary Figure S3