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# Hepatocyte-specific deletion of XBP1 sensitizes mice to liver injury through hyperactivation of IRE1 $\alpha$

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## Abstract

X-box binding protein-1 (XBP1) is a transcription factor that plays a central role in controlling cellular responses to endoplasmic reticulum (ER) stress. Under stress conditions, the transcriptionally active form of XBP1 is generated via splicing of *Xbp1* mRNA by the ER-resident protein inositol-requiring enzyme-1 (IRE1 $\alpha$ ). Genetic deletion of XBP1 has multiple consequences: some resulting from the loss of the transcription factor per se, and others related to compensatory activation of IRE1 $\alpha$ . The objective of the current study was to investigate the effects of XBP1 deletion in adult mouse liver and determine to what extent they are direct or indirect. XBP1 was deleted from hepatocytes in adult *Xbp1*<sup>f/f</sup> mice using AAV8-Transthyretin-Cre (*Xbp1* <sup>$\Delta$ hep</sup>). *Xbp1* <sup>$\Delta$ hep</sup> mice exhibited no liver disease at baseline, but developed acute biochemical and histologic liver injury in response to a dietary challenge with fructose for 4 weeks. Fructose-mediated liver injury in *Xbp1* <sup>$\Delta$ hep</sup> mice coincided with heightened IRE1 $\alpha$  activity, as demonstrated by *Xbp1* mRNA splicing, JNK activation, and regulated IRE1 $\alpha$ -dependent RNA decay (RIDD). Activation of eIF2 $\alpha$  was also evident, with associated up-regulation of the pro-apoptotic molecules CHOP, BIM, and PUMA. To determine whether the adverse consequences of liver-specific XBP1 deletion were due to XBP1 loss or heightened IRE1 $\alpha$  activity, we repeated a fructose challenge in mice with liver-specific deletion of both XBP1 and IRE1 $\alpha$  (*Xbp1* <sup>$\Delta$ hep</sup>;*Ire1a* <sup>$\Delta$ hep</sup>). *Xbp1* <sup>$\Delta$ hep</sup>;*Ire1a* <sup>$\Delta$ hep</sup> mice were protected from fructose-mediated liver injury and failed to exhibit any of the signs of ER stress seen in mice lacking XBP1 alone. The protective effect of IRE1 $\alpha$  deletion persisted even with long-term exposure to fructose. *Xbp1* <sup>$\Delta$ hep</sup> mice developed liver fibrosis at 16 weeks, but *Xbp1* <sup>$\Delta$ hep</sup>;*Ire1a* <sup>$\Delta$ hep</sup> mice did not. Overall, the results indicate that the deleterious effects of hepatocyte-specific XBP1 deletion are due primarily to hyperactivation of IRE1 $\alpha$ . They support further exploration of IRE1 $\alpha$  as a contributor to acute and chronic liver diseases.

## Introduction

X-box binding protein-1 (XBP1) is an important component of the signal transduction network that protects cells against

ER stress. XBP1 is positioned downstream of IRE1 $\alpha$  (inositol-requiring enzyme-1), one of three canonical ER stress sensors (IRE1 $\alpha$ , ATF6, PERK) residing in the ER membrane. IRE1 $\alpha$  has kinase and endoribonuclease activities that are unleashed under conditions of ER stress. When IRE1 $\alpha$  is activated, its endoribonuclease acts upon *Xbp1* mRNA by splicing a 26-nucleotide fragment that enables translation of a protein termed XBP1s. XBP1s is a transcription factor that induces genes involved in chaperoning proteins through the ER and degrading proteins that cannot

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be properly folded in the ER [1]. XBP1s also induces genes pertinent to phospholipid synthesis, which enhance ER membrane biogenesis and increase the capacity of the organelle [2, 3].

Cell-specific deletion of XBP1 often results in adverse consequences. Deletion of XBP1 from lymphoid precursors prevents the maturation of B cells into plasma cells [4]; deletion from intestinal epithelia predisposes to inflammatory bowel disease [5]; and deletion from CNS neurons promotes leptin resistance and obesity [6]. In these situations, the targeted loss of XBP1 causes enhanced ER stress in the affected cells, which can lead to cell death and associated inflammation. Targeted disruption of XBP1, however, can in some cases lead to mixed positive and negative outcomes. This is true of the liver, in which XBP1 deletion from hepatocytes improves hepatic insulin sensitivity [7] and reduces the hepatic contribution to circulating lipids [8] but sensitizes the liver to pharmacologic ER stress [9] and impairs liver regeneration [10].

One important consequence of XBP1 deletion is hyperactivation of IRE1 $\alpha$  [8]. Hyperactivation of IRE1 $\alpha$  leads to significant broadening of its endoribonuclease activity, which in turn prompts large-scale degradation of mRNAs in a process called regulated IRE1 $\alpha$ -dependent decay (RIDD) [11]. IRE1 $\alpha$  hyperactivation also accentuates its kinase activity toward TRAF2, initiating a cascade of events culminating in the activation of JNK and downstream targets such as cJun [12]. Both of these events can trigger cell death. The goal of the current study was to examine the impact of hepatocyte-specific XBP1 deletion in the adult mouse liver, under basal conditions and in response to a mild metabolic stress (fructose feeding). We wished to gain insight into metabolic outcomes as well as cell survival, and to dissect whether the phenotype of XBP1-deficient mice was due primarily to XBP1 loss or to up-regulation of other ER stress pathways.

## Methods

### Mice and experimental diets

XBP1 conditional knockout mice on a C57BL/6 background (*Xbp1*<sup>fl/fl</sup>) were obtained from Drs. Ann-Hwee Lee and Laurie Glimcher [8]. IRE1 $\alpha$  conditional knockout mice (*Ire1a*<sup>fl/fl</sup>) were generated as previously described [13] and back-crossed for 10 generations to C57BL/6. The two strains were cross-bred to generate *Xbp1*<sup>fl/fl</sup>;*Ire1a*<sup>fl/fl</sup> conditional knockout mice. At 8 weeks of age, male *Xbp1*<sup>fl/fl</sup> or *Xbp1*<sup>fl/fl</sup>;*Ire1a*<sup>fl/fl</sup> mice were injected IV with either  $4 \times 10^{11}$  GC AAV8-Transthyretin-Cre or  $4 \times 10^{11}$  GC AAV8-CMV-null as a control (Vector Biolabs, Malvern, PA).

Gene-deleted mice are designated *Xbp1*<sup>Δhep</sup> and *Xbp1*<sup>Δhep</sup>;*Ire1a*<sup>Δhep</sup>. Animals were housed for 2 weeks after AAV8 treatment before initiating experimental studies. At 10 weeks of age, mice were placed on either a chow diet (Pico Lab Diets #5053) or a fructose-enriched diet (Envigo TD.89247) for intervals up to 16 weeks. At the end of each experiment, mice were fasted for 4 h before killing. All diet studies contained 4 mice per group; some were repeated for a total of 8 per group. No formal randomization protocol was applied, and investigators were not blinded to the treatment groups. Positive controls for ER stress were generated by injecting adult C57BL/6 mice with tunicamycin (1 mg/kg) and killing them 3 h later. All mouse experiments were performed in accordance with guidelines set by the America Veterinary Medical Association. All mouse studies were reviewed and approved by the Committee on Animal Research at the University of California San Francisco.

