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Role of Transglutaminase 2 in Liver Injury via Cross-linking and Silencing of Transcription Factor Sp1

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Background & Aims: **Despite high morbidity and mortality of alcoholic liver disease worldwide, the molecular mechanisms underlying alcohol-induced liver cell death are not fully understood. Transglutaminase 2 (TG2) is a cross-linking enzyme implicated in apoptosis. TG2 levels and activity are increased in association with various types of liver injury. However, how TG2 induces hepatic apoptosis is not known.** ***Methods:*** **Human hepatic cells or primary hepatocytes from rats or TG2$^{+/+}$ and TG2$^{-/-}$ mice were treated with ethanol. Mice were administered anti-Fas antibody or alcohol. Liver sections were prepared from patients with alcoholic steatohepatitis. Changes in TG2 levels, Sp1 cross-linking and its activities, expression of hepatocyte growth factor receptor, c-Met, and hepatic apoptosis were measured.** ***Results:*** **Ethanol induced apoptosis in hepatic cells, enhanced activity and nuclear accumulation of TG2 as well as accumulation of cross-linked and inactivated Sp1, and reduced expression of the Sp1-responsive gene, *c-Met*. These effects were rescued by TG2 knockdown, restoration of functional Sp1, or addition of hepatocyte growth factor, whereas apoptosis was reproduced by Sp1 knockdown or TG2 overexpression. Compared with TG2$^{+/+}$ mice, TG2$^{-/-}$ mice showed markedly reduced hepatocyte apoptosis and Sp1 cross-linking following ethanol or anti-Fas treatment. Treatment of TG2$^{+/+}$ mice with the TG2 inhibitors putrescine or cystamine blocked anti-Fas–induced hepatic apoptosis and Sp1 silencing. Moreover, enhanced expression of cross-linked Sp1 and TG2 was evident in hepatocyte nuclei of patients with alcoholic steatohepatitis.** ***Conclusions:*** **TG2 induces hepatocyte apoptosis via Sp1 cross-linking and inactivation, with resultant inhibition of the expression of *c-Met* required for hepatic cell viability.**

Alcohol-induced liver damage is a major cause of morbidity and mortality worldwide. Ethanol-induced hepatocyte death is believed to result from a combination of oxidative stress and a cytotoxic cytokine cascade, with injury being secondary to apoptosis and necrosis. However, the mechanisms involved remain incompletely defined.[1]

Transglutaminase 2 (TG2) is a member of a family of cross-linking enzymes that catalyze posttranslational modification of proteins by calcium-dependent cross-linking to form N$^{\varepsilon}$(γ-glutamyl) lysine bonds.[2–4] In addition, it has many other functions, such as serving as a guanosine triphosphate binding protein involved in receptor signaling[2–4] and as a scaffolding protein involved in stabilizing fibronectin binding by β-integrins.[2–4]

TG2 has been implicated in the regulation of cell growth and differentiation, and its cross-linking activity has been associated with apoptosis.[2–4] For example, accumulating evidence suggests a link between enhanced transamidating activity of TG2 and neuronal cell death, for example, enhanced expression of TG2 in patients with Alzheimer's disease and enhanced TG activity observed in patients with Huntington's disease.[2–4] We showed previously that TG2 cross-linking activity was significantly increased with carbon tetrachloride–induced liver injury

Abbreviations used in this paper: 5-BAPA, 5-(biotinamido)pentylamine; CL-Sp1, cross-linked Sp1; HGF, hepatocyte growth factor; siRNA, small interfering RNA; TG2, transglutaminase 2.

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in rats and in acute human liver injury.[5] However, its involvement in apoptosis remains incompletely defined and controversial. For example, enhanced expression of a short form of TG2, which is encoded by an alternatively spliced transcript, lacks guanosine triphosphatase activity, has poor cross-linking activity, and is proapoptotic,[6] although, thus far, short-form TG2 expression has only been reported in the human brain.[7,8] We also observed previously that enhancing α_1-adrenergic receptor–coupled signaling via the G protein activity of TG2 results in decreased TG2 cross-linking and enhanced hepatocyte proliferation. Moreover, activation of TG2 cross-linking either by ethanol treatment or by Fas activation resulted in increased apoptosis and decreased hepatocyte proliferation.[9] Mice with homozygous inactivation of *Tgm2*, the TG2 gene ($TG2^{-/-}$ mice),[10,11] are more susceptible to toxin-induced hepatobiliary injury[12] and CCl_4-induced liver injury.[13] Moreover, TG2 has been reported to be antiapoptotic, an effect linked both to its guanosine triphosphate binding and transamidating activity.[13–16] Thus, it is still unclear if TG2 is causative for apoptosis induction or antiapoptotic, or if its apparent involvement in apoptosis is merely an epiphenomenon, and clarification of the molecular mechanism by which TG2 induces hepatic apoptosis will help answer these questions.

By using loss- and gain-of-function approaches for TG2 and Sp1 in in vitro and in vivo models, we show that TG2 plays a causative role in hepatocyte apoptosis induced by ethanol and Fas. As an underlying molecular mechanism, we show that the transcription factor Sp1 is cross-linked, oligomerized, and inactivated by nuclear TG2, resulting in reduced expression of growth factor receptors, such as *c-Met*, required for cell integrity. These effects define a novel proapoptotic pathway resulting in caspase-independent apoptosis accompanied by chromatin condensation, responses that can be rescued by Sp1 overexpression or by growth factor receptor activation.

Materials and Methods

Materials

Recombinant human Sp1 and guinea pig TG2 were purchased from Promega (Madison, WI) and Oriental Co (Osaka, Japan), respectively. Histidine-tagged recombinant human Sp1 was made using Rapid Translation System from Roche Molecular Biochemicals (Mannheim, Germany) by coupled transcription/translation and purified using Ni-NTA agarose (Qiagen, Hilden, Germany) at 4°C. Anti-Sp1 and *c-Met* antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti–cross-linked Sp1 (CL-Sp1) and anti-TG2 polyclonal antibody was made in rabbits and purified as detailed in Supplementary Figure 1 and text. Anti-TG2 monoclonal antibody was from Lab Vision (Fremont, CA). Cy5-/horseradish peroxidase–conjugate anti-rabbit immunoglobulin (Ig) G and tetramethylrhodamine isothiocyanate–conjugate streptavidin were from Jackson ImmunoResearch Laboratories (West Grove, PA). 5-(biotinamido) pentylamine (5-BAPA), a biotinylated primary amine substrate for TG2, was from Pierce (Rockford, IL). A broad-spectrum caspase inhibitor, zVAD-fmk, and caspase-3 specific inhibitor, zDEVD-fmk, as well as Hoechst 33258 were from Calbiochem-Novabiochem (La Jolla, CA).

The expression vector for human Sp1 (Sp1-pCIneo) was constructed as described previously.[17] A TG2 and Sp1 small interfering RNA (siRNA)-expressing lentiviral vector was constructed in the pSIH-H1 short hairpin RNA vector (SBI System Biosciences, Mountain View, CA) as detailed in Supplementary Figure 2 and text. A GC3-Luc vector, which contains 3 sequential repeats of the GC box motifs derived from the *c-Met* promoter and its TATA box sequence[18] upstream of the luciferase complementary DNA, was generated by inserting a synthesized oligodeoxynucleotide cassette into the pGL3 vector (Promega Corp).

Transient Transfection and Luciferase Assays

Cells were cultured as described in the Supplementary text (see Supplementary Material and Methods). Transfections using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) and luciferase assays using the Dual-Luciferase Reporter Assay System (Promega) were performed as described previously.[19] Transfection of 5×10^5 HepG2 cells with 50 ng of Sp1-expressing vector, Sp1-pCIneo, or empty vector was performed using a microporator, MP-100, from AR Brown (Tokyo, Japan).

Preparation of Nuclear Extracts

Nuclear proteins were isolated and nuclear extracts prepared from liver tissue as previously described.[19]

Immunoprecipitation

Reaction mixtures of Sp1, TG2, and 5-BAPA, as well as nuclear extracts from TG2-transfected and 5-BAPA–treated cells, were incubated with 40 μL of nonimmune rabbit IgG-conjugated agarose beads (Sigma, St Louis, MO) for 16 hours at 4°C on a rotating mixer as described previously.[17]

Western Blotting

Western blotting was performed as described previously.[19] Detailed conditions are described in the Supplementary text. (see supplemental material online at www.gastrojournal.org).

Assays of TG2 Activity

Assays of total TG2 enzymatic capacity (activity) of extracts and in situ TG2 activity were performed as described in the Supplementary text (see Supplementary Material and Methods).

Gel Shift Assays

Gel shift assays were performed as described[19] using end-labeled oligonucleotides (consensus GC box,

5′-ATTCGATCGGGGCGGGGCGAGC-3′; or AP-1 consensus sequence, 5′-CGCTTGATGACTCAGCCGGAA-3′) and nuclear proteins (6 μg) or Sp1 (25 ng), preincubated with or without TG2.

Staining of Cells and Liver Sections

Primary hepatocytes and HepG2 cells on coverslips were fixed with 10% formalin in culture medium. Cells were permeabilized and stained with various antibodies as detailed in the figure legends. Apoptotic cells of liver sections were detected using a DeadEnd Colorimetric TUNEL System (Promega). Digital images of cells and liver sections were obtained by confocal microscopy (Carl Zeiss, Inc, Oberkochen, Germany) and digital images were recorded.

Flow Cytometry and Cell Image Analysis

Ethanol-treated hepatocytes and HepG2 cells were stained with fluorescein isothiocyanate–labeled Annexin V using Annexin V-FITC Kit (Beckman Coulter, Marseille, France). They were analyzed with a fluorescence activated cell sorter (Epics Elite ESP; Beckman Coulter) or using a cell-based imaging system (Celaview RS-100; Olympus, Tokyo, Japan) after fixing and staining with both Hoechst 33258 and anti-TG2 polyclonal antibody (10 μg/mL) plus Cy5-conjugate anti-rabbit IgG (1:1000).

Animals

Sprague–Dawley rats (200–250 g) and C57BL/6 × 129 SvJ mice were purchased from Japan SLC Inc (Hamamatsu, Sizuoka, Japan) or Charles River Laboratory (Wilmington, MA). $TG2^{-/-}$ mice and their $TG2^{+/+}$ littermates derived from heterozygous crosses[10] were used for the isolation of hepatocytes and in vivo liver injury experiments. All animal experiments were performed in accordance with protocols approved by the University of California Davis, University of Southern California, and RIKEN Institutional Animal Use and Care Administrative Advisory Committees. Detailed conditions for each animal experiment are described in Supplementary text.

Human Resource

Human alcohol steatohepatitis and control liver samples were obtained after informed consent and approval by the Ethics Committee for Biomedical Research, Jikei University School of Medicine (Tokyo, Japan).

Statistical Analysis

Quantitative data are shown as mean ± SD or SEM. Statistical analyses were evaluated by single-factor analysis of variance test. Newman–Keuls test was used for multiple comparisons among groups. The 2-tailed Student *t* test was used to evaluate differences between 2 groups. $P < .05$ was considered statistically significant.

