

University of Massachusetts Medical School

eScholarship@UMMS

GSBS Student Publications

Graduate School of Biomedical Sciences

2016-11-03

Endoplasmic reticulum stress-induced hepatocellular death pathways mediate liver injury and fibrosis via Stimulator of Interferon Genes.

Arvin Iracheta-Vellve
University of Massachusetts Medical School

Et al.

Let us know how access to this document benefits you.

Follow this and additional works at: https://escholarship.umassmed.edu/gsbs_sp

 Part of the [Biochemistry Commons](#), [Immunity Commons](#), and the [Molecular Biology Commons](#)

Repository Citation

Iracheta-Vellve A, Petrasek J, Gyongyosi B, Satishchandran A, Lowe P, Kodys K, Catalano D, Calenda CD, Kurt-Jones EA, Fitzgerald KA, Szabo G. (2016). Endoplasmic reticulum stress-induced hepatocellular death pathways mediate liver injury and fibrosis via Stimulator of Interferon Genes.. GSBS Student Publications. <https://doi.org/10.1074/jbc.M116.736991>. Retrieved from https://escholarship.umassmed.edu/gsbs_sp/1997

This material is brought to you by eScholarship@UMMS. It has been accepted for inclusion in GSBS Student Publications by an authorized administrator of eScholarship@UMMS. For more information, please contact Lisa.Palmer@umassmed.edu.

Endoplasmic reticulum stress-induced hepatocellular death pathways mediate liver injury and fibrosis via Stimulator of Interferon Genes

Arvin Iracheta-Vellve, Jan Petrasek, Benedek Gyongyosi, Abhishek Satishchandran,
Patrick Lowe, Karen Kodys, Donna Catalano, Charles D. Calenda, Evelyn A. Kurt-Jones,
Katherine A. Fitzgerald, Gyongyi Szabo

From the Department of Medicine, University of Massachusetts Medical School, Worcester, MA 01605

Running title: *STING-IRF3 signaling in liver fibrosis*

To whom correspondence should be addressed: Professor, Gyongyi Szabo M.D., Ph.D., University of Massachusetts Medical School, Department of Medicine, LRB208, 364 Plantation Street, Worcester, MA 01605 Tel: (508) 856-5275; Fax: (508) 856-4770. Email: gyongyi.szabo@umassmed.edu

Key words: carbon tetrachloride; apoptosis; ER stress; fibrosis; STING; IRF3; IRF7

ABSTRACT

Fibrosis, driven by inflammation, marks the transition from benign to progressive stages of chronic liver diseases. Although inflammation promotes fibrogenesis, it is not known whether other events, such as hepatocyte death, are required for the development of fibrosis. Interferon Regulatory Factor 3 (IRF3) regulates hepatocyte apoptosis and production of Type-I interferons (IFNs). In the liver, IRF3 is activated via Toll-like receptor 4 (TLR4) signaling or the ER adapter, Stimulator of Interferon Genes (STING). We hypothesized that IRF3-mediated hepatocyte death is an independent determinant of chemically-induced liver fibrogenesis. To test this, we performed acute or chronic carbon-tetrachloride (CCl₄) administration to WT, IRF3-, TRAM-, TRIF-, and STING-deficient mice. We report that acute CCl₄ administration to WT mice resulted in early ER stress, activation of IRF3 and Type-I IFNs, followed by hepatocyte apoptosis and liver injury, accompanied by liver fibrosis upon repeated administration of CCl₄. Deficiency of IRF3 or STING prevented hepatocyte death and fibrosis both in acute or chronic CCl₄. In contrast, mice deficient in Type-I IFN receptors or in TLR4-signaling adaptors, TRAM or TRIF,

upstream of IRF3, were not protected from hepatocyte death and/or fibrosis suggesting that the pro-apoptotic role of IRF3 is independent of TLR signaling in fibrosis. Hepatocyte death is required for liver fibrosis with causal involvement of STING and IRF3. Thus, our results identify that IRF3, by its association with STING in the presence of ER stress, couples hepatocyte apoptosis with liver fibrosis, and indicate that innate immune signaling regulates outcomes of liver fibrosis via modulation of hepatocyte death in the liver.

Fibrosis represents a late-stage of liver disease that is common to all chronic liver diseases including viral hepatitis, alcoholic and non-alcoholic fatty liver disease, biliary liver diseases, and some genetic liver disease. In spite of their specific etiologies, common denominators of fibrosis are shared among all these liver diseases, including liver inflammation and hepatocyte death. Fibrosis results from chronic unresolved liver inflammation and may progress from fibrotic scarring to cirrhosis that ultimately leads to liver failure. Inflammation triggers liver fibrosis via a signaling event in which liver resident macrophages (Kupffer cells, KC) activate hepatic

stellate cells (HSC) to deposit collagen (1, 2). Liver dysfunction results from the fibrotic tissue distorting the liver parenchyma. This process involves a dynamic and complex series of multicellular events, involving inflammation and HSC activation. The hepatocyte is primarily responsible for metabolism and detoxification, and as such, it is often exposed to damage due to toxic metabolites and reactive oxygen species. However, the role of hepatocyte death in liver fibrosis has been only partially elucidated. Data available up to date demonstrate that hepatocyte apoptosis activates HSC via paracrine mechanisms. Alternatively, HSC can be directly activated by apoptotic bodies (3). Apoptosis in hepatocytes results from displacement or down-regulation of the Bcl-2 family of anti-apoptotic proteins and subsequent activation of pro-apoptotic BH3-only proteins such as Bax and Bak (4). These events lead to oligomerization of Bax and Bak, resulting in permeabilization of the outer mitochondrial membrane and execution of the intrinsic apoptotic process (5). Recently, IRF3, a transcription factor that induces interferon- β (IFN- β), has been shown to play a major role in cell death by way of its newly characterized BH3-only domain. IRF3 associated with the pro-apoptotic adaptor Bax and triggered apoptosis in murine embryonic fibroblasts and murine hepatocytes (6–8). However, it is not known whether other key signal transduction events in hepatocytes are required for the pathogenesis of fibrosis. For this

reason, we sought to understand the nature of pro-fibrogenic hepatocyte damage and specifically assess the potential role of hepatocyte apoptosis in determining the onset of fibrosis. In this study, we investigated the role of IRF3 in hepatocyte death and liver fibrosis. We used carbon-tetrachloride (CCl₄), a chemical that, in the short term, induces hepatocyte apoptosis (9) which is followed by secondary necrosis (10, 11). When administered repetitively, CCl₄ induces liver fibrosis (12). Using two models of CCl₄-induced liver injury, we investigated the complex multi-cellular events associated with fibrosis in a chronic model, as well as the tightly-controlled signal transduction events associated with early disruption of homeostasis and cell death in the acute model. We report that hepatocyte-specific endoplasmic reticulum (ER) stress and activation of IRF3 occurs and is mediated by the ER adaptor, STING. Activated IRF3 associates with the pro-apoptotic adaptor Bax and induces hepatocyte death. We initially hypothesized that IRF3 will utilize established pathways of antiviral response, including Type-I IFNs, to induce hepatocyte apoptosis. However, our results demonstrate that the pro-apoptotic role of IRF3 is independent of its function to induce Type-I IFNs such as IFN- β . Taken together, our data demonstrate that hepatocyte death is indispensable in liver fibrosis and that the pathogenic role of IRF3 in liver fibrosis is mediated via its pro-apoptotic effect in hepatocytes.

RESULTS

IRF3 deficiency attenuates chronic CCl₄-mediated liver injury and fibrosis—Repetitive episodes of liver injury, such as those induced by administration of CCl₄ (a chemical inducer of hepatocyte death), result in the development of liver fibrosis, which is a hallmark of advanced and often irreversible liver disease in humans. We previously showed that IRF3-deficient mice were protected from alcohol-induced liver disease and that IRF3 deficiency in hepatocytes prevented fibrogenesis in the liver in alcoholic liver disease (8). We evaluated this hypothesis using a mouse model of liver injury and fibrosis using CCl₄ or vehicle, in a six-week regimen of bi-weekly intraperitoneal injections. Deficiency of IRF3 significantly attenuated liver damage, as

demonstrated by significantly lower levels of serum ALT and substantially improved histological findings compared to CCl₄-treated wild-type controls (**Fig.1A,B**), IRF3-deficient mice had decreased TUNEL positive staining in the liver compared to WT mice, suggestive of decreased apoptosis (**Fig.1C**). Interestingly, TUNEL positive staining was predominantly present in hepatocytes. IRF3-deficient mice showed significant protection from liver fibrosis, indicated by reduced alpha-smooth muscle actin (α -SMA) immunohistochemistry and Sirius-red staining compared to WT mice (**Fig.1D,E**). The protection from liver fibrosis in IRF3-deficient mice was accompanied by significantly lower expression of *Acta2* and *Colla2* (**Fig.1F**),

encoding α -SMA and Type-I collagen, and by decreased expression of *Ifnb* and *Isg15*, two commonly used markers of IRF3-mediated transcriptional response (**Fig.1G**).

Acute CCl₄ induces ER stress and phosphorylation of IRF3 in hepatocytes—Disruption of intracellular homeostasis can lead to an accumulation of misfolded proteins associated with the unfolded protein response (UPR) in the ER, also known as ER stress. Excessive ER stress is associated with pro-apoptotic signaling and can be pathogenic (13, 14). To test if CCl₄-induced acute hepatocyte injury induces an early ER stress response, we analyzed livers of mice at various time points after a single administration of CCl₄. We found an early upregulation of *Xbp1* mRNA splicing (*sXbp1*), a marker of early ER stress (15, 16) at one and two hours after administration of CCl₄ (**Fig.2A**). Analysis of cell populations from murine livers showed that CCl₄ selectively upregulated *Xbp1* mRNA splicing in hepatocytes but not in liver mononuclear cells (LMNCs) (**Fig.2B**).