### Gene expression

RNA was extracted from whole liver in TRIzol (Invitrogen, Carlsbad, CA). RNA was then purified using a Direct-zol RNA Miniprep kit (ZymoResearch, Irvine, CA) and cDNA synthesized as previously described [14]. Gene expression was assessed by quantitative PCR using PrimeTime qPCR assays (Integrated DNA Technologies, Coralville, IA), E@sy Oligo primers (Millipore-Sigma, Burlington, MA) or TaqMan Assays (Life Technology, Carlsbad, CA), followed by normalization to mouse  $\beta$ -glucuronidase. A complete list of primers is cataloged in Table S1. *Xbp1* mRNA splicing was quantitated by measuring spliced and total *Xbp1* mRNA independently in tissue homogenates by QPCR and expressing the ratio as a percentage, as described previously [8, 15].

### Histology and immunohistochemistry

Formalin-fixed sections of liver tissue were stained with hematoxylin and eosin. Cell death and proliferation were evaluated by immunohistochemical staining for cleaved caspase-3 and Ki-67. CD68 and myeloperoxidase staining were used to assess the abundance of inflammatory cells in the liver. Stained tissue sections were photographed using a Nikon Microphot microscope (Nikon, Melville, NY) equipped with a SPOT digital camera (Diagnostic Instruments, Inc., Sterling Heights, MI). Caspase-3- and Ki-67-positive cells were counted manually in 10 microscopic fields per liver, and data were reported as the average number of cells per microscopic field. CD68 and myeloperoxidase staining were quantitated as the mean % stained area in 10 microscopic fields per liver (Simple PCI, Hamamatsu Corporation, Sewickly, PA).

## Quantitation of hepatic lipids

Lipids were extracted from fresh liver tissue using the Folch method [16]. Total triglyceride was measured spectrophotometrically as previously described (TR0100; Millipore-Sigma) [17].

## Quantitation of hepatic fibrosis

Hepatic fibrosis was assessed morphometrically in Sirius Red-stained tissue sections using LAS X (Leica Microsystems, Wetzlar, Germany). The fibrosis area (%) for each liver was assessed as the mean measurement of 6 microscopic fields. Fibrosis was also quantitated by measuring the amount of hydroxyproline in tissue homogenates [18]. Values are reported as mg hydroxyproline/g liver.

## Serum tests

Alanine aminotransferase (ALT), total cholesterol, and total triglycerides were measured in mouse serum using an ADVIA 1800 autoanalyzer (Siemens Healthcare Diagnostics, Deerfield, IL) in the clinical chemistry laboratory at the Zuckerberg San Francisco General Hospital.

## Western blotting

Livers were homogenized in RIPA buffer containing protease and phosphatase inhibitors. Aliquots were separated by electrophoresis (Bio-Rad TGX, Hercules, CA) and transferred to PVDF membranes (Bio-Rad). Proteins were identified using the following primary antibodies: activating transcription factor 3 (ATF3), activating transcription factor 4 (ATF4), the BH3-only proteins BIM and Bcl-XL, C/EBP homologous protein (CHOP), eukaryotic translation initiating factor 2 $\alpha$  (eIF2 $\alpha$ ), P-eIF2 $\alpha$ , cJun, P-cJun, JNK, P-JNK, IRE1 $\alpha$ , P-IRE1 $\alpha$ , lamin B1, p53 upregulated modulator of apoptosis (PUMA), p38 mitogen-activated protein kinase (p38), P-p38, tubulin, and XBP1. The proteins of interest were visualized by chemiluminescence using a FluorChem FC2 system (Protein Simple, San Jose, CA) and Super Signal West Dura (Thermo Scientific). A complete list of antibodies used for western blotting is included in Table S2.

## Statistical analysis

All experimental results were reported as mean  $\pm$  SEM. Results were compared using analysis of variance (ANOVA) followed by Tukey's multiple comparisons test unless otherwise stated. *P* values < 0.05 were considered significant. Taking into consideration all measurements in the study, variance was similar among the experimental

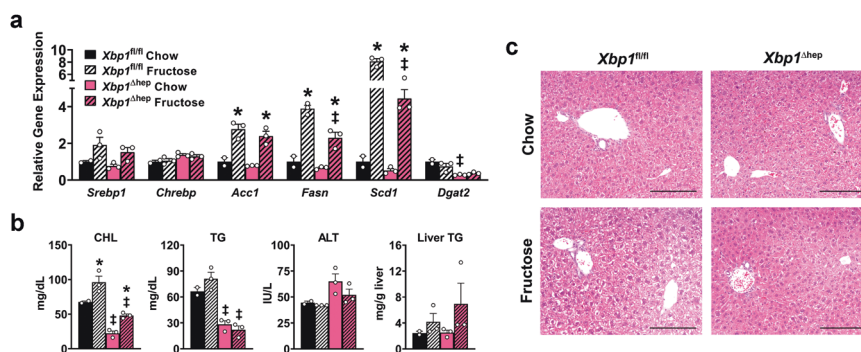
groups. Statistical analyses were performed with Prism 8.4.3 software (GraphPad Software, San Diego, CA).

## Results

AAV8-Transthyretin-Cre successfully deleted XBP1 from hepatocytes in *Xbp1<sup>fl/fl</sup>* mice, as shown by the near-complete absence of nuclear XBP1s in the livers of *Xbp1<sup>Δhep</sup>* mice exposed to inducers of ER stress (Fig. S1A, B). *Xbp1<sup>Δhep</sup>* mice had low levels of serum lipids (Fig. S1C), which has been reported previously and attributed to impaired hepatic lipid secretion [8, 19, 20]. Liver histology in *Xbp1<sup>Δhep</sup>* mice was normal (Fig. S1D). When *Xbp1<sup>Δhep</sup>* mice were fed a fructose-enriched diet for 1 week, lipogenic genes were induced in the liver, although to a lesser extent than *Xbp1<sup>fl/fl</sup>* controls (Fig. 1a). Serum cholesterol and hepatic triglyceride levels increased modestly in both groups of mice in response to fructose feeding, but serum triglycerides were unchanged and liver histology and ALT remained normal (Fig. 1b, c).