Results

Ethanol Increases Hepatocyte Nuclear TG2 Activity

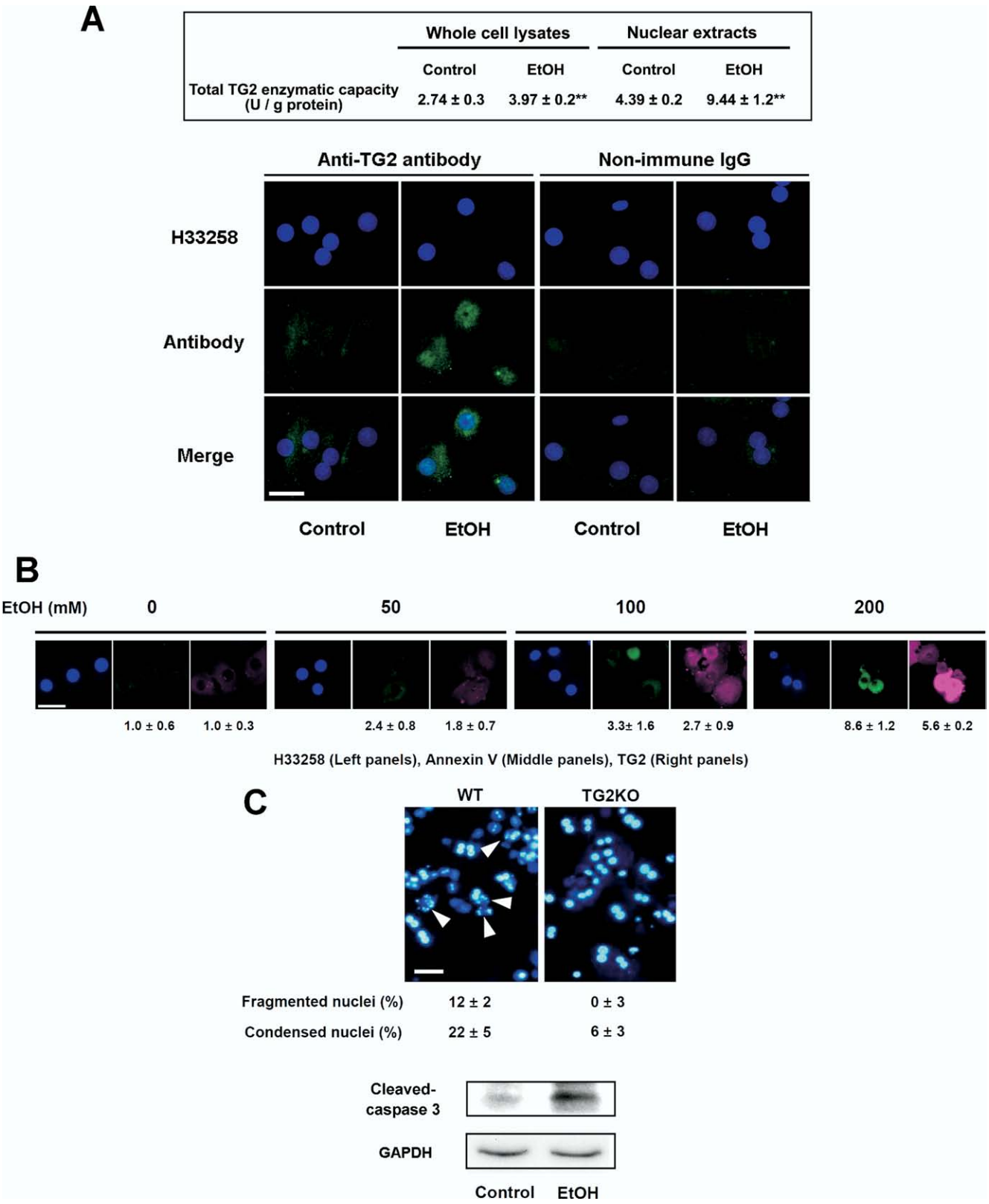
Treatment of primary rat hepatocytes with ethanol overnight increased total TG2 enzymatic capacity (activity) both in whole cell extracts (1.5-fold, $P < .01$) and in nuclear fractions (2.1-fold, $P < .01$) (Figure 1*A*). Accumulation of TG2 to the nucleus was also observed, as evidenced by the appearance of dense clumps of fluorescent material (Figure 1*A*, *lower panels*) and Western blot analysis (Supplementary Figure 3*A*). Similar effects were observed with ethanol-treated or TG2-overexpressing human hepatocyte cell lines (Supplementary Figure 3*B* and *C*). Ethanol-treatment dose-dependently increased the number of Annexin V– and nuclear TG2-positive cells (Figure 1*B*). Acetaldehyde, an ethanol metabolite, mimicked the effect at 1/1000 concentration (Supplementary Figure 4). Ethanol also enhanced TG2 messenger RNA expression and the expression of importin-α3 (Supplementary Figure 5), which has been implicated in TG2 nuclear translocation.[20] Furthermore, whereas apoptosis was readily apparent in ethanol-treated hepatocytes from $TG2^{+/+}$ mice (12% of cells showing fragmented nuclei), it was not observed in $TG2^{-/-}$ hepatocytes (Figure 1*C*). Consistent with this morphological evidence of apoptosis, active caspase-3 levels increased in ethanol-treated $TG2^{+/+}$ hepatocytes (Figure 1*C*, *lower panels*).

TG2 Cross-links and Inactivates Sp1 in Cell-Free Systems

We next attempted to identify the nuclear substrate(s) that could mediate TG2-induced apoptosis. Microarray analysis of hepatocytes isolated from ethanol-treated $TG2^{+/+}$ and $TG2^{-/-}$ mice showed that ethanol increased the expression of 248 genes by >2.5-fold in a TG2-dependent manner, whereas that of 157 genes was TG2-dependently decreased (Supplementary Figure 6*A*). Because the latter included genes, such as *c-Met*,[18] that are regulated by the transcription factor Sp1, a glutamine-rich protein, we investigated if Sp1 is a target of TG2 cross-linking. Incubation of human Sp1 with guinea pig liver TG2 resulted in large CL-Sp1 complexes, as detected by Western blot analysis using a commercial anti-Sp1 antibody (Figure 2*A*, *lanes 2–4*). Further evidence for Sp1 being a TG2 substrate was its ability to modify Sp1 by incorporation of 5-BAPA (Figure 2*A*, *lanes 7–10*). In this study, an anti-biotin antibody recognized not only the high-molecular-weight CL-Sp1 complex identified with an anti-Sp1 antibody (*lane 8*) but also a 184-kilodalton band corresponding to Sp1 dimer and a fainter 92-kilodalton band corresponding to Sp1 monomer (*lane 10*). We attribute the greater sensitivity of the anti-biotin antibody to the incorporation of multiple 5-BAPA molecules into Sp1. Interestingly, with prolonged incubation of Sp1 with TG2, CL-Sp1 was no longer apparent with the anti-Sp1 antibody

(*lane 5*), suggesting that heavy cross-linking masks the epitope identified by this antiserum.

Consistent with the ability of TG2 to mediate cross-linking of both the activation and DNA-binding domains of Sp1 (data not shown), after incubation with TG2, Sp1 lost both its ability to bind to its target DNA motif, the GC box (Figure 2*B, column 3*), and to drive GC box-dependent transcription (Figure 2*C, lane 5*).



Ethanol-Induced Hepatic Cell Apoptosis Involves Cross-linking and Inactivation of Sp1

To test if TG2-induced Sp1 cross-linking and inactivation are responsible for ethanol-induced apoptosis, we next studied the HepG2 human hepatoma cell line. Ethanol dose-dependently induced apoptosis coincident with enhanced TG2 expression and activity, accumulation of CL-Sp1, enhanced Annexin V positivity, and Sp1 transcriptional inactivation (Figure 3*A–C*). Apoptosis was caspase independent as it was observed even in the presence of the caspase inhibitor z-DEVD (Figure 3*D*, *lane 3*) and was mimicked by siRNA-mediated Sp1 knockdown (Figure 3*D*, *lane 7*). Moreover, whereas ethanol treatment resulted in a marked increase in Sp1 cross-linking and impaired DNA binding in hepatocytes from $TG2^{+/+}$ mice or control HepG2 cells, these effects were minimal in cells from $TG2^{-/-}$ mice or HepG2 cells overexpressing an siRNA to knockdown TG2 (Figure 3*B* and *C* and Supplementary Figure 2*A*).

TG2-Mediated Sp1 Cross-linking and Inactivation Causes Hepatic Apoptosis In Vivo

To confirm the biological relevance of these in vitro findings, we evaluated Fas-mediated hepatocellular apoptosis in vivo, because this mode of apoptosis induction contributes importantly to alcohol-induced liver damage.[21] In these studies, administration of the anti-Fas (CD95) antibody Jo2 caused liver injury in $TG2^{+/+}$ but not in $TG2^{-/-}$ mice, as evidenced by marked increases in serum alanine aminotransferase (ALT) levels from 45 U/L in untreated $TG2^{+/+}$ animals (Figure 4*A*, *lane 1*) to >10,000 U/L at 1 day postinjection (Figure 4*A*, *lane 2*). Pretreatment with either putrescine or cystamine, having an inhibitory activity against TG2, attenuated this increase in serum ALT level (*lanes 3* and *4*, respectively). In $TG2^{-/-}$ mice, Jo2 caused only a very minor increase in serum ALT level from 45 U/mL in untreated animals to 143 U/mL (Figure 4*A*, *lane 6*). Consistent with these ALT changes, in situ terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick-end labeling staining of livers from Jo2-treated $TG2^{+/+}$ mice showed abundant apoptotic cells (Figure 4*B*, *panel a*), as well as massive cell death and extensive infiltration of inflammatory cells (Figure 4*B, panel c*), which appeared to be mostly F4/80-positive mononuclear cells (Supplementary Figure 7*A*). These effects were not evident in $TG2^{-/-}$ mice (Figure 4*B*, *panels b* and *d*) and were largely prevented by pretreatment with putrescine or cystamine (data not shown). Pretreatment with an inhibitor to the protein disulfide isomerase, bacitracin, did not affect serum ALT level (18,517 ± 3371 U/L).

Next, we investigated if TG2-mediated CL-Sp1 was accompanied by hepatic apoptosis. After treatment of $TG2^{+/+}$ animals with increasing doses of Jo2, the amount of Sp1 monomer present in liver extracts decreased (Figure 4*C*, *lanes 1–3*), whereas Sp1 oligomers increased (Figure 4*C*, *lanes 4–6*, *7*, and *8*). In contrast, the amount of Sp1 multimers remained unchanged or decreased in the livers of Jo2-treated $TG2^{-/-}$ mice (Figure 4*C*, *lanes 9* and *10*). Furthermore, hepatic Sp1 DNA binding activity was lost with Jo2 treatment of $TG2^{+/+}$ but not $TG2^{-/-}$ mice (Figure 4*D*). Consistent with these findings, increased hepatic nuclear TG2 and CL-Sp1 were also observed in a mouse model in which alcoholic steatohepatitis was induced by forced intragastric administration of ethanol and a high-fat diet (Figure 4*E* and Supplementary Figure 7*B*) and in liver specimens from patients with alcoholic steatohepatitis (Figure 4*F*). In normal mice, Sp1 knockdown induced by injection of a lentiviral vector encoding an Sp1 siRNA, but not lentivirus alone, resulted in hepatic apoptosis (Supplementary Figure 7*C*, *arrows*).

TG2-Dependent Apoptosis Accompanies Reduced Expression of Sp1 Transcriptional Targets

Finally, to obtain insights into the molecular mechanisms by which cross-linking of Sp1 results in apoptosis, we investigated if the expression of several genes whose transcription is controlled by Sp1, including Bcl-2, Bcl-xL, p21, and growth factor receptors, is altered in ethanol-treated HepG2 cells. Expression of Bcl-2 and Bcl-X_L remained unchanged (data not shown), and in contrast to a previous report,[22] transactivation of a p21 responsive reporter gene did not increase with ethanol treatment (Supplementary Figure 8*A*). Additionally, the nuclear localization of apoptosis-inducing factor, whose release from mitochondria was implicated in TG2-induced apoptosis, as well as induction of activating

Figure 1. Augmented production and nuclear translocation of TG2 in ethanol-treated hepatocytes. (*A*) Rat hepatocytes were treated with 0 or 100 mmol/L ethanol overnight and total TG2 enzymatic capacity (activity) in whole cell lysates and in nuclear protein extracts was determined. $^{**}P < .01$ compared with controls. Representative confocal images of parallel cultures fixed and stained with either anti-TG2 polyclonal antibody or rabbit nonimmune IgG plus Alexa 488 conjugate anti-rabbit IgG. *Scale bar* = 50 μm. (*B*) Rat hepatocytes were treated with 0–200 mmol/L ethanol overnight. After staining using Annexin V/fluorescein isothiocyanate (*middle panels*), cells were fixed and stained with Hoechst 33258 (*left panels*) and anti-TG2 polyclonal antibody (*right panels*). The intensity of cell imaging more than 3×10^3 was quantified by the cell-based imaging system Celaview. The percentage of apoptotic (Annexin V–positive) and nuclear TG2-positive cells are indicated under each micrograph. *Scale bar* = 50 μm. (*C*) Hepatocytes from either $TG2^{+/+}$ or $TG2^{-/-}$ mice were treated with 50 mmol/L ethanol for 4 hours and nuclei stained with Hoechst 33258. *Arrowheads* show fragmented nuclei. *Scale bar* = 50 μm. The percentage of nuclei undergoing fragmentation or condensation versus total nuclei is indicated. *Lower panels* indicate the levels of cleaved caspase-3 and glyceraldehyde-3-phosphate dehydrogenase in $TG2^{+/+}$ hepatocytes. *A–C* show representative findings from 3 different experiments.

