HHS Public Access

Author manuscript

Hepatology. Author manuscript; available in PMC 2022 September 01.

Published in final edited form as:

Hepatology. 2021 September ; 74(3): 1234–1250. doi:10.1002/hep.31800.

Stress-responsive gene FKBP5 mediates alcohol-induced liver injury through the hippo pathway and CXCL1 signaling

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Abstract

Chronic alcohol drinking is a major risk factor for alcohol-associated liver disease (ALD). FK506-binding protein 51 (FKBP5), a co-chaperone protein, is involved in many key regulatory pathways. It is known to be involved in stress-related disorders but there are no reports regarding its role in ALD. This present study aimed to examine the molecular mechanism of FKBP5 in ALD. We found a significant increase in hepatic FKBP5 transcripts and protein expression in patients with ALD and mice fed with chronic-plus-single binge ethanol. Loss of *Fkbp5* in mice protected against alcohol-induced hepatic steatosis and inflammation. Transcriptomic analysis revealed a significant reduction of *Tead1* and *Cxcl1* mRNA in ethanol-fed *Fkbp5*$^{-/-}$ mice. Ethanol-induced *Fkbp5* expression was secondary to downregulation of methylation level at its 5′ UTR promoter region. The increase in *Fkbp5* expression led to induction in transcription factor Tead1 through Hippo signaling pathway. Fkbp5 can interact with YAP upstream kinase, MST1, affecting its ability to phosphorylate YAP and the inhibitory effect of hepatic YAP phosphorylation by ethanol leading to YAP nuclear translocation and TEAD1 activation. Activation of TEAD1 led to increased expression of its novel target, CXCL1, a chemokine-mediated neutrophil recruitment, causing hepatic inflammation and neutrophil infiltration in our mouse model.

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Author's contribution

PK, ZY, SL: study concept and design; PK, TL, TZ, RAR, SH, KC, AO, YJ, NH, and JM: acquisition of data and performed the experiments; PK, TL, ZY, SL: analysis and interpretation of data; PK and SL: drafting of the manuscript; PK, NS, AD, RAH, ZY, JM, and SL: a critical review of the manuscript, PK, ZY, and SL: Finalizing the manuscript. All authors have read and approved the manuscript for submission.

Conflict of interest: None of the authors have any conflicts of interest with this work

Conclusion: We identified a novel FKBP5-YAP-TEAD1-CXCL1 axis in the pathogenesis of ALD. Loss of FKBP5 ameliorates alcohol-induced liver injury through the Hippo pathway and CXCL1 signaling, suggesting its potential role as a target for the treatment of ALD.

Keywords

FKBP5; alcohol-associated liver disease; YAP/TAZ

Introduction

Alcohol-associated liver disease (ALD) consists of a spectrum of histopathological abnormalities ranging from steatosis, steatohepatitis, and cirrhosis.[1] The cornerstone in the management of ALD is abstinence; however, no approved pharmacological therapies specifically for ALD are currently available. Understanding the underlying mechanism in ALD pathogenesis is of importance, as this may lead to targeted intervention for the treatment of ALD.[2]

FK506-binding protein-51 (FKBP51, encoded by the *FKBP5* gene, is also called FKBP5) belongs to the FKBP family of immunophilins.[3] It is a co-chaperone protein best known as a negative regulator of the glucocorticoid receptor and mediates the stress response.[4] FKBP5 is an important protein involved in the regulation of many key signaling cascades in the cell. The increased expression of FKBP5 has been linked to stress-related and psychiatric disorders such as depression and post-traumatic stress disorder.[5–7] In fact, the *FKBP5* gene was found to be highly expressed in particular brain regions responsible for stress response.[8, 9] Several lines of evidence suggested the role of FKBP5 as a metabolic stressor. First, the increase in expression of hypothalamic *Fkbp5* was observed in mice fed with high-fat diet.[10, 11] Second, its expression was observed in tissues such as the liver, adipocytes, and skeletal muscle, which are responsible for metabolic regulation.[12, 13] *FKBP5* gene expression levels in subcutaneous adipose tissue positively correlated with indices of insulin resistance.[12] Overexpression of *Fkbp5* increased the susceptibility of diet-induced obesity in mice, while *Fkbp5* knockout (KO) mice fed with a high-fat diet were protected against hepatic steatosis.[11] In a recent study the role of FKBP5 in regulating metabolic disease was illustrated; *Fkbp5* KO mice showed improvement in glucose and insulin sensitivity and were protected from high-fat diet-induced weight gain.[14] Treatment with a novel FKBP5 antagonist, SAFit2, had the similar effect of *Fkbp5* deletion in restoring metabolic derangements in mice fed with high-fat diet suggesting the potential therapeutic role of FKBP5 in metabolic abnormalities.[14]

The *FKBP5* gene has been reported to enhance sensitivity to alcohol consumption and alcohol withdrawal.[8, 15] Alcohol is considered as a metabolic and cellular stressor [16]; however, the role of FKBP5 in the pathogenesis of ALD has not been reported. We found that FKBP5 knockdown ameliorates alcohol-induced liver injury through the hippo pathway and CXCL1 signaling. Our study provides a new line of evidence that FKBP5 may be a therapeutic target for ALD.

Materials and Methods

Animal experiments

Full details are outlined in Supplementary Materials and Supplementary Fig. S1.

Total RNA extraction and quantitative real-time PCR analysis

Full details are outlined in Supplementary Materials. The primer sequences are provided in Supplementary Table 1.

Bisulfite conversion and PCR

Genomic DNA was extracted from the WT and WT-E mice liver (n=3). DNA (500 ng) was converted using EZ-DNA methylation Direct Kit (Cat# D5020, Zymo Research, Irvine, CA) following the manufacturer's instructions. Bisulfite converted DNA samples were purified and subjected to qPCR for quantification. For *in vitro* experiments, AML-12 cells and primary mouse hepatocytes were treated with 5-aza-2′-deoxycytidine (5-aza-dC), an agent inducing DNA hypomethylation (Cat# A3656, Millipore Sigma, St.Louis, MO), at indicated concentrations for 1 hour, then further treated with or without ethanol for another 24 hrs. The AML-12 and primary mouse hepatocytes genomic DNAs were then purified for bisulfite treatment as described above. Primers used for detecting methylation are shown in Supplementary Table 2.

Chromatin immunoprecipitation

Full details are outlined in Supplementary Materials. The primers which were used are listed in Supplementary Table 3.

Supplementary materials

Detailed methods for cell culture and treatment, human liver samples, histopathological analysis, immunohistochemistry, immunofluorescence staining, serum alanine, aspartate aminotransferase, hepatic triglyceride measurement, hepatic cytokine measurements, RNASeq, lipidomics, and data analysis, *Fkbp5* gene overexpression and silencing, primary mouse hepatocyte isolation, immunoblotting and co-immunoprecipitation, gRNAs selections and cloning, and luciferase assay, can be found in Supplementary materials.

Statistical Analysis

All data are presented as means ± standard error of the mean (SEM) analyzed with Graph Pad Prism 9 (San Diego, CA). Statistical evaluation was performed by Student's t-test or one-way ANOVA with Tukey's post hoc test for multiple comparisons as appropriate.

Results

Hepatic FKBP5 is significantly elevated in patients with alcohol-associated liver disease

To investigate the role of FKBP5 in alcohol-associated liver diseases, we first analyzed the level of *FKBP5* expression in a total of ten human liver specimens from patients with alcoholic cirrhosis and five control liver samples. We observed a two-fold upregulation

of *FKBP5* mRNA expression in the liver samples from patients with alcoholic cirrhosis compared to controls (p=0.01, Fig. 1A). We also found a significant increase of FKBP5 protein expression by three-fold (p=0.001, Fig. 1B). Immunohistochemistry staining of FKBP5 in the liver sections also confirmed a higher expression of hepatic FKBP5 in patients with alcoholic cirrhosis (Fig. 1C).

Hepatic Fkbp5 expression is significantly elevated in chronic-plus-single binge ethanol-fed mice and deletion of Fkbp5 ameliorates hepatic phenotypes of ALD

To elucidate the role of FKBP5 in the pathogenesis of ALD, wild type, and *Fkbp5*$^{-/-}$ mice were fed with either control or ethanol-containing diet for 10 days followed by a single binge of maltose or ethanol. As per the pair feeding design, the calorie intake was similar during the feeding experiments in each group (Supplementary Fig. S1). There was no difference in the mean daily alcohol consumption and body weight at the end of experiments in WT and *Fkbp5*$^{-/-}$ mice fed with ethanol (Supplementary Fig. S2 A **and** B).

Real-time RT-PCR analysis demonstrated that hepatic *Fkbp5* mRNA and its protein expression were significantly elevated in WT-E fed mice when compared to pair-fed controls (p<0.0001, Figs. 2A and 2B). The increase in the expression was primarily FKBP5 subfamily 51 (51 kDa) (Supplementary Fig. S3). Histologically, WT-E mice exhibited hepatic steatosis with large vesicles displacing the nucleus and enlarging the hepatocytes when compared to WT (Fig. 2C).[17, 18] The level of hepatic triglyceride and serum transaminases were markedly elevated in WT mice fed with ethanol when compared to pair-fed WT controls (Figs. 2D and 2E). Interestingly, loss of *Fkbp5* protected against alcohol-induced hepatic steatosis, as illustrated by a lesser degree of hepatic steatosis by Oil Red-O staining and hepatic triglyceride measurement in *Fkbp5*$^{-/-}$ mice fed with ethanol (*Fkbp5*$^{-/-}$-E) (Figs. 2C **and** 2D). Ethanol feeding in WT mice induced hepatic neutrophil infiltration as demonstrated by the MPO staining, however, there was no significant increase in macrophage (F4/80 staining) infiltration. Hepatic neutrophil infiltration was markedly reduced in *Fkbp5*$^{-/-}$ mice when compared to WT mice fed with ethanol (Fig. 2C **and** Supplementary Fig. S4 A **and** B). Additionally, pro-inflammatory cytokines such as TNF-α and IL-6 were markedly reduced in *Fkbp5*$^{-/-}$ mice fed with ethanol compared to WT-E (Fig. 2F). Taken together, we found that FKBP5 plays a significant role in the pathogenesis of ALD and that loss of FKBP5 abrogates alcohol-induced hepatic steatosis and inflammation.