When fructose feeding was continued for 4 weeks, lipogenic gene expression remained elevated in the livers of *Xbp1<sup>fl/fl</sup>* and *Xbp1<sup>Δhep</sup>* mice with a persistent difference in the magnitude of gene induction between the two groups (Fig. 2a). This was true despite comparable up-regulation of mRNA encoding the transcription factor carbohydrate-response element-binding protein  $\beta$  (ChREBP $\beta$ ) in *Xbp1<sup>fl/fl</sup>* and *Xbp1<sup>Δhep</sup>* livers upon fructose feeding. Despite their weaker lipogenic gene induction in response to fructose, *Xbp1<sup>Δhep</sup>* mice displayed evidence of mild hepatic lipid accumulation at the 4-week time point (Fig. 2b, c). Serum lipids in these mice were significantly lower than those measured in fructose-fed *Xbp1<sup>fl/fl</sup>* control mice (Fig. 2c). Importantly, fructose-fed *Xbp1<sup>Δhep</sup>* mice exhibited several features of liver cell death and regeneration. Serum ALT levels rose to three times normal after 4 weeks of fructose feeding; liver histology demonstrated marked lobular disarray with evidence of cell death and regeneration. These were confirmed by immunostaining for cleaved caspase-3 and Ki-67 (Fig. 2b, c). Despite the presence of liver cell death in *Xbp1<sup>Δhep</sup>* mice, we found little evidence of CD68 immunoreactivity, a marker of macrophage infiltration, at the 4-week time point (Fig. 2b). Liver fibrosis was also absent at this interval (data not shown). The findings in fructose-fed *Xbp1<sup>Δhep</sup>* mice contrasted with those in fructose-fed *Xbp1<sup>fl/fl</sup>* control mice. In control mice, ALT and liver histology remained normal, although their hepatic triglyceride levels did rise above the chow-fed baseline (Fig. 2b, c).

To explore the connection between hepatocyte XBP1 deletion and the development of liver injury in fructose-fed *Xbp1<sup>Δhep</sup>* mice, we investigated the influence of fructose feeding on the IRE1 $\alpha$ -XBP1 axis and the IRE1 $\alpha$  target



**Fig. 1** Response of *Xbp1<sup>Δhep</sup>* mice to fructose feeding for 1 week. **a** Histogram demonstrates lipogenic gene expression in *Xbp1<sup>fl/fl</sup>* and *Xbp1<sup>Δhep</sup>* livers after 1 week of fructose feeding. *Srebp1* sterol regulatory element-binding protein-1, *Chrebp* carbohydrate-response element-binding protein, *Acc1* acetyl-CoA carboxylase-1, *Fasn* fatty acid synthase, *Scd1* stearoyl-CoA desaturase-1, *Dgat2* diacylglycerol O-acyl transferase-2. **b** Graphs depict the corresponding hepatic

triglyceride levels after fructose feeding, as well as serum levels of cholesterol (CHL), triglyceride (TG) and ALT. **c** Liver histology following 1 week of fructose feeding. Bar = 200  $\mu$ m. Values represent mean  $\pm$  SEM.  $P < 0.05$  by ANOVA for *Acc1*, *Fasn*, *Scd1*, CHL and TG. Using Tukey's multiple comparisons test,  $*P < 0.05$  for fructose vs. chow of same genotype and  $^{\ddagger}P < 0.05$  for *Xbp1<sup>fl/fl</sup>* vs. *Xbp1<sup>Δhep</sup>*.

JNK, which can promote cell death [12, 21]. In *Xbp1<sup>fl/fl</sup>* control mice, fructose feeding stimulated nuclear translocation of spliced XBP1 (XBP1s) in the liver at 1 week, which disappeared by 4 weeks (Fig. 3a). The early increase in nuclear XBP1s in *Xbp1<sup>fl/fl</sup>* livers coincided with mild activation of IRE1 $\alpha$ , JNK, and cJun but was not associated with significant liver injury (Fig. 2b, c). *Xbp1<sup>Δhep</sup>* mice, by contrast, contained no detectable XBP1s in the liver at either 1 week or 4 weeks regardless of diet (Fig. 3a). The absence of nuclear XBP1s resulted in significant suppression of several XBP1 target genes as expected [19] (Fig. 3b). Importantly, these same mice displayed robust activation of IRE1 $\alpha$  as determined by prominent IRE1 $\alpha$  phosphorylation, high levels of *Xbp1* mRNA splicing and the suppression of RIDD target genes (*Xbp1* mRNA splicing remains detectable in *Xbp1<sup>Δhep</sup>* mice because the splice site is not within the sequence deleted by Cre recombinase [8]) (Fig. 3a, c). In *Xbp1<sup>Δhep</sup>* mice, IRE1 $\alpha$  activation was accompanied by the activation of JNK and cJun. P-cJun in particular increased with prolonged fructose feeding and coincided with the development of liver injury (Fig. 3a). This contrasted with p38 MAPK, which was active in all *Xbp1<sup>Δhep</sup>* livers but also in fructose-fed *Xbp1<sup>fl/fl</sup>* livers at 4 weeks, independent of IRE1 $\alpha$  and JNK activity and liver damage (Fig. 3a).

Because cell death is not exclusively linked to IRE1 $\alpha$  and JNK in the setting of ER stress [12, 21], we also investigated the influence of fructose feeding on another death pathway that may be operative in *Xbp1<sup>Δhep</sup>* mice. We focused specifically on eIF2 $\alpha$ , whose activation by eIF2 $\alpha$  kinases can lead to cell death by up-regulating apoptotic proteins and downregulating survival proteins. We observed that XBP1 deletion by itself promoted phosphorylation of eIF2 $\alpha$  even with chow feeding, as has been reported previously [22]. In chow-fed *Xbp1<sup>Δhep</sup>* mice, however, eIF2 $\alpha$

