STING-IRF3 pathway links endoplasmic reticulum stress with hepatocyte apoptosis in early alcoholic liver disease

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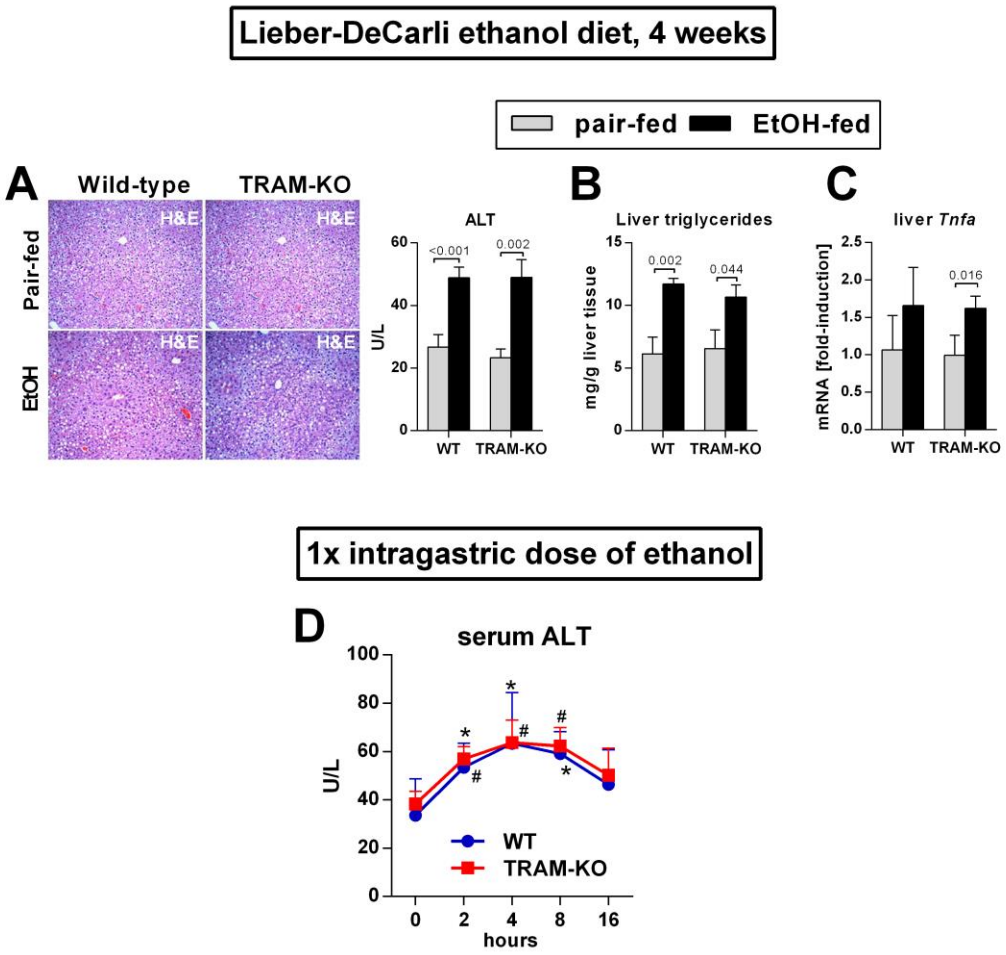
SI Appendix