Previous reports have shown that ER stress results in IRF3 activation in mouse embryonic fibroblast, and that *Xbp1* splicing enhances the IFN- β response in immune cells (17, 18). In our study, acute administration of CCl₄ led to an early activation of IRF3, a pro-apoptotic BH3-only protein, as seen by the phosphorylation in whole cell liver lysates (**Fig.2C**), at the same time as splicing of *Xbp1* mRNA (**Fig.2A**). The phosphorylation of IRF3 had a functional effect, as liver *Ifnb1* mRNA and serum IFN- β , transcriptional targets of IRF3, showed an early upregulation (**Fig.2D**). To test if direct induction of ER stress can lead to IRF3 phosphorylation, we treated primary hepatocytes with thapsigargin *in vitro* and found an early increase in phosphorylated IRF3 (**Fig.2E**). Furthermore, inhibition of Tank-binding kinase 1 (TBK1), a kinase responsible for IRF3 phosphorylation, with BX795 showed a dose-dependent reduction in IRF3 phosphorylation in thapsigargin-treated hepatocytes (**Fig.2E**). Building upon previous findings, our data indicate that acute administration of CCl₄ results in hepatocyte-specific ER stress and phosphorylation of IRF3 *in vivo*, and that *in vitro* inhibition of a kinase upstream of IRF3 inhibited phosphorylation of IRF3 induced by ER stress. Our data support the

hypothesis that CCl₄ activates IRF3 in hepatocytes via ER stress.

Acute CCl₄-induced liver injury is ameliorated by the deficiency of IRF3 independent of TRIF or TRAM, or Type-I IFN signaling—Next, we asked if IRF3 plays a role in liver injury after acute CCl₄ administration. We found that IRF3-KO mice were partially protected from liver injury after acute CCl₄ administration, as indicated by the significant attenuation in serum ALT compared to WT controls (**Fig.3A**) and Caspase-3 cleavage in the liver (**Fig.3B**). To test if the pathogenic phenotype of IRF3 involvement was mediated by its function as a transcription factor, we tested mice deficient in Type-I IFN receptor (IFNAR1) that lack Type-I IFN signaling and found that IFNAR1-KO mice had no protection from CCl₄-mediated liver injury, phosphorylation of IRF3 or apoptotic signaling in the liver compared to WT mice (**Fig.3C,D**). This data suggested that the pathogenic role of IRF3 may be independent of its transcriptional role.

CCl₄-induced liver fibrosis activates hepatic stellate cells via TLR4 (19, 20), enhanced by signal transduction emanating from the TLR4 complex induced by gut bacterial products and LPS (1). To investigate if activation of IRF3 in acute CCl₄-liver injury was mediated by the intracellular MyD88-independent TLR4 adaptors TIR-domain-containing adapter-inducing interferon- β (TRIF) and TRIF-related adaptor molecule (TRAM), or the kinase TBK1, we first inhibited activity of their downstream kinase, TBK1, by the chemical inhibitor BX795 *in vivo*. Pre-treatment of mice with BX795 significantly attenuated the extent of liver injury and apoptotic signaling in the liver after administration of CCl₄ (**Fig.3E,F**). The decrease in phosphorylated IRF3 (**Fig.3F**) with BX795 pre-treatment further suggests that IRF3 requires activation and phosphorylation of TBK1 in order to exert a pathogenic effect in CCl₄-induced liver injury. In contrast, TRAM- and TRIF-KO mice were each equally as susceptible to acute CCl₄ damage and apoptosis in the liver as WT mice (**Fig.3G,H**). Importantly, TRAM- and TRIF-KO mice had similar levels of phosphorylated IRF3 compared to WT mice (**Fig.3H**), suggesting that IRF3 activation by acute CCl₄ occurs independent of the TLR4 receptor complex and its adaptors. Together this data suggested that TBK1-mediated activation

of IRF3 is pathogenic, leading to increased liver injury. Furthermore, Type-I IFNs do not have a significant role in acute liver injury after CCl₄ administration in spite of being activated by IRF3. Lastly, activation of IRF3 by acute CCl₄ is independent of the canonical MyD88-independent TLR4 adaptor-mediated receptor signaling. Therefore, we hypothesized that activation of IRF3 in the liver by acute CCl₄ likely involves different signaling pathways.

CCl₄ induces early association of IRF3 with STING in the ER and association of IRF3 with Bax in the mitochondria in the liver—Given the robust protection from acute or chronic CCl₄-induced liver injury in mice deficient in IRF3, we next investigated subcellular localization and mechanisms of early IRF3 activation. First, we evaluated the kinetics of IRF3 activation in WT mice upon acute CCl₄-mediated injury and found an early phosphorylation of IRF3 at two and four hours in whole cell liver lysates (Fig.4A). Next, we analyzed the intracellular distribution of IRF3. Analysis of subcellular liver fractions revealed that administration of CCl₄ resulted in localization of phosphorylation of IRF3 not only in the cytoplasmic (Fig.4B) and nuclear extracts (Fig.4C), but also in the ER (Fig.4D) and mitochondrial fractions (Fig.4E). The finding of phosphorylated IRF3 in cytoplasmic and nuclear extracts (Fig.4A,B) was consistent with the transcriptional role of IRF3 in induction of *Ifnb*. However, the finding of phosphorylated IRF3 in the ER (Fig.4D) and mitochondria (Fig.4E) was unexpected and prompted us to search for ER- and mitochondria-specific binding partners of IRF3.

It has been reported that during viral infections, IRF3 associates with ER via binding to Stimulator of IFN Gene (STING, alias Tmem173 or MPYS), an adaptor protein residing in the ER membrane. We found that a single administration of CCl₄ *in vivo* resulted in a significant increase in STING in whole cell liver lysates (Fig.4F) and in ER extracts (Fig.4H) after a pull-down with IRF3 antibody, suggesting an association between IRF3 and STING in the liver of mice exposed to CCl₄.

Based on the recent report that IRF3 plays a key role in viral-mediated apoptosis mediated by Bax (6), and on our previous report data that IRF3 is required for hepatocyte apoptosis triggered by FasL (8), we hypothesized that IRF3 would associate with pro-apoptotic proteins involved in

the intrinsic, mitochondrial pathway of hepatocytes apoptosis. We found an increased association between IRF3 and Bax at two hours after administration of CCl₄ (Fig.4I), followed by Casp-8 activation and apoptosis (Fig.4J,K). The timing of this association correlates with the increased presence of phosphorylated IRF3 in the mitochondrial fraction, suggesting that only active and phosphorylated IRF3 has a pro-apoptotic role when found in the mitochondria. Moreover, the presence of phosphorylated IRF3 in the mitochondria and its association with BAX coincided with activation and cleavage of the initiator Caspase-8 and the executioner Caspase-3 (Fig.4J). Our data suggest that ER stress and phosphorylation of IRF3 (Fig.2A,C), which occurred within one hour, was followed by pro-apoptotic signaling and hepatocyte damage (Fig.4I,J,K), which were established within two hours. Taken together, our data indicated that following acute administration of CCl₄, IRF3 interacts with the ER-associated protein, STING, and with the pro-apoptotic molecule, Bax. Importantly, the association of IRF3 with STING or phospho-TBK1 in the ER occurred within the first two hours after CCl₄ administration (Fig.4F,H,G), concurrent with phosphorylation of IRF3 and upregulation of *Ifnb* in the liver (Fig.2C,D); all of these events occurred prior to the development of significant liver injury (Fig.3).

Deficiency in STING, but not in Type-I IFN signaling, attenuates liver fibrosis—Acute CCl₄-induced liver injury resulted in ER stress and IRF3 activation, as well as early localization of IRF3 in the ER in association with STING, an ER-adaptor protein. Furthermore, IRF3 demonstrated a pathogenic role, independent of the TLR4-adaptor complex, in models of acute or chronic CCl₄-induced liver injuries, we then hypothesized that activation of IRF3 was through STING after chronic CCl₄ administration in mice. In support of this hypothesis, we observed that Tmem173^{Gt} mice, which lack the STING protein (21) had significantly attenuated liver injury induced by repetitive injections of CCl₄ (Fig.5A,B) to a similar extent to that observed in IRF3-deficient mice (Fig.1A). Further, STING-deficient mice (Tmem173^{Gt}) showed protection from liver fibrosis (Fig.5C) as well as a decrease in deposition α -SMA and Type1-Collagen protein in

the liver (**Fig. 5D,E**) and expression of pro-fibrogenic markers (**Fig.5F**).

IRF3 is the transcription factor responsible for Type-I IFN production, a pro-inflammatory cytokine important in creating an antiviral state. In addition to IRF3, IRF7 is another major regulator of Type-I IFN production (22). Administration of CCl₄ resulted in significantly upregulated *Ifnb1* and the IFN inducible gene *Isg15* in the liver in WT mice, while IRF3-deficient mice lacked induction of Type-I IFN genes (**Fig.1F**). However, IRF7 or Type I IFNs did not mediate the pathogenic effect of IRF3 in the liver as demonstrated by lack of protection from liver injury (**Fig.5H,I**) and fibrosis (**Fig.5J,K**) in IRF7- or IFNAR1-KO mice, respectively. The lack of Type-I IFN induction or signaling in IFNAR1-KO mice resulted in aggravated liver fibrosis, as demonstrated by increased Sirius-red area (**Fig.5J**) and augmented expression of *Acta2* and *Colla2* (**Fig.5K**) compared to WT mice, which is consistent with the previously reported anti-fibrotic role of Type-I IFNs (23, 24). Taken together, this data implied that IRF3, but not IRF7 or Type-I IFN signaling, play a pathogenic role in chronic liver injury and fibrosis induced by chronic CCl₄ administration. In addition, the data demonstrate the pathogenic role of STING, a binding partner of IRF3 (**Fig.4** and (25)), as a determinant in the development of liver injury and fibrosis in this model.