We next performed the lipidomic analysis to gain further insight into the inhibitory effect of FKBP5 on alcohol-induced steatosis. Inherent differences in metabolomic profiles were visualized using Principal Component Analysis (PCA), which showed a clear separation between ethanol-fed WT and *Fkbp5*$^{-/-}$ mice (Fig. 3A). A heat map was generated to identify hepatic lipid metabolites that contributed to the distinct separation as observed in the PCA analysis. We found downregulation of di- and tri-acylglycerols (DG and TAG) in the liver samples of *Fkbp5*$^{-/-}$ mice when compared to wild-type mice fed on an ethanol diet (Fig. 3B and Supplementary Fig. 5A). We also observed an increase in hepatic phosphatidylcholine (PC) and phosphatidylethanolamines (PE), the most abundant phospholipids regulating the size of lipid droplets[17, 19, 20] in ethanol-fed *Fkbp5*$^{-/-}$ mice when compared to wild type mice on ethanol diet (Figs. 3B **and** Supplementary Fig. S5B **and** S5C). However, the levels

of several hepatic PCs were significantly lower in wild type ethanol diet mice compared to those in *Fkbp5*$^{-/-}$ mice on ethanol diet (marked in a red box, Fig. 3B). In the liver, PCs are biosynthesized from choline via cytidine diphosphate (CDP)-choline pathway and from phosphatidylethanolamine *N*-methyltransferase (PEMT); a transferase enzyme that synthesizes PC via the choline pathway or by methylation of PE. We found an increase in the mRNA expression of genes involved in the CDP-choline pathway [choline kinase-α (*Chk α*), choline kinase-β (*Chk β*) and choline phosphate cytidylyltransferase 2 (*Cct2*)] and *Pemt* in ethanol-fed *Fkbp5*$^{-/-}$ compared to that of wild-type mice (Supplementary Fig. S5D). Both human and animal studies have shown that lower phosphatidylcholine (PC) contributed to hepatic steatosis and inflammation[21]. Taken together, our data suggested that FKBP5 affects the biosynthesis of hepatic PCs; worsening hepatic steatosis may be associated with lower hepatic PCs in ethanol-fed WT compared to ethanol-fed *Fkbp5*$^{-/-}$ mice.

Identifying the novel target genes underlying the protective effect of FKBP5 on ALD utilizing RNA sequencing

To better our understanding and obtain a mechanistic insight on the role of FKBP5 on alcohol-induced liver injury, we performed RNA-seq analysis and compared the alterations in gene expression from WT and *Fkbp5*$^{-/-}$ mice on ethanol and pair-fed diet. The normalized unit of transcript expression [reads per kilobase of transcript per million mapped reads (RPKM)], as illustrated in Figure 4A, showed a higher peak on *Fkbp5* gene exons; confirming the increase in hepatic *Fkbp5* expression in WT-E. Volcano plots represented differential expression analysis of genes in both WT and *Fkbp5*$^{-/-}$ mice on ethanol and pair-fed diet (Fig. 4B). Right and left points marked the genes with significantly increased and decreased expression respectively in each group comparison with qval (false discovery rate adjusted p-value) <0.05. The x-axis shows $\log_2$ fold-changes in expression and the y-axis for the -log10 (p value), while the blue dots represent genes being differentially expressed (DEGs). The distributions of the numbers of DEGs among mice in each experimental group were shown as a Venn diagram (Fig. 4C). A total of 1,199 (94.2%) differentially expressed genes were found between WT and WT-E and 45 (3.5%) unique genes were observed between WT-E and *Fkbp5*$^{-/-}$- E (Supplementary Figs S6). Only 29 (2.3%) genes shared common differential expression and the hierarchical clustering of all the DEGs based on the RPKMs in all 4 experimental groups are shown in the heap map (Fig. 4D). In addition to *Fkbp5*, we observed an increase in expression of TEA Domain Transcription Factor 1 (*Tead1*) and chemokine (C-X-C motif) ligand 1 (*Cxcl1*) in WT-E, and its expression was significantly downregulated in *Fkbp5*$^{-/-}$ mice fed with ethanol diet. The increase in the expression of *Cxcl1* and *Tead1* from the RNASeq was confirmed by qRT-PCR analysis of the liver samples (Fig. 4E). We also performed qRT-PCR analysis for other genes as shown in the heat map analysis (Fig. 4D) from the liver tissues of mice and patients with alcoholic cirrhosis (Supplementary Figs. S7A **and** S7B). Interestingly, we found a similar pattern of gene expression in the liver of WT-E mice and those with alcoholic cirrhosis.

Alcohol-induced FKBP5 expression is mediated by methylation at its promoter region

There is a wide range of mechanisms in regulating gene expression. Among them is DNA methylation which can alter DNA accessibility and chromatin structure, thereby regulating

patterns of gene expression.[22] DNA methylation of the *FKBP5* gene is assumed to alter FKBP5 expression and subsequently its protein expression; methylation of the *FKBP5* gene has been reported in patients with depression and aging.[23, 24]

We, therefore, reasoned that ethanol-induced FKBP5 expression is mediated through the methylation process. We first examined FKBP5-specific DNA methylation in the peripheral blood of patients with alcoholic cirrhosis using 23 different promoter region-specific probes (Supplementary Table 4). The results showed 74% of *FKBP5* promotor (17 of the 23 probes) were hypomethylated while 26% (6 out of the 23 probes) were hypermethylated (Fig. 5A); suggesting that patients with alcoholic cirrhosis had significant downregulation in FKBP5 methylation levels at its promoter region (Supplementary Table 4 **and** Fig. 5A).

To further investigate if the methylation at the *Fkbp5* promoter region affects its expression, we designed methylation-specific (MSP) primers targeting the CpG island at the 5′ UTR promoter region of the *Fkbp5* (Fig. 5B, Supplementary Fig. S8, Supplementary Table 2). The quantification changes in methylation level from these primers reflect the overall *Fkbp5* CpG island status change. To validate these primers, we treated AML-12 cells with different concentrations of 5-aza-dC, a known inhibitor of DNA methylation. The MSP qPCR demonstrated 60% and 90% reduction in *Fkbp5* DNA methylation at 1 μM and 10 μM 5-aza-dC, respectively (Fig. 5C). We also performed the experiments in primary mouse hepatocytes and observed the reduction in *Fkbp5* DNA methylation with different concentrations of 5-aza-dC (supplementary Fig. S9A), similar to what we observed in AML-12 cells.

To determine if hepatic *Fkbp5* methylation was altered in mice fed with ethanol, liver genomic DNA isolated from WT and WT-E was converted using EZ-DNA methylation Kit. Bisulfite converted DNA samples were purified and subjected to qPCR for quantification (Supplementary Table 2). We found that ethanol feeding led to a significant reduction in *Fkbp5* methylation levels by qPCR (Fig. 5D). Our findings led us to hypothesize that reduced methylation levels of *Fkbp5* at the CpG island located at its 5′ UTR promoter region by ethanol led to the induction in its transcript and protein expression. To test this hypothesis, we treated AML-12 cells (Fig. 5E) and primary mouse hepatocytes (supplementary Fig. S9B), with 5-aza-dC in the presence and absence of ethanol at indicated concentrations and determined the expression of *Fkbp5* mRNA by qRT-PCR. The expression of *Fkbp5* mRNA increased by two-fold and 1.5-fold when AML-12 cells and primary mouse hepatocytes were treated with 5-aza-dC at 1 μM, respectively. Similar to what we observed in patients with alcoholic cirrhosis and ethanol-fed mice, the expression of *Fkbp5* mRNA increased by eight- and six-fold when AML-12 cells were treated with ethanol at 50 and 100 mM, respectively. Its expression was significantly increased when AML-12 cells (Fig. 5E) and primary mouse hepatocytes (Supplementary Fig. S9 B) were treated with a combination of 5-aza-dC and ethanol when compared to controls. Taken together, our data suggest that the upregulation of *Fkbp5* expression by ethanol is secondary to the downregulation of methylation level at its 5′ UTR promoter region.

To further examine the mechanism of FKBP5 methylation, we set out the experiments to test if its methylation was directly regulated by DNA methyltransferases (DNMTs),

a key enzyme in transferring a methyl group to DNA. In mammalian cells, DNMTs modifies cytosine to 5-methylcytosine (5mC) leading to DNA methylation, while ten-eleven translocation methylcytosine dioxygenase (TET) oxidizes the conversion of 5mC to 5-hydroxymethylcytosine; the process leading to DNA demethylation.[25] We adopted a method previously developed to investigate the specific methylation events using guide RNAs and fusion of a catalytically inactive Cas9 (lost its cutting ability but retains the guide RNA binding ability) with DNA (cytosine-5)-methyltransferase 3A (DNMT3A) or TET. The fused DNMT or TET can specifically target the genomic site and thereby edit the DNA methylation (Fig. 5F).[26] We designed two guide RNA (gRNA) plasmids as described in the method section and co-transfected these plasmids with fused dCas9-DNMT3A or dCas9-TET (Fig 5G). AML-12 cells transfected with dCas9-TET or DNMA3A without any gRNAs did not show any significant changes compared with control (first line). Both gRNAs transfected with dCas9-TET increased the expression of *Fkbp5*, while gRNAs co-transfected with dCas9-DNMT3A inhibited *Fkbp5* mRNA expression. Our results confirmed that the reduction in methylation levels of *Fkbp5* at its promoter region led to an increase in the expression of *Fkbp5* mRNA and protein expression.

Increased Fkbp5 expression leads to increased expression of transcription factor Tead1 through the Hippo signaling pathway

We next set out the experiments to explore the downstream pathway of FKBP5 which led to the pathogenesis of ALD. We observed an increase in the expression of *Tead1* in ethanol-fed mice and its expression was significantly downregulated in *Fkbp5*$^{-/-}$ mice. From the STRING analysis of 29 genes in which the expression was significantly changed in WT-E vs WT and *Fkbp5*$^{-/-}$-E vs WT-E (Figs. 4C **and** 4D), we observed a protein-protein interaction network involving TEAD1 (transcription factor), YAP (co-transcription factor) and CXCL1 (Supplementary Fig. S10A). The GO terms from the categories ‘cellular component’ and ‘KEGG pathways analysis’ also confirmed the enrichment in the Hippo signaling pathway, to which YAP and TEAD1 belong (Supplementary Fig. S10B). In fact, YAP is a known transcriptional co-activator and its function is driven by its association with the transcription factor, TEAD. The expression level of *TEAD1* was significantly elevated in ALD patients (Supplementary Fig. S11). To confirm and extend if these changes were triggered by Fkbp5 and ethanol, we first assessed the expression and activation of YAP by Western blot analysis. We first examined the YAP phosphorylation status in the liver of WT and *Fkbp5*$^{-/-}$ mice fed with ethanol and compared it to those of controls. We observed a decrease in YAP phosphorylation at S127, in WT but not in *Fkbp5*$^{-/-}$ mice fed with ethanol compared to pair-fed controls (Fig. 6A). We next explored the upstream kinases which are responsible for YAP phosphorylation, MST1, and LATS1/2, and found a significant reduction in phosphorylation of MST1/2 but not LATS1/2 (Supplementary Fig. S12A **and** B). Interestingly, the inhibitory effect of ethanol on YAP phosphorylation was abrogated in *Fkbp5*$^{-/-}$ mice (Figs. 6A). We next determined if the loss of YAP phosphorylation by ethanol promoted its nuclear translocation from the cytosol. We found an increase in nuclear to cytosol YAP ratio when AML-12 cells were treated with 50 mM ethanol at the indicated time points (Fig. 6C). This observation was also confirmed by immunofluorescence (Fig. 6B). We also performed the experiments in primary mouse hepatocytes and observed a similar observation as those in AML-12 cells (Supplementary Fig. S12C).