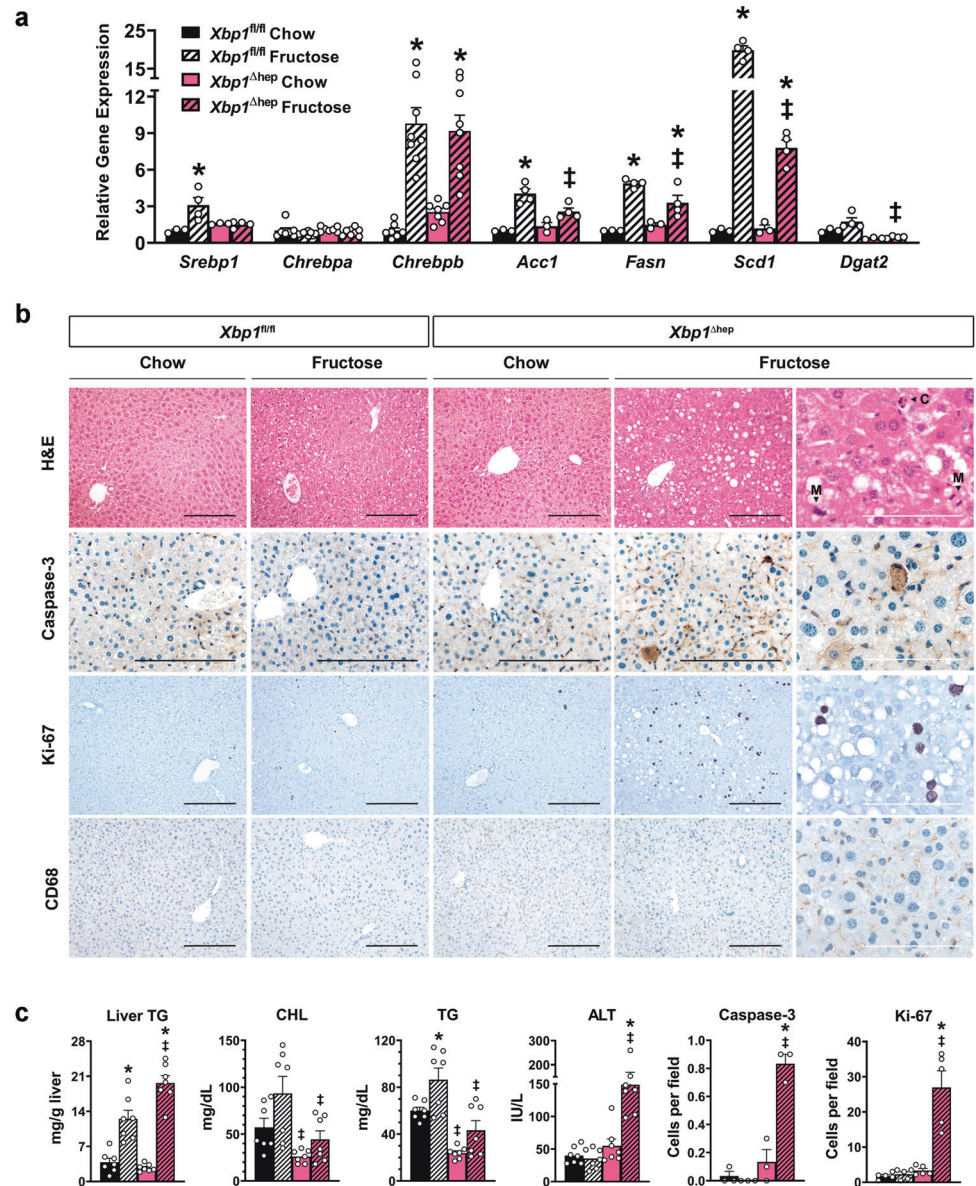
phosphorylation did not trigger any downstream events such as up-regulation of ATF 3/4 or CHOP (Fig. 4a). The failure of eIF2 $\alpha$  to signal induction of ATF3/4 or CHOP has also been reported previously in the livers of *Xbp1<sup>Δhep</sup>* mice, and attributed to IRE1 $\alpha$ -mediated suppression of the eIF2 $\alpha$  phosphatase PPP1r15b, via RIDD [22]. When XBP1 deletion was coupled with fructose feeding, eIF2 $\alpha$  phosphorylation appeared comparable to that in chow-fed mice. However, in this case, it was accompanied by the induction of ATF3 and ATF4 and up-regulation of the apoptotic proteins CHOP, BIM, and PUMA, without any apparent change in the anti-apoptotic protein Bcl-XL. Together these findings indicate that hepatocyte-specific XBP1 deletion is sufficient to induce activation of IRE1 $\alpha$  and eIF2 $\alpha$ , but the alterations are without consequence until an exogenous stress such as fructose feeding is applied. This is completely in line with previously published observations [22]. In the presence of fructose, the downstream consequences of XBP1 deletion are associated with hepatic lipid accumulation and liver cell death within 4 weeks. These findings are noteworthy because fructose feeding per se is a mild metabolic challenge, insufficient to provoke a robust or sustained ER stress response in control mice. The experimental results underscore that hepatocyte-specific XBP1 deletion sensitizes the liver to what otherwise would be an innocuous metabolic insult.

Because the activation of IRE1 $\alpha$  was so pronounced in the livers of *Xbp1<sup>Δhep</sup>* mice, we reasoned that it was central to their sensitization to liver injury. To test this directly, we generated mice with AAV8-mediated deletion of XBP1 as well as the RNase domain of IRE1 $\alpha$  (*Xbp1<sup>Δhep</sup>;Ire1a<sup>Δhep</sup>*) [13]. *Xbp1<sup>Δhep</sup>;Ire1a<sup>Δhep</sup>* mice expressed a truncated form of IRE1 $\alpha$  indicative of the RNase domain deletion [23] (Fig. S2). In contrast to *Xbp1<sup>Δhep</sup>* mice, *Xbp1<sup>Δhep</sup>;Ire1a<sup>Δhep</sup>* mice exhibited no significant *Xbp1* mRNA splicing, even



**Fig. 2 Response of *Xbp1* <sup>$\Delta$ hep</sup> mice to fructose feeding for 4 weeks.** **a** Histogram depicts lipogenic gene expression in the livers of *Xbp1*<sup>fl/fl</sup> and *Xbp1* <sup>$\Delta$ hep</sup> mice after 4 weeks of fructose feeding. Values represent mean  $\pm$  SEM.  $P < 0.05$  by ANOVA for *Chrebp*, *Acc1*, *Fasn*, and *Scd1*. Using Tukey's multiple comparisons test,  $*P < 0.05$  for fructose vs. chow of same genotype;  $^{\ddagger}P < 0.05$  for *Xbp1*<sup>fl/fl</sup> vs. *Xbp1* <sup>$\Delta$ hep</sup>.

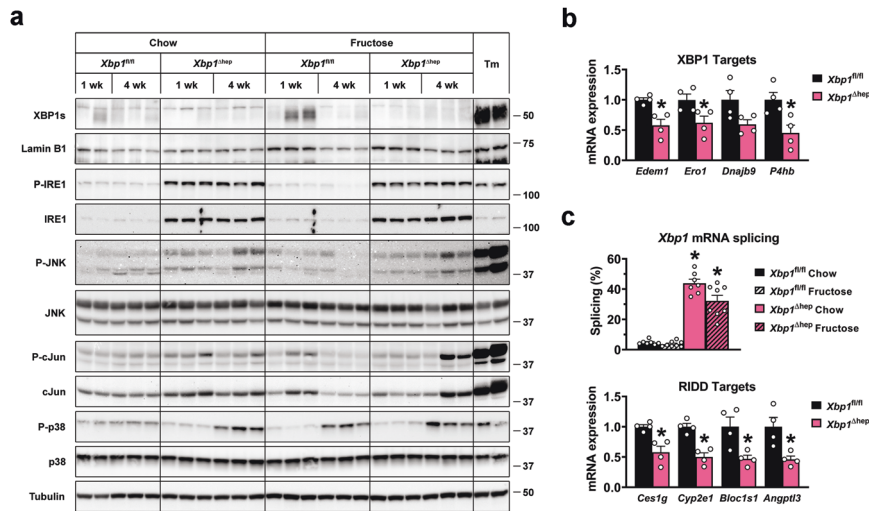
**b** Photomicrographs illustrate H&E-stained liver sections as well as immunohistochemistry for cleaved caspase-3, Ki-67 and CD68 in *Xbp1*<sup>fl/fl</sup> and *Xbp1* <sup>$\Delta$ hep</sup> livers after 4 weeks of fructose feeding. High-power H&E photo illustrates cell death (C) and mitoses (M). Black bar = 200  $\mu$ m; white bar = 100  $\mu$ m. **c** Graphs depict quantitative measures of hepatic triglyceride, serum lipids, ALT levels and cell counts for cleaved caspase-3 and Ki-67. Legend as in **a**. Values represent mean  $\pm$  SEM.  $P < 0.05$  by ANOVA for all measurements. Using Tukey's multiple comparisons test,  $*P < 0.05$  for fructose vs. chow of same genotype and  $^{\ddagger}P < 0.05$  for *Xbp1*<sup>fl/fl</sup> vs. *Xbp1* <sup>$\Delta$ hep</sup>.