STING mediates pro-apoptotic activation of IRF3 in a chronic CCl₄-induced model of fibrosis—To further characterize the role of STING in mediating the CCl₄-induced liver injury in the chronic CCl₄ model, we evaluated phosphorylation of TBK1 and IRF3. After six weeks of exposure to CCl₄, STING-deficient mice had decreased phosphorylation of TBK1 (**Fig.6A**) as well as decreased phosphorylation of IRF3 (**Fig.6B**) compared to WT mice. Previous reports have studied the pro-apoptotic association of IRF3 with Bax. To test if STING-mediated activation of IRF3 results in pro-apoptotic signaling, we tested Caspase activation in STING-deficient mice using the chronic CCl₄-mediated liver injury model. We found that STING-deficient mice (Tmem173^{Gt}) given a six-week regimen of CCl₄ exhibited a significant reduction in the cleavage and activation of the apoptosis initiator Caspase-8 (**Fig.6C**) and

the apoptosis executioner Caspase-3 (**Fig.6D**) compared to WT mice.

This data was consistent with the association between IRF3, STING and Bax that we found in the liver following a single exposure to CCl₄ (**Fig.4H,I**) and indicated the possibility that ER stress may be a source of IRF3 activation in this model. In addition, this data also supported the possibility that in the chronic model of CCl₄-induced liver injury and fibrosis, IRF3 represents an activator of pro-apoptotic signaling in the liver.

Early pro-apoptotic activation of IRF3 by CCl₄ is hepatocyte-specific and mediated by STING—To further explore the mechanism of cell death and liver injury and to test whether induction of cell death in the acute CCl₄ liver injury model was mediated by STING, we evaluated activation of Caspase-8 and Caspase-3 in mouse livers. We found that Caspase-8 and -3 were both activated at 8 and 12 hours after CCl₄ injection in WT mice (**Fig.7A,B**), while the STING-deficient mice (Tmem173^{Gt}) had complete absence of cleaved caspases (**Fig.7C,D**).

To better understand the underlying mechanism behind the protection observed in mice deficient in either IRF3 or STING in a chronic CCl₄ injury, we tested the dynamics of hepatocyte damage by comparing WT mice with Tmem173^{Gt} mice during an acute CCl₄ liver injury. We found protection from liver injury in STING-deficient mice (Tmem173^{Gt}) after CCl₄ administration as seen by serum ALT and H&E histology (**Fig.7E,F**). WT mice exhibited extensive necrosis at 12 hours, peaking at 24 hours, as seen by irregular histological morphology, while the STING-deficient mice did not (**Fig.7F**). Surprisingly, we found that one dose of CCl₄ was sufficient to elicit a pro-fibrogenic response in WT mice as early as 24 hours after injection (**Fig.7G,H**). In comparison to WT mice, the STING-deficient mice (Tmem173^{Gt}) were protected from this early pro-fibrogenic response in the liver.

Our data established that CCl₄-mediated ER stress occurred primarily in hepatocytes and that phospho-IRF3 after ER stress occurs in primary hepatocytes (**Fig.2B,C**). To assess which liver cell type is responsible for IRF3 phosphorylation and caspase activation after CCl₄ administration *in vivo*, we isolated liver mononuclear cells (LMNCs), hepatic stellate cells

(HSCs) and primary hepatocytes from mouse livers 9 hours post CCl₄ injection. We found that phosphorylation of IRF3 occurred predominantly in hepatocytes, and not in LMNCs or HSCs (**Fig.7I**). In assessing cell type-specific contribution to apoptotic signaling, we found increased Casp-8 cleavage in hepatocytes but not in HSCs or LMNCs. Similarly, there was an increase in Caspase-3 cleavage predominantly in hepatocytes, albeit some cleaved Caspase-3 was detected in HSCs (**Fig.7I**). The phosphorylation of IRF3 and Caspase-8 cleavage found exclusively in the hepatocyte population was consistent with our previous findings of TUNEL positive staining in hepatocytes (**Fig.1C**) and in IRF3 association with Caspase-8 and BAX in total liver lysates (**Fig.4I**).

Taken together, this data demonstrated that activation of IRF3 in acute or chronic liver damage is triggered in hepatocytes via the adaptor STING. Deficiency in either STING or IRF3 resulted in significant decreased hepatocyte death and fibrosis.

DISCUSSION Previous studies described the role of STING activation in viral infections in immune cells (26–30). In hepatocytes, we demonstrated earlier that STING and IRF3 mediate the pathogenesis in alcoholic liver disease, a role independent of microbial components (8). In the present study, our data demonstrate a hepatocyte-specific role of STING and IRF3 in mediating hepatic apoptosis as an early event in the development of liver fibrosis.

Hepatocytes are responsible for metabolism and for detoxifying portal blood, which can be rich in bacterial products. For this reason, innate immunity plays an important role in the liver; even though this organ is rich in immune cells, parenchymal cells have an abundance of receptors and are capable of initiating immune signaling (31–34). In our study, TBK1-mediated ER stress was detected early after CCl₄-administration only in hepatocytes and not in LMNCs. Similarly, phosphorylated IRF3 was detected in hepatocytes exposed to CCl₄ *in vivo* but not in LMNCs or HSCs. Furthermore, in support of our hypothesis of the pro-apoptotic role of IRF3, CCl₄-mediated Caspase-8 cleavage was detected only in hepatocytes and was absent from LMNCs and HSCs. Lastly, compared to hepatocytes exposed to CCl₄, Caspase-3 cleavage

was reduced in HSCs and absent in LMNCs. The decreased level of Caspase-3 cleavage in HSCs compared to hepatocytes is likely due to the absence of concurrent phosphorylated IRF3 and Caspase-8 cleavage, suggesting of an alternate mechanism of toxicity and cell death signaling independent of IRF3 in HSCs. It is possible that the mechanisms that tightly regulate apoptotic signaling may also be responsible for hepatic stellate cell activation, leading to fibrogenesis.

Regulated IRE1-dependent decay of mRNA (RIDD), a key component of the unfolded protein response, results in degradation of viral mRNA transcripts and spliced *Xbp1* (13, 35). However, there are multiple lines of evidence suggesting that IRF3 activation can result from other forms of noninfectious cell damage that involve *Xbp1* splicing (17, 18, 36).

Here we show that STING activation may provide the link between induction of ER stress and IRF3. Moreover, the rapid nature of the phosphorylation of IRF3 and *Xbp1* splicing in our experiments due to an acute CCl₄-induced liver injury underscores the importance of STING and its location in the ER.

Viral infections as well as noninfectious injuries are associated with induction of cell death signals, emphasizing the importance of the BH3-domain within IRF3 as responsible for its pro-apoptotic properties in the mitochondria. It is also possible that CCl₄-induced cell death signaling from STING activation of IRF3 and BAX may trigger ER membrane permeability (37) in addition to the effects on the mitochondrial membrane integrity.

A previous study by Knockaert et al. (38) of CCl₄ in mouse livers interestingly found no apoptosis or pro-apoptotic caspase activity at three or 24 hours, the only two time-points they tested. It is important to highlight that our data showed Caspase-8 and -3 cleavage peaking between eight and twelve hours in WT mice, while apoptotic signaling had resolved by 24 hours (**Fig.7A,B**). Importantly, the STING-deficient mice had no detectable Caspase-8 or -3 cleavage (**Fig.7C,D**), indicating protection from apoptosis and secondary cell death.

Moreover, the Knockaert et al. study similarly found CCl₄-mediated necrosis at 24 hours (**Fig.4K,7F**), further supporting our hypothesis that early, and extensive pro-apoptotic

signaling leads to necrosis, or secondary necrosis, which have overlapping components (39). In support of the study by Roychowdhury et al. (40), our data indicate that an absence of early apoptosis is also associated with an absence in early fibrogenesis and detectable necrosis at the level of liver histology in STING-deficient mice.

Pro-apoptotic signaling may play a beneficial role in the resolution of inflammation after liver injury, specifically when it is targeted to infiltrating immune cells such as neutrophils (41). Uncontrolled parenchymal apoptosis, however, although typically controlled and tightly regulated, may be the tipping point responsible for subsequent necrosis.

As summarized in **Fig.7J**, our data suggest that overwhelming levels of IRF3-mediated apoptosis may eventually lead to a form of secondary necrosis. Taken together, our data provides strong evidence that inhibition of parenchymal apoptotic signaling is sufficient to reduce necrosis and amplified liver damage.

In conclusion, our study builds on the previous paradigm of fibrogenesis. The CCl₄-induced liver damage, ER stress and death of hepatocytes is mediated by the engagement of the ER adaptor protein, STING, with phosphorylated IRF3, resulting in activation of the mitochondrial pathway of hepatocyte death. As such, fibrosis is more than a pathology strictly driven by inflammation. Rather, hepatocyte death appears to be a separate key component in the process of liver fibrosis.

EXPERIMENTAL PROCEDURES

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) and IRF7-KO mice (provided by T. Taniguchi, Tokyo, Japan), all on C57Bl/6 background, were used. The STING-deficient (*Tmem173^{Gt}*) mice (provided by R. Vance, University of California, Berkeley, CA) and the TRAM- and TRIF-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 strains. Some mice received a single intraperitoneal (i.p.) injection of carbon tetrachloride (CCl₄), 0.6 μ L/gram body weight, diluted 1:3 in corn oil (Sigma, St. Louis, MO).

Other mice received a six-week regiment of CCl₄ injections, given twice per week. 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.

In vitro experiments—Primary hepatocytes and liver mononuclear cells were isolated as described previously (42). 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 BX795 (10 μ M or 100 μ M) and thapsigargin (1 μ M) were purchased from Sigma (St. Louis, MO).

RNA Analysis—RNA analysis and PCR primers are described in Table 1. *sXbp1* PCR was performed and products were separated on 3% agarose gel as described previously (8).

Protein quantification—Liver whole-cell lysates and nuclear preparations were extracted as described previously (8). 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 (43). Antibodies specific for phospho-IRF3, phospho-TBK1, STING, Casp-8, Casp-3, cleaved Casp-8, cleaved Casp-3 and Bax were from Cell Signaling (Danvers, MA). 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). The TrueBlot system from eBioscience (San Diego, CA) was used for immunoprecipitation assays and performed as per manufacturer's instructions.