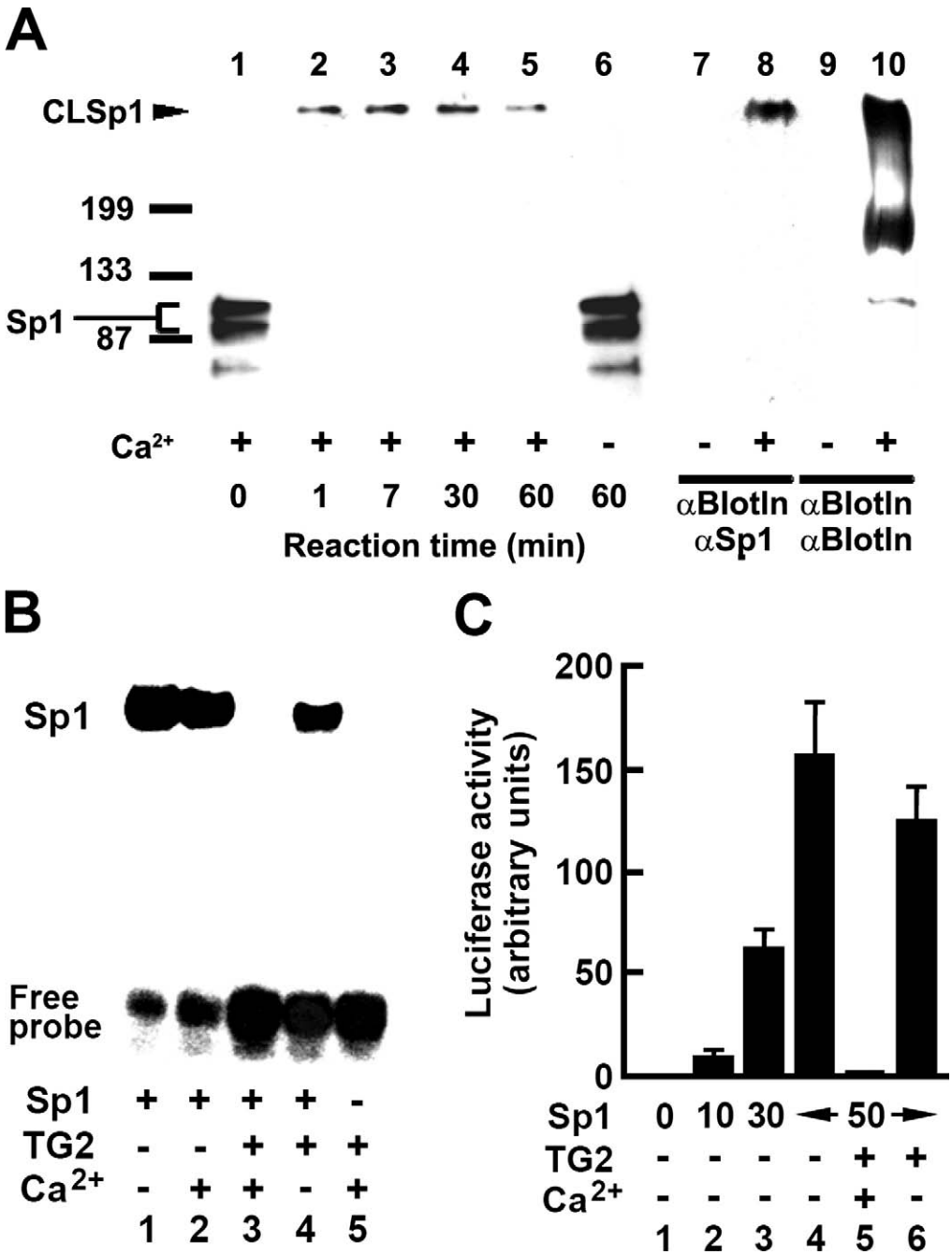


Figure 2. Inactivation of Sp1 by TG2-mediated cross-linking in vitro. (*A*) Sp1 and guinea pig TG2 (400 nmol/L each) were incubated at 37°C for the times indicated in the presence or absence of 10 mmol/L Ca^{2+} (*lanes 1–6*) or mixed and incubated with 100 μmol/L 5-BAPA for 3 hours in the absence or presence of 10 mmol/L Ca^{2+} in HEPES-buffered saline followed by immunoprecipitation of 5-BAPA–incorporated proteins with anti-biotin antibody–conjugated agarose beads (*lanes 7–10*). Changes in the molecular size of Sp1 were analyzed by Western blotting with either anti-Sp1 antibody (αSp1, *lanes 1–8*) or anti-biotin antibody (αbiotin, *lanes 9 and 10*). Molecular weight markers (kilodaltons) are indicated. (*B*) After incubating Sp1 for 1 hour with or without TG2 as described in *A*, and in the absence or presence of 10 mmol/L Ca^{2+}, as indicated, changes in DNA binding activity were examined by gel shift assays using the consensus GC box oligonucleotide as a probe. (*C*) Sp1 (0–50 ng) was incubated with 20 nmol/L TG2 in the absence or presence of 10 mmol/L Ca^{2+} overnight in HEPES-buffered saline. The reaction mixture was dialyzed and incubated (90 minutes at 30°C) with 20 μL of the TNT T7 Quick Master Mix solution and GC3-Luc vector. Sp1-dependent transcription was determined by monitoring luciferase activity. *Lanes 1–4*, increase in reporter activity with increasing Sp1 amount; *lanes 5 vs 6*, disappearance of Sp1 (50 ng)-dependent transactivation activity following preincubation with TG2. Results shown are means ± SD (n = 3). *A–C* show representative findings from 3 different experiments.

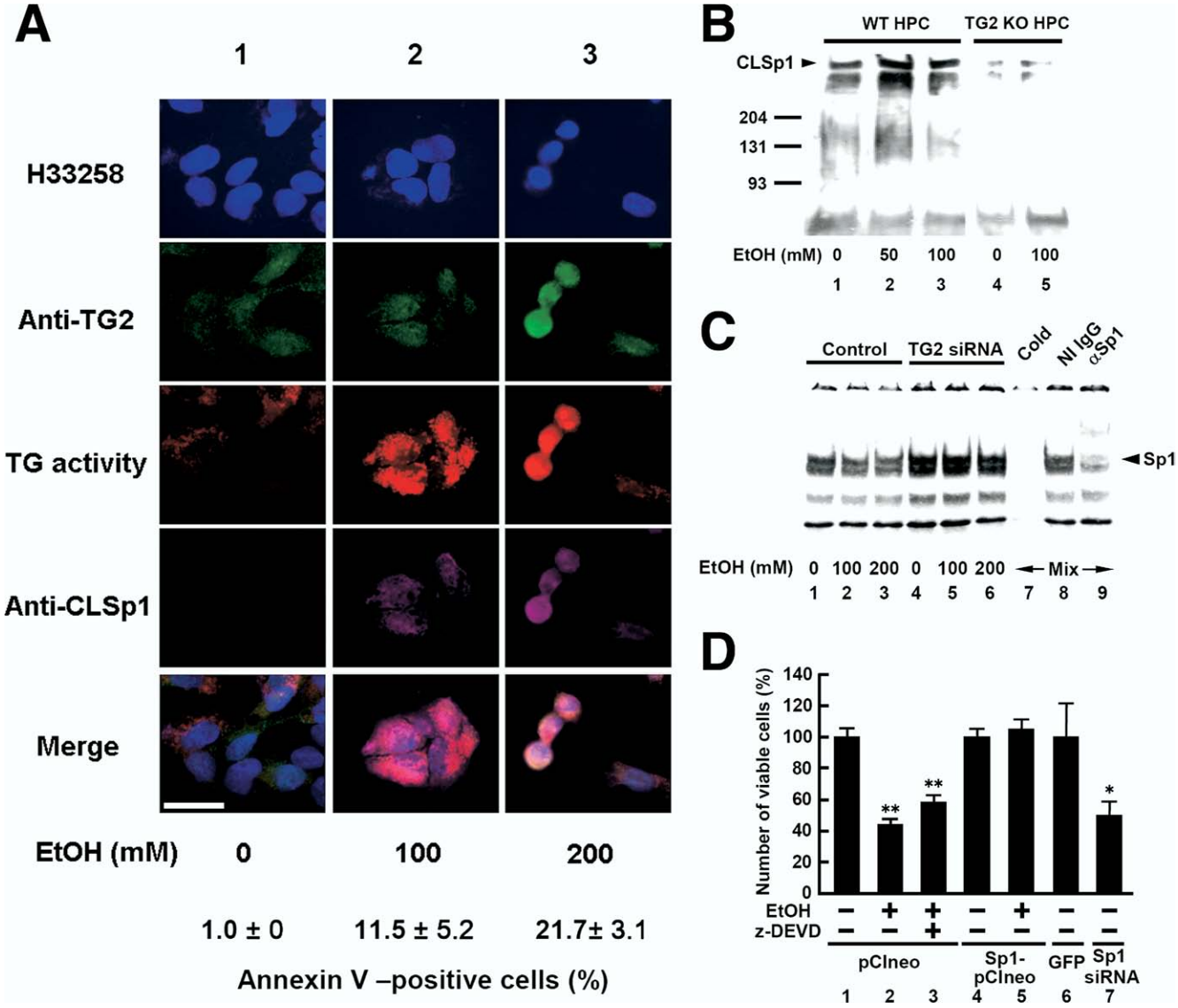
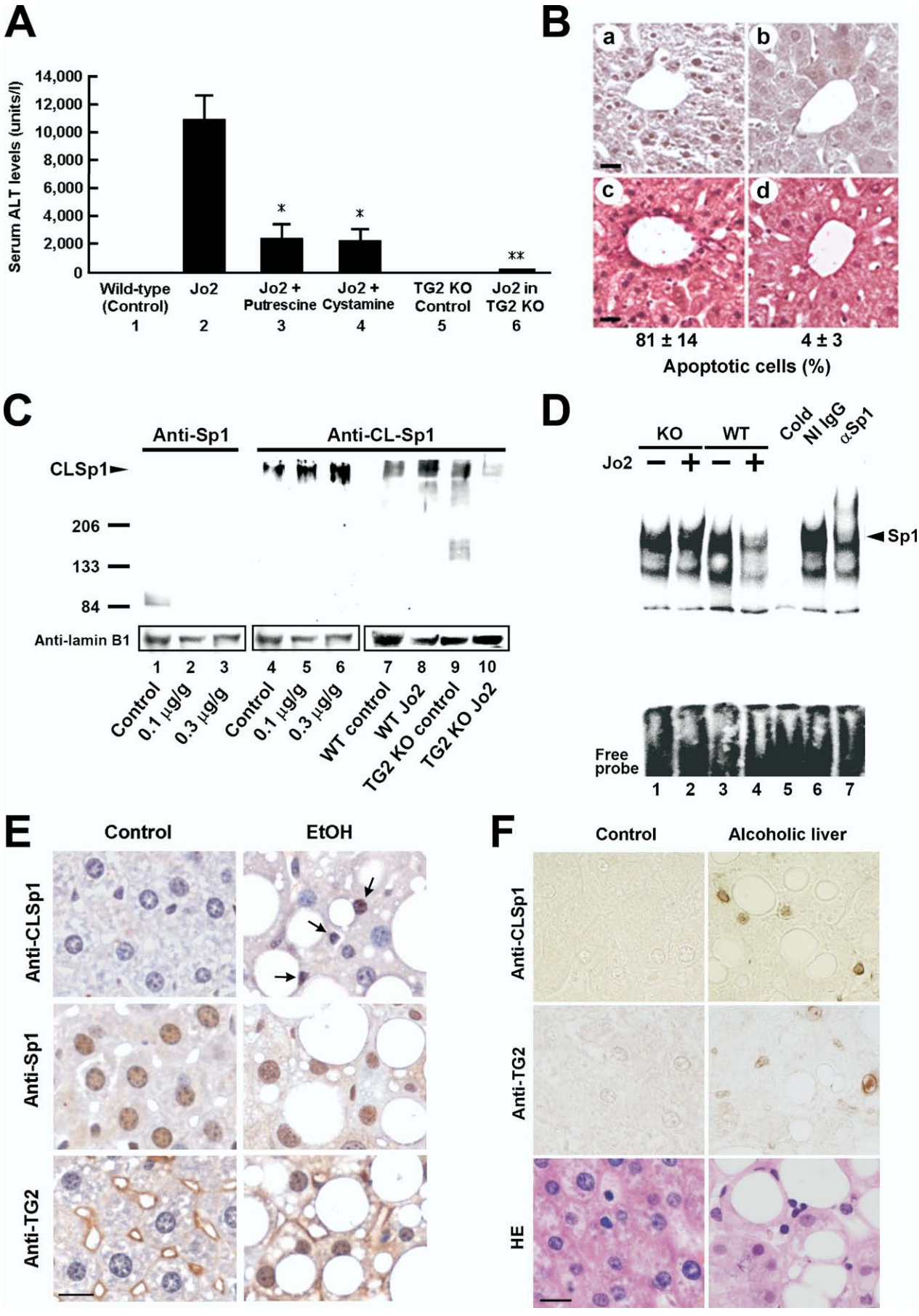