To determine the mechanistic role of Fkbp5 and Yap phosphorylation, we next performed co-immunoprecipitation (Co-IP) experiments to determine if FKBP5 can bind with upstream kinases of Yap, based on its property as a co-chaperone protein. AML-12 cells and primary mouse hepatocytes were treated with and without ethanol (50 mM) and Co-IP was performed using FKBP5, MST1, and LATS1/2 antibodies (Fig. 6D **and** Supplementary Fig. S12D). We found an interaction between FKBP5 and MST1 (but not with LATS1/2) in AML-12 cells or primary mouse hepatocytes treated with ethanol (Fig. 6D **and** Supplementary Fig. S12D). We next asked if the interaction between FKBP5 and MST1 affected MST1 kinase activity by employing the gain- and loss of function approach for FKBP5 (using AAV-FKBP5 and AAV-shFKBP5) and siRNA MST1 in AML-12 cell and lentivirus shMST1 in primary mouse hepatocytes. We found that ethanol treatment did not change total MST1 expression; however, ethanol and siMST (or a combination) significantly inhibited YAP phosphorylation in AML-12 cells and primary mouse hepatocytes (Fig. 6E **and** Supplementary Fig. 12E). Next, we overexpressed FKBP5 with AAV-FKBP5 in AML-12 cells and primary mouse hepatocytes with and without siMST1 (in AML-12 cells) or lentivirus shMST1 (in primary mouse hepatocytes), respectively (Fig. 6F **and** Supplementary Fig. 12F). YAP phosphorylation was inhibited in the cells treated with siMST and AAV-FKBP5 (or a combination) when compared to controls (Fig. 6F). Similar results were observed in the experiments with primary mouse hepatocytes (Supplementary Fig. 12F). To further determine the effect of FKBP5 on YAP phosphorylation, we knocked down FKBP5 with sh-FKBP5 in the presence and absence of siMST1 (in AML-12 cells) or lentivirus shMST1 (in primary mouse hepatocytes). We found that loss of FKBP5 increased YAP phosphorylation; however, its effect was ameliorated in the presence of siMST1 or lentivirus shMST1 (Fig. 6G **and** Supplementary Fig. 12G). Taken together, our results suggested that (i) FKBP5 can interact with YAP upstream kinase, MST1, affecting its ability to phosphorylate YAP, and (ii) the inhibitory effect of hepatic YAP phosphorylation by ethanol led to YAP nuclear translocation and TEAD activation through its role as TEAD transcriptional co-activator[27].

YAP binds to Tead1 transcription factor mediating increased Cxcl1 gene expression

The STRING analysis also predicted a strong association between YAP-TEAD1-CXCL1 (Supplementary Fig. S10A). We reasoned that the FKBP5-YAP-TEAD1-CXCL1 axis plays an important role in the pathogenesis of ALD as observed in our mouse model. To test our hypothesis, we sought to determine whether TEAD, together with its transcriptional coactivator YAP, can bind directly to the promoter of *Cxcl1* and activate the expression of its transcript. We searched the online database and found that the *Cxcl1* promoter has possible putative *Tead1* binding sequences (CATTCCT), at ~400bp and ~560bp from the TSS site (transcription starting site) (Figs. 7A, 7B, and Supplementary Table.3).

We next conducted chromatin immunoprecipitation (ChIP) assays using an anti-TEAD1 antibody and observed DNA enrichment as measured by real-time PCR. The relative enrichment of DNA binding was normalized with the background signal in the negative control (IgG) group. The relative DNA enrichment on both Tead1 binding sites was increased approximately 6-fold in cells treated with ethanol when compared to controls ($p<0.0001$, Fig. 7C). ChIP analysis was also performed using liver tissues of WT and

Fkbp5$^{-/-}$ mice and we found a significant increase in relative DNA binding between *Tead1* and *Cxcl1* promotor, notably in ethanol-fed WT mice, but completely diminished by *Fkbp5* deficiency (p<0.005, Fig. 7D). Our results suggested the direct binding of TEAD1 to the promoter of *Cxcl1* genes in the presence of ethanol, and FKBP5 is critical for ethanol to enhance TEAD1 binding ability. To further explore the biological function of this binding, we performed luciferase assays using a reporter gene carrying a DNA fragment with the *Cxcl1* promoters. We found a significant increase in luciferase activity when we co-transfected AML-12 cells with pCMV-Flag-YAP and pRK-Myc-TEAD1 in the presence of 50 mM ethanol. Interestingly, luciferase activity was further increased with the addition of pcDNA3.1-FKBP5 when compared to individual plasmid transfection (Fig. 7E). As a consequence of the transcriptional activation, the *Cxcl1* mRNA expression was also increased in AML-12 cells transfected with Fkbp5 or Yap/Tead1, and its expression was further enhanced with the addition of Fkbp5 (Fig. 7F). Additionally, the expression of well-known Tead1/Yap targets, *Axl* (AXL Receptor Tyrosine Kinase) and *Areg* (amphiregulin) were also induced by ethanol feeding, and the inductions were abrogated in *Fkbp5*$^{-/-}$ mice (Supplementary Fig. S13). Taken together, our data suggested that (i) FKBP5 regulates TEAD1 through its transcriptional co-activator, YAP, and (ii) CXCL1 is a novel target of TEAD1.

Discussion

The mechanism of alcohol-induced liver injury is complex. Here we describe the role of Fkbp5 in mediating liver injury caused by ethanol intake. Ethanol caused the reduction in methylation levels of *Fkbp5* at its promoter region leading to an increase in the expression of *Fkbp5* mRNA and protein expression in patients with ALD and also in ethanol-fed mice. FKBP5 affected the biosynthesis of PCs and loss of *Fkbp5* protected against alcohol-induced hepatic steatosis. As a co-chaperon protein, FKBP5 can bind to MST1 and inhibit YAP phosphorylation causing its nuclear translocation to activate its known transcription factor TEAD1. Activation of TEAD1 led to increased expression of its novel target, CXCL1, a chemokine-mediated neutrophil recruitment, causing hepatic inflammation and neutrophil infiltration in our mouse model. Of importance, loss of FKBP5 markedly ameliorated alcohol-induced liver injury; suggesting its potential role as the therapeutic strategy for ALD. Our working model of the FKBP5-YAP-TEAD1-CXCL1 axis in the pathogenesis of ALD is shown in Fig. 8.

FKBP5, a co-chaperone protein, is known for its interaction with steroid receptors and is considered an important modulator of stress responses.[28, 29] FKBP5 belongs to a subclass of immunophilin proteins, and it has peptidyl-prolyl *cis—trans* isomerase (PPIase) activity which is essential for protein folding and protein complex formation.[30, 31] FKBP5 is well studied as a co-chaperone of heat shock protein 90 (HSP90) and forms a glucocorticoid receptor (GR) complex with other components. Both *GR* and *FKBP5* are associated with alcohol use disorder.[8, 32] FKBP5 plays an important role in hypothalamic-pituitary-adrenal (HPA) axis negative feedback regulation by reducing GR activity [33], and directly affects the stress response to various stimuli.[34, 35] It has been associated with multiple stress-related psychiatric disorders, addiction, as well as metabolic diseases such as obesity.[10, 14]

FKBP5 is an important regulator in energy and glucose homeostasis and is implicated in the pathogenesis of metabolic disorder in response to nutrient overload.[10, 14] Mice lacking *Fkbp5* are resistant to diet-induced obesity and hepatic steatosis.[10] They also had markedly reduced plasma triglyceride and free fatty acid compared to wild type counterparts when they were fed with high-fat diet. They had improved glucose tolerance and increased insulin signaling in skeletal muscle through the activation of Akt-AS160 signaling, GLUT4 expression at the plasma membrane, and glucose uptake into primary myotubes.[14]

FKBP5 has been associated with alcohol drinking in humans and plays a role in alcohol withdrawal severity.[8, 15] Its transcript expression was significantly increased in the prefrontal cortex and hypothalamus of patients with alcohol use disorder.[36] A previous study employing a free choice, two bottle feeding method, of water and alcohol showed an increase in the average alcohol consumption per day in *Fkbp5*$^{-/-}$ mice[8]. In our study using a single housing and single bottle feeding, we did not observe a significant difference in the amount of alcohol consumed in WT and *Fkbp5*$^{-/-}$ mice (Supplementary Fig. S2 A). A genome-wide analysis of problematic alcohol use individuals has been reported[37]; however, the genetic variants for alcohol use disorder are quite different than those related to alcoholic cirrhosis[38]. While FKBP5 may be associated with alcohol consumption, its role in ALD is not known. We observed that hepatic *FKBP5* mRNA and protein expression were significantly elevated in patients with ALD and mice fed with ethanol. *FKBP5* transcription is known to be induced by GR and a recent report found that DNA methylation levels within regulatory regions of the *FKBP5* locus demonstrated dynamic changes following a glucocorticoid challenge.[39] We indeed found that ethanol-induced the expression of Fkbp5 by epigenetic regulation, causing the reduction in methylation levels of *Fkbp5* at its promoter region through its effect on DNA methyltransferases. Our data suggested that aberrant alteration in the methylation of the stress-related gene, *FKBP5*, is fundamental in the pathogenesis of ALD.