after fructose feeding (Fig. 5a). Several XBP1 target genes were suppressed in the livers of *Xbp1* <sup>$\Delta$ hep</sup>;*Irela* <sup>$\Delta$ hep</sup> mice as expected due to the absence of XBP1. RIDD target genes were not suppressed, indicating that RIDD was inactive in the doubly-deficient mice (Fig. 5a). When *Xbp1* <sup>$\Delta$ hep</sup>;*Irela* <sup>$\Delta$ hep</sup> mice were challenged with a fructose-enriched diet for 4 weeks, they exhibited a similar profile of lipogenic gene induction as *Xbp1* <sup>$\Delta$ hep</sup> mice (Fig. 5b) but did not display the features of ER stress noted previously in *Xbp1* <sup>$\Delta$ hep</sup> mice (Fig. 5c). *Xbp1* <sup>$\Delta$ hep</sup>;*Irela* <sup>$\Delta$ hep</sup> mice were also spared from fructose-induced hepatic lipid accumulation and liver injury at 4 weeks. Their hepatic triglyceride levels were no higher than those in chow-fed controls, their serum transaminases were normal, and they displayed no evidence of hepatocellular injury by caspase-3 immunostaining (Fig. 6).

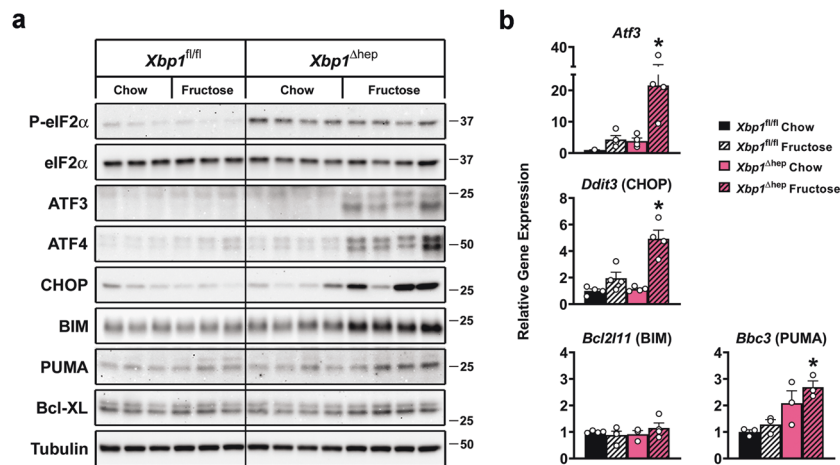
This was true despite the presence of hypolipidemia, indicative of effective gene knockout.

To determine the long-term consequences of XBP1 deletion in hepatocytes, we challenged *Xbp1* <sup>$\Delta$ hep</sup> and *Xbp1* <sup>$\Delta$ hep</sup>;*Irela* <sup>$\Delta$ hep</sup> mice with fructose vs. chow for 16 weeks. IRE $\alpha$  remained active in *Xbp1* <sup>$\Delta$ hep</sup> livers and inactive in *Xbp1* <sup>$\Delta$ hep</sup>;*Irela* <sup>$\Delta$ hep</sup> livers at 16 weeks (Fig. S3). Single- and double-knockout mice continued to display dampened induction of lipogenic gene expression in response to long-term fructose feeding compared to their respective floxed controls despite similar fructose-mediated induction of *Chrebbp* (Fig. 7a). At 16 weeks, fructose feeding caused modest hepatic steatosis in all mice including the floxed controls (Fig. 7b). Only *Xbp1* <sup>$\Delta$ hep</sup> mice, however, developed signs of liver injury, including ALT elevation, macrophage infiltration, and early



**Fig. 3** Effect of XBP1 deletion and fructose feeding on the IRE1 $\alpha$ -XBP1 axis. **a** Western blots illustrate the effects of XBP1 deletion, with or without fructose feeding for 1 or 4 weeks, on activity of the IRE1 $\alpha$ -XBP1 axis. Tunicamycin treatment (Tm) served as a positive control. XBP1s and lamin B1 were measured in nuclear extracts; other proteins were measured in whole liver homogenates. In *Xbp1*<sup>fl/fl</sup> mice, fructose feeding stimulated nuclear translocation of XBP1s at 1 week but not 4 weeks. This coincided with modest and transient up-regulation of IRE1 $\alpha$  and phosphorylation of JNK and cJun. In *Xbp1* <sup>$\Delta$ hep</sup> mice, XBP1s was undetectable; IRE1 $\alpha$  was strongly activated regardless of diet or duration. This coincided with activation of JNK and cJun, the latter particularly after 4 weeks of fructose feeding. **b** Graph depicts relative mRNA expression for a panel of direct XBP1

target genes in fructose-fed liver at 4 weeks. *Edem1* ER degradation enhancing alpha-mannosidase like protein 1, *Ero1* ER oxidoreductin 1, *Dnajb9* DnaJ heat shock protein family member B9, *P4hb* protein disulfide isomerase. **c** Graphs depict *Xbp1* mRNA splicing and mRNA expression for a panel of RIDD target genes in the liver at 4 weeks. *Ces1g* carboxylesterase 1g, *Cyp2e1* cytochrome P4502E1, *Bloc1s1* biogenesis of lysosomal organelles complex 1 subunit 1, *Angptl3* angiopoietin-like protein 3. Values represent mean  $\pm$  SEM. For XBP1 and RIDD targets, \* $P < 0.05$  for *Xbp1* <sup>$\Delta$ hep</sup> vs. *Xbp1*<sup>fl/fl</sup> by unpaired *t*-test. For *Xbp1* mRNA splicing,  $P < 0.05$  by ANOVA. Using Tukey's multiple comparisons test, \* $P < 0.05$  for chow vs. fructose of the same genotype.