Figure 3. Ethanol induces TG2-dependent apoptosis via cross-linking and inactivation of Sp1 in HepG2 cells. (*A*) HepG2 cells treated with 0–200 mmol/L ethanol plus both 0.2 mmol/L 5-BAPA and 100 μmol/L aminoguanidine for 24 hours were fixed and stained with Hoechst 33258 (*upper row*), anti-TG2 polyclonal antibody (*second row*), tetramethylrhodamine isothiocyanate–conjugate streptavidin (*third row*), or anti–CL-Sp1 antibody (*fourth row*). The results of merging the 4 images are shown in the *bottom panels* (Merge). Apoptotic (Annexin V–positive) cells, determined by flow cytometry and expressed a percent of control, are indicated under each *column*. *Scale bar* = 50 μm. (*B*) Hepatocytes isolated from $TG2^{+/+}$ (WT; *lanes 1–3*) or $TG2^{-/-}$ (*lanes 4 and 5*) mice were treated with 0–100 mmol/L ethanol for 24 hours in the presence of 5-BAPA. Nuclear extracts were prepared and in situ incorporation of 5-BAPA into CL-Sp1 was assessed by Western blotting with an anti-Sp1 antibody. (*C*) HepG2 cells stably overexpressing green fluorescent protein (control; *lanes 1–3*) or TG2 siRNA (*lanes 4–6*) were treated with 0–200 mmol/L ethanol for 24 hours. Sp1 DNA binding activity of nuclear extracts was determined by gel shift assay using a consensus GC box as a probe. *Lane 7*, mixed nuclear extracts + 50-fold excess of unlabeled probe (cold); *lane 8*, + nonimmune (NI) IgG; *lane 9*, + anti-Sp1 IgG (αSp1). Detailed information about the cells used is described in Supplementary Figure 2*A* (see supplemental material online at www.gastrojournal.org). (*D*) HepG2 cells transfected with pCIneo vector (controls, *lanes 1–3*) or Sp1-pCIneo (*lanes 4 and 5*), or HepG2 cells stably overexpressing green fluorescent protein (*lane* 6) or Sp1 siRNA (*lane* 7), were seeded in 1×10^5 cells/3.5-cm dishes and treated with 0 or 200 mmol/L ethanol in the absence or presence of the caspase inhibitor Z-DEVD for 48 hours. Results shown are the number of viable cells relative to the controls expressed as percent ± S.D. $^{*}P < .05$, $^{**}P < .01$ compared with control cells (*lane 1*). Detailed information about the cells used is described in Supplementary Figure 2*B* (see supplemental material online at www.gastrojournal.org). *A–D* show representative findings from 3 different experiments.

transcription factor 6, which regulates glucose-regulated protein genes encoding endoplasmic reticulum chaperones, were unchanged or minimally altered, respectively (Supplementary Figure 8*B*). Of several growth factor receptors examined, steady state messenger RNA expression of *c-Met* (the receptor for hepatocyte growth factor [HGF]) decreased significantly with ethanol (100 mmol/L) treatment. This was confirmed ex vivo in ethanol-treated hepatocytes from $TG2^{+/+}$ but not $TG2^{-/-}$ mice; the former but not the latter showing reduced *c-Met*



BASIC–LIVER, PANCREAS, AND BILIARY TRACT

messenger RNA expression (Figure 5*A*), and in vivo in hepatocytes prepared from Jo2-treated animals, which showed reduced expression of *c-Met* protein only in TG2$^{+/+}$ cells (Figure 5*B* and Supplementary Figure 8*C*). Further, microarray analysis showed a 27-fold reduction in *c-Met* expression by ethanol treatment in TG2$^{+/+}$ but not TG2$^{-/-}$ hepatocytes, whereas TG2-dependent inhibition of tumor necrosis factor receptor and epidermal growth factor receptor expression by ethanol were only 4- and 2-fold, respectively (Supplementary Figure 6*B*).

To determine if reduced expression of *c-Met* was due to Sp1 inactivation, we monitored transactivation of a chimeric reporter gene construct in which expression was driven by 3 tandem functional GC box motifs in the *c-Met* promoter. As shown in Figure 5*C*, ethanol-treatment dose-dependently decreased luciferase activity from this reporter gene (*lanes 1–3*) in HepG2 cells, this cell line being used to obviate the need to transfect primary hepatocytes, which is problematic. This reduced transactivation of the *c-Met* promoter was not prevented by caspase inhibition (*lanes 4–9*), but was prevented by overexpression of Sp1 (*lanes 10–12*), and was mimicked by Sp1 knockdown with siRNA (*lanes 13–15*; see also Figure 5*B*, *lanes 5* and *6*, which confirm Sp1 siRNA-induced reduction of c-Met expression). In contrast, AP-1 luciferase activity decreased only marginally with ethanol treatment (*lanes 16–18*). Finally, we found that HGF treatment not only increased phospho-*c-Met* levels (Figure 5*D*) but markedly inhibited ethanol-induced death of rat hepatocytes (Figure 5*E*), indicating that reduced Sp1-mediated transcription of *c-Met* may at least in part directly mediate ethanol-induced apoptosis.

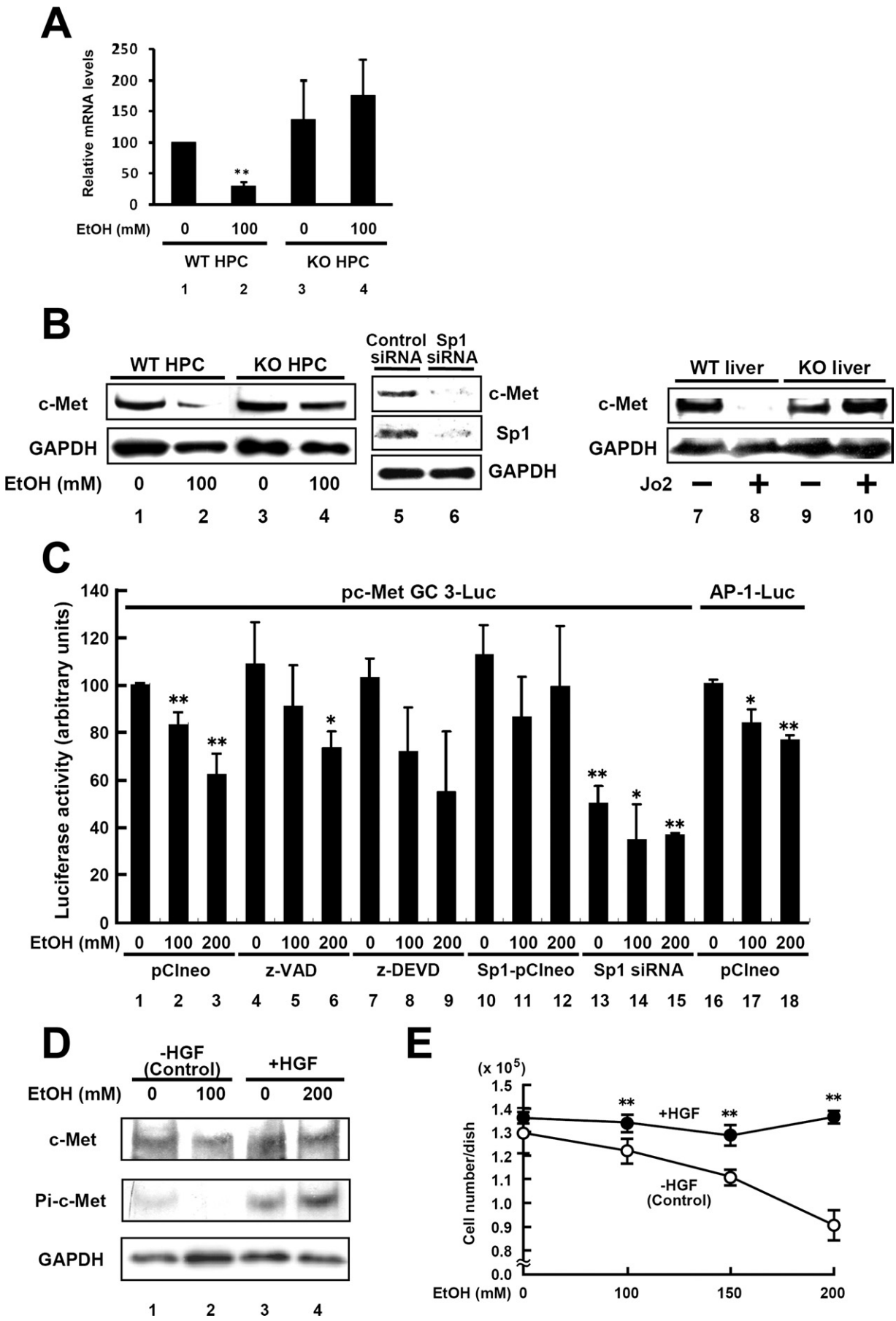
Discussion

Using TG2$^{+/+}$ and TG2$^{-/-}$ mice, we provide evidence that TG2 plays a primary role in hepatic injury-induced hepatocyte apoptosis in vivo and that this effect is mediated by accumulation of TG2 in the nucleus (Figure 6). This results in cross-linking and transcriptional inactivation of Sp1 and, as a consequence, impairs expression of Sp1-regulated target genes, such as the HGF receptor c-Met, which is critical for cell growth and survival (Figure 6). These conclusions are supported by several lines of evidence including that (1) TG2 overexpression results in apoptosis, Sp1 cross-linking, and inactivation, effects that can be mimicked by siRNA-induced Sp1 knockdown and rescued by TG2 knockout or knockdown, or by Sp1 overexpression, (2) apoptosis induced by ethanol or Jo2 treatment is accompanied by TG2-dependent Sp1 cross-linking and inactivation, and (3) treatment with HGF markedly decreases cell death.

Sarang et al[15] reported that TG2 protects against high-dose (1 μg/g body wt) Jo2-induced liver injury, based on the finding that TG2$^{-/-}$ mice were more sensitive to Jo2-mediated necrosis than TG2$^{+/+}$ animals. In those studies,[15] FVB mice were used as the wild-type controls. In our hands, using wild-type littermates from heterozygous TG2$^{+/-}$ crosses, the high dose of Jo2 used by Sarang et al[15] caused massive hepatic necrosis, both in TG2$^{+/+}$ and TG2$^{-/-}$ mice (data not shown). Thus, the different genetic backgrounds of the TG2$^{+/+}$ versus TG2$^{-/-}$ mice used by Sarang et al[15] could have contributed to the discrepancy in our findings. However, we believe Jo2 dosage is another important factor, because we can reproduce the protective effect of TG2 silencing on hepatic injury caused by low-dose Jo2 (ie, the dose used in the present study: 0.1 μg/g body wt) in the exact same TG2$^{-/-}$ mice used by Sarang et al[15] (Supplementary Figure 9). Thus, TG2 may promote hepatic apoptosis caused by relatively low doses of Jo2, but not by higher doses. The detailed mechanisms underlying these distinct effects remain to be elucidated.