We found that loss of Fkbp5 protected against alcohol-induced hepatic steatosis. FKBP5 has been reported to regulate lipid metabolism; it is an important regulator of cellular adipogenesis that is necessary for the transformation of preadipocytes into lipid bearing cells and serves as a pro-adipogenic factor essential to lipid accumulation in mature adipocytes[10]. This intrinsic factor on the functional effect of FKBP5 in mediating adipogenesis may, in part, play a role in protecting against lipid accumulation in our knockout mouse model. In addition to the reduction in hepatic triglycerides, our lipidomic analysis provided an interesting observation on the role of FKBP5 on hepatic phospholipids biosynthesis (Figs. 3B **and** Supplementary Fig. S5B **and** S5C). Mechanistic studies demonstrated the induction in the expression of several genes in the PC biosynthesis pathway, notably in *Fkbp5*$^{-/-}$ mice fed with ethanol. Our data suggest the protective role of FKBP5 in alcohol-induced steatosis through the modulation of PC synthesis; the notion deserving additional investigations in future studies.

To explore the downstream effects of FKBP5, we analyzed the STRING analysis of our RNAseq data. The STRING analysis is a web-based bioinformatic tool that uses the biological database to summarize and predict protein-protein interactions[40]. We found a novel relationship between FKBP5 and YAP/TEAD1 in the Hippo signaling pathway.

Protein-protein interaction between FKBP5 and MST1 in ethanol-fed mice led to a significant decrease in YAP phosphorylation and promoted its nuclear translocation. Interestingly, we only observed a significant reduction in phosphorylation of MST1, one of the upstream kinase of YAP, and no significant changes in the phosphorylation state of LATS1/2; suggesting that the regulation of the ethanol-FKBP5-YAP axis was independent of LATS.[41] YAP, a TEAD transcriptional co-activator, can then activate TEAD and its novel target gene identified in our study, *CXCL1*, leading to an increase in neutrophil infiltration, as observed in our model of alcohol-induced liver injury. In addition to the FKBP5-YAP axis, the STRING analysis also suggested the possible association of FKBP5 with Lepr and Socs3, future exploration on the role of FKBP5 and these genes in ALD pathogenesis is warranted.

It is known that FKBP5 can regulate signaling pathways via direct protein-protein interactions[14], as shown by the effect of FKBP5 on the Hippo pathway and CXCL1 in our experimental model. Follow-up studies should address the differential effect of FKBP5 on hepatic and non-hepatic parenchymal cells in the pathogenesis of ALD; the questions which cannot be addressed in the present study because of the global loss of *Fkbp5* in our mouse model. Furthermore, it will be of interest to determine how the interaction between FKBP5 and MST1 affects its phosphorylation states, as a recent study found that the FKBP5 protein complex may enhance protein phosphatase activity.[14] Additionally, the cross-talk between tissues known to be affected by FKBP5, such as adipose tissues and muscle, and the liver in the pathogenesis of ALD should be further explored. Lastly, our study offers an insight into the potential therapeutic role of FKBP5 in ALD. The discovery of the potent and highly selective inhibitors of FKBP5 (notably FKBP51) has recently been reported.[42] This new class of ligands achieved selectivity for Fkbp5 by an induced-fit mechanism and the compounds can improve neuroendocrine feedback and stress-coping behavior in mice.[42] The use of this novel antagonist recapitulated the effects of FKBP51 deletion on body weight regulation and glucose tolerance.[14] It will be of interest to determine the effect of these selective inhibitors in the mouse models of ALD. If these compounds yield a positive result, they will demonstrate their potential applications in a clinical setting in patients with ALD.

Together, our present study defines a novel role of FKBP5 in the pathogenesis of ALD. Loss of FKBP5 ameliorates alcohol-induced liver injury through the hippo pathway and CXCL1 signaling, suggesting its potential role as the target for the treatment of ALD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgment

ZY is supported by NIH K01AA26385, Indiana University Research Support Fund Grant (IU RSFG), the Ralph W. and Grace M. Showalter Research Trust Indiana University School of Medicine, and Indiana Institute for Medical Research; SL is supported in part by R01 DK107682, R01 AA025208, U01 AA026917, UH2 AA026903, VA Merit Award 1I01CX000361, Dean's Scholar from Indiana University School of Medicine, and Indiana Clinical and Translational Sciences Institute, UL1TR002529, National Center for Advancing Translational Sciences, Clinical and Translational Sciences Award and Showalter Scholar Indiana University School of Medicine; PK and TL are supported by the grant from Indiana Institute for Medical Research (IIMR).

Abbreviation

ALD	Alcoholic liver disease
ALT	Alanine transaminase
AST	Aspartate aminotransferase
FKBP5	FK506-binding protein 51
IL	Interleukin
KO	knockout
MPO	Myeloperoxidase
NIAAA	National Institute on Alcohol Abuse and Alcoholism
TNF-α	Tumor necrosis factor-α
TG	Triglyceride
WT	Wild type

References

1. Liangpunsakul S, Haber P, McCaughan GW. Alcoholic Liver Disease in Asia, Europe, and North America. Gastroenterology2016;150:1786–97. [PubMed: 26924091]
2. Gao B, Bataller R. Alcoholic liver disease: pathogenesis and new therapeutic targets. Gastroenterology2011;141:1572–1585. [PubMed: 21920463]
3. Kang CB, Hong Y, Dhe-Paganon S, et al.FKBP family proteins: immunophilins with versatile biological functions. Neurosignals2008;16:318–25. [PubMed: 18635947]
4. Li H, Su P, Lai TK, et al.The glucocorticoid receptor-FKBP51 complex contributes to fear conditioning and posttraumatic stress disorder. J Clin Invest2020;130:877–889. [PubMed: 31929189]
5. Ellsworth KA, Eckloff BW, Li L, et al.Contribution of FKBP5 genetic variation to gemcitabine treatment and survival in pancreatic adenocarcinoma. PLoS One2013;8:e70216. [PubMed: 23936393]
6. Hauger RL, Olivares-Reyes JA, Dautzenberg FM, et al.Molecular and cell signaling targets for PTSD pathophysiology and pharmacotherapy. Neuropharmacology2012;62:705–14. [PubMed: 22122881]
7. Rao S, Yao Y, Ryan J, et al.Common variants in FKBP5 gene and major depressive disorder (MDD) susceptibility: a comprehensive meta-analysis. Sci Rep2016;6:32687. [PubMed: 27601205]
8. Qiu B, Luczak SE, Wall TL, et al.The FKBP5 Gene Affects Alcohol Drinking in Knockout Mice and Is Implicated in Alcohol Drinking in Humans. Int J Mol Sci2016;17.
9. Connelly KL, Unterwald EM. Chronic cocaine administration upregulates FKBP5 in the extended amygdala of male and female rats. Drug Alcohol Depend2019;199:101–105. [PubMed: 31029877]
10. Stechschulte LA, Qiu B, Warrier M, et al.FKBP51 Null Mice Are Resistant to Diet-Induced Obesity and the PPARgamma Agonist Rosiglitazone. Endocrinology2016;157:3888–3900. [PubMed: 27442117]
11. Yang L, Isoda F, Yen K, et al.Hypothalamic Fkbp51 is induced by fasting, and elevated hypothalamic expression promotes obese phenotypes. Am J Physiol Endocrinol Metab2012;302:E987–91. [PubMed: 22318949]
12. Cherno O, Sidibeh MJP, Abalo Xesus M., Boersma Gretha J., Skrtic Stanko, Lundkvist Per, Katsogiannos Petros, Hausch Felix, Castillejo-López Casimiro, and Eriksson Jan W.. FKBP5

expression in human adipose tissue: potential role in glucose and lipid metabolism, adipogenesis and type 2 diabetes. Endocrine2018;62:116–128. [PubMed: 30032404]

13. Shimoide T, Kawao N, Tamura Y, et al.Novel roles of FKBP5 in muscle alteration induced by gravity change in mice. Biochem Biophys Res Commun2016;479:602–606. [PubMed: 27680313]
14. Balsevich G, Hausl AS, Meyer CW, et al.Stress-responsive FKBP51 regulates AKT2-AS160 signaling and metabolic function. Nat Commun2017;8:1725. [PubMed: 29170369]
15. Huang MC, Schwandt ML, Chester JA, et al.FKBP5 moderates alcohol withdrawal severity: human genetic association and functional validation in knockout mice. Neuropsychopharmacology2014;39:2029–38. [PubMed: 24603855]
16. Manzo-Avalos S, Saavedra-Molina A. Cellular and mitochondrial effects of alcohol consumption. Int J Environ Res Public Health2010;7:4281–304. [PubMed: 21318009]
17. Schulze RJ, Ding W-X. Lipid droplet dynamics in alcoholic fatty liver disease. Liver Research2019;3:185–190. [PubMed: 33664985]
18. You M, Arteel GE. Effect of ethanol on lipid metabolism. J Hepatol2019;70:237–248. [PubMed: 30658725]
19. Clugston RD, Gao MA, Blaner WS. The Hepatic Lipidome: A Gateway to Understanding the Pathogenes is of Alcohol-Induced Fatty Liver. Current molecular pharmacology2017;10:195–206. [PubMed: 26278391]
20. Clugston RD, Jiang H, Lee MX, et al.Altered hepatic lipid metabolism in C57BL/6 mice fed alcohol: a targeted lipidomic and gene expression study. Journal of lipid research2011;52:2021–2031. [PubMed: 21856784]
21. Arendt BM, Ma DW, Simons B, et al.Nonalcoholic fatty liver disease is associated with lower hepatic and erythrocyte ratios of phosphatidylcholine to phosphatidylethanolamine. Appl Physiol Nutr Metab2013;38:334–40. [PubMed: 23537027]
22. Jaenisch R, Bird A. Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals. Nat Genet2003;33Suppl:245–54. [PubMed: 12610534]
23. Klinger-Konig J, Hertel J, Van der Auwera S, et al.Methylation of the FKBP5 gene in association with FKBP5 genotypes, childhood maltreatment and depression. Neuropsychopharmacology2019;44:930–938. [PubMed: 30700816]
24. Zannas AS, Jia M, Hafner K, et al.Epigenetic upregulation of FKBP5 by aging and stress contributes to NF-kappaB-driven inflammation and cardiovascular risk. Proc Natl Acad Sci U S A2019;116:11370–11379. [PubMed: 31113877]
25. Gu T, Lin X, Cullen SM, et al.DNMT3A and TET1 cooperate to regulate promoter epigenetic landscapes in mouse embryonic stem cells. Genome Biol2018;19:88. [PubMed: 30001199]
26. Liu XS, Wu H, Ji X, et al.Editing DNA Methylation in the Mammalian Genome. Cell2016;167:233–247 e17. [PubMed: 27662091]
27. Lin KC, Park HW, Guan KL. Regulation of the Hippo Pathway Transcription Factor TEAD. Trends Biochem Sci2017;42:862–872. [PubMed: 28964625]
28. Storer CL, Dickey CA, Galigniana MD, et al.FKBP51 and FKBP52 in signaling and disease. Trends Endocrinol Metab2011;22:481–90. [PubMed: 21889356]
29. Zannas AS, Wiechmann T, Gassen NC, et al.Gene-Stress-Epigenetic Regulation of FKBP5: Clinical and Translational Implications. Neuropsychopharmacology2016;41:261–74. [PubMed: 26250598]
30. Quinta HR, Maschi D, Gomez-Sanchez C, et al.Subcellular rearrangement of hsp90-binding immunophilins accompanies neuronal differentiation and neurite outgrowth. J Neurochem2010;115:716–34. [PubMed: 20796173]
31. Gassen NC, Hartmann J, Zannas AS, et al.FKBP51 inhibits GSK3beta and augments the effects of distinct psychotropic medications. Mol Psychiatry2016;21:277–89. [PubMed: 25849320]
32. Desrivieres S, Lourdusamy A, Muller C, et al.Glucocorticoid receptor (NR3C1) gene polymorphisms and onset of alcohol abuse in adolescents. Addict Biol2011;16:510–3. [PubMed: 20731635]
33. Ising M, Depping AM, Siebertz A, et al.Polymorphisms in the FKBP5 gene region modulate recovery from psychosocial stress in healthy controls. Eur J Neurosci2008;28:389–98. [PubMed: 18702710]