**Fig. 4** Effect of XBP1 deletion and fructose feeding on eIF2 $\alpha$  and downstream targets. **a** Western blots illustrate the effects XBP1 deletion, with or without fructose feeding for 4 weeks, on the activity of eIF2 $\alpha$  and the expression of several eIF2 $\alpha$  targets. *Xbp1* <sup>$\Delta$ hep</sup> mice exhibited phosphorylation of eIF2 $\alpha$  with chow feeding as well as fructose feeding at 4 weeks. Up-regulation of molecules downstream

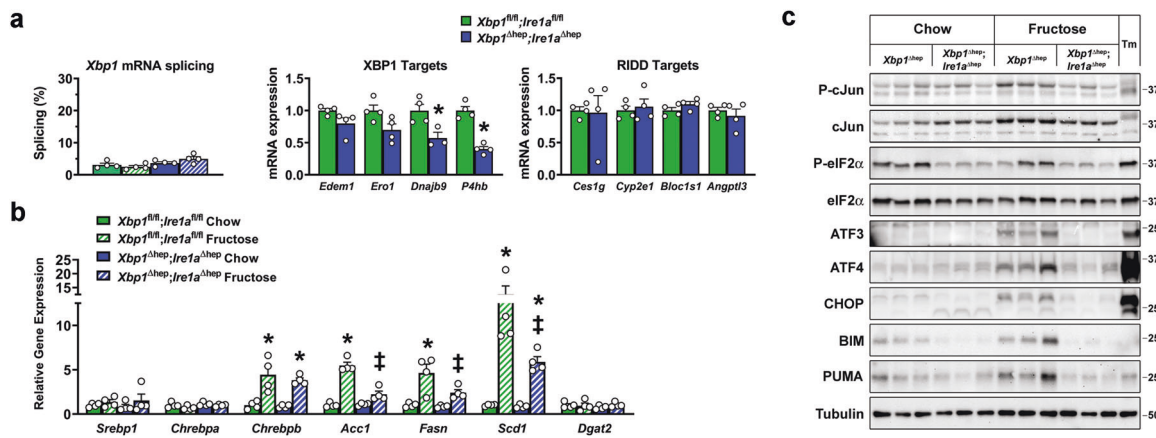
of eIF2 $\alpha$  occurred only after fructose feeding in *Xbp1* <sup>$\Delta$ hep</sup> mice. **b** Graphs demonstrate the transcriptional up-regulation of *Atf3*, *Ddit3*, and *Bbc3* but not *Bcl2l11* in fructose-fed *Xbp1* <sup>$\Delta$ hep</sup> mice after 4 weeks. Values represent mean  $\pm$  SEM.  $P < 0.05$  by ANOVA for *Atf3*, *Ddit3*, and *Bbc3*. \*  $P < 0.05$  for chow vs. fructose of the same genotype.

liver fibrosis (Fig. 7b, c, Fig. S4). Expression of select pro-inflammatory genes was also increased in *Xbp1* <sup>$\Delta$ hep</sup> mice (Fig. S4). *Xbp1* <sup>$\Delta$ hep</sup>; *Irel1* <sup>$\Delta$ hep</sup> mice were protected from the adverse outcomes seen in *Xbp1* <sup>$\Delta$ hep</sup> mice (Fig. 7b, c).

## Discussion

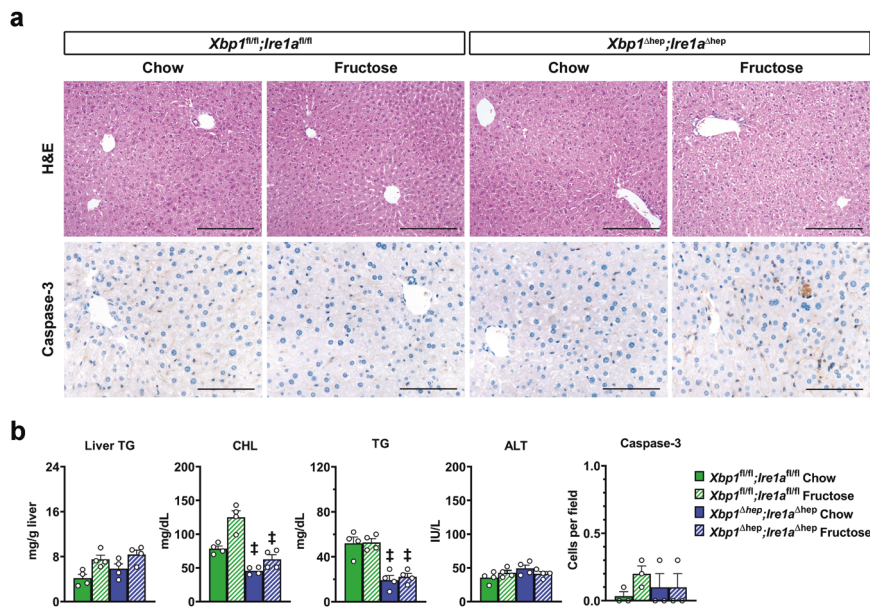
This study demonstrates that hepatocyte-specific deletion of XBP1 in adult mice renders them susceptible to acute





**Fig. 5** Characteristics of *Xbp1<sup>Δhep</sup>;Ire1α<sup>Δhep</sup>* mice after 4 weeks of fructose feeding. **a** Graphs depict *Xbp1* mRNA splicing and the relative expression of XBP1 and RIDD target genes in *Xbp1<sup>fl/fl</sup>;Ire1α<sup>fl/fl</sup>* and *Xbp1<sup>Δhep</sup>;Ire1α<sup>Δhep</sup>* mice after 4 weeks of fructose feeding. Legend for left panel as in **b**. **b** Lipogenic gene expression after 4 weeks of chow or fructose feeding. Values represent mean  $\pm$  SEM. For lipogenic genes,  $P < 0.05$  by ANOVA for *Chrebp*, *Acc1*, *Fasn*, and *Scd1*. Using Tukey's multiple comparisons test,  $*P < 0.05$  for chow vs. fructose of

the same genotype and  $^{\ddagger}P < 0.05$  for *Xbp1<sup>fl/fl</sup>* vs. *Xbp1<sup>Δhep</sup>*. For XBP1 and RIDD targets,  $*P < 0.05$  for *Xbp1<sup>fl/fl</sup>* vs. *Xbp1<sup>Δhep</sup>* by unpaired *t*-test. **c** Western blots illustrate the expression of ER stress-related molecules in liver homogenates from *Xbp1<sup>Δhep</sup>* and *Xbp1<sup>Δhep</sup>;Ire1α<sup>Δhep</sup>* mice after 4 weeks of chow or fructose feeding. Fructose-fed *Xbp1<sup>Δhep</sup>* mice display phosphorylation of cJun and eIF2α as well as up-regulation of several eIF2α targets. These molecules are not induced in fructose-fed *Xbp1<sup>Δhep</sup>;Ire1α<sup>Δhep</sup>* mice.