Using the same TG2 knockout mice as used by Sarang et al,[15] Nardacci et al[13] reported that TG2 was protective

Figure 4. TG2$^{-/-}$ mice are resistant to hepatic injury due to reduced formation of cross-linked and inactivated Sp1. (*A*) Jo2 (0.1 μg/g) was administered to TG2$^{+/+}$ (*lanes 2–4*) and TG2$^{-/-}$ (*lane* 6) mice. Some animals intraperitoneally received putrescine (5 mg/kg) or cystamine (18 μg/kg) 30 minutes before Jo2 administration, as indicated. Serum ALT levels were determined as described in Materials and Methods 24 hours after Jo2 treatment. $^{*}P < .05$, $^{**}P < .01$ compared with Jo2 treatment alone in TG2$^{+/+}$ mice. (*B*) Twenty-four hours after administration of Jo2 to TG2$^{+/+}$ (*panels a and c*) and TG2$^{-/-}$ (*panels b* and *d*) mice, livers were sectioned and either frozen for double staining of terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick-end labeling and hematoxylin or fixed, embedded in paraffin, and stained with H&E. The percentage of apoptotic cells observed for each sample was indicated. *Scale bars* = 50 μm. (*C*) Twenty-four hours after administration of the indicated doses of Jo2 to TG2$^{+/+}$ (*lanes 1–8*) or TG2$^{-/-}$ mice (*lanes 9 and 10*), liver extracts were prepared and assessed by Western blotting for Sp1 monomer or CL-Sp1 using the antisera indicated. Polyvinylidene difluoride membranes from the studies in *lanes 1–10* were reprobed with anti-lamin B1. (*D*) Twenty-four hours after administration of Jo2 to TG2$^{-/-}$ (*lanes 1 and 2*) or TG2$^{+/+}$ mice (*lanes 3–7*), DNA binding activity of Sp1 in liver extracts was assessed by gel shift assay using a consensus GC box as the probe. *Lanes 1, 3, and 5–7*, controls; *lanes 2 and 4*, Jo2; *lane 5*, +50-fold excess of unlabeled probe (cold); *lane 6*, + nonimmune antibody (NI IgG); *lane 7*, + anti-Sp1 IgG (αSp1). (*E*) Liver sections from mice with alcoholic steatohepatitis were stained with anti–CL-Sp1 (30 μg/mL), anti-Sp1 (5 μg/mL), or polyclonal anti-TG2 (30 μg/mL) antibodies, and the signals were enhanced using an ABC kit and developed with DAB substrate. Sections were counterstained with hematoxylin. Serum ALT levels (mean ± SD, n = 5) were 14 ± 2 U/L (controls) and 299 ± 121 U/L (ethanol treated). *Scale bar* = 50 μm. (*F*) Human alcoholic liver and control samples were stained with anti–CL-Sp1 (30 μg/mL), monoclonal anti-TG2 (30 μg/mL), or H&E. Noncancerous regions of the liver from a patient with uterine cancer with hepatic metastases were used as the controls. Staining signals were enhanced using the Envision+ system, horseradish peroxidase, detection (Dako, Carpinteria, CA) and developed with DAB substrate. Sections were counterstained with hematoxylin. *Scale bar* = 50 μm. More than 6 mice were used for each experiment in *A–E*. *A–F* show representative findings from 3 different experiments that all gave similar results.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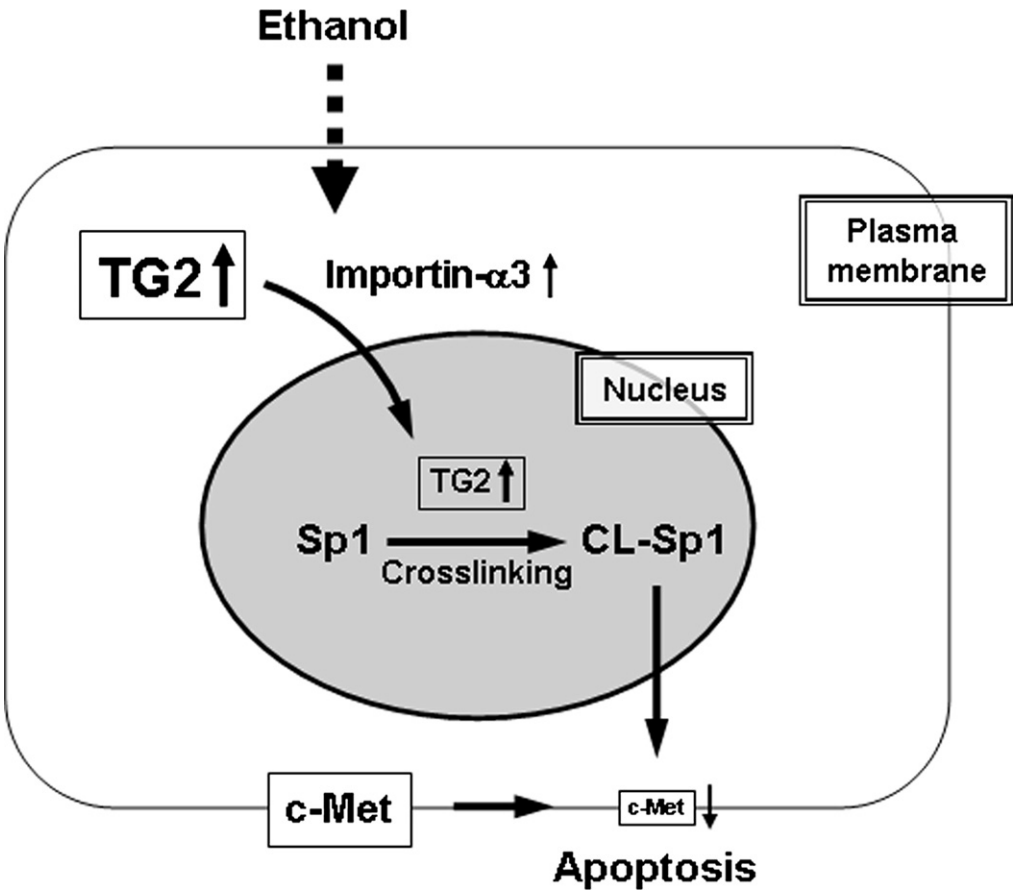

Figure 6. Schematic showing the molecular mechanism by which ethanol causes hepatic apoptosis via TG2-mediated cross-linking and silencing of Sp1, thereby reducing *c-Met*–mediated HGF signaling.

in CCl_4-mediated liver injury and speculated that the observed increase in TG2 expression during the initial stages of liver fibrosis in hepatitis C virus–infected patients may protect against liver injury. However, another explanation may be that high levels of TG2 contribute to liver injury in these patients, in which case, as we have shown here in an animal model (Figure 4*A*), TG2 inhibitors may be a useful treatment for the prevention of hepatic apoptosis.

Han and Park reported that 293T cells overexpressing TG2 showed increased binding of Sp1 to its target DNA, as measured by gel electrophoretic mobility shift assay and p21 reporter construct.[22] We speculate that differences in cell types and experimental conditions, including the extent of Sp1 cross-linking, could account for these contradictory results.

Expression of a number of major apoptotic and anti-apoptotic genes, such as those for caspase family mem-

Figure 5. Ethanol impairs the expression of Sp1-regulated growth factor receptor genes. (*A*) Hepatocytes from $TG2^{+/+}$ (*lanes 1 and 2*) or $TG2^{-/-}$ mice (*lanes 3 and 4*) were treated with 0 or 100 mmol/L ethanol for 15 hours, and *c-Met* messenger RNA in cell lysates was quantitated by real-time polymerase chain reaction. Values show mean ± SD. $^{**}P < .01$ compared with controls. (*B*) Hepatocytes from $TG2^{+/+}$ (*lanes 1 and 2*) or $TG2^{-/-}$ mice (*lanes 3 and 4*) were treated with 0 or 100 mmol/L ethanol for 48 hours, or HepG2 cells were transfected with scrambled Sp1 siRNA oligo (control, *lane 5*) or Sp1 siRNA (*lane 6*) and incubated for 48 hours. Hepatocyte or HepG2 total cell extracts were then prepared. *Lanes 7–10*: Twenty-four hours after administration of either saline (-) or Jo2 (+) to $TG2^{+/+}$ (*lanes 7 and 8*) or $TG2^{-/-}$ mice (*lanes 9 and 10*), animals were killed and liver extracts prepared. c-Met, glyceraldehyde-3-phosphate dehydrogenase, and Sp1 protein expression in total cell extracts or liver whole extracts was assessed by Western blotting. (*C*) One day after transfection of HepG2 cells with *c-Met* promoter GC3-Luc (*lanes 1–15*) or AP-1 Luc reporter (*lanes 16–18*), cells were treated with either 0–200 mmol/L ethanol for 24 hours in the absence (*lanes 1–3*) or presence of 50 μg/ml z-VAD (*lanes 4–6*) or z-DEVD (*lanes 7–9*) or cotransfected with Sp1-pCIneo (*lanes 10–12*) or Sp1 siRNA (*lanes 13–15*) vector. Results shown are means ± SD (n = 3). $^{*}P < .05$, $^{**}P < .01$ compared with controls. (*D*) After treatment of HepG2 cells with 0–100 mmol/L ethanol for 24 hours in the absence (*lanes 1 and 2*) or presence of 10 ng/mL HGF (*lanes 3 and 4*), cell lysates were prepared and *c-Met* (*upper row*), phospho (Pi)-*c-Met* protein (*middle row*), and glyceraldehyde-3-phosphate dehydrogenase (*bottom row*) expression were determined by Western blotting. (*E*) Rat hepatocytes were treated with 0–200 mmol/L ethanol for 24 hours in the absence (*open circles*) or presence (*closed circles*) of 10 ng/mL HGF and the number of viable cells were determined after trypsinization by Trypan blue dye exclusion. Results shown are means ± SD (n = 3). $^{**}P < .01$ for HGF-treated vs untreated cells. *A–E* show representative findings from 3 different experiments that all gave similar results.

bers, Bcl proteins, apoptosis-inducing factor, and activating transcription factor 6, was unchanged or only minimally (<2-fold) altered (Supplementary Figure 8*B*), indicating hepatocyte apoptosis induced by TG2-mediated CL-Sp1 is a novel cell death pathway basically independent from apoptosis-inducing factor and caspase. Ingenuity pathway analysis of genes determined by microarray analysis to be up-regulated or down-regulated in a TG2-dependent manner by >2.5-fold following ethanol treatment showed highly significant activation ($P < 1 \times 10^{-6}$) of both hepatic cell death and disease pathways, including TG2-dependent inhibition of *c-Met* expression. Our finding that inactivation of Sp1 by TG2-mediated cross-linking impairs transcription of Sp1-regulated target genes, such as those for growth factors that are required for normal cell functioning, is consistent with the finding that inactivation of Sp1 in neuronal cells down-regulates both dopamine and nerve growth factor receptor expression,[23–25] and that down-regulation of *c-Met* induces apoptosis.[26] Further, in rat hepatocytes, which are less sensitive than HepG2 cells or mouse hepatocytes to the inhibitory effects of ethanol on *c-Met* expression (~50% reduction in *c-Met*), excess HGF induces significant c-Met phosphorylation of residual c-Met receptors (Figure 5*D*) and limited ethanol-induced apoptosis (Figure 5*E*). We cannot explain why Sp1 cross-linking down-regulates *c-Met* expression, while other Sp1-dependent genes are much less affected. We speculate that differences in Sp1 dependence may depend on the cell type and culture conditions used and that other member(s) of Sp/KLF family may compensate for the function of Sp1 in the transactivation of less affected genes. Indeed, we recently found that in TG2- and CLSp1-dependent apoptosis of acyclic retinoid-induced hepatocarcinoma, the putative target was epidermal growth factor receptor rather than *c-Met* (Tatsukawa et al, unpublished data, February 2008). The result of pathway analysis also suggested potential modulation of the PI3K/Akt, Toll-like receptor, and pTEN signaling pathways.

In summary, our findings in liver cells, in in vivo models of hepatic apoptosis, and in patients with alcoholic steatohepatitis indicate that TG2 enhances apoptosis and may, indeed, underlie its initiation. The transcription factor Sp1 is cross-linked, oligomerized, and inactivated by nuclear TG2, leading to activation of a caspase-independent apoptotic process as a result of reduced expression of critical growth factor receptor genes, such as c-Met. It would also be of interest to evaluate the contribution of TG2 and Sp1 in ethanol/FasL-induced apoptosis of stellate cells in the context of liver fibrosis.

Supplementary Data

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at doi: 10.1053/j.gastro.2009.01.007.

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Reprint requests

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Conflicts of interest

The authors disclose no conflicts.

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Supplementary Materials and Methods

Microarray Analysis

After treatment of $TG2^{+/+}$ and $TG2^{-/-}$ mice with ethanol for 15 hours, total RNA was isolated from hepatocytes. Conversion to complementary RNA and biotin labeling were performed according to manufacturers' protocols. Calculation of signal intensities in each array (Affymetrix Mouse Genome 430 2.0 Array, Santa Clara, CA) and comparative analyses were performed using GeneSpring GX software (Agilent Technologies, Santa Clara, CA). After fragmentation of complementary RNA, microarray studies were performed by the RIKEN Research Resource Center.

Cell Culture

Hepatocytes, isolated from both rats and mice by 2-step collagenase digestion, were purified and cultured as described.[1] HepG2 cells were grown in RPMI 1640 medium and Dulbecco's modified Eagle medium (Sigma–Aldrich, Inc, St Louis, MO) supplemented with 10% fetal calf serum, respectively.

Detailed Condition of Western Blotting

Western blotting was performed as described previously[2] using anti-TG2 monoclonal (1:200 dilution), anti-TG2 polyclonal (final 2.5 μg/mL), anti-Sp1 (1:200 dilution), anti–cross-linked Sp1 (final 2.5 μg/mL), and anti-cleaved caspase-3 antibody (1:1000 dilution; Cell Signaling, Beverly, MA) that were probed with either horseradish peroxidase–conjugated goat anti-rabbit/mouse IgG (1:1000 dilution; Jackson ImmunoResearch Laboratories) or horseradish peroxidase–conjugated anti-biotin antibody (1:1000 dilution; Sigma). Anti-lamin B1 antibody (1:500 dilution; Santa Cruz Biotechnology) and anti–glyceraldehyde-3-phosphate dehydrogenase antibody (1:2000 dilution; Chemicon International, Temecala, CA) were used to normalize for sample loading. Reactants were detected using enhanced chemiluminescence reagents (GE Healthcare, Buckinghamshire, UK).