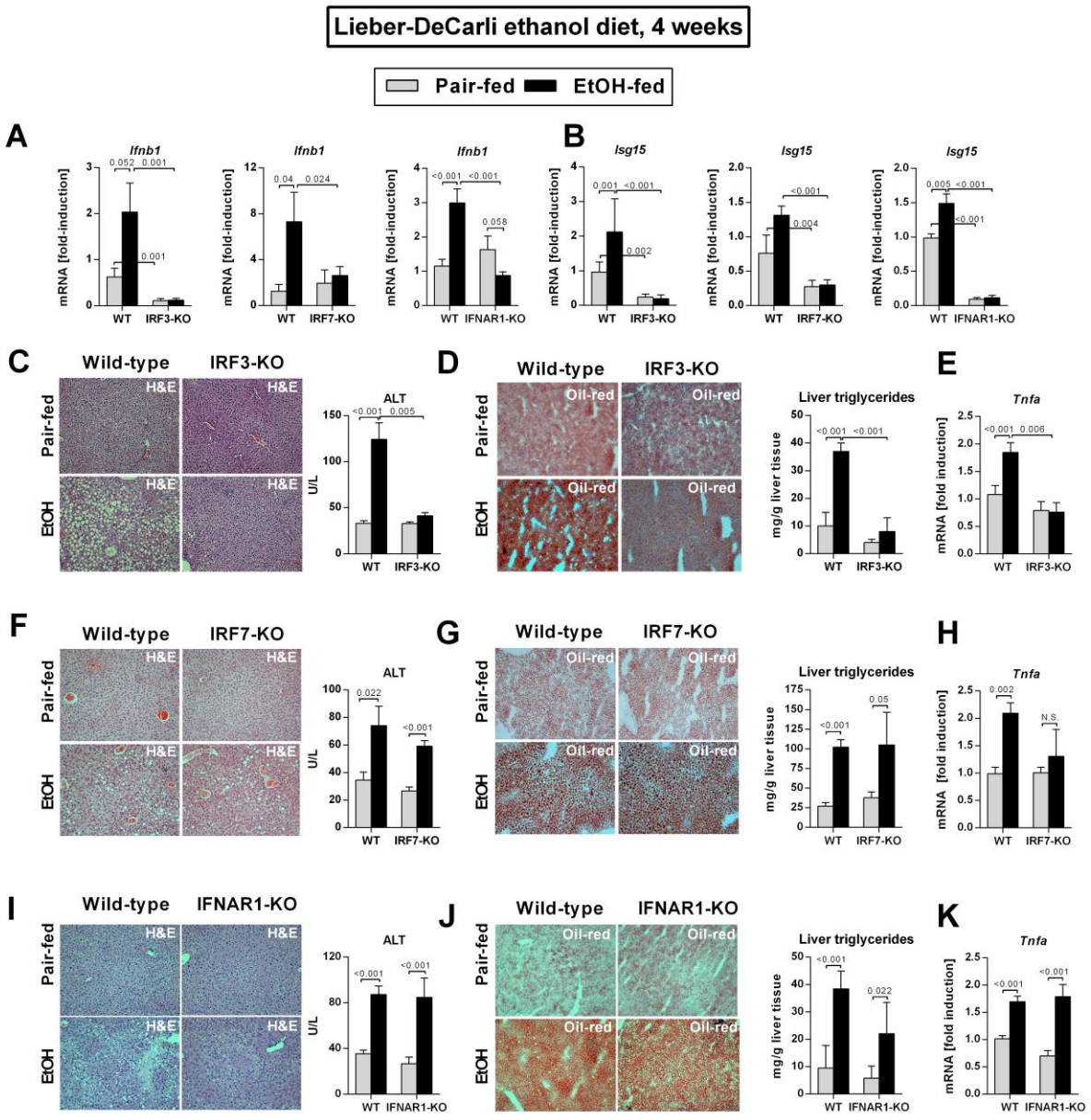
Supplementary figures

Supplementary methods



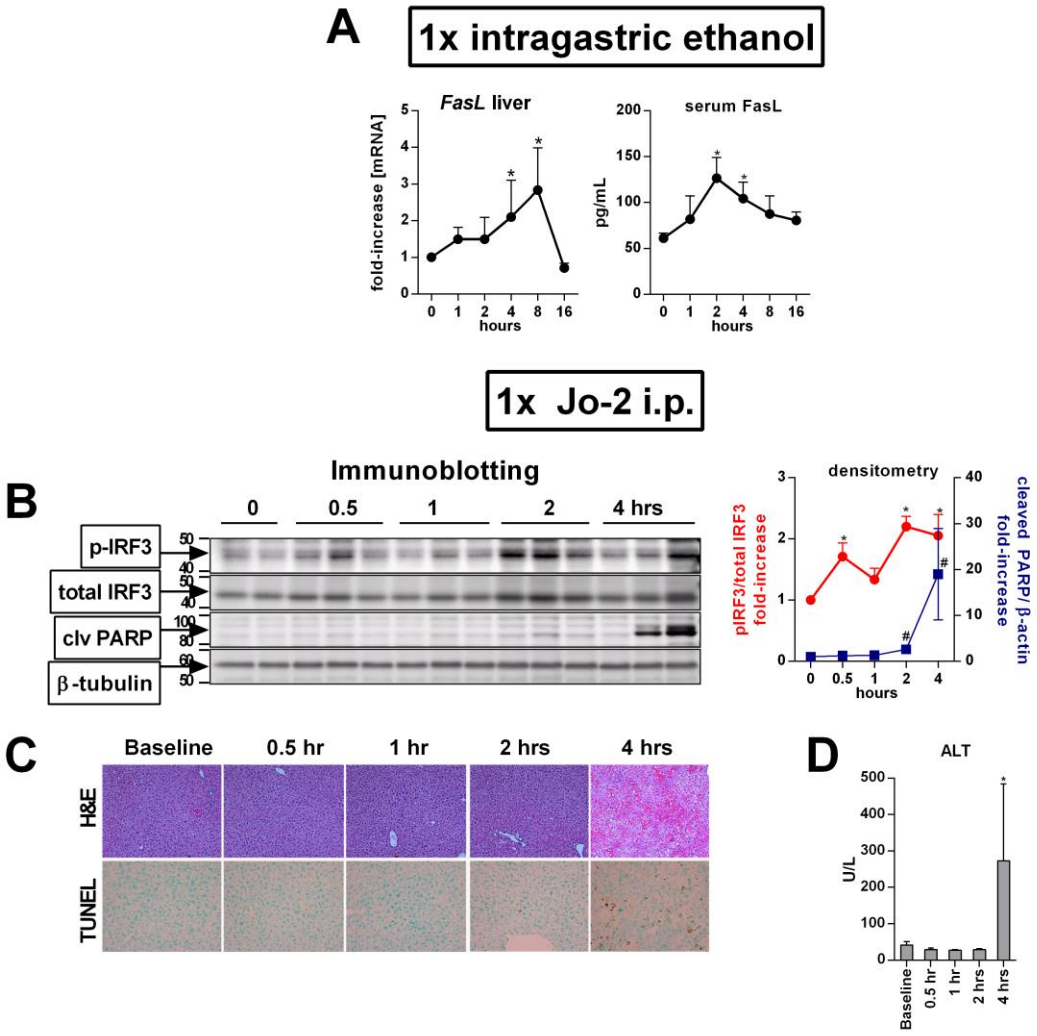
Supplementary Figure 1. *Deficiency of TRAM does not protect from ALD*