34. Guidotti G, Calabrese F, Anacker C, et al.Glucocorticoid receptor and FKBP5 expression is altered following exposure to chronic stress: modulation by antidepressant treatment. Neuropsychopharmacology2013;38:616–27. [PubMed: 23169346]

35. Schmidt U, Buell DR, Ionescu IA, et al.A role for synapsin in FKBP51 modulation of stress responsiveness: Convergent evidence from animal and human studies. Psychoneuroendocrinology2015;52:43–58. [PubMed: 25459892]

36. Gatta E, Grayson DR, Auta J, et al.Genome-wide methylation in alcohol use disorder subjects: implications for an epigenetic regulation of the cortico-limbic glucocorticoid receptors (NR3C1). Mol Psychiatry2019.

37. Zhou H, Sealock JM, Sanchez-Roige S, et al.Genome-wide meta-analysis of problematic alcohol use in 435,563 individuals yields insights into biology and relationships with other traits. Nat Neurosci2020;23:809–818. [PubMed: 32451486]

38. Schwantes-An TH, Darlay R, Mathurin P, et al.Genome-wide association study and meta-analysis on alcohol-related liver cirrhosis identifies novel genetic risk factors. Hepatology2020.

39. Wiechmann T, Roh S, Sauer S, et al.Identification of dynamic glucocorticoid-induced methylation changes at the FKBP5 locus. Clin Epigenetics2019;11:83. [PubMed: 31122292]

40. Szklarczyk D, Gable AL, Lyon D, et al.STRING v11: protein-protein association networks with increased coverage, supporting functional discovery in genome-wide experimental datasets. Nucleic Acids Res2019;47:D607–D613. [PubMed: 30476243]

41. Sugihara T, Werneburg NW, Hernandez MC, et al.YAP Tyrosine Phosphorylation and Nuclear Localization in Cholangiocarcinoma Cells Are Regulated by LCK and Independent of LATS Activity. Mol Cancer Res2018;16:1556–1567. [PubMed: 29903769]

42. Gaali S, Kirschner A, Cuboni S, et al.Selective inhibitors of the FK506-binding protein 51 by induced fit. Nat Chem Biol2015;11:33–7. [PubMed: 25436518]

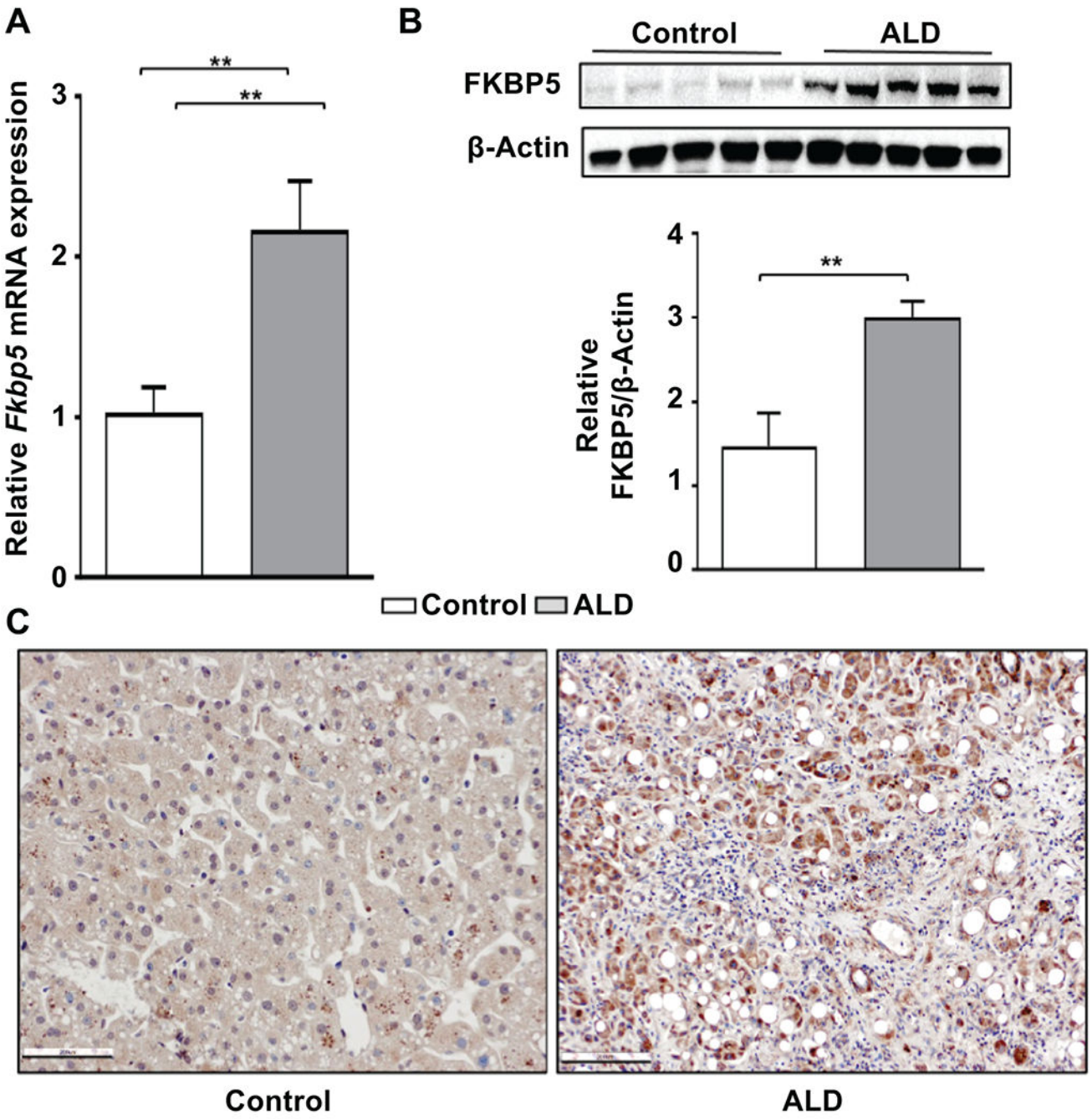

Figure 1. Hepatic FKBP5 is significantly elevated in patients with alcohol-associated liver disease.

(A) Relative mRNA levels of *FKBP5* were determined by real-time qPCR. The total RNA was isolated from liver samples of healthy controls (n=5) and alcoholic liver disease patients (n=10), as described in the method section. (B) Representative Western blot analysis and (C) immunohistochemistry of FKBP5 expression at 20× magnification in liver samples from control and ALD patients. Data are presented as means ± SEM per group. Statistical evaluation was performed by Student T-test. ** $p < 0.005$. Scale bars represent 200 μm.

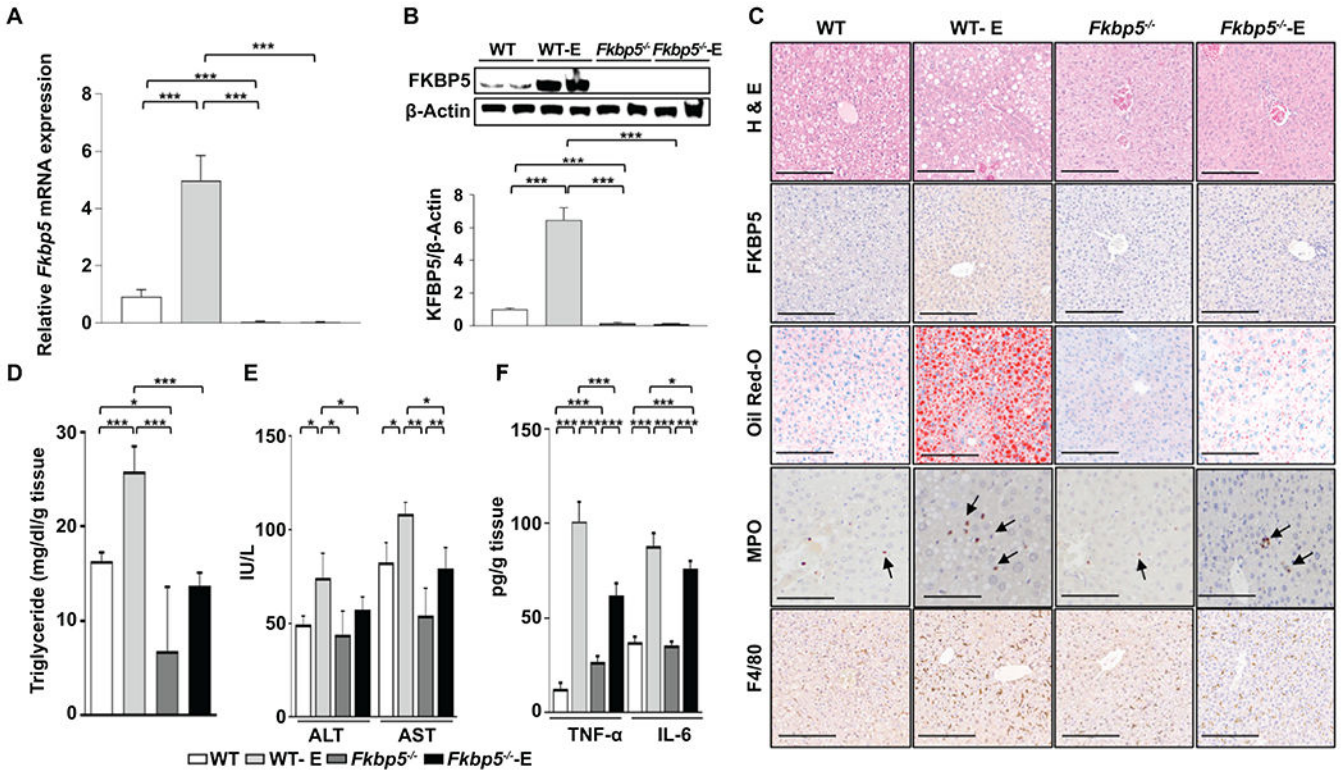

Figure 2. Hepatic FKBP5 expression is significantly elevated in chronic-plus-single binge ethanol-fed mice and deletion of FKBP5 ameliorates hepatic phenotypes of ALD.