Detailed Conditions for Assays of TG Activity

Hepatocytes seeded on 6-well Costar plates were lysed and used to determine total TG2 enzymatic capacity (activity), with 1 U defined as the incorporation of 1 μmol of [1,4-^{14}C]-putrescine (GE Healthcare) into 1 mg of dimethylcasein (Sigma) per minute as previously described.[3] In situ TG2 activity was detected by incorporation of 5-BAPA into the cells. Hepatocytes were incubated with 0.2 mmol/L 5-BAPA and 100 μmol/L aminoguanidine for 24 hours. After washing with culture medium, cells were lysed to perform the Western blotting using anti-biotin antibodies, or cells were fixed, permeabilized, and stained with tetramethylrhodamine isothiocyanate–conjugate streptavidin (1:500 dilution).

TG2 and Sp1 siRNA Stable Cell Line

A TG2 siRNA stable cell line was made by infection of lentivirus, which expresses the pSIH-H1 short hairpin RNA vector containing TG2 short hairpin RNA (SBI System Biosciences). The siRNA oligonucleotide or a lentivirus-based short hairpin RNA construct was used to silence Sp1 and TG2. The sequence of siRNA oligonucleotides is as follows: Sp1 siRNA, 5′-TGTACCAGTGGCCCTGAAT-3′; TG2 siRNA, 5′-GATGGGATCCTAGACATCT-3′. The lentivirus-based vector was prepared in 293T cells and used to infect HepG2 cells. Forty-eight to 72 hours after infection, HepG2 cells were used for several experiments.

Anti–CL-Sp1 Antibody Production

Anti–CL-Sp1 antibodies were elicited by immunization of a rabbit and the antiserum purified using protein A/agarose. The cross-linked antigen used for immunization was produced by incubating purified Sp1 with guinea pig liver TG2 and then separating the resultant highly cross-linked product from Sp1 monomer and TG2 by preparative sodium dodecyl sulfate/polyacrylamide gel electrophoresis. The bands were excised, homogenized, and mixed with MPL+TDM+CWS Emulsion (Ribi Adjuvant System). Immunization and bleeding were continued every week for 3 months. IgGs in the resulting immune serum that recognized un–cross-linked Sp1, as well as TG2, were removed by passing the IgG fraction through an Sp1-Sepharose column and then a TG2-Sepharose column. The concentration of IgG remaining, which specifically recognized only cross-linked Sp1, was determined using a BCA Assay Kit (Pierce, Rockford, IL).

Anti-TG2 Polyclonal Antibody

Anti-TG2 antibodies were elicited by immunization of a rabbit and the antiserum purified using protein A/agarose. TG2 from guinea pig liver used for immunization was from Oriental Co and then was separated by preparative sodium dodecyl sulfate/polyacrylamide gel electrophoresis. The bands were excised, homogenized, and mixed with MPL+TDM+CWS Emulsion (Ribi Adjuvant System). Immunization and bleeding were continued every week for 3 months. IgG fraction was purified by protein A/agarose (Sigma) from serum. The concentration of IgG was determined using a BCA Assay Kit (Pierce).

Jo2-Induced Acute Liver Apoptosis

Jo2, an anti-Fas antibody (0.1 μg/kg in normal saline; Pharmingen Corp, San Diego, CA[4]), was given intravenously to wild-type (C57 BL/6 × 129 SvJ) or $TG2^{-/-}$ mice. Some animals were treated intraperitoneally with putrescine (5 mg/kg) or cystamine (18 μg/kg) 30 minutes before Jo2 injection. Animals were

anesthetized 24 hours after Jo2 injection. More than 6 mice were used for each experiment, and all animals were fed a commercial diet (CE2, Clea Japan; or Purina Chow 5001, Rodent Laboratory) and water ad libitum. Blood was collected for ALT assays by a kit from Sigma Diagnostics (St Louis, MO), and liver samples were fixed for frozen sectioning in a cryostat or for preparation of paraffin-embedded sections.

Alcohol Liver Injury Model

The alcohol-induced liver injury model for the early stage of steatohepatitis was made as described previously.[5] Briefly, mice were implanted with gastrostomy catheters under general anesthesia. The animals received continuous intragastric infusion of a high-fat (35% calories as corn oil; Dyets Inc, Bethlehem, PA) diet plus ethanol for 9 weeks. More than 6 mice were used for each experiment. Intragastric ethanol feeding was performed by the Animal Core of the Southern California Research Center for ALPD and Cirrhosis at the University of Southern California. Livers were removed, fixed in 10% formalin, and embedded into paraffin. Sections were made and stained with anti-CLSp1 antibody, anti-Sp1 antibody, and anti-TG2 polyclonal antibody before hematoxylin staining. Staining signals were enhanced using an ABC kit (Vectastain; Vector Laboratories, Burlingame, CA) and developed with DAB substrate.

Reverse-Transcription Polymerase Chain Reaction

Reverse-transcription polymerase chain reaction was performed as described[6] using sets of specific primers summarized in Supplementary Table 1.

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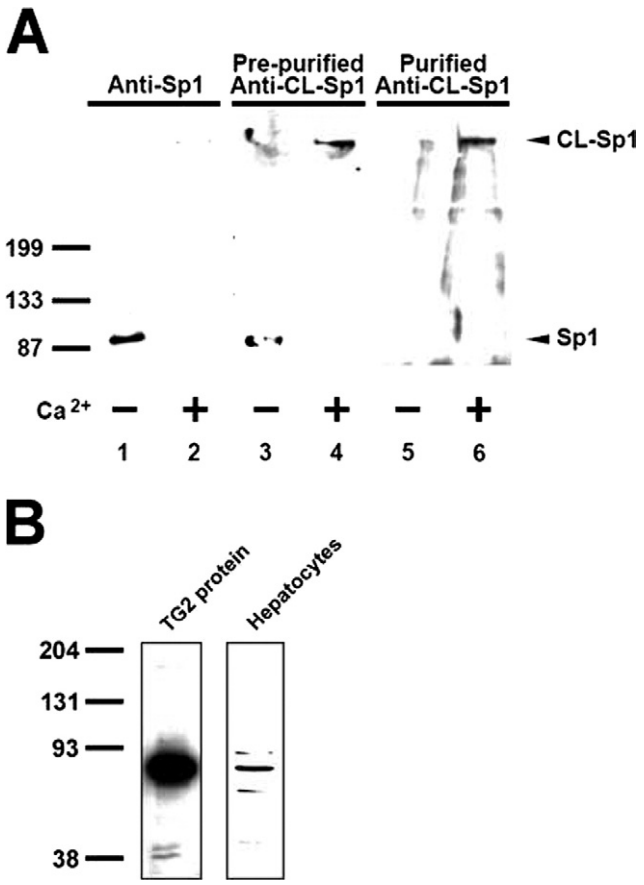
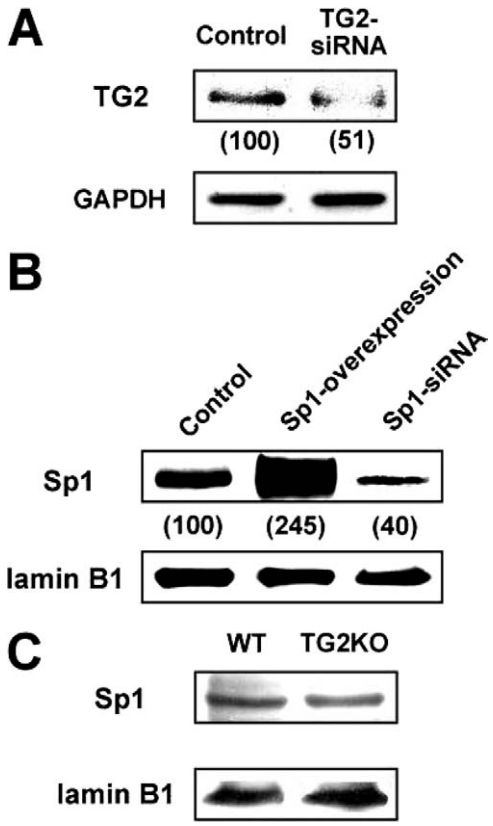
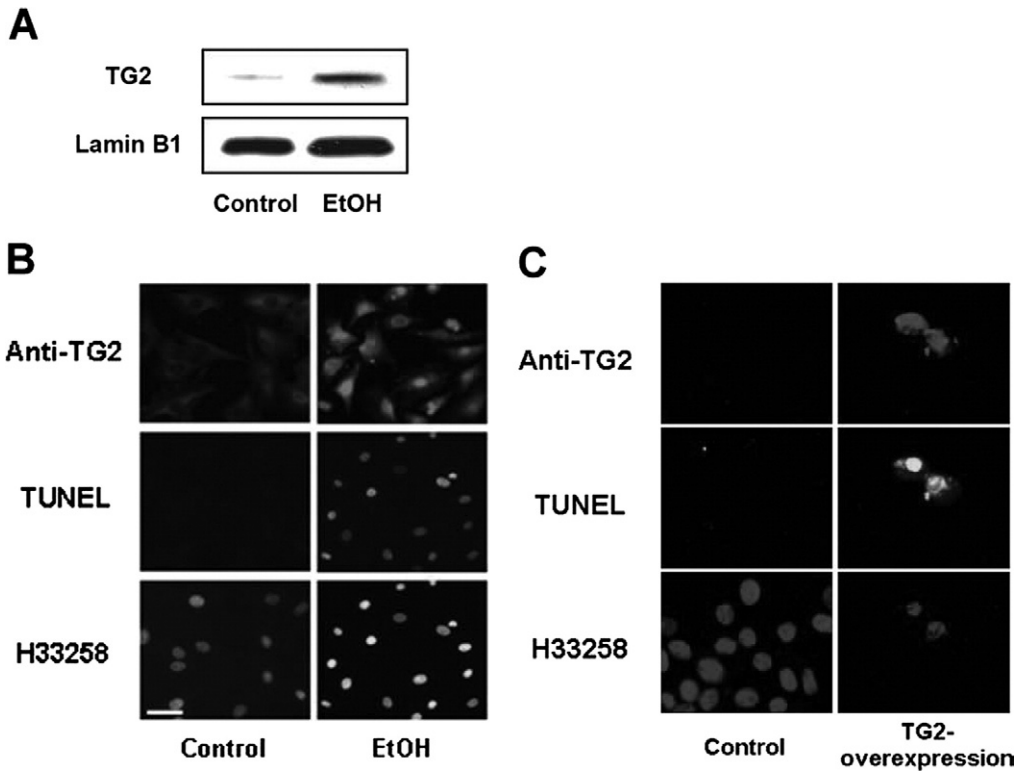