WT or TRAM-KO mice were fed control or alcohol diet and sacrificed 4 weeks later. Liver injury was assessed by H&E staining and serum ALT **(A)**. Steatosis was evaluated by measuring liver triglycerides **(B)**. Expression of *Tnfa* gene was evaluated by qPCR **(C)**. A single dose of ethanol was administered to WT or TRAM-KO mice, and liver injury was evaluated by serum ALT **(D)**. N=7 (pair-fed, per genotype), N=8 (alcohol-fed, per genotype) in (**A-C**); N=5 mice per genotype in **(D)**. Numbers in graphs denote *P* values. *,# $P < 0.05$ vs. baseline. Original magnification, x200.



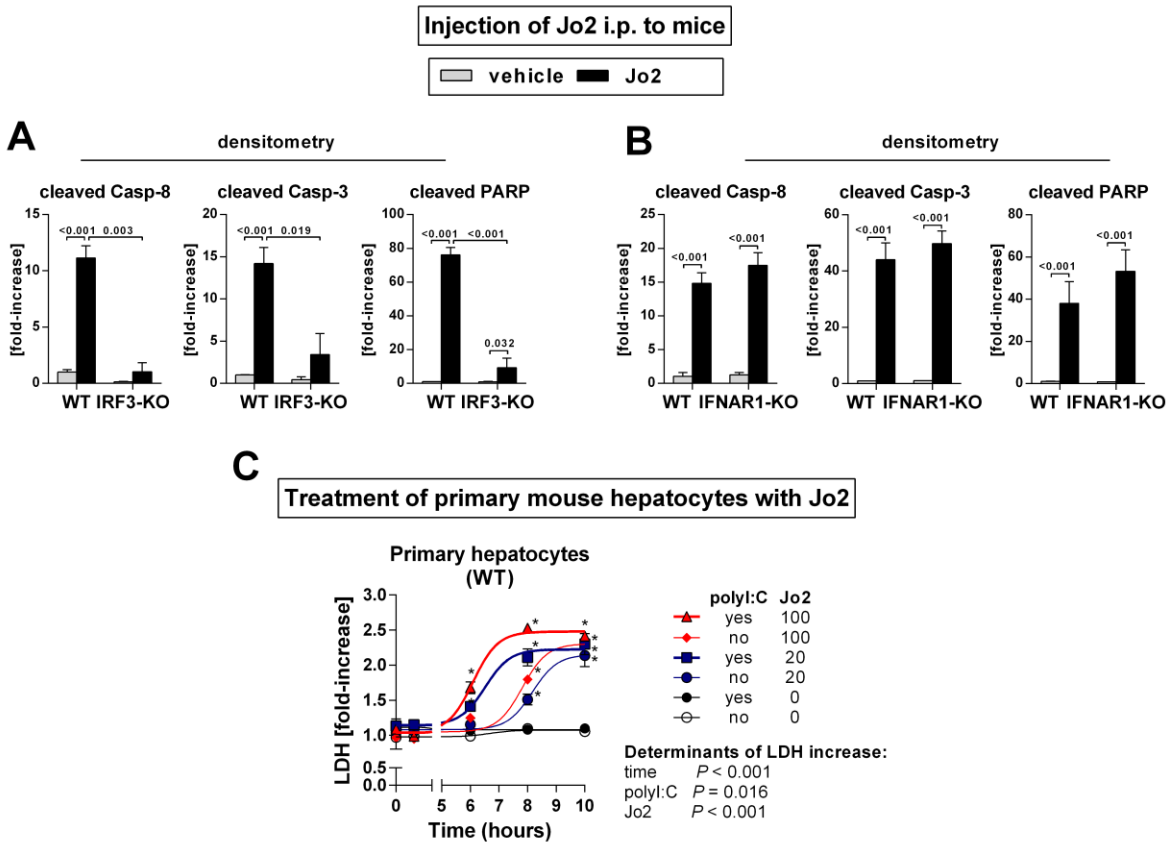
Supplementary Figure 2. *Deficiency of Type-I IFN signaling does not protect from ALD*

