Role of Yes-Associated Protein (YAP) in Liver Injury and Regeneration following Acetaminophen Overdose

By

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Submitted to the graduate degree program in Toxicology and the Graduate Faculty of the University of Kansas Medical Center in partial fulfillment of the requirement for the degree of Master of Science.

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Date Defended: August 2, 2016

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Abstract

Acetaminophen (APAP) overdose is the major cause of Acute Liver Failure (ALF) in the Western world. Treatment options for APAP-induced ALF are limited. Studies have shown that stimulating liver regeneration could be a potential treatment for ALF after APAP overdose. However, the mechanisms of liver regeneration after APAP overdose are not completely understood, which is the focus of research in our laboratory. We investigated the role of Yes-associated protein (YAP), the downstream co-activator of Hippo Signaling Pathway, in liver injury and regeneration following APAP overdose. Previous studies have shown that YAP is involved in stimulation of hepatocyte proliferation during postnatal liver growth, regeneration after partial hepatectomy and during liver cancer pathogenesis. Treatment of two-three month old C57BL/6J male mice with 300 mg/kg APAP (APAP 300) resulted in significant liver injury and regeneration over a time course of 0 to 96 hr. Increased activation of YAP was observed during initial time points (3, 6 and 12 hr) after APAP administration which coincided with both injury and regeneration. To determine whether YAP activation plays a role in injury or regeneration, we generated hepatocyte specific YAP knockout mice (hYAP-KO) by treating two-three month old male YAP-floxed mice with AAV8.TGB.Cre. Hepatocyte specific deletion of YAP neither caused liver injury, nor did it change hepatic CYP2E1 expression and hepatic glutathione (GSH) levels. Consistent with these data, no difference in APAP-protein adducts was observed between control and hYAP-KO mice after APAP treatment. Liver injury measured by serum ALT and histopathology showed extensive and similar liver injury up to 12 hr after APAP treatment in both control and hYAP-KO mice. However, the progression of the liver injury beyond 12 hr

after APAP administration was significantly lower in hYAP-KO mice as compared to control mice. The decrease in progression of liver injury was accompanied with an earlier decrease in JNK activation and faster GSH recovery. Additionally, the hYAP-KO mice showed an early induction of proliferative markers demonstrating early liver regeneration as compared to the control mice. These data indicate that hepatocyte specific activation of YAP may be involved in progression of liver injury. Linked to that, hepatocytes specific deletion of YAP results in earlier onset of regeneration after APAP overdose. These findings indicate a differential role of YAP in APAP overdose and highlight YAP as a potential therapeutic target for the treatment of APAP-induced ALF.

Acknowledgments

I would like to thank my mentor, Dr. Udayan Apte, for giving me an opportunity to be the part of the awesome lab (APTE lab), and for the guidance and support throughout the course of my research. I would also like to acknowledge my committee members, Dr. Hartmut Jaeschke and Dr. Michele Pritchard, for offering valuable comments and suggestions on my thesis. Thank you my current and former lab members: Bharat, Prachi, Steve, Ian, Kevin and Michael, and all my friends. You all are great! I would also like to thank my FAMILY and relatives in Kansas. Kansas is a home to me because of you all. And, a special thanks to my mother.

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Introduction

Acetaminophen (APAP) Overdose and Hepatotoxicity

Acetaminophen (APAP) is a safe analgesic and antipyretic over-the-counter drug[1]. Whereas it is safe at therapeutic doses, overdose of APAP causes severe hepatic necrosis leading to acute liver failure (ALF). APAP-induced ALF is the most common cause of ALF in the United States[2, 3]. The reasons for APAP overdose are suicide attempts with the drug and unintentional therapeutic misadventures during treatment for pain and fever exceeding the recommended 4g/day dose[2]. There are an estimated 60,000 emergency visits and 26,000 hospitalizations yearly associated with APAP overdoses, with most cases being intentional overdose. Nearly 500 patients die of the overdose annually, and about 20% death occur with unintentional APAP overdose[4]. The most common treatment option available at present is N-acetylcysteine (NAC), which replenishes glutathione levels if administered within 12 hours of the overdose[2, 5]. The other option is liver transplantation, which is complicated by donor availability, donor and recipient age, donor and recipient ABO mismatching, high costs and post-transplantation psychological issues[6, 7].