(A) Relative mRNA levels of *Fkbp5* were assessed in WT and *Fkbp5*$^{-/-}$ mice after pair-fed and ethanol feeding. (B) Hepatic FKBP5 expression was determined by Western blot analysis and the protein expression levels were normalized to the levels of β-Actin. (C) Representative IHC staining at 20× magnification (Bar, 200 μm), showed an increase in FKBP5 expression in WT-E. Representative liver sections with hematoxylin and eosin demonstrated evidence of hepatic steatosis in WT-E; which was ameliorated in ethanol-fed *Fkbp5*$^{-/-}$ mice (shown in Oil Red O staining). MPO immunohistochemistry staining as a marker of neutrophils and F4/80 immunohistochemistry staining as a marker of macrophage showed a significant increase in neutrophil infiltration in WT-E but not in *Fkbp5*$^{-/-}$ mice fed with ethanol (as indicated by arrows). (D) Hepatic TG levels. (E) Serum ALT and AST levels. (F) The pro-inflammatory cytokines (TNF-α, IL-6) were tested using an ELISA kit. Data are presented as mean ± SEM per group. Statistical evaluation was performed by one-way ANOVA with Tukey's post hoc test for multiple comparisons. *$p<0.05$, ** $p< 0.01$, ***$p< 0.005$.

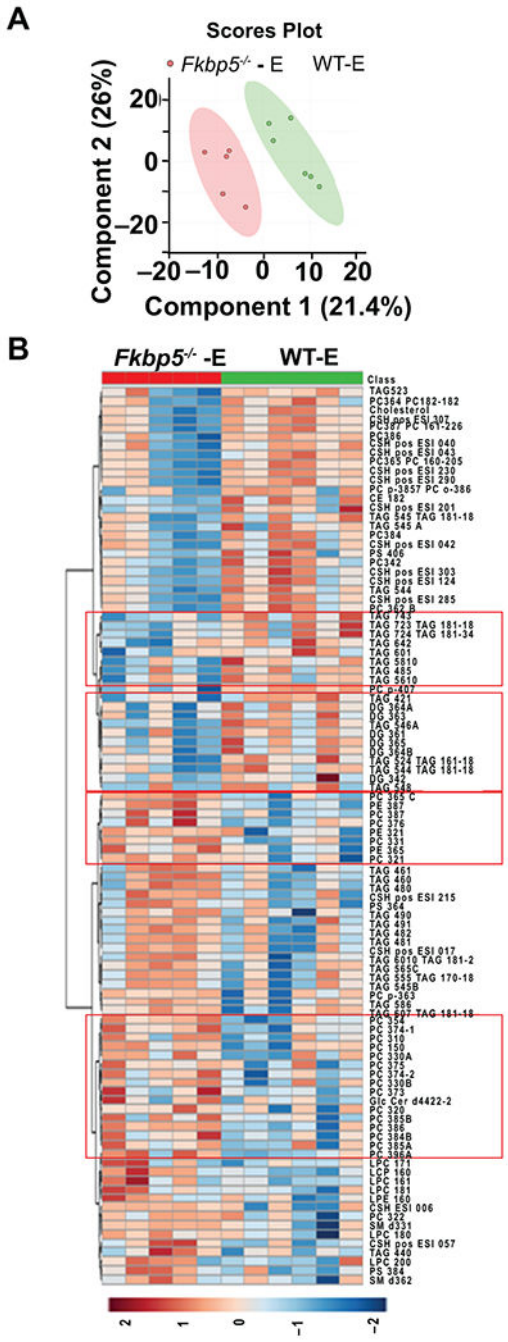

Figure 3. Lipidomic analysis in the liver samples of WT and *Fkbp5*$^{-/-}$ mice fed with ethanol.

(A) Principal Component Analysis (PCA) plots showed the separation between WT-E tissue samples (green) and *Fkbp5*$^{-/-}$-E tissue samples (red). (B) Heat map analysis of the top 100 of significantly changed in the levels of lipid between both groups, corresponding to their relative concentrations (intensity) for positive MS ionization mode. Red rectangles and letters indicated the clustered lipids and types. TAG: Triglyceride; DG: Diglyceride; PC: Phosphatidylcholine; PE: Phosphatidylethanolamine.

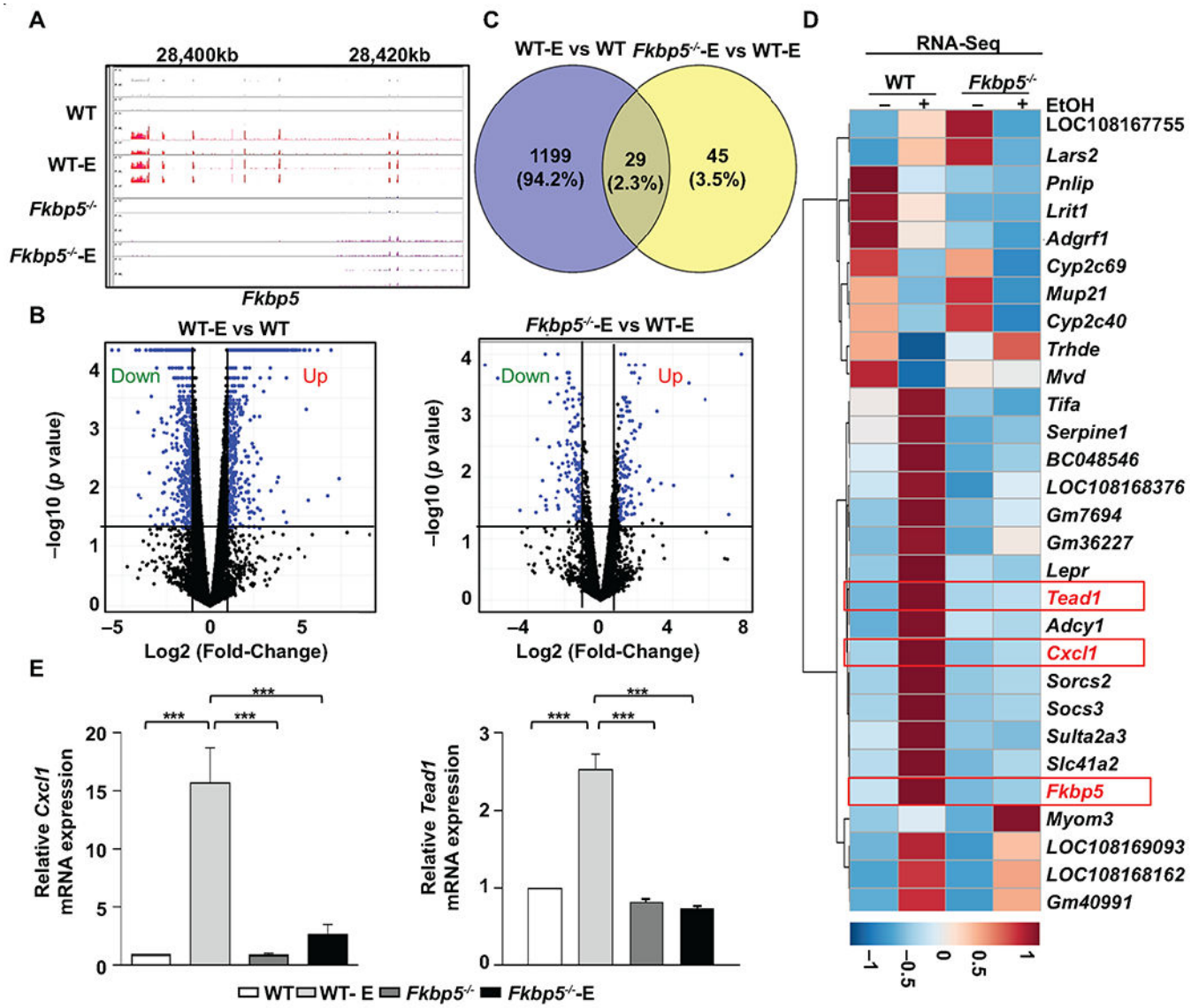

Figure 4. Identifying the novel target genes underlying the protective effect of FKBP5 on ALD utilizing RNA sequencing.

RNA-seq identified distinct differences in hepatic gene expression in WT and *Fkbp5*$^{-/-}$ mice fed with control or ethanol-containing diet. (A) Gene track showing *Fkbp5* was knocked out in the *Fkbp5*$^{-/-}$ mice. (B) Volcano plot from RNA-Seq data revealed variance in gene expression to fold change in WT versus WT-E and *Fkbp5*$^{-/-}$ -E versus WT-E mice. Vertical lines indicate the threshold for a relative expression fold change of > 2 or < −2-fold compared to WT and qval <0.05. The blue points in the top left and top right sectors are significantly down-regulated and up-regulated, respectively. Each gene was represented by a single point. (C) Venn diagram of significantly changed genes overlapped between two groups, WT versus WT-E and *Fkbp5*$^{-/-}$ -E versus WT-E. The same threshold in (B) was used. Numbers in parenthesis represented the percent of all the genes in the segment. (D) Heat map of mRNA representing the 29 genes that overlapped in the Venn diagram (C), between WT, WT-E, *Fkbp5*$^{-/-}$ and *Fkbp5*$^{-/-}$ -E (n=4). Color-coding is based

on log-transformed read count values. mRNA changes between WT and *Fkbp5*$^{-/-}$ mice pair-fed or ethanol-fed (n=4). Using uncentered Pearson correlation as the distance metric, an unsupervised hierarchical heat map was generated. Light blue, dark blue, orange, and red denotes very low, low, median, and relatively high mRNA expression respectively. (E) Hepatic mRNA expression levels of *Cxcl1* and *Tead1* of mice in each group. Data are presented as means ± SEM per group. ***$p < 0.005$.