WT, IRF3-KO, IRF7-KO or IFNAR1-KO mice were fed control or alcohol diet and sacrificed 4 weeks later. Induction of Type-I IFNs was evaluated by measuring expression of *Ifnb* gene **(A)**, and *Isg15* gene expression was measured to evaluate Type-I IFN signaling **(B)**. Liver injury was assessed by H&E staining and serum ALT **(C,F,I)**, steatosis was evaluated by Oil-red-O staining and liver triglycerides **(D,G,J)**. Expression of *Tnfa* gene was evaluated by qPCR **(E,H,K)**. N=6 (pair-fed, per genotype) and 7-10 (ethanol-fed, per genotype) in **(C-E)**; 5 per group in **(F-H)**; 8 (pair-fed, per genotype) and 7-15 (ethanol-fed, per genotype) in **(I-K)**. Numbers in graphs denote *P* values. Original magnification, x200.



Supplementary Figure 3. *Phosphorylation of IRF3 precedes liver injury induced by Fas ligand*

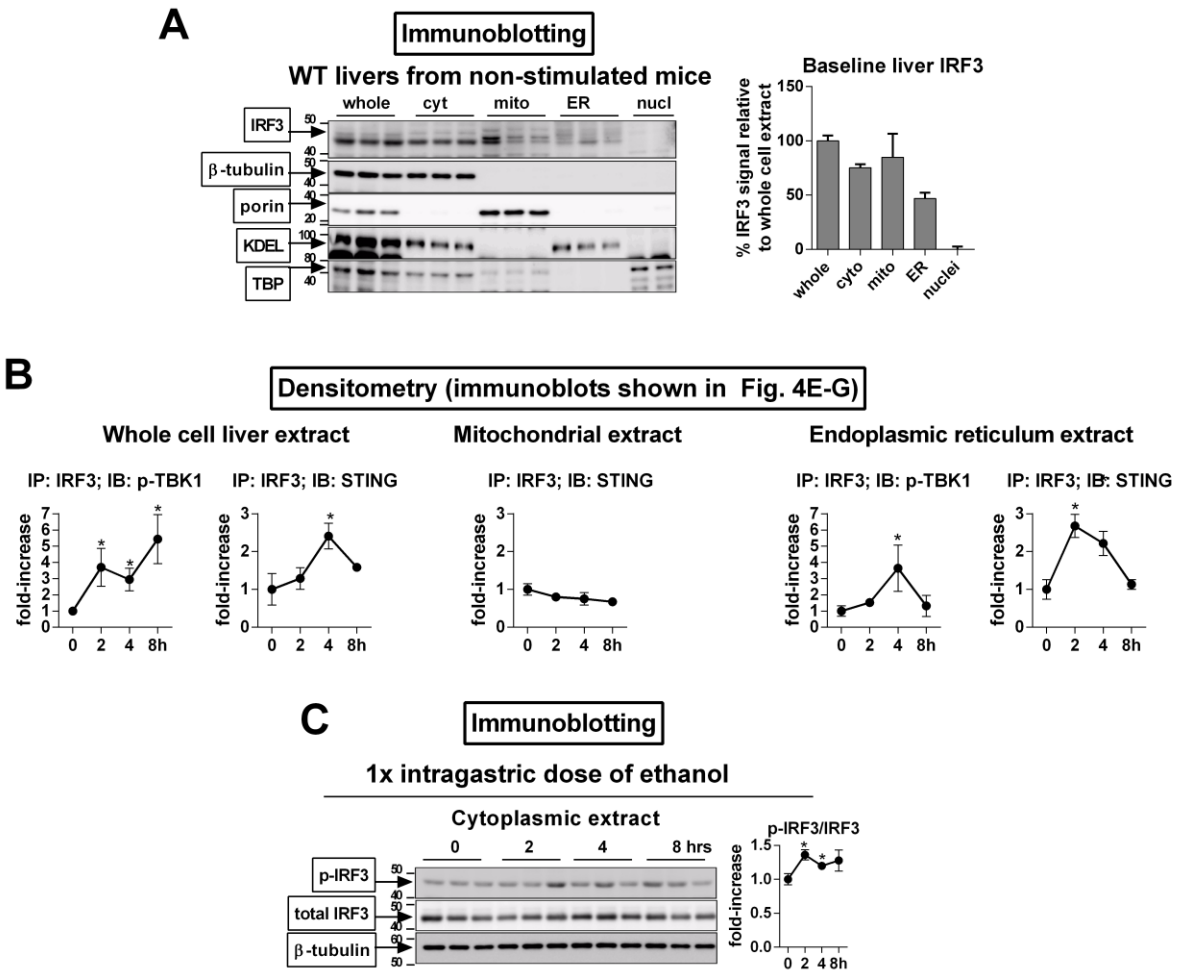
WT mice received a single intragastric dose of ethanol. At indicated time points, the expression of *FasL* gene was measured in the liver by qPCR **(A)**, and concentration of FasL protein was measured in the serum by specific ELISA **(B)**. WT mice received i.p. injection of Jo2. Phosphorylation of IRF3 was evaluated by immunoblotting **(B)**, and apoptosis in the liver was evaluated by measuring cleaved PARP **(B)** and TUNEL staining **(C)**. Liver injury was evaluated by H&E staining **(C)** and serum ALT **(D)**. N=5 mice per time point in **(A)**; 3 per time point in **(B-D).** **P* < 0.05 vs. baseline. Original magnification, x200.