The mechanisms of APAP induced hepatotoxicity have been studied in rodent models for over four decades[8-10]. When a therapeutic dose of APAP is taken, the majority of the dose (about 70-80%) is glucuronidated or sulfated and then excreted. A small percentage (about 5-10 %) of APAP is metabolized by cytochrome P450 enzymes to the reactive metabolite N-acetyl-p-benzoquinone imine (NAPQI). NAPQI conjugates with the cellular nucleophile glutathione (GSH) and is then excreted[10, 11]. The higher doses saturate the conjugation pathways, resulting in the formation of excessive

NAPQI. The excess NAPQI lowers the GSH levels and binds with the cellular proteins, especially mitochondrial proteins, forming adducts[12, 13]. NAPQI binding to mitochondrial proteins leads to oxidative stress in mitochondria, which ultimately results in the phosphorylation and activation of the c-jun N-terminal kinase (JNK). The activated JNK then translocate to the mitochondria resulting in amplification of the oxidative stress[14]. JNK does not seem to be directly activated by early events of APAP toxicity. A number of upstream kinases have been identified, which have shown to activate JNK. These kinases include apoptosis signal-regulating kinase 1 (ASK1), glycogen synthase kinase-3β (GSK-3β), mixed-lineage kinase 3 (MLK 3), receptor interacting protein kinase (RIP) 1 and RIP3[15]. The extensive oxidative stress results in the opening of mitochondrial membrane permeability transition (MPT) pore and release of apoptosis inducing factor (AIF) and endonuclease G (EndoG) from mitochondria[16, 17]. These proteins translocate to the nucleus causing nuclear DNA fragmentation, ultimately leading to necrotic liver cell death[18] (Fig.1).

Mechanisms of Regeneration following APAP overdose

The liver has a remarkable capacity to regenerate after drug-induced injury. It is known that liver regeneration is a critical determinant of final outcome, i.e. survival or death, after drug overdose[19]. A previous study has shown an increase in alpha-fetoprotein (AFP) in surviving patients with APAP- induced liver injury, which is considered an indicator of liver regeneration[20]. Studies of liver regeneration in mouse models suggest stimulating liver regeneration improves survival after APAP overdose in mice[21, 22]. These studies indicate that stimulating liver regeneration could be the therapeutic option

in the treatment of APAP -induced ALF. The mechanisms of liver regeneration have been studied mainly using partial hepatectomy or relatively less following toxicant-induced injury with carbon tetrachloride, chloroform and thioacetamide or drug induced liver injury with acetaminophen[21-23]. Specifically, the mechanisms of liver regeneration after APAP-induced ALF are not completely understood[22].