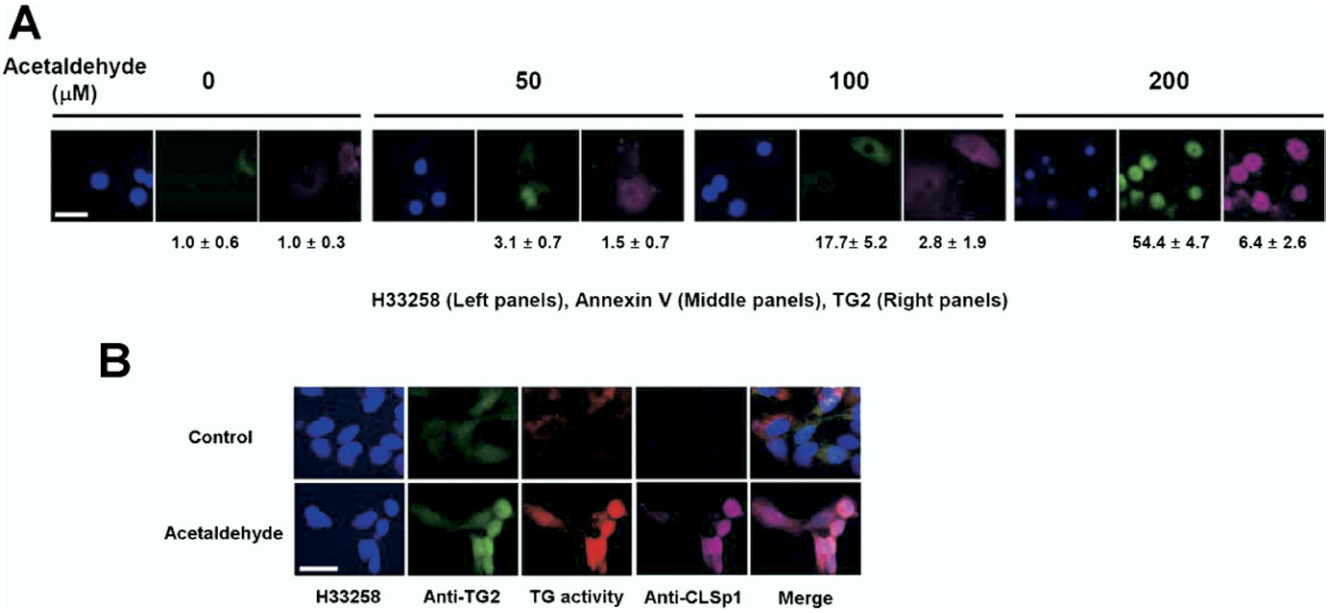
Supplementary Figure 1. Characterization of homemade anti–cross-linked Sp1 (CL-Sp1) and anti-TG2 polyclonal antibodies. (*A*) Sp1 (200 nmol/L) and guinea pig TG2 (5 nmol/L) were mixed and incubated for 48 hours in the absence (*lanes 1*, *3*, and *5*) or presence (*lanes 2*, *4*, and *6*) of 10 mmol/L Ca^{2+} in HEPES-buffered saline containing 100 mmol/L dithiothreitol and subjected to Western blot analysis using the antisera indicated. Monomeric Sp1 identified with an anti-Sp1 antibody (*lane 1*) is no longer evident after incubation of Sp1 with TG2 (*lane 2*). CL-Sp1 was not detected with this anti-Sp1 antibody (*lane 2*). A rabbit polyclonal antiserum was developed that specifically recognizes highly CL-Sp1. This CL-Sp1–specific antibody was isolated from the crude antiserum, the latter recognizing both monomeric and cross-linked Sp1 (*lanes 3* and *4*, respectively). After purification of the anti–CL-Sp1—specific fraction, the antiserum barely detected monomeric Sp1 monomer (*lane 5*) but readily detected CL-Sp1 (*lane 6*). Comparison of the findings shown in *lanes 2* and *6* indicates that the antigenic determinants recognized by the commercial anti-Sp1 antibody are masked by TG2-mediated cross-linking. Molecular weight markers (kD) are shown on the left. (*B*) Western blot of TG2 protein from guinea pig liver and whole cell lysates of hepatocytes, developed with polyclonal anti-TG2 antibody, mainly showing a strong band of TG2 (80 kD).



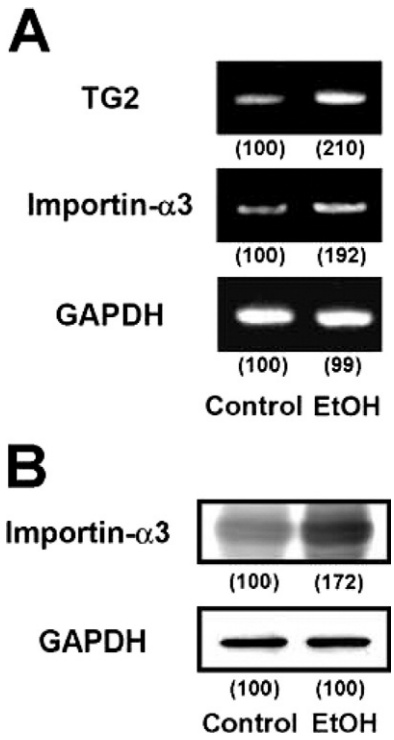
Supplementary Figure 2. Measurement of protein levels in TG2 and Sp1 siRNA stable cell lines. (*A*) TG2 siRNA stable cell lines were made by lentivirus infection as described in Supplementary Materials and Methods. Cell lysates were used 48 hours later for Western blot. The values in *parentheses* indicate TG2 protein levels. (*B*) Sp1 siRNA stable cell lines were also made by lentivirus infection as described in Supplementary Materials and Methods. Sp1-pCIneo was transfected using Lipofectamine (Invitrogen). Cell lysates were used 48 hours later for Western blot. The values in *parentheses* indicate Sp1 protein levels. Expression of lamin B1 is shown as a control for loading. (*C*) Western blot of nuclear extracts of $TG2^{+/+}$ and $TG2^{-/-}$ hepatocytes, developed with anti-Sp1 antibody, showing few changes of protein levels in the nucleus. Expression of lamin B1 is shown as a control for loading.



Supplementary Figure 3. Induction of apoptosis in ethanol-treated or TG2-overexpressing human hepatocyte cell lines. (*A*) Western blot of nuclear extracts of untreated or ethanol-treated (100 mmol/L for 18 hours) hepatocytes, developed with monoclonal anti-TG2 antibody, showing increased TG2 protein in the nucleus. Expression of lamin B1 is shown as a control for loading. (*B*) Hc cells were incubated for 48 hours in CS-C medium containing 1% serum, followed by treatment with either 0 or 200 mmol/L ethanol overnight in serum-free medium. Replicate samples of fixed cells were stained with anti-TG2 polyclonal antibody (*upper panels*), terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick-end labeling (*middle panels*), and Hoechst 33258 (*bottom panels*). *Scale bar* = 50 μm. (*C*) HepG2 cells were transfected with either control or a TG2-expressing vector. After 48 hours, cells were fixed and stained with anti-TG2 polyclonal antibody (*upper panels*), terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick-end labeling (*middle panels*), and Hoechst 33258 (*bottom panels*). *Scale bar* = 50 μm. Cells overexpressing TG2 fell into apoptosis.

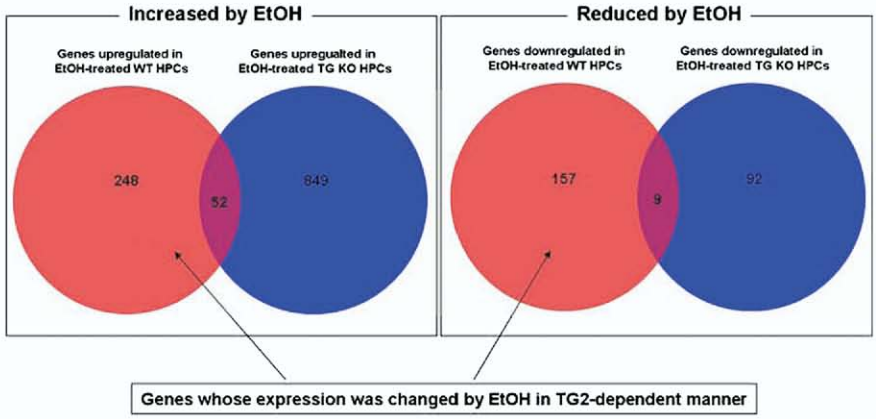


Supplementary Figure 4. Flow cytometric analysis and immunostaining of rat hepatocytes treated with acetaldehyde. (*A*) Rat hepatocytes were treated with 0–200 μmol/L acetaldehyde overnight. After staining using Annexin V/fluorescein isothiocyanate (*middle panels*), cells were fixed and stained with Hoechst 33258 (*left panels*) and anti-TG2 polyclonal antibody (*right panels*). The intensity of cell imaging was quantitated using a cell-based imaging system (Celaview RS-100). The percentages of apoptotic (Annexin V–positive) cells and nuclear TG2-positive cells are indicated below each micrograph. *Scale bar* = 50 μm. (*B*) HepG2 cells treated with 200 μmol/L acetaldehyde plus both 0.2 mmol/L 5-BAPA and 100 μmol/L aminoguanidine for 24 hours were fixed and stained with Hoechst 33258 (*left column*), anti-TG2 polyclonal antibody (*second column*), tetramethylrhodamine isothiocyanate–conjugate streptavidin (*third column*), or anti–CL-Sp1 antibody (*fourth column*). The results of merging the 4 images are shown in the *right most panel* (Merge). *Scale bar* = 50 μm.



Supplementary Figure 5. Increased expression of TG2 and importin-α in ethanol (EtOH)-treated hepatocytes. (*A*) HepG2 cells were treated with 0 or 200 mmol/L ethanol for 24 hours and expression of TG2, importin-α3, and glyceraldehyde-3-phosphate dehydrogenase messenger RNA determined by reverse-transcription polymerase chain reaction. Values in *parentheses* show densitometrically determined relative messenger RNA abundance. (*B*) HepG2 cells were treated with 0 or 200 mmol/L ethanol for 24 hours and cell lysates used for Western blot. Values in *parentheses* show densitometrically determined protein levels of importin-α3. Expression of glyceraldehyde-3-phosphate dehydrogenase is shown as a control for loading.

A



B **Receptor-related genes whose expression was consistently altered by at least 2.5-fold with EtOH treatment**

Genes up-regulated by EtOH treatment

Fold Change	Gene
70.39	Peroxisome proliferator activated receptor alpha
30.87	Ectonucleoside triphosphate diphosphohydrolase 2
6.70	Coiled-coil domain containing 21
6.16	Ankyrin repeat and KH domain containing 1
5.90	Sema domain, transmembrane domain (TM), and cytoplasmic domain, (semaphorin) 6B
5.43	EP300 interacting inhibitor of differentiation 2
5.10	G protein-coupled receptor 116
5.04	Protein tyrosine phosphatase, non-receptor type 6
4.75	Aldo-keto reductase family 1, member B3 (aldose reductase)
4.60	Nuclear receptor subfamily 1, group H, member 4
4.43	Protein tyrosine phosphatase, receptor type, D
3.98	Interleukin 11 receptor, alpha chain 1
3.86	Suppressor of cytokine signaling 3
3.75	Ring finger and SPRY domain containing 1
3.47	Neogenin
3.15	Progestin and adipoQ receptor family member VII
3.02	Attractin like 1
2.94	Nuclear receptor binding protein 2
2.80	Regulator of G-protein signaling 16
2.60	Suppressor of cytokine signaling 3
2.60	Neuropilin 1

Genes down-regulated by EtOH treatment

Fold Change	Gene
99.75	Integrin alpha 3
78.79	Low density lipoprotein receptor
28.45	Guanine nucleotide binding protein, alpha 12
27.03	Met proto-oncogene (Met)
17.84	Lysyl oxidase-like 2
10.85	3-monooxygenase/tryptophan 5-monooxygenase activation protein, gamma polypeptide
7.51	A disintegrin and metallopeptidase domain 10
6.43	Fibronectin 1
6.29	poliovirus receptor
5.93	Conserved helix-loop-helix ubiquitous kinase
5.32	Guanine nucleotide binding protein, alpha 12
4.67	Opioid receptor, sigma 1
4.00	Tumor necrosis factor receptor superfamily, member 22
3.91	Tumor necrosis factor receptor superfamily, member 23
3.77	Toll interacting protein
3.46	Transmembrane emp24 protein transport domain containing 5
3.45	Catenin (cadherin associated protein), beta 1
3.43	Mitogen activated protein kinase 14
3.38	Inhibitor of kappaB kinase beta
3.36	High mobility group box transcription factor 1
3.25	Protein tyrosine phosphatase, receptor type, F
3.11	Protein tyrosine phosphatase, non-receptor type 12
3.11	Activin A receptor, type 1
3.05	Src homology 2 domain-containing transforming protein C1
2.83	Nuclear receptor coactivator 4
2.82	Cardiotrophin-like cytokine factor 1 (Clcf1)
2.71	ADP-ribosylation factor 1
2.67	Transcribed locus
2.61	ERBB receptor feedback inhibitor 1
2.55	Thyroid hormone receptor associated protein 3
2.52	WAS protein family, member 2

C **Apoptosis-related genes whose expression was consistently altered by at least 2.5-fold with EtOH treatment**