Supplementary Figure 4. *IRF3 is required for apoptosis of hepatocytes*

(A,B) WT, IRF3-KO or IFNAR1-KO mice received a single i.p. dose of Jo2 (0.5 mg/kg) or saline and were sacrificed after 8 hours**.** Apoptosis was evaluated by cleavage of PARP, Casp- 3 and Casp-8 in the liver. Densitometry data presented. Original immunoblots are shown in main Figure 3D, I.

(C) Primary hepatocytes were isolated from WT mice and *ex vivo* treated with indicated doses of Jo2 for 10 hours. Some hepatocytes were pretreated for 30 minutes with lipofectamin plus polyI:C (10 μg/mL), which induces phosphorylation of IRF3 via RIG-I (Yamamoto M, et al Science 2003:301(5633): 640-643) . Hepatocyte death was evaluated by measuring the levels of LDH in cell culture supernatant. Stimulations were performed in triplicates. Regression analysis was used to calculate P values for the determinants of LDH increase**.** Numbers in the graphs indicate *P* values. **P* < 0.05 vs. baseline.



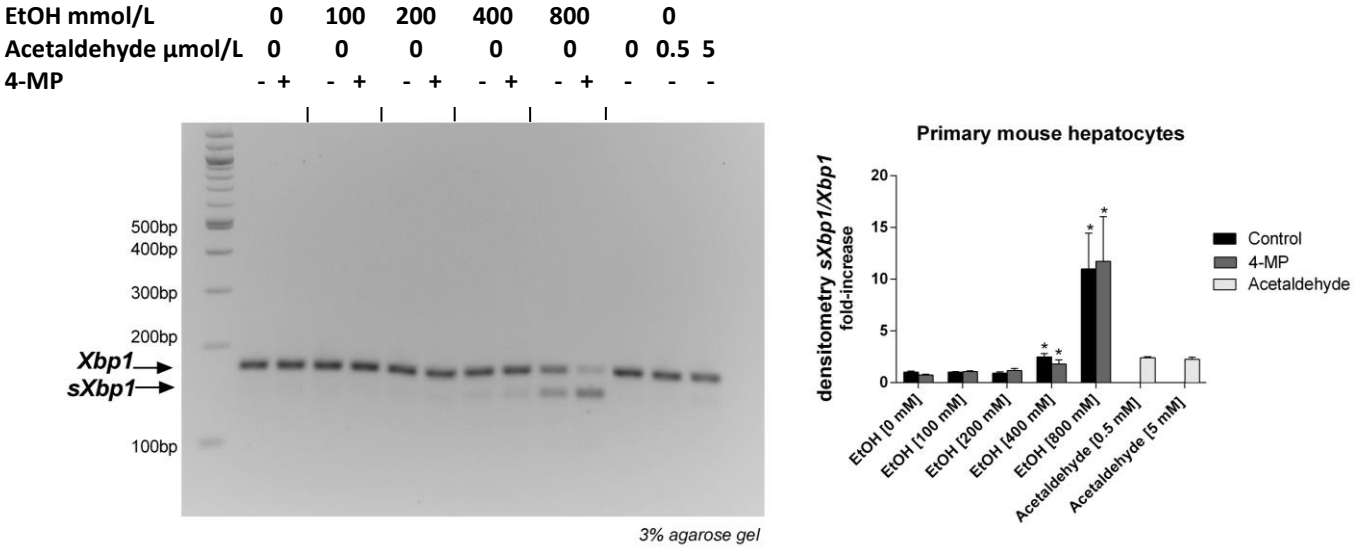
Supplementary Figure 5. *IRF3 associates with the endoplasmic reticulum adaptor STING in the liver.*