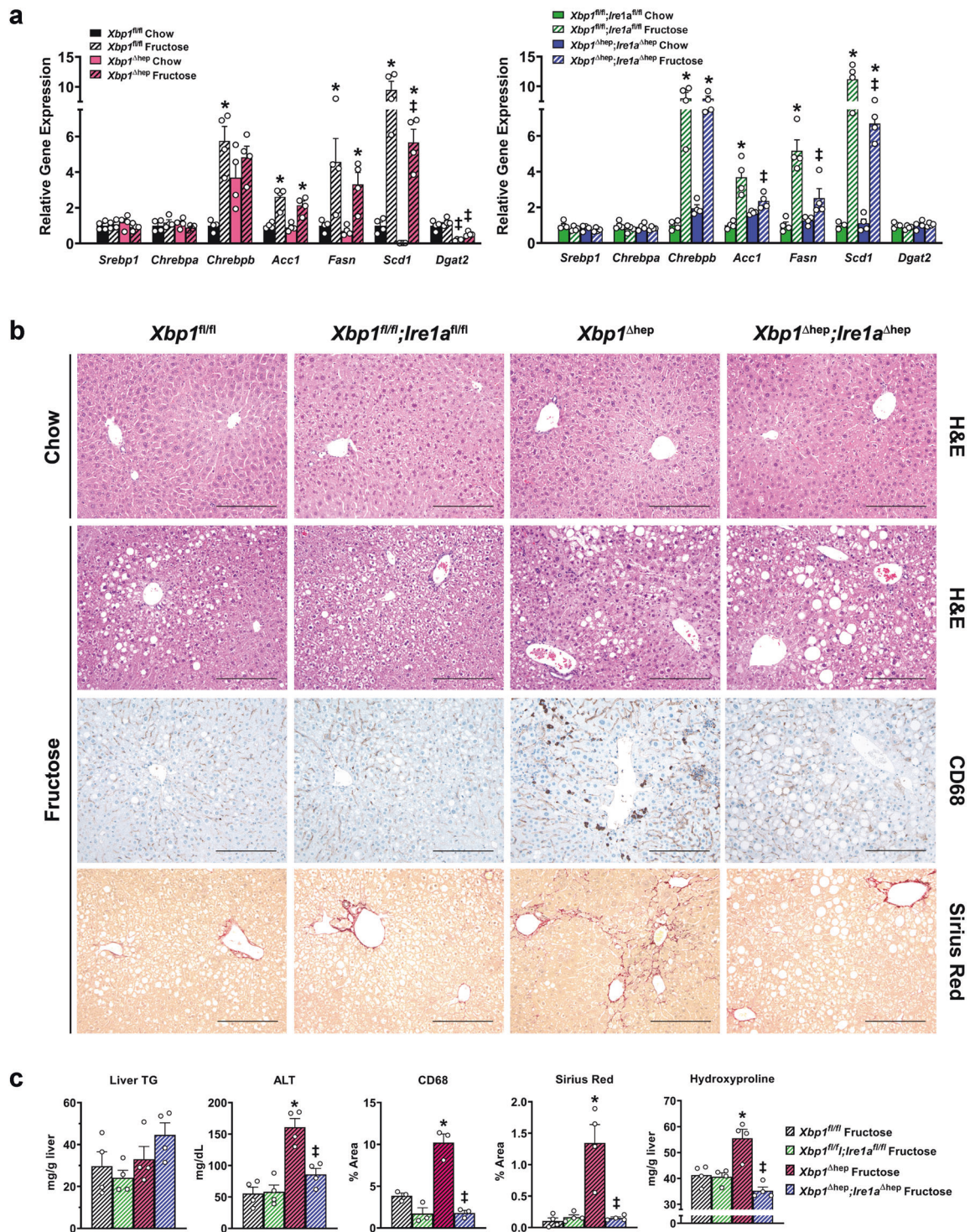
**Fig. 6** Fructose feeding does not induce liver injury in mice with dual deletion of XBP1 and IRE1α. **a** Photomicrographs depict liver histology in *Xbp1<sup>fl/fl</sup>;Ire1α<sup>fl/fl</sup>* and *Xbp1<sup>Δhep</sup>;Ire1α<sup>Δhep</sup>* mice after 4 weeks of chow or fructose feeding. There is no evidence of hepatic lipid accumulation and no evidence of liver cell death as assessed by cleaved caspase-3 staining. Bar = 200  $\mu$ m. **b** Measurements of liver

TG, serum ALT, and cleaved caspase-3-positive cell counts confirm the absence of hepatic steatosis or liver injury in *Xbp1<sup>Δhep</sup>;Ire1α<sup>Δhep</sup>* mice. Serum lipids in *Xbp1<sup>Δhep</sup>;Ire1α<sup>Δhep</sup>* mice are suppressed below the values in *Xbp1<sup>fl/fl</sup>;Ire1α<sup>fl/fl</sup>* mice. Values represent mean  $\pm$  SEM.  $P < 0.05$  by ANOVA for CHL and TG. Using Tukey's multiple comparisons test,  $^{\ddagger}P < 0.05$  for *Xbp1<sup>fl/fl</sup>;Ire1α<sup>fl/fl</sup>* vs. *Xbp1<sup>Δhep</sup>;Ire1α<sup>Δhep</sup>*.

and chronic liver injury in response to fructose feeding. In *Xbp1<sup>Δhep</sup>* mice, a mild dietary challenge that was insufficient to cause liver disease in control mice induced significant disease after gene deletion, characterized by hepatic lipid accumulation, liver cell death, and ultimately fibrosis.

Importantly, the susceptibility of *Xbp1<sup>Δhep</sup>* mice to liver disease was not the direct consequence of XBP1 deletion. Instead, it was due to compensatory up-regulation of the upstream ER stress transducer IRE1α. This was confirmed by liver-specific deletion of both XBP1 and IRE1α, which





protected mice from the injury observed in mice lacking XBP1 alone.

Fructose-mediated liver injury in *Xbp1<sup>Δhep</sup>* mice was associated with several features of heightened ER stress, including activation of IRE1 $\alpha$  and eIF2 $\alpha$  and the induction of the pro-apoptotic molecules JNK and CHOP. All of these stress responses were abrogated by IRE1 $\alpha$  deletion, indicating

they were triggered by IRE1 $\alpha$  activation. Whether IRE1 $\alpha$  provoked liver injury through its kinase or endoribonuclease functions or both is difficult to pinpoint: both were upregulated in our mice, based on the activation of JNK and RIDD. We suspect JNK was activated as a result of IRE1 $\alpha$  kinase activity and eIF2 $\alpha$  activated as an indirect consequence of IRE1 $\alpha$  endonuclease activity (RIDD) [22]. Still, we

**◀ Fig. 7 Long-term consequences of fructose feeding in *Xbp1* <sup>$\Delta$ hep</sup> and *Xbp1* <sup>$\Delta$ hep</sup>;*Ire1a* <sup>$\Delta$ hep</sup> mice.** **a** Histograms depict lipogenic gene expression in the livers of *Xbp1*<sup>fl/fl</sup> and *Xbp1* <sup>$\Delta$ hep</sup> mice or *Xbp1*<sup>fl/fl</sup>; *Ire1a*<sup>fl/fl</sup> and *Xbp1* <sup>$\Delta$ hep</sup>;*Ire1a* <sup>$\Delta$ hep</sup> mice after 4 weeks of fructose feeding. Values represent mean  $\pm$  SEM.  $P < 0.05$  by ANOVA for *Chrebbp*, *Acc1*, *Fasn*, and *Scd1*; and for *Dgat2* in single knockout only. Using Tukey's multiple comparisons test,  $*P < 0.05$  for chow vs. fructose of the same genotype and  $^{\ddagger}P < 0.05$  for floxed vs. knockout. **b** Photomicrographs demonstrate liver histology in *Xbp1* <sup>$\Delta$ hep</sup> and *Xbp1* <sup>$\Delta$ hep</sup>;*Ire1a* <sup>$\Delta$ hep</sup> mice and their floxed controls after 16 weeks of chow or fructose feeding. Chow-fed mice have no obvious histologic abnormalities; fructose feeding induced moderate steatosis in all four groups of mice. CD68 staining shows hepatic inflammation only in fructose-fed *Xbp1* <sup>$\Delta$ hep</sup> mice. Sirius red staining shows perisinusoidal and bridging fibrosis only in fructose-fed *Xbp1* <sup>$\Delta$ hep</sup> mice. Bar = 200  $\mu$ m. **c** Graphs depict liver TG, serum ALT, CD68-stained area and hepatic fibrosis assessed by Sirius Red morphometry and hydroxyproline measurement in control, *Xbp1* <sup>$\Delta$ hep</sup> and *Xbp1* <sup>$\Delta$ hep</sup>;*Ire1a* <sup>$\Delta$ hep</sup> mice. The highest values of ALT, CD68 immunoreactivity and fibrosis are in fructose-fed *Xbp1* <sup>$\Delta$ hep</sup> mice at 16 weeks. Values represent mean  $\pm$  SEM.  $P < 0.05$  by ANOVA for ALT, CD68, Sirius Red morphometry and hydroxyproline.  $*P < 0.05$  for floxed vs. knockout;  $^{\ddagger}P < 0.05$  for *Xbp1* <sup>$\Delta$ hep</sup> vs. *Xbp1* <sup>$\Delta$ hep</sup>;*Ire1a* <sup>$\Delta$ hep</sup>.