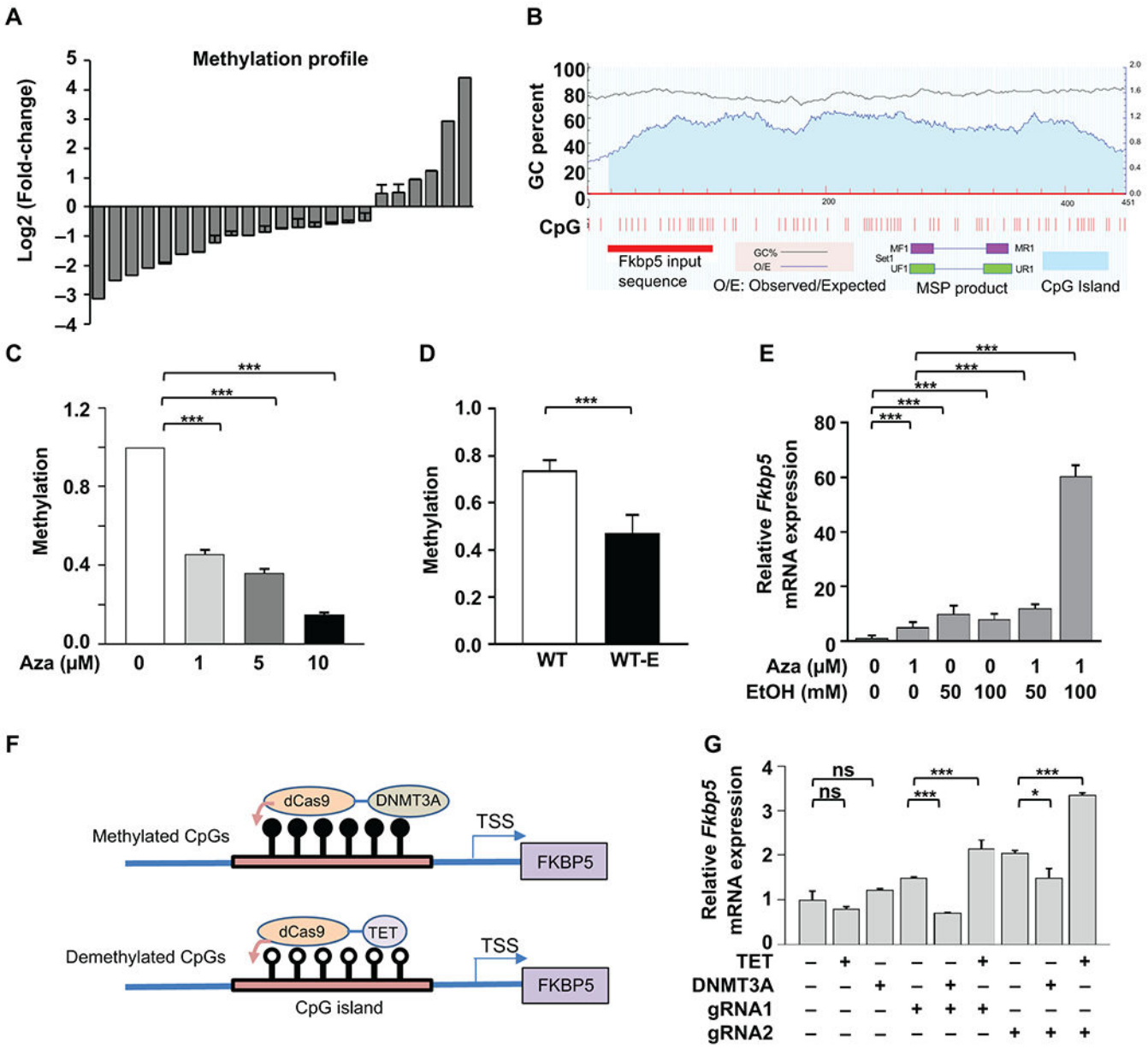

Figure 5. Alcohol-induced FKBP5 expression is mediated by methylation at its promoter region

(A) The *Fkbp5* DNA methylation profile in ALD blood samples using 23 probes covering multiple regions at 5′UTR. (B) 5′UTR promoter region of the *Fkbp5* gene was used for the prediction of CpG island. Two sets of MSP (Methylation-Specific PCR) primers were designed to detect the original sequences (UF/UR) or bisulfate converted sequences (MF/MR). (C) *In vitro* methylation in AML-12 cell lines treated with 5-aza-2′-deoxycytidine (Aza), an agent inducing DNA hypomethylation with different doses for 24 hours. The methylation levels of *Fkbp5* 5′UTR were detected using the MSP primers designed from (B). (D) The promoter region of hepatic *Fkbp5* was significantly hypomethylated in ethanol-fed WT mice liver (WT-E) compared to WT mice fed with a control diet (n=3/group). (E) *Fkbp5* mRNA expression in AML-12 cells after Aza treatment with and without ethanol.

(F) Schematic representation of editing the *Fkbp5* promoter region methylation levels by dCas9-TET or dCas9-DNMT3A with specific gRNAs to erase methylation or methylate the promoter controlling the *Fkbp5* expression. (G) AML-12 cells were transfected with plasmids of dCas9-TET or dCas9-DNMT3A with gRNA1 or gRNA2 targeting the *Fkbp5* promoter region. RNA was isolated and subjected to qRT-PCR for *Fkbp5* expression. Data are presented as means ± SEM per group. *p<0.05, ****p*< 0.005. Abbreviations: Aza, 5-aza-2′-deoxycytidine; TET, ten-eleven translocation; DNMT, DNA methyltransferase; dCas9, catalytically inactive mutant Cas 9; gRNA, guild RNA.

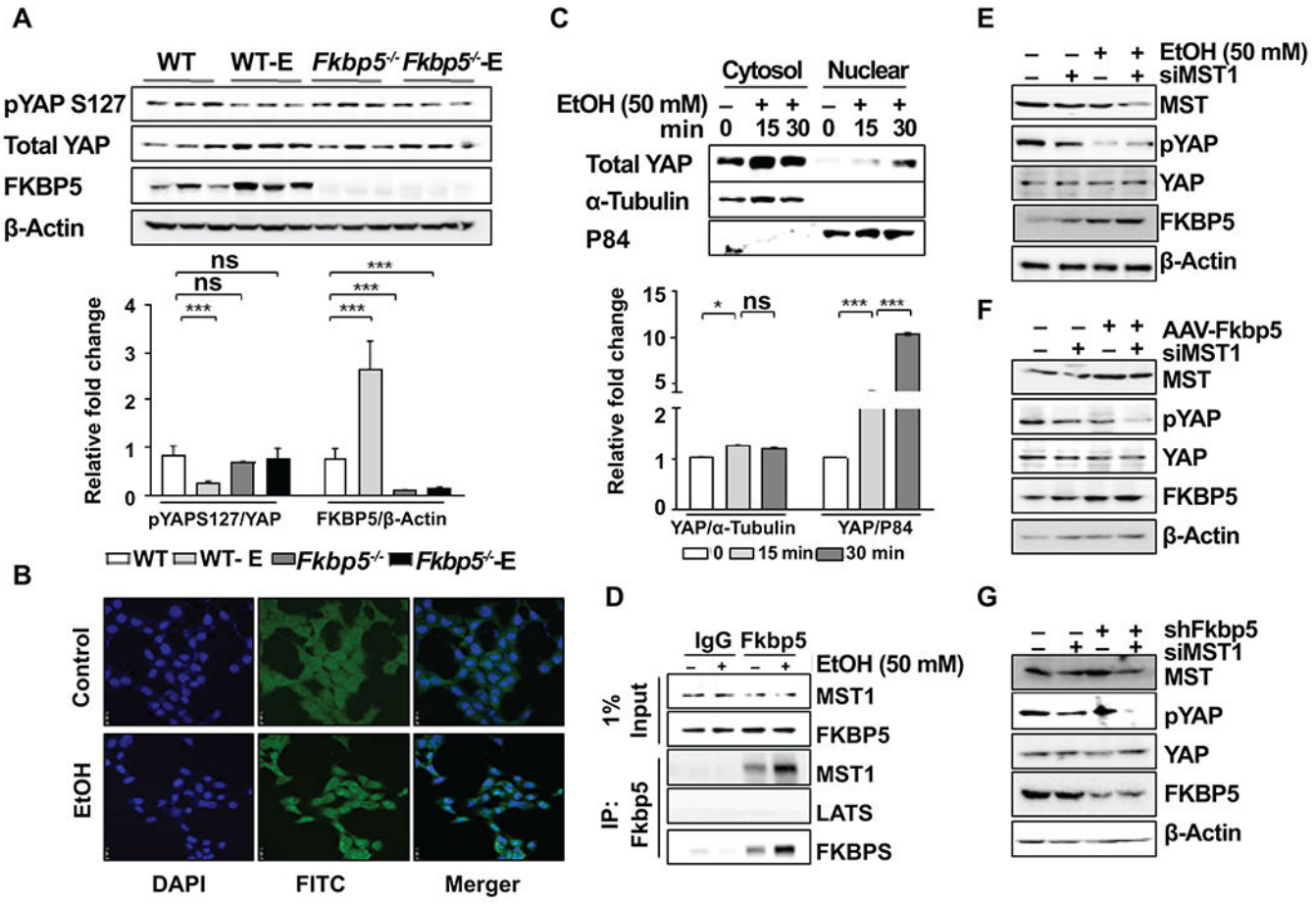

Figure 6. Increased Fkbp5 expression leads to increased expression of transcription factor Tead1 through Hippo signaling pathway

(A) Total liver protein (three individual samples from each group are shown as a representative of the group) were isolated from pair-fed and ethanol-fed WT and *Fkbp5*$^{-/-}$ mice and analyzed for pYAP, total YAP, and FKBP5. The protein expression levels were normalized to the levels of total YAP or β-ACTIN. (B) Immunofluorescence of AML12 shows nuclear localization of YAP on ethanol treatment. (C) Nuclear localization of YAP on ethanol treatment. Ethanol treatment of AML-12 cells showed an increase in nuclear YAP protein compared to control. (D) Cell extracts from AML-12 cells were precipitated with an anti-FKBP5 antibody. The immunoprecipitated proteins were assayed by Western blot analysis with MST1, LATS, and FKBP5 antibodies. 1% of the total lysate used for immunoprecipitation was used as an input as shown by Western blot analysis for MST1 and FKBP5. (E-G) AML-12 cells were transfected with or without siMST1 and subsequently treated with 50 mM ethanol or co-transfected with AAV-Fkbp5 or AAV-shFkbp5 and analyzed by Western blot for MST1, pYAP, total YAP, FKBP5, and β-ACTIN.