(A) Whole-cell, cytoplasmic, mitochondrial, endoplasmic reticulum (ER) and nuclear extracts were isolated from the livers of WT non-stimulated mice and analyzed by immunoblotting for the presence of IRF3. We used β-tubulin as loading control for cytoplasm, porin for mitochondria, KDEL for ER and TATA-binding protein (TBP) for nuclear extract.

(B) WT mice received a single dose of ethanol and livers were harvested at indicated time points. We used immunoprecipitation with anti-total IRF3 antibody for protein pull-down to evaluate association between total IRF3 and STING or phosphorylated TBK1 in the whole-cell, mitochondrial and ER extracts. Original immunoblots are shown in main Figure 4E-G.

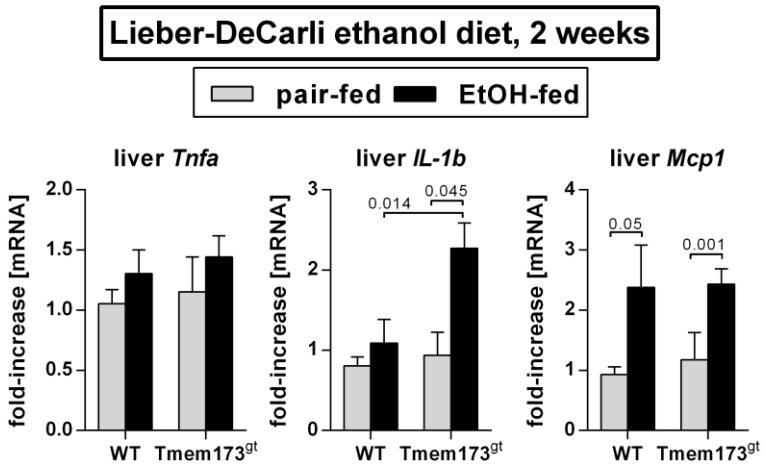
(C) WT mice received a single dose of ethanol and livers were harvested at indicated time points. Phosphorylation of IRF3 was assessed by immunoblotting in liver cytoplasmic extracts. This data is complementary to data shown in Figure 4A-D.

N=3 mice per time point. *P < 0.05 vs. baseline.



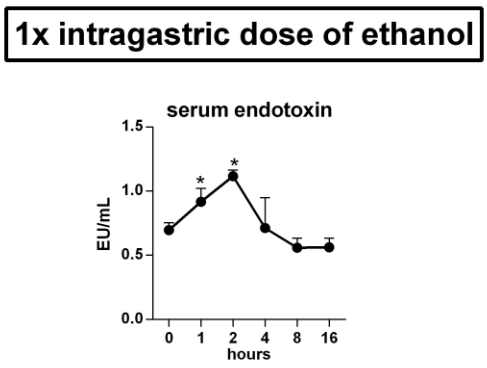
Supplementary Figure 6. *Ethanol induces ER stress in primary mouse hepatocytes independently of acetaldehyde*

Primary hepatocytes isolated from WT mice were *ex vivo* treated with indicated concentrations of ethanol or acetaldehyde and harvested after 4 hours. Some hepatocytes were concurrently treated with 4-methylpyrazole (4-MP, 3 mM), an inhibitor of alcohol dehydrogenase. After 2 hours, RNA was extracted and ER stress total spliced form of Xbp1 (*sXbp1*) was analyzed to evaluate ER stress. Stimulations were performed in triplicates and the experiment was repeated twice. *$P < 0.05$ vs. baseline.



Supplementary Figure 7. *Expression of inflammatory cytokines in the livers of alcohol-fed WT and Tmem173^{gt} mice*

WT or Tmem173^{gt} mice were fed control or alcohol diet. After 2 weeks expression of inflammatory cytokine genes *Tnfa*, *Il-1b* and *Mcp1* was evaluated by qPCR. N=10-15 (ethanol-fed, per genotype); 3-6 (pair-fed, per genotype). Numbers in the graphs indicate *P* values.