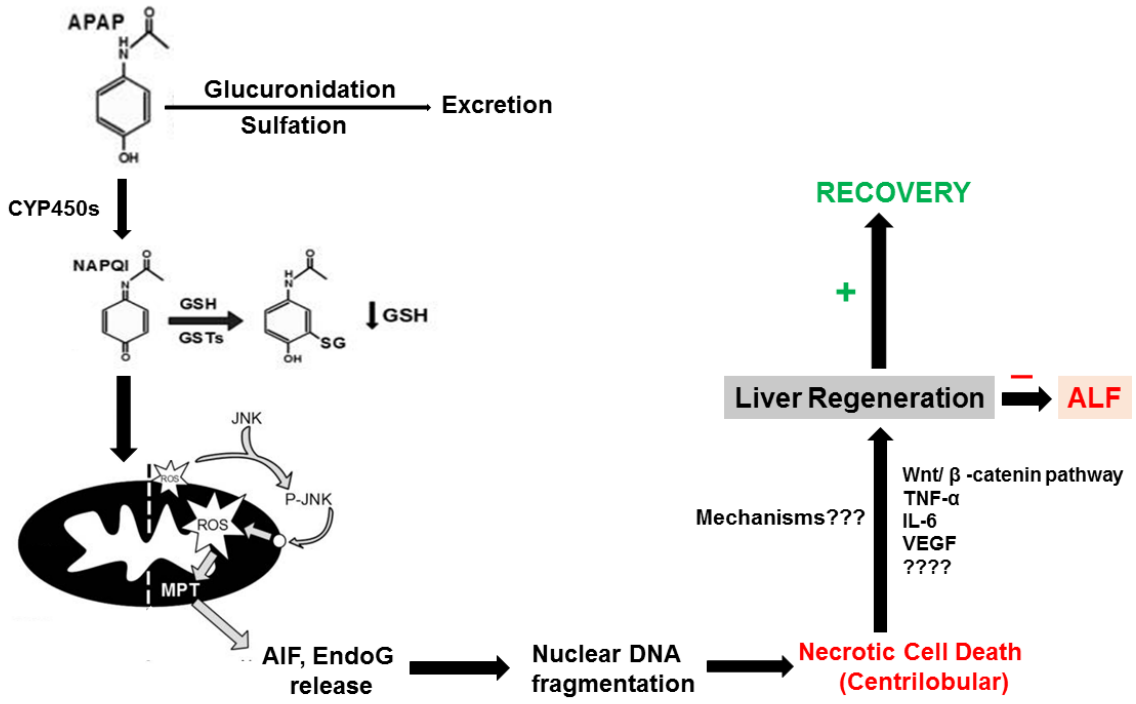

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Figure 1: Mechanism of APAP metabolism and toxicity, and compensatory liver regeneration

The therapeutics dose of APAP is glucuronidated and sulfated and excreted from the body. The reactive metabolite NAPQI formed from the metabolism of APAP is conjugated with glutathione and excreted. However, the toxic/overdose of APAP saturates the conjugation pathway leading to excessive accumulation of NAPQI, which binds, with the mitochondrial protein increasing the oxidative stress in

mitochondria. The increased oxidative stress phosphorylates and activates JNK and leads to its mitochondrial translocation. The activated JNK further amplifies oxidative stress leading to the opening MPT pore and release of AIF and EndoG. These proteins translocate to the nucleus causing nuclear DNA fragmentation and ultimately leading to necrotic cell death. Liver has remarkable capacity to regenerate. If the regeneration is impaired, it leads to acute liver failure (ALF). However, if regeneration follows, then it leads to recovery.

Role of cytokines and growth factors have been studied in liver regeneration following APAP induced hepatotoxicity. Tumor necrosis factor -α (TNF-α) mediated proliferative signaling in hepatocytes is majorly mediated by TNF receptor 1 (TNFR1). Deletion of TNFR1 results in decreased liver regeneration after APAP overdose in mice[24]. TNF-α binds to its receptor leading to stabilization and nuclear translocation of the transcription factor NF-κB. Reduced NF-κB DNA binding is associated with reduced liver regeneration in mice[25]. Similarly, IL-6 also plays a significant role in regeneration after APAP-induced liver injury. Impairment of liver regeneration is seen in IL-6 knockout mice shown by decreased PCNA positive cells. However, pre-treatment of the knockout mice with IL-6 restores the regeneration[26]. These studies show that cytokines are essential for the liver regeneration following APAP overdose.

The epidermal growth factor (EGF) and hepatocyte growth factor (HGF), which act through phosphorylation or activation of EGF receptor (EGFR) and c-Met receptor respectively, are critical for liver regeneration after PH[23]. However, administration of TGF-α (ligand of EGFR) along with other hepatic stimulatory substances and liver growth factors in beagle dogs did not affect survival or regeneration following lethal dose of APAP[27]. The other study shows elevated plasma level of HGF in the non-

surviving APAP- induced ALF patients[28]. These studies show that EGF and HGF may not be beneficial for liver regeneration after APAP overdose and additional studies are required to elucidate additional mechanisms of liver regeneration after APAP overdose. In contrast to EGF and HGF, vascular endothelial growth factor (VEGF) is important in liver regeneration after APAP overdose. Expression of VEGF and its receptors (VEGFR1, VEGFR 2, and VEGFR 3) increase after APAP overdose in mice. In addition, treatment with a VEGF inhibitor decreases regeneration in those mice after APAP overdose showing the advantageous role of VEGF in liver regeneration[29].