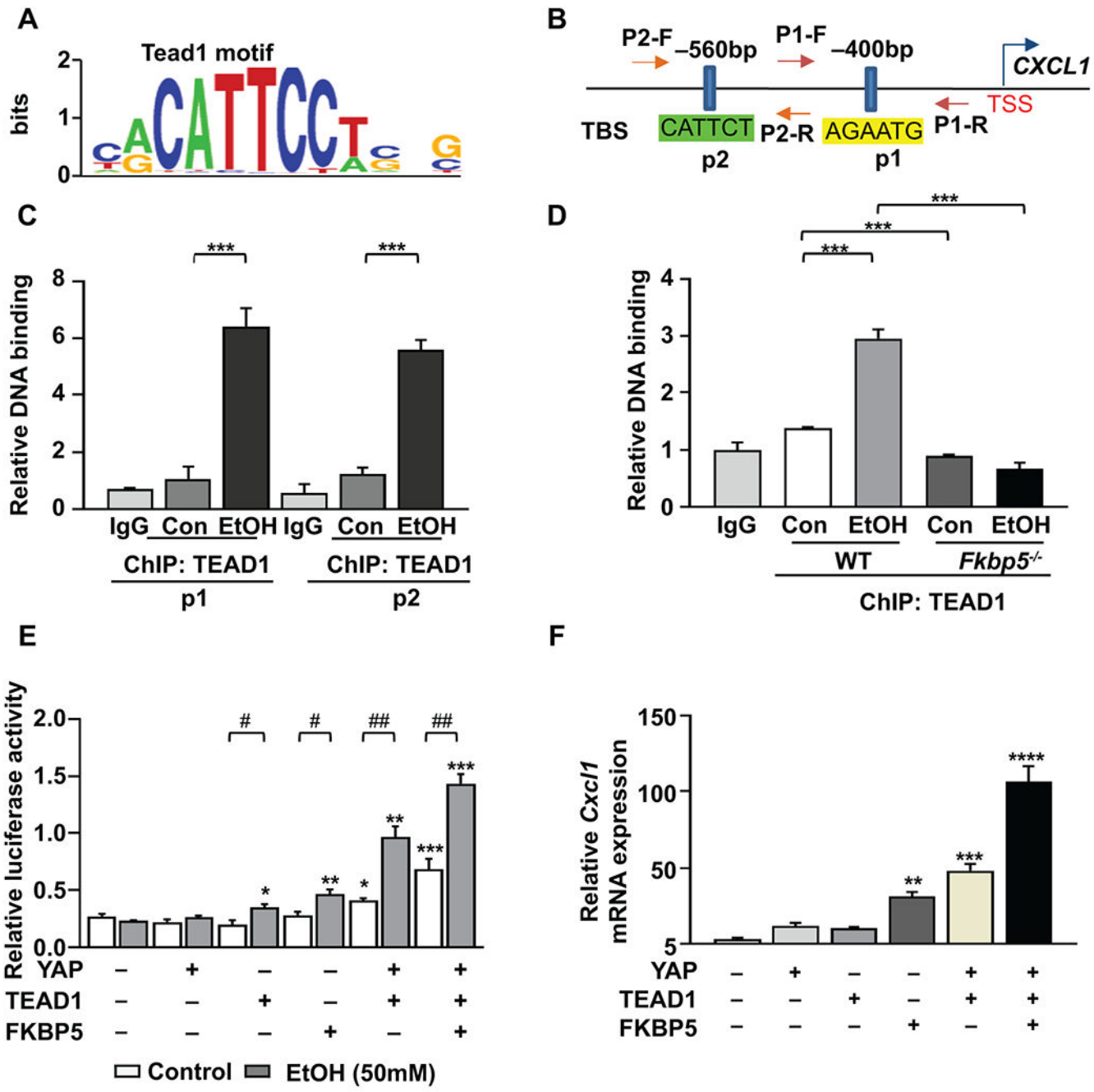

Figure 7. YAP binds to the Tead1 transcription factor mediating increased Cxcl1 gene expression.

(A) Tead1 transcription factor binding motif sequence from JASPAR (http://jaspar.genereg.net). (B) Schematic representation of *CXCl1* promoter regions for primer design to be used in ChIP assay. ChIP assay was done in AML-12 cells with TEAD1 antibody. Recruitment of YAP and TEAD1 proteins to the CXCL1 promoter was analyzed using primers specific to the *Cxcl1* promoter. Immunoglobulin G was used as an internal control. (C) ChIP samples were used for RT-PCR for quantification of the binding in the AML-12 cells (D) RT-PCR showing quantification of relative DNA binding from

ChIP analysis from the liver of WT and *Fkbp5*$^{-/-}$ mice fed with and without ethanol (E) AML-12 cells were transiently transfected with reporter plasmids, and luciferase activity was measured by a luminometer (Promega). (F) AML-12 cells transfected or co-transfected with pCMV-Flag-YAP (YAP), pRK-Myc-TEAD1 (TEAD1), pCMV-FKBP5 (FKBP5) for *Cxcl1* mRNA expression. Data are presented as mean ± SEM. Abbreviations: IgG, immunoglobulin G; TBS, TEAD binding site; TSS, transcription start site; P1 and P2, ChIP primer 1, and primer 2 respectively. All experiments were performed in duplicate for a minimum of three individual occasions, with specific replicate numbers shown in each legend *** $P<0.001$, ** or ## $P<0.01$, * or # $P<0.05$.

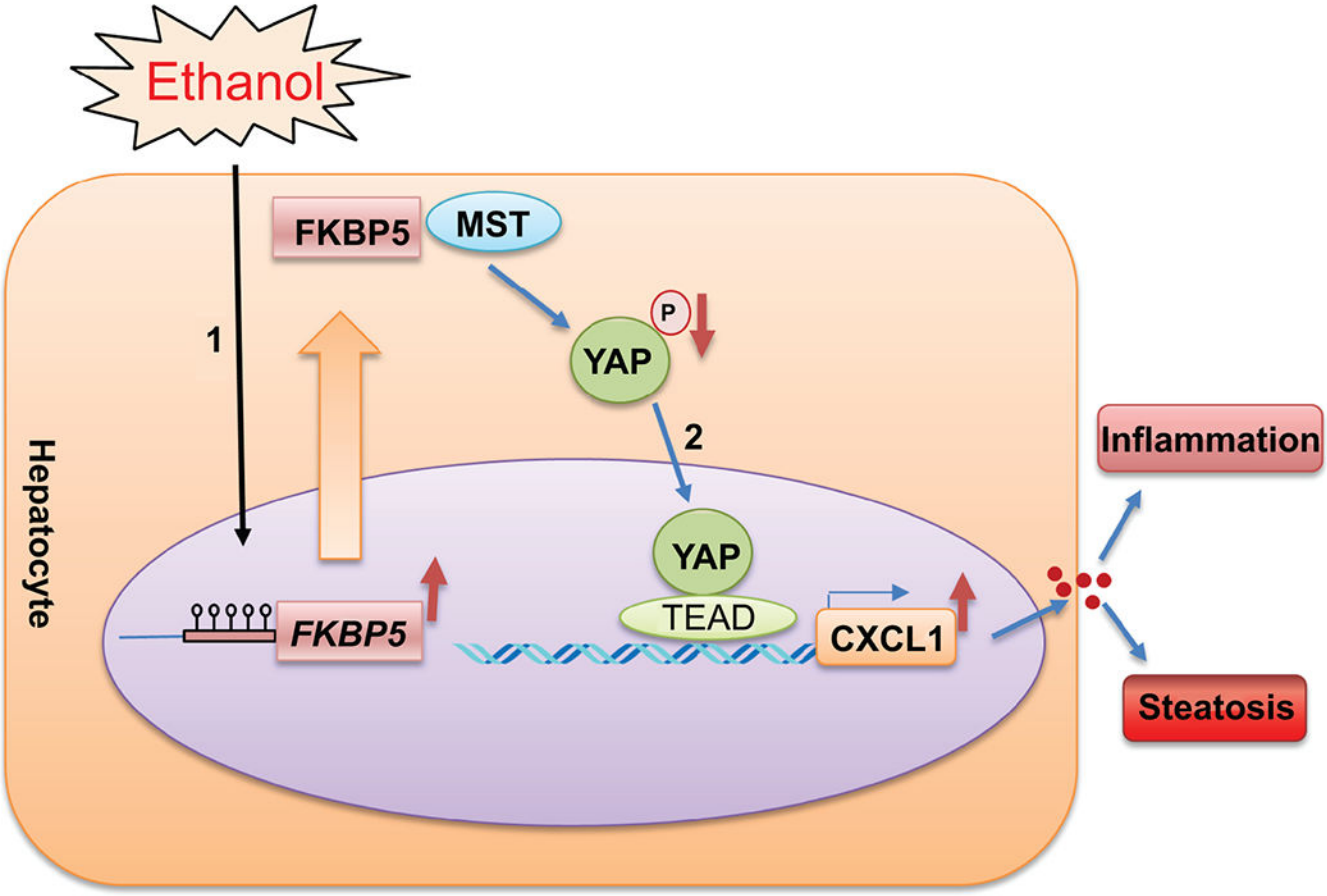

Figure 8. Schematic working model on the role of FKBP5-YAP-TEAD1-CXCL1 axis in the pathogenesis of ALD.

Upregulation of *Fkbp5* expression by ethanol is secondary to downregulation of methylation level at its 5′UTR promoter region. FKBP5 is associated with an increase in hepatic di- and tri-acylglycerols. It also affects the biosynthesis of hepatic PCs leading to worsening hepatic steatosis. FKBP5 can interact with YAP upstream kinase, MST1, affecting its ability to phosphorylate YAP and the inhibitory effect of hepatic YAP phosphorylation by ethanol leading to YAP nuclear translocation and TEAD activation. Activation of TEAD1 led to increased expression of its novel target, CXCL1, a chemokine-mediated neutrophil recruitment, causing hepatic inflammation and neutrophil infiltration. Taken together, we identified a novel role of the FKBP5-YAP-TEAD1-CXCL1 axis in the pathogenesis of ALD.