Supplementary Figure 8. *Increased levels of serum endotoxin in mice treated with a single dose of ethanol*

WT mice received a single dose of ethanol. At indicated time points, levels of endotoxin in the serum were analyzed using colorimetric assay. N=5 per time point. *$P < 0.05$ vs. baseline.

Supplementary methods

Animal studies. 6- to 8-week-old female C57Bl/6 WT (Jackson Laboratory, Bar Harbor, ME), IRF3-deficient (IRF3-KO) or type I IFN α/β receptor 1-(IFNAR1)-KO mice (provided by J. Sprent, Scripps Research Institute, La Jolla, CA, USA), Casp-1-KO (gift of A. Hise, Case Western Reserve University, Cleveland, Ohio, USA) and IRF7-KO mice (provided by T. Taniguchi, Tokyo, Japan), all on C57Bl/6 background, were used. The STING-deficient Tmem173^{gt} (goldenticket) mice (provided by R. Vance, University of California, Berkeley, CA, USA, (5)) and the TRAM-KO mice (provided by S. Akira, Osaka University, Japan) were on the B6.129sf2 background; we used B6.129sf2 WT mice (Jackson Laboratory) as controls for these two strains. Some animals were fed with the Lieber-DeCarli ad libitum diet (chronic ALD) or received intragastric ethanol (5g/kg body weight, acute model), as described in (3). *In vivo treatment of mice.* Some animals received a single dose of 25 mg/kg recombinant IL-1 receptor antagonist (IL-1Ra, Anakinra; Amgen) as described (3). Anti-CD95 antibody, clone: Jo2, 0.5 mg/kg (BD biosciences, San Jose, CA, USA), or saline was administered as a single i.p. injection. All animals received proper care in agreement with animal protocols approved by the Institutional Animal Use and Care Committee of the University of Massachusetts Medical School.

Kupffer-cell depletion protocol. WT mice were injected with 200 µL liposomal clodronate or liposomal PBS (purchased from N. van Rooijen, Free University Amsterdam, Amsterdam, the Netherlands) via tail vein injection. 48 hours later, mice were sacrificed to evaluate KC depletion using F4/80 staining (CI:A3-1, AbD Serotec, Raleigh, NC, USA), or administered a single intragastric dose of ethanol.

Biochemical assays. Serum ALT was determined using a kinetic method (D-Tek LLC., Bensalem, PA). Colorimetric assays were used to measure liver triglycerides (Wako Chemicals, VA, USA) and the LDH activity in cell culture supernatants (Abcam, Cambridge, MA, USA).

Cytokine measurement. Levels of TNF-α were measured using specific anti-mouse ELISA from Biolegend (San Diego, CA, USA). IFN-β was measured using specific anti-mouse ELISA from PBL (Piscataway, NJ, USA) and cytochrome *c* and serum FasL was measured by ELISA kit from R&D (R&D systems, Inc., Minneapolis, MN).

Protein quantification. Liver whole-cell lysates and nuclear preparations were extracted as described previously (1,2). Mitochondrial and total ER fractions were prepared using specific extraction kits from Imgenex (San Diego, CA) as per manufacturer's protocol. Immunoblotting was performed as described in (4). Antibodies specific for phospho-IRF3, phospho-TBK1, STING, Casp-8, Casp-3, cleaved Casp-8, cleaved Casp-3, PARP and Bax were from Cell Signaling (Danvers, MA, USA). Antibodies against the total IRF3, GRP78 and KDEL, and control IgG were from Santa Cruz (Santa Cruz Biotechnology, Santa Cruz, CA). Beta-actin, beta-tubulin, porin and TATA-binding protein (TBP) antibodies were from Abcam (Cambridge,

MA, USA). The TrueBlot system from eBioscience (San Diego, CA, USA) was used for immunoprecipitation assays and performed as per manufacturer's instructions.

RNA Analysis. RNA analysis and PCR primers are described in Supplementary Table 1. s*Xbp1* PCR was performed as described previously (3) and products were separated on 3% agarose gel.

Histopathological analysis. Liver sections were stained with H&E or Oil-red-O and analyzed by microscopy, as we previously described (4). Immunohistochemistry staining for F4/80 (CI:A3-1, AbD Serotec, Raleigh, NC, USA) or TUNEL staining (Roche, Indianapolis, IN, USA), were performed in formalin-fixed, paraffin-embedded livers. ImageJ (NIH) was used for image analysis.

Isolation of primary mouse hepatocytes and liver mononuclear cells was performed as described previously in (1).