Genes up-regulated by EtOH treatment

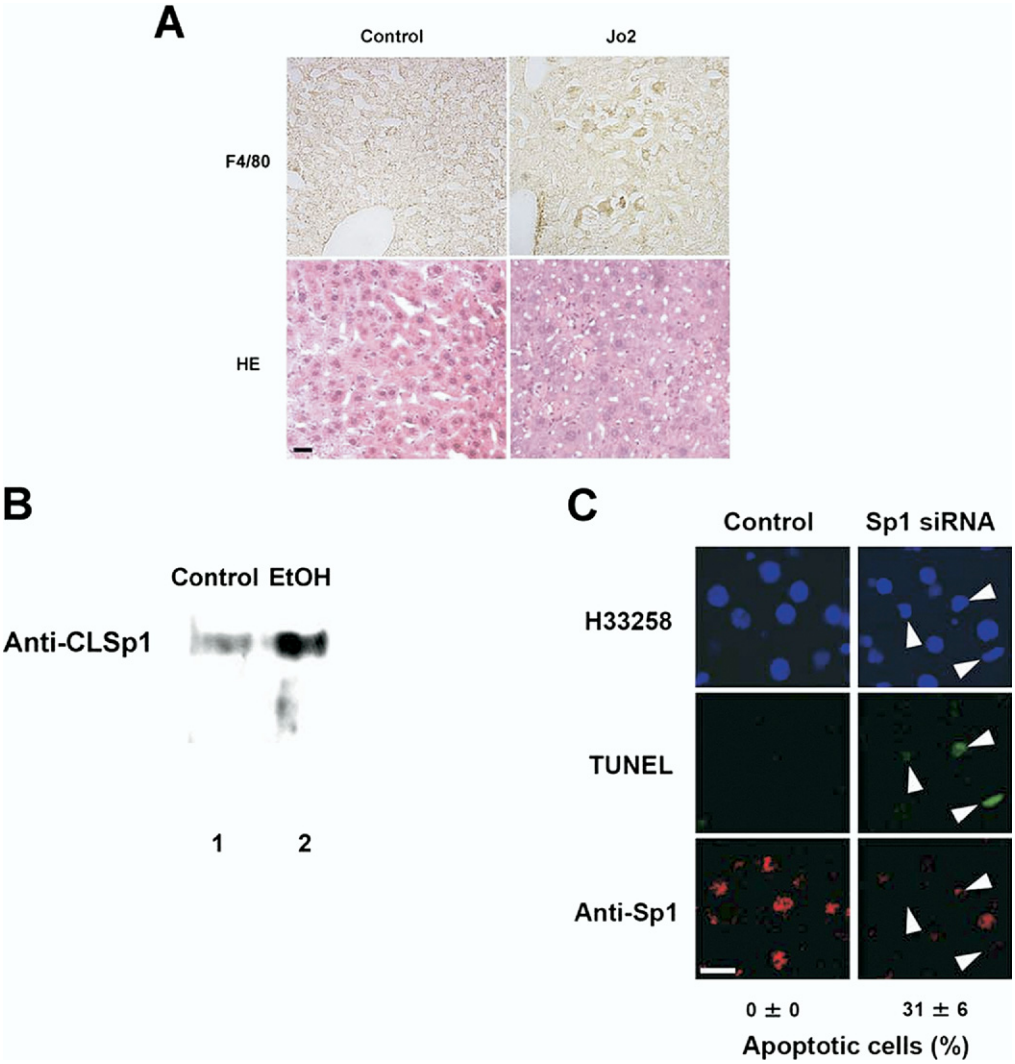
Fold Change	Gene
28.77	RNA binding motif and ELMO domain 1
17.27	Cell death-inducing DNA fragmentation factor, alpha subunit-like effector A
4.59	F-box protein 21
4.50	DNA-damage inducible transcript 3
4.05	Expressed sequence AA591059
3.61	MutL homolog 1 (E. coli)
3.20	THAP domain containing, apoptosis associated protein 3
2.82	Engulfment and cell motility 3, ced-12 homolog (C. elegans)

Genes down-regulated by EtOH treatment

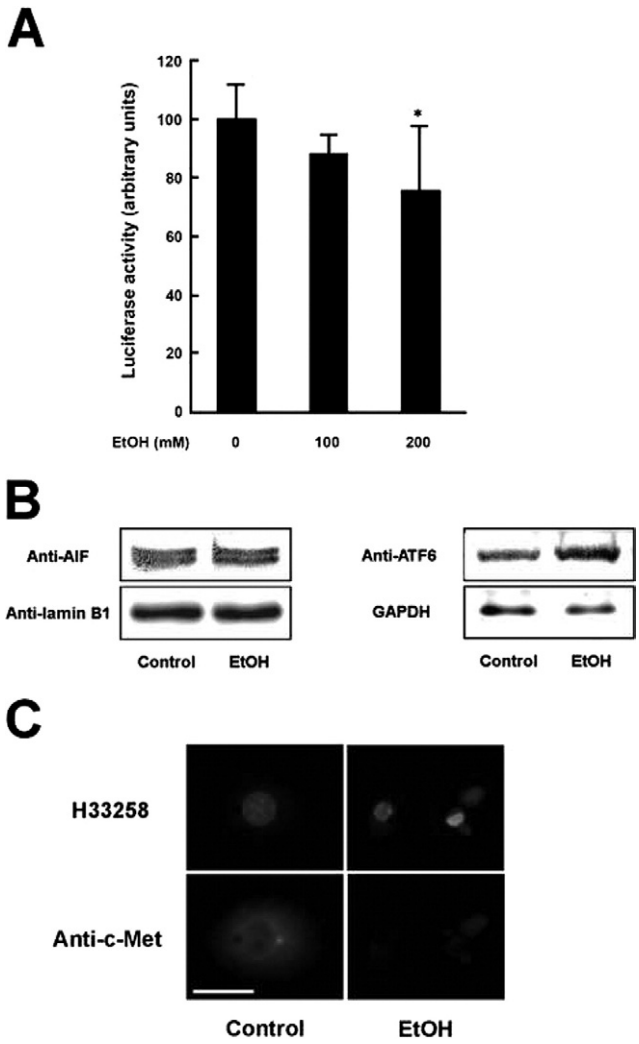
Fold Change	Gene
35.31	Programmed cell death 6 interacting protein
10.85	3-monooxygenase/tryptophan 5-monooxygenase activation protein, gamma polypeptide
8.50	Transformation related protein 53
6.07	Serine incorporator 3
5.93	Conserved helix-loop-helix ubiquitous kinase
5.66	Thymoma viral proto-oncogene 1
4.31	B-cell leukemia/lymphoma 10
4.00	Tumor necrosis factor receptor superfamily, member 22
3.91	Tumor necrosis factor receptor superfamily, member 23
3.62	Bcl2-like 1
3.47	BH3 interacting domain death agonist
3.38	Inhibitor of kappaB kinase beta
2.95	Transformation related protein 53
2.82	Cardiotrophin-like cytokine factor 1 (Clcf1), mRNA.
2.77	Mitogen activated protein kinase kinase 4
2.56	RAD21 homolog (S. pombe)

←

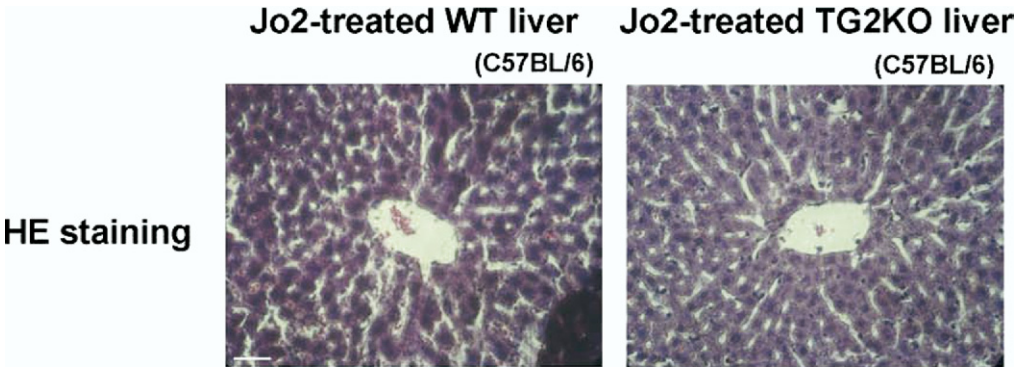
Supplementary Figure 6. Microarray analyses of genes whose expression was altered in mouse hepatocytes with ethanol treatment. After hepatocytes from $TG2^{+/+}$ or $TG2^{-/-}$ mice had been treated with 0 or 100 mmol/L ethanol for 15 hours, cell lysates were prepared, RNA was extracted, and changes in whole gene expression were analyzed using the Affymetrix Mouse Genome 430 2.0 Array. (*A*) Numbers of genes whose expression levels were up-regulated or down-regulated by at least 2.5-fold are presented. (*B*) Receptor-related genes whose expression was consistently altered by at least 2.5-fold with ethanol treatment are listed. (*C*) Apoptosis-related genes whose expression was consistently altered by at least 2.5-fold with ethanol treatment are listed. *B* and *C* list genes whose expression was down-regulated or up-regulated in a TG2-dependent manner after 100 mmol/L ethanol treatment of hepatocytes. The result showed that *c-Met* expression was selectively down-regulated in $TG2^{+/+}$ mice but not in $TG2^{-/-}$ mice.



Supplementary Figure 7. Infiltration of mononuclear cells following Jo2 administration, formation of CL-Sp1 in an alcohol steatohepatitis model, and hepatic apoptosis mimicked by Sp1 knockdown with siRNA. (*A*) Infiltration of mononuclear cells following Jo2 administration. Twenty-four hours after administration of Jo2 to wild-type mice, livers were sectioned, fixed, embedded in paraffin, and stained with F4/80 antibodies (1:50; BMA Biomedicals AG, Augst, Switzerland) and H&E (HE). *Scale bars* = 50 μm. The results suggest that leukocytes accumulated in Jo2-treated mouse liver are mostly mononuclear cells. (*B*) Formation of CL-Sp1 in alcohol steatohepatitis model. Nuclear extracts were prepared from livers of control (*lane 1*) and steatohepatitis (*lane 2*) mice, and the amounts of CL-Sp1 present in each sample were determined semiquantitatively by immunoblotting with anti-CL Sp1 antibody, as before. (*C*) Hepatic apoptosis mimicked by Sp1 knockdown with siRNA. Forty-eight hours after administration of control green fluorescent protein–expressing lentiviral vector or Sp1 siRNA-expressing lentiviral vector to $TG2^{+/+}$ mice, livers were harvested and fixed in Tissue-Mount frozen sectioning solution. Frozen sections were prepared and stained with Hoechst 33258, terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick-end labeling, or anti-Sp1. *Arrowheads* indicate terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick-end labeling –positive cells with diminished Sp1 expression and chromatin condensation. *Scale bar* = 50 μm.



Supplementary Figure 8. Transactivation and protein levels of several factors related to induction of apoptosis in ethanol-treated hepatocytes. (*A*) One day after transfection of HepG2 cells (culture in 35-mm dishes) with *p21* promoter, pp21-Luc reporter (a generous gift from Dr Friedman[7]; 0.75 μg/dish) cells were treated with 0–200 mmol/L ethanol in serum-free Dulbecco's modified Eagle medium for 24 hours. Cell lysates were prepared and luciferase activity was determined as described in Materials and Methods. Results shown are means ± SD (n = 3). $^{*}P < .05$. (*B*) Rat hepatocytes were treated without or with 100 mmol/L ethanol for 24 hours. Nuclear extracts (*left panels*) and whole cell lysates (*right panels*) were prepared and nuclear localization of apoptosis-inducing factor (AIF) as well as induction of activating transcription factor 6 (ATF6) were determined by Western blotting with anti-AIF antibodies (1:1000; Santa Cruz Biotechnology) or anti-ATF6 antibodies (1:200; Santa Cruz Biotechnology). Expressions of lamin B1 and glyceraldehyde-3-phosphate dehydrogenase are shown as respective controls for loading. (*C*) Rat hepatocytes were treated with 0 or 100 mmol/L ethanol for 24 hours and cells fixed, permeabilized, and stained with Hoechst 33258 and anti–c-Met antibody. *Scale bar* = 50 μm.



Supplementary Figure 9. Reproduction of the protective effect of TG2 silencing on hepatic injury caused by Jo2 in mice used by Sarang et al. Twenty-four hours after administration of Jo2 to $TG2^{+/+}$ and $TG2^{-/-}$ mice, livers were fixed, embedded in paraffin, and stained with H&E. *Scale bars* = 50 μm.

Supplementary Table 1. Primers for RT-PCR experiments

Gene	Sequence	Nucleotide no.
Human *TG2*		
Sense	AACCCCAAGTTCCTGAAG	597–614
Antisense	AGGTTGCTGTTCTGGTC	916–932
Human *importin-α3*		
Sense	TGGGCATTGGGAAATATCAT	685–704
Antisense	CAATGTTGCCCACAGCTCTA	1065–1084
Human *GAPDH*		
Sense	GCAGGGGGGAGCCAAAAGGG	395–414
Antisense	TGCCAGCCCCAGCGTCAAAG	942–961
Mouse *c-Met*		
Sense	TTGGTGCGGTCTCAATATCA	2803–2822
Antisense	GACCAGCTCTGGATTTAGAG	3164–3183
Mouse *GAPDH*		
Sense	AACTTTGGCATTGTGGAAGG	493–512
Antisense	ACACATTGGGGGTAGGAACA	696–715