Statistical Analysis—Statistical significance was determined using two-sided t-test; Two-way ANOVA was used in Figures 3A-D and Figure 7E to determine the global effect of BX795 on serum ALT. Numbers in the graphs indicate global *P* values. Data are shown as scatter plots of individual data plots \pm S.D. to represent error and were considered statistically significant at *P* <

0.05. Symbols *P < 0.05 vs. baseline; **<0.001 vs. baseline; #P < 0.05 vs. WT CCl₄ condition; ##P < 0.001 vs. WT CCl₄ condition. We used SPSS 19.0 (IBM SPSS, Chicago, IL) and GraphPad Prism 6.0 (San Diego, CA) for calculations.

Acknowledgements: The authors thank members of the Szabo Lab, as well as the Programs in Translational Science and Biochemistry and Molecular Pharmacology at the University of Massachusetts Medical School for their support.

Financial support: This work was supported by grant AA017729 from NIAAA (to GS) and PA-12-149 from NIAAA (to AIV) as supplement to grant 1R01AA020744-02 (to GS). Core resources supported by Diabetes Endocrinology Research Center grant DK32520 were also used. Research reported in this manuscript was supported by the National Institute on Alcohol Abuse and Alcoholism of the National Institutes of Health under Award Number F31AA025545 (to AIV). The content is solely the responsibility of the authors and does not necessarily represent official views of the National Institutes of Health.

Conflicts of interest: The authors declare that they have no conflicts of interest with the contents of this article.

Author contributions: AIV, JP and GS designed research; AIV, JP, BG, AS, PL, CDC, KK, and DC performed research; EAKJ and KAF contributed new reagents/analytic tools; AIV, JP, and GS analyzed data; AIV, JP, and GS wrote manuscript; all authors reviewed the results and approved the final version of the manuscript.

REFERENCES

1. Seki, E., Minicis, S., Osterreicher, C., Kluwe, J., Osawa, Y., Brenner, D., and Schwabe, R. (2007) TLR4 enhances TGF-beta signaling and hepatic fibrosis. *Nature medicine* **13**, 1324–32
2. Rivera, Bradford, and Hunt (2001) Attenuation of CCl₄-induced hepatic fibrosis by GdCl₃ treatment or dietary glycine.
3. Malhi, H., and Gores, G. J. (2008) Cellular and molecular mechanisms of liver injury. *Gastroenterology* **134**, 1641–54
4. Danial, N., and Korsmeyer, S. (2004) Cell death: critical control points. *Cell* **116**, 205–19
5. Jiang, X., and Wang, X. (2004) Cytochrome C-mediated apoptosis. *Annual review of biochemistry* **73**, 87–106
6. Chattopadhyay, S., Marques, J. T., Yamashita, M., Peters, K. L., Smith, K., Desai, A., Williams, B. R., and Sen, G. C. (2010) Viral apoptosis is induced by IRF-3-mediated activation of Bax. *EMBO J.* **29**, 1762–73
7. Chattopadhyay, S., Fensterl, V., Zhang, Y., Veleparambil, M., Yamashita, M., and Sen, G. C. (2013) Role of interferon regulatory factor 3-mediated apoptosis in the establishment and maintenance of persistent infection by Sendai virus. *J. Virol.* **87**, 16–24
8. Petrasek, J., Iracheta-Vellve, A., Csak, T., Satischchandran, A., Kodys, K., Kurt-Jones, E. A., Fitzgerald, K. A., and Szabo, G. (2013) STING-IRF3 pathway links endoplasmic reticulum stress with hepatocyte apoptosis in early alcoholic liver disease. *Proc. Natl. Acad. Sci. U.S.A.* **110**, 16544–9
9. Shi, Aisaki, Ikawa, and Wake (1998) Evidence of hepatocyte apoptosis in rat liver after the administration of carbon tetrachloride.
10. Zakim, D., and Boyer, T. D. (2002) *Hepatology: A Textbook of Liver Diseases*, Saunders; 4 edition, p.746
11. Sun, Hamagawa, Tsutsui, Ono, Ogiri, and Kojo (2001) Evaluation of oxidative stress during apoptosis and necrosis caused by carbon tetrachloride in rat liver. *Biochimica et biophysica acta* **1535**,

186–91

12. Nakatsukasa, Nagy, Evarts, Hsia, Marsden, and Thorgeirsson (1990) Cellular distribution of transforming growth factor-beta 1 and procollagen types I, III, and IV transcripts in carbon tetrachloride-induced rat liver fibrosis. *The Journal of clinical investigation* **85**, 1833–43
13. Maurel, Chevet, Tavernier, and Gerlo (2014) Getting RIDD of RNA: IRE1 in cell fate regulation. *Trends in biochemical sciences* **39**, 245–54
14. Upton, J.-P. P., Wang, L., Han, D., Wang, E. S., Huskey, N. E., Lim, L., Truitt, M., McManus, M. T., Ruggero, D., Goga, A., Papa, F. R., and Oakes, S. A. (2012) IRE1 α cleaves select microRNAs during ER stress to derepress translation of proapoptotic Caspase-2. *Science* **338**, 818–22 [online] <http://www.ncbi.nlm.nih.gov/pubmed/23042294>.
15. Walter, P., and Ron, D. (2011) The unfolded protein response: from stress pathway to homeostatic regulation. *Science (New York, N.Y.)* **334**, 1081–6
16. Schröder, M., and Kaufman, R. (2005) The mammalian unfolded protein response. *Annual review of biochemistry* **74**, 739–89
17. Liu, Y.-P. P., Zeng, L., Tian, A., Bomkamp, A., Rivera, D., Gutman, D., Barber, G. N., Olson, J. K., and Smith, J. A. (2012) Endoplasmic reticulum stress regulates the innate immunity critical transcription factor IRF3. *J. Immunol.* **189**, 4630–9
18. Hu, F., Yu, X., Wang, H., Zuo, D., Guo, C., Yi, H., Tirosh, B., Subjeck, J., Qiu, X., and Wang, X.-Y. (2011) ER stress and its regulator X-box-binding protein-1 enhance polyIC-induced innate immune response in dendritic cells. *European journal of immunology* **41**, 1086–97
19. Paik, Y.-H., Schwabe, R., Bataller, R., Russo, M., Jobin, C., and Brenner, D. (2003) Toll-like receptor 4 mediates inflammatory signaling by bacterial lipopolysaccharide in human hepatic stellate cells. *Hepatology (Baltimore, Md.)* **37**, 1043–55
20. Soares, J.-B., Pimentel-Nunes, P., Roncon-Albuquerque, R., and Leite-Moreira, A. (2010) The role of lipopolysaccharide/toll-like receptor 4 signaling in chronic liver diseases. *Hepatology international* **4**, 659–72
21. Sauer, J.-D. D., Sotelo-Troha, K., von Moltke, J., Monroe, K. M., Rae, C. S., Brubaker, S. W., Hyodo, M., Hayakawa, Y., Woodward, J. J., Portnoy, D. A., and Vance, R. E. (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**, 688–94
22. Servant, M., Tenoever, B., and Lin, R. (2002) Overlapping and distinct mechanisms regulating IRF-3 and IRF-7 function. *Journal of interferon & cytokine research : the official journal of the International Society for Interferon and Cytokine Research* **22**, 49–58
23. Roh, Y., Park, S., Kim, J., Lim, C., Seki, E., and Kim, B. (2014) Toll-like receptor 7-mediated type I interferon signaling prevents cholestasis- and hepatotoxin-induced liver fibrosis. *Hepatology (Baltimore, Md.)* **60**, 237–49
24. Petrasek, J., Dolganiuc, A., Csak, T., Nath, B., Hritz, I., Kodys, K., Catalano, D., Kurt-Jones, E., Mandrekar, P., and Szabo, G. (2011) Interferon regulatory factor 3 and type I interferons are protective in alcoholic liver injury in mice by way of crosstalk of parenchymal and myeloid cells. *Hepatology* **53**, 649–60
25. Liu, S., Cai, X., Wu, J., Cong, Q., Chen, X., Li, T., Du, F., Ren, J., Wu, Y.-T. T., Grishin, N. V., and Chen, Z. J. (2015) Phosphorylation of innate immune adaptor proteins MAVS, STING, and TRIF induces IRF3 activation. *Science* **347**, aaa2630
26. Tanaka, Y., and Chen, Z. J. (2012) STING specifies IRF3 phosphorylation by TBK1 in the cytosolic DNA signaling pathway. *Sci Signal* **5**, ra20
27. Abe, T., Harashima, A., Xia, T., Konno, H., Konno, K., Morales, A., Ahn, J., Gutman, D., and Barber, G. N. (2013) STING Recognition of Cytoplasmic DNA Instigates Cellular Defense. *Mol. Cell* [online] <http://www.ncbi.nlm.nih.gov/pubmed/23478444>.
28. Ishikawa, H., Ma, Z., and Barber, G. N. (2009) STING regulates intracellular DNA-mediated, type I interferon-dependent innate immunity. *Nature* **461**, 788–92
29. Burdette, D., Monroe, K., Sotelo-Troha, K., Iwig, J., Eckert, B., Hyodo, M., Hayakawa, Y., and