In vitro experiments. Primary hepatocytes were cultured in Waymouth's medium supplemented with 10% fetal bovine serum and 1% insulin, transferrin, selenium solution. Primary hepatocytes were seeded in 6-well collagen-coated plates (Biocoat, Becton Dickinson, Bedford, MA). Before starting stimulation experiments, hepatocytes were rested for 4 hours. Subsequently culture media was replaced, and stimulation was performed as indicated in the figure legends. The Jo2 antibody was purchased from BD biosciences (San Jose, CA, USA), BX795 (6), polyI:C and lipofectamine from Invitrogen (Life Technologies Corp., Grand Island, NY, USA), and thapsigargin (1μM) from Sigma (St. Louis, MO, USA).

Statistical Analysis. Statistical significance was determined using two-sided t-test; ANOVA and Dunnett's multiple comparison post-test were used to compare the means of multiple groups. Two-way ANOVA was used in Figures 1C and 5F to determine the global effect of genotype on serum ALT. Regression analysis with calculation of standardized coefficients beta (influence statistics) was used in Figures 1A and 1B to determine the extent by which deficiencies of investigated genes protected from alcohol-induced increase in TNF-α or ALT. Data are shown as mean ± SEM and were considered statistically significant at $P < 0.05$. We used SPSS 19.0 (IBM SPSS, Chicago, IL, USA) and GraphPad Prism 5.00 (San Diego, CA, USA) for calculations.

References (supplementary methods)

1. Hritz I, *et al* (2008) The critical role of toll-like receptor (TLR) 4 in alcoholic liver disease is independent of the common TLR adapter MyD88. *Hepatology* 48(4): 1224-1231.
2. Nath B, *et al* (2011) Hepatocyte-specific hypoxia-inducible factor-1alpha is a determinant of lipid accumulation and liver injury in alcohol-induced steatosis in mice. *Hepatology* 53(5): 1526-1537.

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4. Iwakoshi NN, *et al* (2003) Plasma cell differentiation and the unfolded protein response intersect at the transcription factor XBP-1. *Nat Immunol* 4(4): 321-329.
5. Sauer JD, *et al* (2011) The N-ethyl-N-nitrosourea-induced goldenticket mouse mutant reveals an essential function of sting in the in vivo interferon response to listeria monocytogenes and cyclic dinucleotides. *Infect Immun* 79(2): 688-694.
6. McIver EG, *et al* (2012) Synthesis and structure-activity relationships of a novel series of pyrimidines as potent inhibitors of TBK1/IKKε kinases. *Bioorg Med Chem Lett.* (23):7169-7173.

Supplementary table

qPCR primers

Target gene	**Forward primer (5'→ 3')**	**Reverse primer (5'→ 3')**
18S	gta acc cgt tga acc cca tt	cca tcc aat cgg tag tag cg
Tnfa	cac cac cat caa gga ctc aa	agg caa cct gac cac tct cc
Ifnb	agc tcc aag aaa gga cga aca t	gcc ctg tag gtg agg gtt gat ct
Isg15	cag gac ggt ctt acc ctt tcc	agg ctc gct gca gtt ctg tac
Xbp1	aca cgc ttg gga atg gac ac	cca tgg gaa gat gtt ctg gg
Irf3	ctg agg ggt ttc tga cgg ac	aac cac aga gtg tag cgt gg

Quantitative polymerase chain reaction (qPCR) was performed using the iCycler (Bio-Rad Laboratories, Hercules, CA). The PCR conditions were: 95°C for 15 minutes followed by 40 cycles at 95°C for 15 seconds, 60°C for 10 seconds, and 72°C for 30 seconds. The reaction mixture for the SYBR Green assay contained 12.5 μL SYBR Green PCR Master Mix (Bio-Rad, Hercules, CA, USA), 0.5 μM of forward and reverse primer and 1 μL of cDNA (corresponding to 100 ng RNA) for a total volume of 25 μL. All amplifications and detections were carried out in a MicroAmp optical 96-well reaction plate with optical tape. At each cycle, accumulation of PCR products was detected by monitoring the increase in fluorescence by double-stranded DNA-binding SYBR Green. After PCR, a dissociation melting curve was constructed in the range of 55°C to 95°C. All data were analyzed using Bio-Rad iCycler software. The 18S was used for normalization of all experiments. Data was analyzed using the comparative Ct method (ΔΔCt method) using the following formula: ΔCt = Ct (target) - Ct (normalizer). The comparative ΔΔCt calculation involved finding the difference between the sample ΔCt and the baseline ΔCt. Fold increase in the expression of specific mRNA compared with 18S was calculated as $2^{-(\Delta\Delta Ct)}$.