A novel incremental dose model has been developed in our laboratory to provide insight into several signaling pathways that are involved in liver regeneration after APAP overdose. In this model, liver injury and regeneration were compared over a time course after administration of 300 mg/kg APAP (APAP 300) and 600 mg/kg APAP (APAP 600) in mice. After APAP 300, liver injury was followed by regeneration. By contrast, after APAP 600, liver injury persisted over the time course. Hence, APAP 300 was defined as the regenerative dose and APAP 600 as the non- regenerative dose[22]. This model revealed that the canonical Wnt/ β-catenin signaling pathway is one of the main pathways involved in liver regeneration after APAP overdose. The pathway was stimulated in the regenerative dose and was inhibited in the non- regenerative dose, suggesting the reason for the inhibited regeneration at the higher APAP dose. The inhibition of Wnt/ β -catenin pathway was evident by decreased nuclear localization of β-catenin and substantial increase in the two inactive forms of β-catenin (Ser45/Thr41 phosphorylated and Ser33/37/Thr41 phosphorylated). The non-phosphorylated or active form of glycogen synthase kinase-3β (GSK3β) regulates β-catenin degradation, and its

expression was increased at the higher dose or the non- regenerative dose. Additionally, liver regeneration was increased in β- catenin overexpressing mice following APAP overdose showing the potential role of β-catenin in regeneration[22].

Hippo Signaling Pathway

The Hippo signaling pathway is highly conserved from *Drosophila* to mammals and is involved in the regulation of organ size[30]. Yes-associated protein (YAP) is the downstream transcriptional coactivator of the Hippo signaling pathway (Fig. 2). The Hippo pathway consists of two serine/threonine kinases Mst1/2 and LATS1/2, both of which act upstream of YAP. Activated Mst phosphorylates and activates LATS kinase. The activated LATS then phosphorylates YAP at serine 127. Once phosphorylated, YAP is inactive and is targeted for 14-3-3 mediated degradation in the cytoplasm. However, when the pathway is inactive, YAP is not phosphorylated by upstream protein kinases. The non-phosphorylated-active YAP enters the nucleus, associates with the transcription factor TEAD and initiates the transcription of genes involved in cell proliferation and survival[30, 31].

Upstream regulators of the Hippo pathway remain elusive. Studies have implicated several proteins including NF2/Mer, Fat cadherin and G-protein coupled receptors as upstream regulators[32-34]. Increased nuclear YAP and elevated YAP protein level is observed in liver specific NF2 knockout mice. The activity of LATS 1/2 is also reduced in these NF2 deficient mice showing that NF2 acts upstream and regulates the Hippo pathway[33]. Fat cadherin, a transmembrane protein in the apical membrane in