acknowledge that these events could occur through other pathways [12, 24, 25]. We also cannot specify which of the several death-promoting molecules induced in *Xbp1* <sup>$\Delta$ hep</sup> livers were responsible for fructose-induced cell death. JNK, CHOP, BIM, and PUMA were all upregulated on a similar timeline, and thus they all likely contributed collectively to the development of liver injury.

One intriguing observation in the current study was that IRE1 $\alpha$  and eIF2 $\alpha$  were upregulated at baseline in the livers of *Xbp1* <sup>$\Delta$ hep</sup> mice, yet there was no evidence of downstream signaling from these two molecules and no liver injury in the absence of an exogenous stimulus. This phenomenon has been reported previously in *Xbp1* <sup>$\Delta$ hep</sup> mice [22], and supports the notion that some threshold of IRE1 $\alpha$  activation must be surpassed, possibly by an exogenous stimulus, in order to effect a cytotoxic response. Some have linked the cytotoxic potential of IRE1 $\alpha$  to stress-induced high-order oligomerization of the protein with resultant activation of RIDD [26]. In the current study, however, RIDD was detectable in XBP1-deleted livers even before fructose feeding, and thus RIDD per se cannot be the sole trigger to liver injury. RIDD-induced cell death may be cell-type specific [27, 28], and hepatocytes may be somewhat resistant [19, 29, 30]. Still, our results support the concept that IRE1 $\alpha$  activation can be pushed to a level that causes hepatotoxicity, and XBP1 deletion nearly achieves this goal.

It is intriguing that hepatic lipid accumulation occurred in *Xbp1* <sup>$\Delta$ hep</sup> but not *Xbp1* <sup>$\Delta$ hep</sup>;*Ire1a* <sup>$\Delta$ hep</sup> mice after short-term fructose feeding, whereas it occurred in both genotypes at 16 weeks. In the single- and double-knockout mice, fructose-induced hepatic lipid accumulation should be influenced in part by diet-induced lipogenesis and in part by

suppression of hepatic lipid secretion, the latter due to down-regulation of the *Xbp1* target gene *P4hb* that plays a role in hepatic VLDL secretion [20]. Our data show no major differences in lipogenic gene induction or *P4hb* suppression between single- and double-knockout mice at 4 weeks (Figs. 2, 3, and 5); however, at this early time point, the amount of lipid measured in the livers of the single-knockout mice was quite small, and thus the mild increase noted in *Xbp1* <sup>$\Delta$ hep</sup> mice is of unclear significance. Long-term fructose feeding caused more pronounced hepatic lipid accumulation, but in this instance the lipid accrual in *Xbp1* <sup>$\Delta$ hep</sup> mice was no worse than that in any other group including the floxed controls (Fig. 7c). Still, *Xbp1* <sup>$\Delta$ hep</sup> mice developed liver injury, inflammation and fibrosis, whereas *Xbp1* <sup>$\Delta$ hep</sup>;*Ire1a* <sup>$\Delta$ hep</sup> mice were protected. These findings suggest that hepatic lipid accumulation, although a feature of the liver injury seen in fructose-fed *Xbp1* <sup>$\Delta$ hep</sup> mice, is not itself central to liver damage in this experimental paradigm.

Another interesting observation from our study is that in control mice (*Xbp1*<sup>fl/fl</sup>), fructose feeding induced ER stress only transiently in the liver. In control livers, fructose feeding stimulated the nuclear translocation of XBP1s at 1 week but no longer at 4 weeks; this is in keeping with concept that fructose is a mild stress stimulus, and may explain in part why fructose feeding does not provoke liver injury in control animals. Although ER stress did not persist with prolonged fructose feeding, over time, fructose induced hepatic steatosis in all mice in the study. The uniform steatosis in all mice at 16 weeks underscores that IRE1 $\alpha$  activation and associated stress signaling are not absolute prerequisites to fructose-induced hepatic lipid accumulation. Fructose did induce *Chrebbp* $\beta$  in the livers of all mice in the study, which suggests that fructose-induced hepatic lipogenesis is a strong stimulus to the observed steatosis. Again, these findings support a mechanistic distinction between hepatic steatosis and liver injury our XBP1-deleted mice.

The current work uniquely supplements an existing body of research examining the impact of IRE1 $\alpha$  and XBP1 on the liver. Previous studies of liver-targeted deletion of XBP1 have yielded mixed results: some showed no deleterious effect on the liver [8], others showed that XBP1 deletion improved hepatic steatosis in obese mice [19, 31], and still others showed that XBP1 deletion worsened liver injury from pharmacologic or dietary insults [9, 32]. Our experiments are in general agreement with the last group of reports indicating that XBP1 deletion is harmful to the liver when combined with another insult. However, our work diverges from these studies in that we attribute the harmful effect of hepatic XBP1 deletion to IRE1 $\alpha$ . When IRE1 $\alpha$  is activated to excess, it sensitizes hepatocytes to injurious stimuli.



In summary, the current work underscores the potential for IRE1 $\alpha$  to induce liver injury when activated above a threshold level in the proper clinical setting. Indeed, heightened IRE1 $\alpha$  activity has been linked to lipotoxic liver injury and nonalcoholic fatty liver disease [33–35]. We believe our experiments have implications extending beyond metabolic liver disease, although they may not be applicable to all forms of liver injury [29]. Nevertheless, our demonstration that IRE1 $\alpha$ -mediated liver injury is separable from hepatic steatosis suggests that excess IRE1 $\alpha$  activation can contribute to the pathogenesis of other types of ER stress-related liver injury [36–38]. Accordingly, therapeutics targeting excess IRE1 $\alpha$  activation may have broad application in the treatment of an array of hepatotoxic diseases.

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### Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interest.

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