- Vance, R. (2011) STING is a direct innate immune sensor of cyclic di-GMP. *Nature* **478**, 515–518
30. Xia, T., Konno, H., Ahn, J., and Barber, G. (2015) Deregulation of STING Signaling in Colorectal Carcinoma Constrains DNA Damage Responses and Correlates With Tumorigenesis. *Cell reports*
31. Gao, B., Jeong, W.-I. I., and Tian, Z. (2008) Liver: An organ with predominant innate immunity. *Hepatology* **47**, 729–36
32. Akira, S., Uematsu, S., and Takeuchi, O. (2006) Pathogen recognition and innate immunity. *Cell* **124**, 783–801
33. Deminice, R., de Castro, G. S., Brosnan, M. E., and Brosnan, J. T. (2016) Creatine supplementation as a possible new therapeutic approach for fatty liver disease: early findings. *Amino Acids*
34. Zhou, Z., Xu, M.-J. J., and Gao, B. (2015) Hepatocytes: a key cell type for innate immunity. *Cell. Mol. Immunol.*
35. Plumb, R., Zhang, Z.-R., Appathurai, S., and Mariappan, M. (2015) A functional link between the co-translational protein translocation pathway and the UPR. *eLife* **4**
36. Smith, J., Turner, M., DeLay, M., Klenk, E., Sowders, D., and Colbert, R. (2008) Endoplasmic reticulum stress and the unfolded protein response are linked to synergistic IFN-beta induction via X-box binding protein 1. *European journal of immunology* **38**, 1194–203
37. Wang, Olberding, White, and Li (2011) Bcl-2 proteins regulate ER membrane permeability to luminal proteins during ER stress-induced apoptosis. *Cell death and differentiation* **18**, 38–47
38. Knockaert, L., Berson, A., Ribault, C., Prost, P.-E. E., Fautrel, A., Pajaud, J., Lepage, S., Lucas-Clerc, C., Bégue, J.-M. M., Fromenty, B., and Robin, M.-A. A. (2012) Carbon tetrachloride-mediated lipid peroxidation induces early mitochondrial alterations in mouse liver. *Lab. Invest.* **92**, 396–410
39. Vandenabeele, P., Galluzzi, L., Berghe, T., and Kroemer, G. (2010) Molecular mechanisms of necroptosis: an ordered cellular explosion. *Nature reviews. Molecular cell biology* **11**, 700–14
40. Roychowdhury, S., Chiang, D., Mandal, P., McMullen, M., Liu, X., Cohen, J., Pollard, J., Feldstein, A., and Nagy, L. (2012) Inhibition of apoptosis protects mice from ethanol-mediated acceleration of early markers of CCl₄-induced fibrosis but not steatosis or inflammation. *Alcoholism, clinical and experimental research* **36**, 1139–47
41. Fox, S., Leitch, A. E., Duffin, R., Haslett, C., and Rossi, A. G. (2010) Neutrophil apoptosis: relevance to the innate immune response and inflammatory disease. *J Innate Immun* **2**, 216–27

FOOTNOTES

The abbreviations used are: Interferon Regulatory Factor 3 (IRF3); interferons (IFNs); Stimulator of Interferon Genes (STING); carbon tetrachloride (CCl₄); hepatic stellate cells (HSC); endoplasmic reticulum (ER); alpha-smooth muscle actin (α -SMA); unfolded protein response (UPR); Type-I IFN receptor (IFNAR1); TIR-domain-containing adapter-inducing interferon- β (TRIF); TRIF-related adaptor molecule (TRAM); Tank-binding Kinase 1 (TBK1); Regulated IRE1-dependent decay of mRNA (RIDD).

FIGURE LEGENDS

FIGURE 1. IRF3 deficiency attenuates chronic CCl₄-mediated liver injury and fibrosis. WT or IRF3-KO mice injected with oil or CCl₄ for six weeks and sacrificed 48 hours after final injection. Liver injury was assessed by serum ALT (A) and H&E staining (B). Liver apoptosis was assessed via TUNEL staining (black arrows) on paraffin-embedded liver sections (C). Liver fibrosis was evaluated by Sirius-red staining (D) and alpha-smooth muscle actin immunohistochemistry (E), and quantified using ImageJ. mRNA expression in liver for *Acta2* and *Colla2* (F) as well as *Ifnb1* and *Isg15* (G) were measured by RT-PCR to assess liver fibrosis and IRF3 activation, respectively. N= 6-7 mice (CCl₄-treated, per genotype); 3-4 mice (oil-treated, per genotype).

FIGURE 2. *Acute CCl₄ induces ER stress and TBK1-mediated phosphorylation of IRF3 in hepatocytes.* WT mice received a single injection of corn oil or CCl₄. At indicated time points, mice were sacrificed, and splicing of *Xbp1* mRNA (*sXBP1*) in the liver, indicating ER stress, was evaluated by PCR, and products were separated on 3% agarose gel (A). Two hours after CCl₄ administration, hepatocytes or LMNCs from WT mice were isolated and analyzed for *sXBP1* by PCR (B). At indicated time points, phosphorylation of IRF3 was assessed by immunoblotting in liver (C) while *Ifnb1* mRNA expression in liver and as serum IFN-β were measured by RT-PCR and ELISA, respectively (D). N= 4 mice per time point. Primary hepatocytes isolated from WT mice were treated with 1μM thapsigargin, and were pretreated with DMSO or BX795 (10μM or 100μM), and analyzed for phospho-IRF3 (E).

FIGURE 3. *Acute CCl₄-induced liver injury is ameliorated by the deficiency of IRF3 or inhibition of TBK1, independent of TRIM or TRAM, Type-I IFN signaling.* WT or IRF3-KO mice received a single injection of CCl₄. Serum ALT levels were assessed (A) and apoptosis was assessed by measuring Caspase-3 cleavage in the liver by immunoblot (B). WT or IFNAR1-KO mice received a single injection of CCl₄. Serum ALT levels were assessed (C) and IRF3 activation and apoptosis were assessed by probing for phospho-IRF3 and Caspase-3 cleavage in the liver by immunoblot (D). WT were pretreated with DMSO or BX795, a TBK1-inhibitor, two hours before receiving a single injection of corn oil (vehicle) or CCl₄. Serum ALT levels were assessed (E) and IRF3 activation and apoptosis were assessed by probing for phospho-IRF3 and Caspase-3 cleavage in the liver by immunoblot (F). WT, TRIF- or TRAM-KO mice received a single injection of CCl₄. Serum ALT levels were assessed (G) and IRF3 activation and apoptosis were assessed by probing for phospho-IRF3 and Caspase-3 cleavage in the liver by immunoblot (H). Mice were bled serially at indicated time points. N= 8-9 mice (CCl₄-treated, per genotype); 3-4 mice (oil-treated, per genotype) (A,C,G). N= 3-5 mice (CCl₄-treated, pre-treated with BX795); 3 mice (oil-treated, pre-treated with DMSO) (E).

FIGURE 4. *Acute CCl₄ induces early association of IRF3 with STING in the ER and association of IRF3 with Bax in the mitochondria in the liver.* WT mice received a single injection of CCl₄. At indicated time points, mice were sacrificed, and phosphorylation of IRF3 was assessed by immunoblotting in liver whole-cell lysate (A) or cytoplasmic (B), nuclear (C), ER (D), and mitochondrial (E) extracts. Using immunoprecipitation with anti-total IRF3 antibody for protein pull-down, we evaluated association between total IRF3 and phosphorylated TBK1 or STING in the whole-cell (F), mitochondrial (G), and ER (H) extracts. Similarly, we evaluated association between total IRF3 and cleaved Caspase-8 p43 fragment or BAX in liver whole-cell lysate (I), with representative IgG control shown (two hours after injection). At indicated time points, cleavage of Caspase-8 and Caspase-3 was assessed by immunoblotting in liver (J). Liver injury and apoptosis was assessed by H&E liver histology and TUNEL staining on paraffin-embedded liver sections (K). N= 4 mice per time point.

FIGURE 5. *Deficiency in STING, but not in Type-I IFN signaling, attenuates liver fibrosis.* WT or STING-deficient mice (*Tmem173^{Gt}*) were injected with oil or CCl₄ for six weeks and sacrificed 48 hours after final injection. Liver injury was assessed by serum ALT (A) and H&E staining (B). Liver fibrosis was evaluated by measuring Sirius-red staining and the percent-positive area stained was quantified using ImageJ (C). Liver fibrosis was also evaluated by immunoblot for alpha-smooth muscle actin (D) or Type-1 collagen (E). mRNA expression in liver for *Acta2* and *Colla2* (F) as well as *Ifnb1* and *Isg15* (G) were measured by PCR to assess liver fibrosis and IRF3 activation, respectively. WT, IRF7- or IFNAR1-KO mice injected with oil or CCl₄ for six weeks and sacrificed 48 hours after final injection. Liver injury was assessed by serum ALT (H) and H&E staining (I). Liver fibrosis was evaluated by Sirius-red staining and quantified using ImageJ (J). mRNA expression in liver for *Acta2* and *Colla2* (K) as well as *Ifnb1* and *Isg15* (L) were measured by RT-PCR. N= 8-9 mice (CCl₄-treated, per genotype); 3-4 mice (oil-treated, per genotype).

FIGURE 6. *STING* mediates pro-apoptotic activation of IRF3 in a chronic CCl₄-induced model of fibrosis. WT or STING-deficient mice (Tmem173^{Gt}) were injected with oil or CCl₄ for six weeks and sacrificed. Whole cell liver lysates were probed for phosphorylated TBK1 (A), phosphorylated IRF3 (B), cleaved Caspase-8 (C), and cleaved Caspase-3 (D) by immunoblot. N= 8-9 mice (CCl₄-treated, per genotype); 3-4 mice (oil-treated, per genotype).