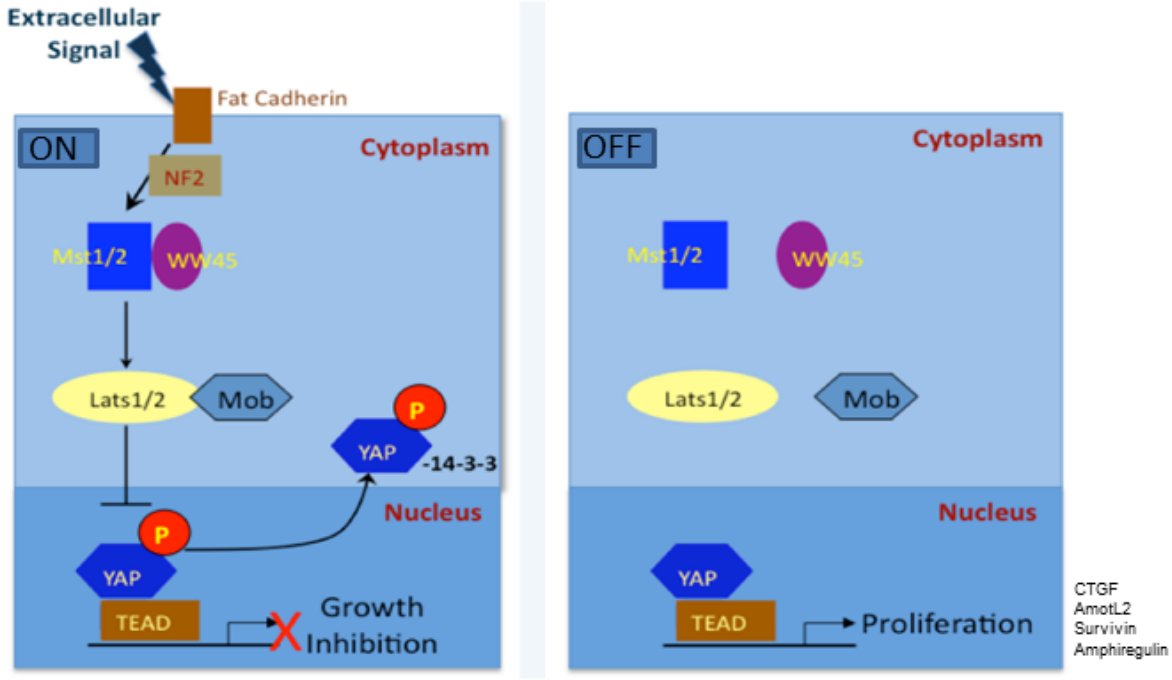

Figure 2: Hippo Signaling Pathway

In the on state, the upstream proteins of the Hippo pathway are active and hence phosphorylates YAP leading to its degradation in cytoplasm. However, in the off state, YAP is in its active form which enters the nucleus, binds with TEAD and initiates the transcription of genes involved in anti-apoptosis and proliferation.

epithelial cells of the drosophila imaginal disc, was also shown to regulate the Hippo pathway. Mutation in fat (ft) cadherin in drosophila imaginal disc results in the increased size of the disc. The disc size is even greater in a ft homozygous and wts (LATS 1/2 ortholog in drosophila) heterozygous double mutant. However, the size of the disc is suppressed and returns back to the size of wild type disc when one copy of yki (drosophila ortholog of YAP) is also mutated in the ft mutant. This change in the disc

size explains that Fat controls organ size by modulating the activity of the Hippo pathway[34]. It has also been shown that the Hippo pathway acts downstream of G-protein coupled receptor (GPCR). Overexpression of GPCRs that mainly activate Gs signaling induce YAP/TAZ phosphorylation. Also, activation of Gs-coupled receptor, by epinephrine or glucagon stimulation, increases LATS 1/2 activity and inhibits YAP function showing GPCR as the modulator of the Hippo pathway[32].

Roles of YAP in Pathophysiology

Studies have shown the roles of YAP in organ size control, liver development, regeneration, stem cell function and cancer development[31, 35-37]. The overexpression of Yorki (Yki, the drosophila ortholog of YAP) in the imaginal disc of the fly during development results in the massive increase in the size of the wing. This overgrowth results because the cells overexpressing Yki multiply faster than the wild-type cells. There is no acceleration of the particular phase of the cell-cycle after Yki overexpression; each phase is proportionally accelerated[38]. Similarly, overexpression of YAP in a normal mouse liver without surgical resection or injury results in massive hepatomegaly because of an increase in cell number, not the cell size[31].

The liver undergoes a maturation process following birth. The proliferation, differentiation of liver, and expression of an adult genes, several drug metabolizing enzymes, transporters and other enzymes involved in the metabolic process occur during the postnatal liver development period[36, 39]. This process takes about 30 days in rodents and up to 5 years in humans[40]. The involvement of YAP in postnatal liver

development has been studied[36]. The activation of YAP and increased proliferation are observed during postnatal liver development in mice. Also, the partial deletion of YAP in mice results in decreased liver to body weight ratio and decreased proliferation during the postnatal period. These findings demonstrate that YAP stimulates cell proliferation and is involved in hepatic differentiation during postnatal liver development[36].