FIGURE 7. Early pro-apoptotic activation of IRF3 by CCl₄ is hepatocyte-specific and mediated by *STING*. WT or STING-deficient mice (Tmem173^{Gt}) received a single injection of CCl₄ or oil (baseline) and sacrificed at indicated time-points. Whole cell liver lysates were probed for cleaved Caspase-8 (A,B), and cleaved Caspase-3 (C,D) by immunoblot. Liver injury was assessed by serum ALT (E) and H&E staining at indicated time points (F). Deposition of alpha-smooth muscle actin (α -SMA) 24hrs after injection was evaluated by immunoblots from whole cell liver lysate (G), while mRNA expression in liver for *Acta2* and *Colla2* (H) was assessed by PCR. N= 5-8 mice per time point, per genotype (A-H). WT mice received a single injection of CCl₄ or oil (baseline) and sacrificed nine hours later. Liver mononuclear cells (LMNC), hepatic stellate cells (HSC) or hepatocytes were isolated from livers and probed for phosphorylated IRF3, total IRF3, cleaved Caspase-8 and cleaved Caspase-3 by immunoblot (I). N=3 per condition (LMNC and HSC were pooled from 3 mice); N=1 (hepatocytes isolated from single mouse per condition); experiment was repeated three times (I). Schematic of pro-apoptotic STING and IRF3 activation from CCl₄ administration in mice (J). In CCl₄-treated hepatocytes, ER stress results in phosphorylation of TBK1 via *STING*, followed by phosphorylation of IRF3. IRF3 associates with BAX in the mitochondria through its BH3-only domain, leading to pro-apoptotic Caspase-3 activation and hepatocyte apoptosis. After chronic CCl₄ administration, hepatocyte apoptosis is associated with secondary necrosis, which results in liver fibrosis.

Table 1
qPCR primers

Target gene	Forward primer(5' → 3')	Reverse primer(5' → 3')
<i>18S</i>	gtaaccggtgaacccatt	ccatccaatcggtagtagcg
<i>Tnfa</i>	caccacatcaaggactcaa	aggcaacctgaccactctcc
<i>Ifnb</i>	agctccaagaaggacgaacat	gcctgtaggtaggggtgatct
<i>Isg15</i>	caggacggtcttacccttcc	aggctcgctgcagttctgtac
<i>Xbp1</i>	acacgcttgggaatggacac	ccatgggaagatgttctggg
<i>Acta2</i>	acacgcttgggaatggacac	ttcctgaccactagaggggg
<i>Colla2</i>	ggagggacggtccacgat	gagtcgcgatccacaa

Fig. 1

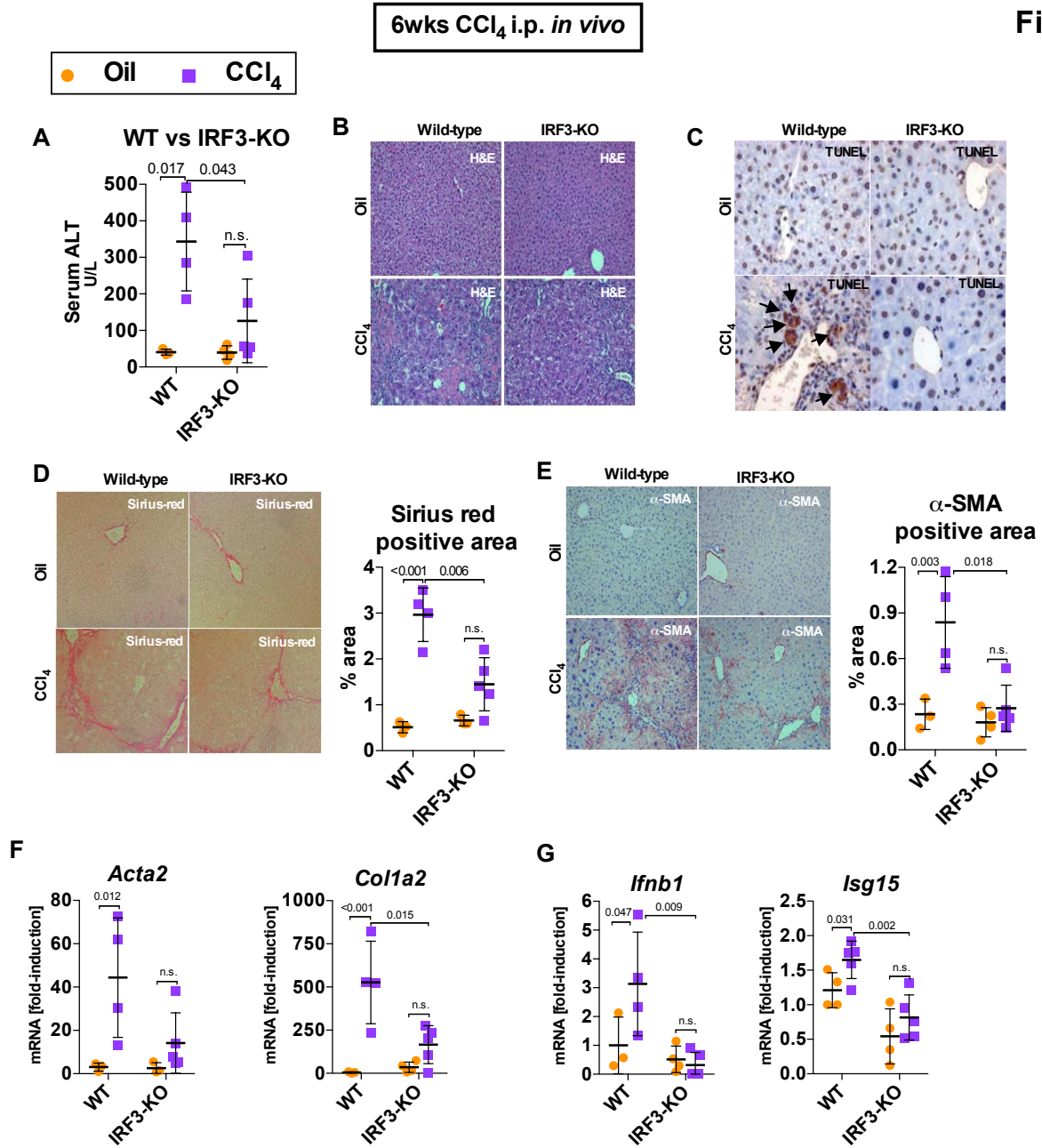


Fig. 2

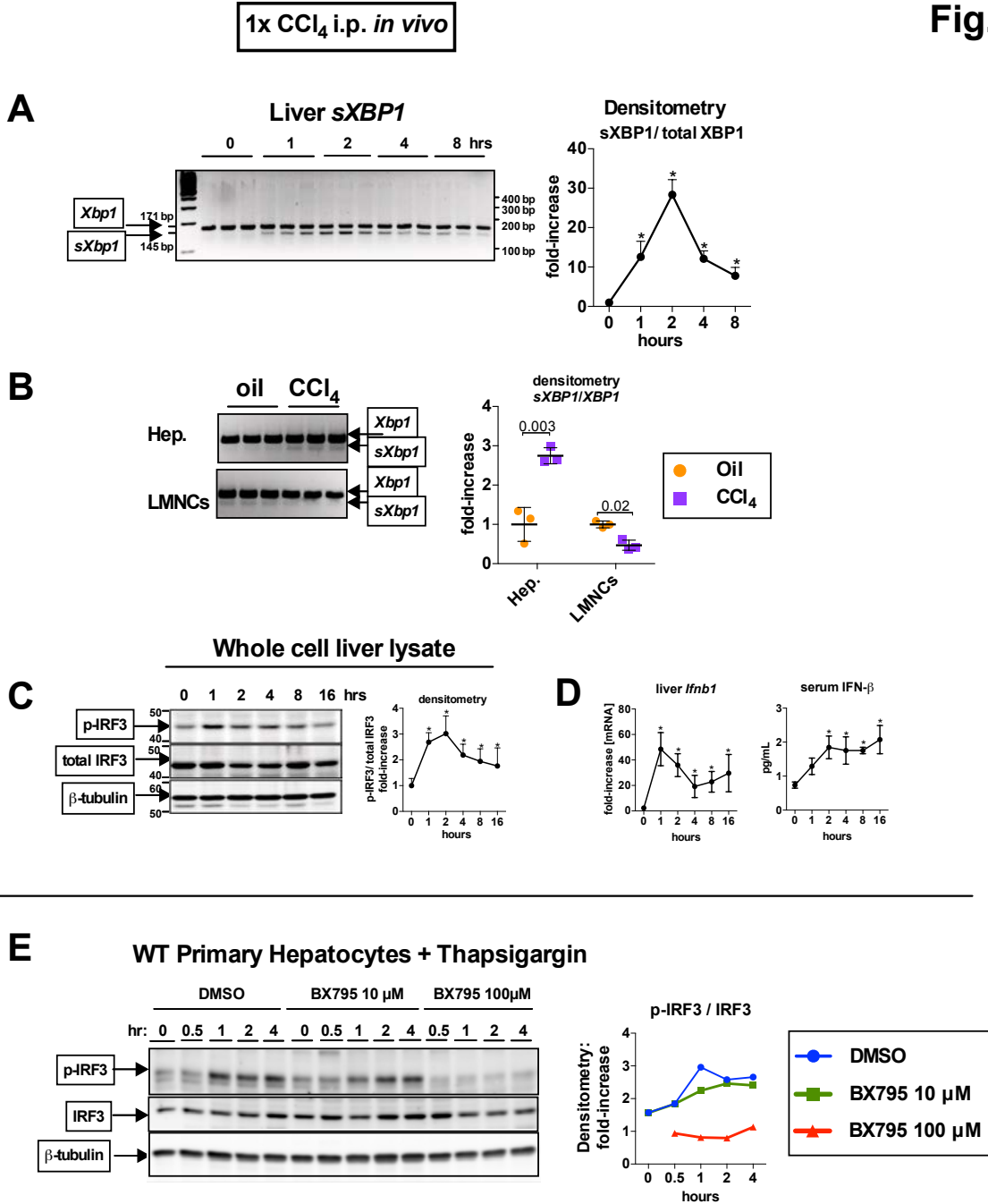


Fig. 3

1x CCl₄ i.p. *in vivo*

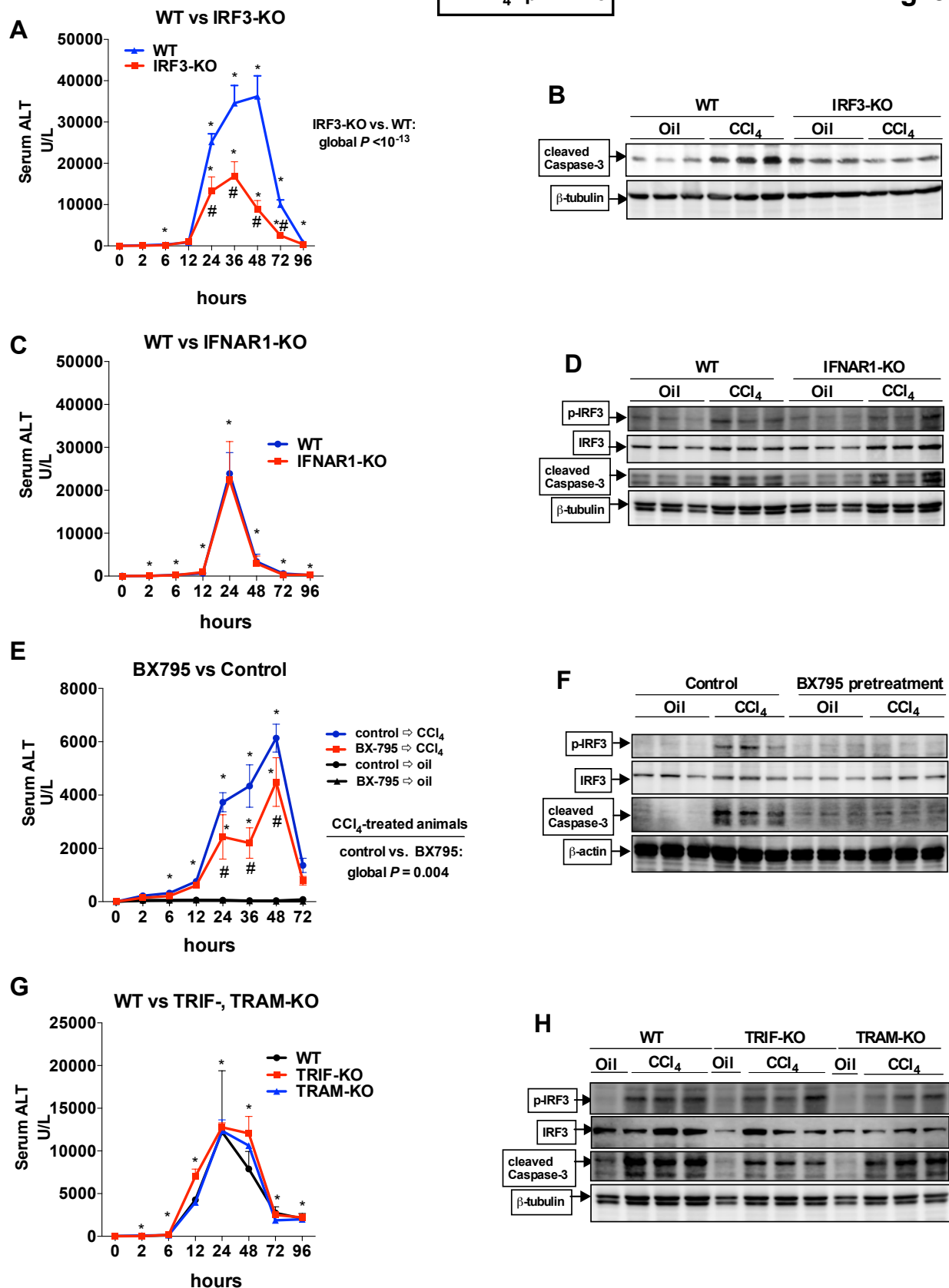


Fig. 4

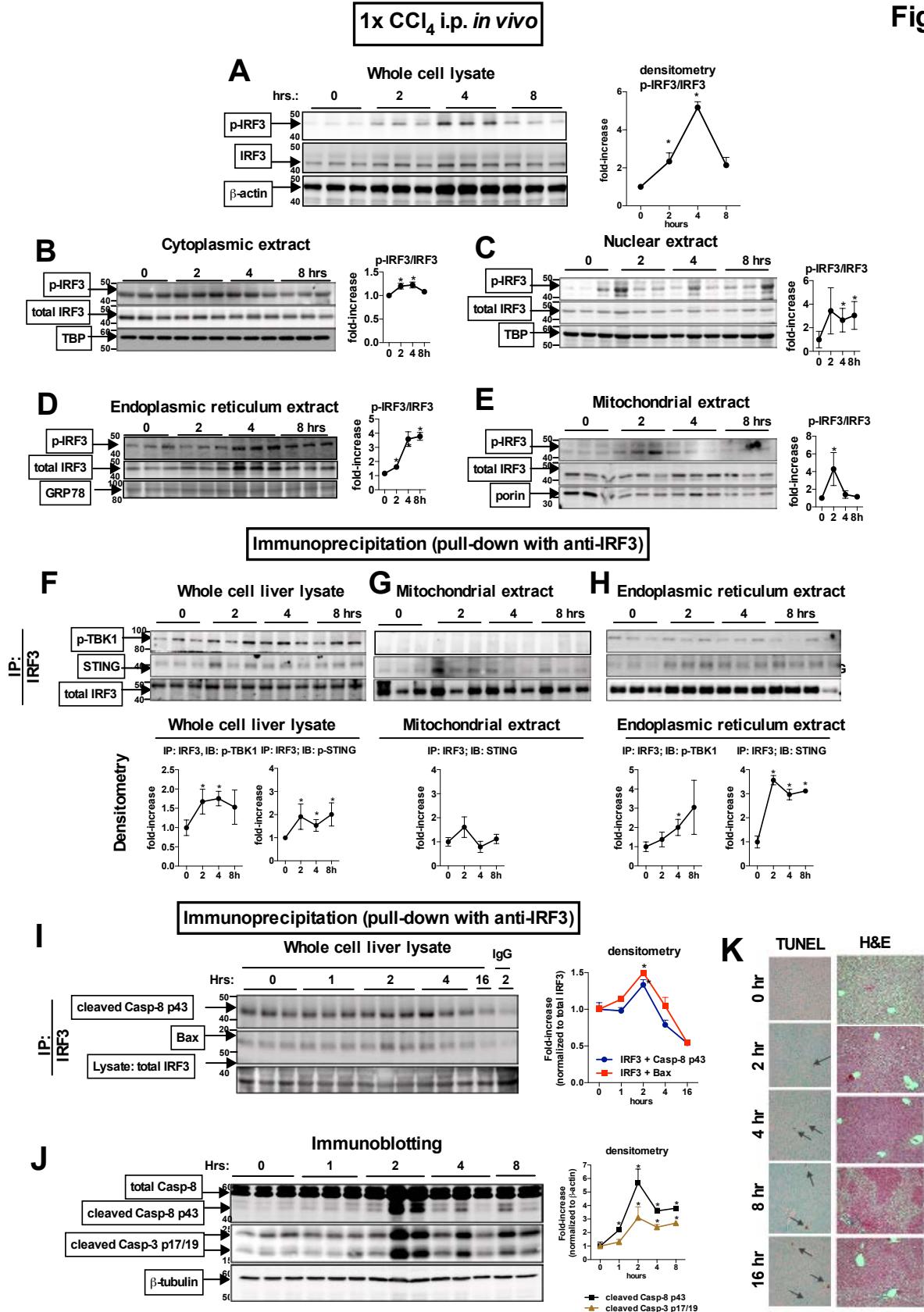


Fig. 5

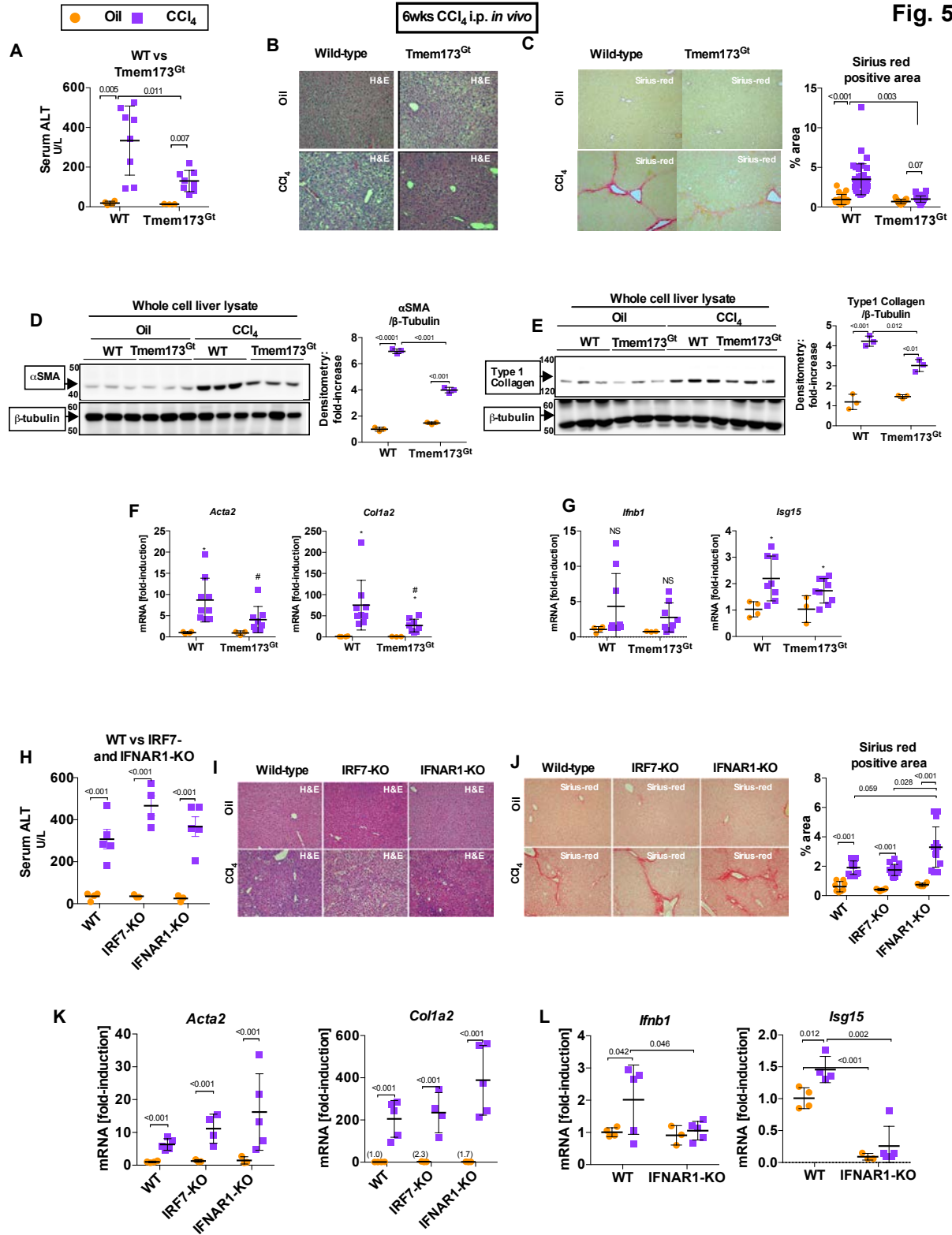
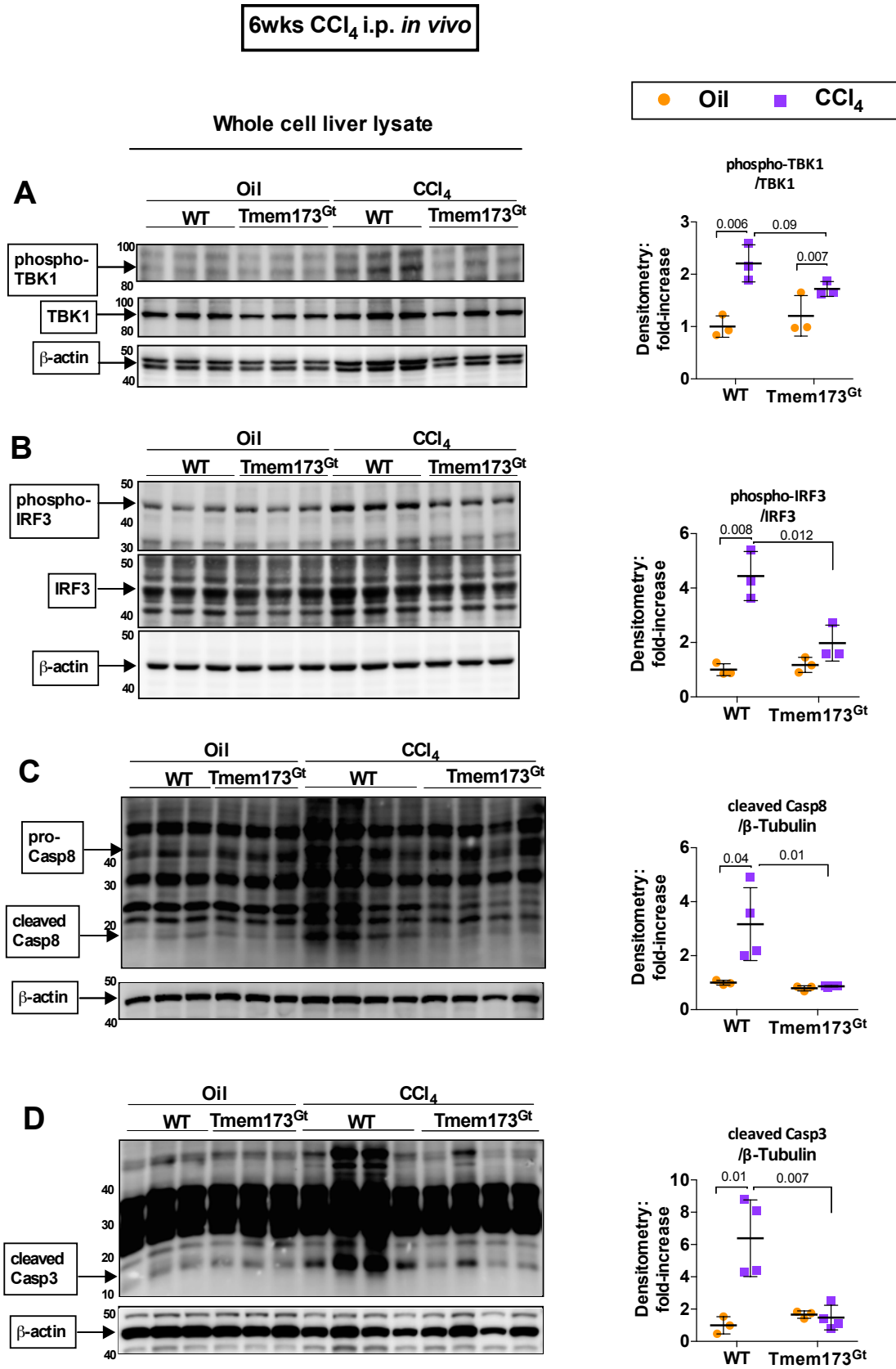


Fig. 6



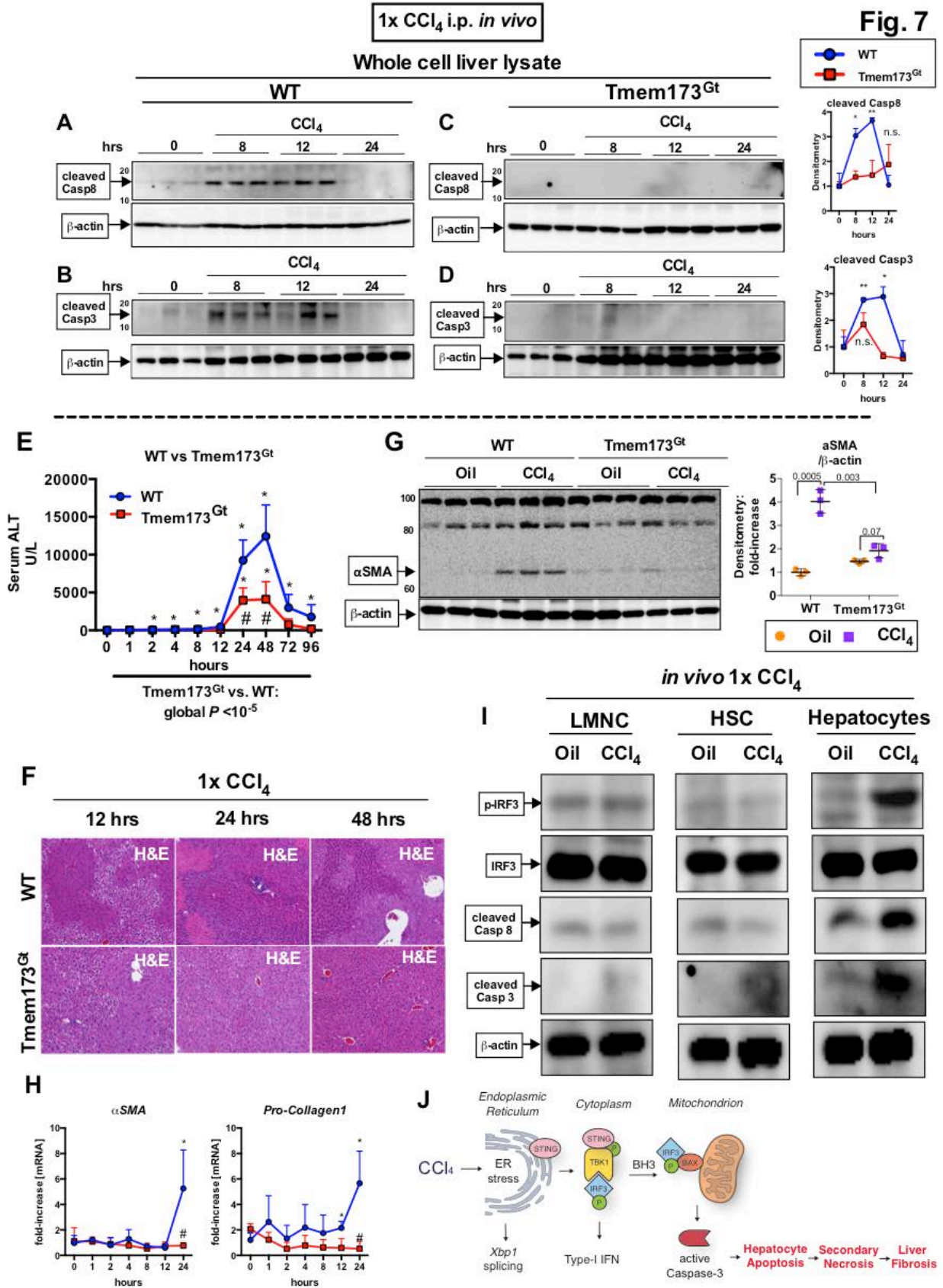


Fig. 7

● WT
■ Tmem173^{Gt}

Endoplasmic reticulum stress-induced hepatocellular death pathways mediate liver injury and fibrosis via Stimulator of Interferon Genes

Arvin Iracheta-Vellve, Jan Petrasek, Benedek Gyongyosi, Abhishek Satishchandran, Patrick Lowe, Karen Kodys, Donna Catalano, Charles D. Calenda, Evelyn A. Kurt-Jones, Katherine A. Fitzgerald and Gyongyi Szabo

J. Biol. Chem. published online November 3, 2016

Access the most updated version of this article at doi: [10.1074/jbc.M116.736991](https://doi.org/10.1074/jbc.M116.736991)

Alerts:

- [When this article is cited](#)
- [When a correction for this article is posted](#)

[Click here](#) to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at <http://www.jbc.org/content/early/2016/11/03/jbc.M116.736991.full.html#ref-list-1>