The mammalian liver has tremendous capacity to regenerate. Following partial hepatectomy (PH), in which approximately 60% to 70% of the liver is removed, the remaining liver regenerates and restores lost liver mass after 5-7 days by proliferation of all the existing mature cellular populations[41]. Involvement of the Hippo signaling pathway has been studied during liver regeneration following PH[35]. During the regeneration period, the expression level of YAP is increased, its inactive form (p-YAP) is decreased and its nuclear localization is increased showing the activation of YAP. Also, YAP target genes, including CTGF, AmotL2 and Cyr61, are also increased. These results support the involvement of YAP during liver regeneration after PH[35]. The proliferation of mature hepatocytes is sufficient for regeneration after acute liver injury. However, during conditions of extreme stress and chronic injury, hepatic progenitor cells (HPCs), which are also called “oval cells”, undergo proliferation and differentiation to restore the liver mass[42,43]. The expression and activation of YAP is higher in biliary cells than in hepatocytes, shown by the increased nuclear localization of YAP in bile ducts. Overexpression of YAP in biliary cells results in hyperplasia, with no activation or appearance of HPCs[44]. However, overexpression of YAP in hepatocytes can transdifferentiate them into HPCs. High levels of YAP in hepatocytes alters the fate of about 75% of the adult hepatocytes which develop into HPCs. In addition, when YAP

overexpression is ceased after the hepatocytes differentiate into HPCs, the HPCs redifferentiate into cells of the hepatocyte lineage[44]. These findings suggest that overexpressing YAP in hepatocytes of chronically injured liver could accelerate the recovery of the lost liver mass.

Because YAP plays a critical role in the regulation of organ size and regeneration, it must have potent growth promoting activity. Indeed, overexpression of YAP is observed in various forms of cancer: lung, ovarian, pancreatic, colorectal, hepatocellular, and prostate carcinomas[31, 37]. Specific to hepatic malignancies, hepatocellular carcinoma (HCC), hepatoblastoma (HB) and cholangiocarcinoma (CC) are the three different types of tumors that arise from the liver cells[45]. HCC is the sixth most common cancer worldwide and the incidence is attributed mostly to the Hepatitis B or C Virus (HBV/ HCV)[46]. Increased YAP expression is seen in samples of HBV-induced HCC and HBV-infected cell lines[47]. Similarly, elevated YAP expression and its nuclear localization is observed in HCC cell lines[48]. Furthermore, induction of YAP in mice results in uniform liver expansion initially and then results in hyperplastic nodule formation throughout the hepatic parenchyma. The nodules are composed of proliferative hepatocytes and display features of HCC[31]. HB is the most frequent pediatric liver tumor and exhibits significant activation of YAP[48]. CC is the cancer that originates from the cholangiocytes of the intra- and extrahepatic biliary tract system. Overexpression of YAP and its nuclear localization is observed in CC cells and human samples[48, 49]. Silencing YAP inhibits CC tumorigenesis and metastasis, while overexpressing YAP promotes CC tumorigenesis and metastasis both *in vivo* and *in vitro*[49]. Thus, downregulating YAP could reduce liver tumor formation and metastasis. A

small molecule inhibitor called verteporfin has been discovered, which prevents the physical association between YAP and TEAD by selectively binding to YAP and changing its conformational structure to prevent any interaction with TEAD. Decreased liver size is observed in the YAP transgenic mice after verteporfin administration, demonstrating the potential use of verteporfin in patients with HCC[50].

In summary, YAP is involved in regulation of organ size in Drosophila as well as in mouse liver[31, 38]. YAP is also considered a candidate oncogene in multiple forms of human cancers[51]. Additionally, activation of YAP is shown in liver regeneration after partial hepatectomy[35]. However, the role of the Hippo Kinase pathway and YAP in liver injury and subsequent regeneration after APAP overdose is not known; this was the focus of my studies. Based on the previous studies, I hypothesized that YAP promotes liver regeneration following APAP overdose, and this was tested as defined briefly in the following specific aims:

Specific Aim 1: To Determine the role of YAP in liver injury and regeneration after 300 mg/kg APAP treatment.

Based on the previous findings, I hypothesized that YAP promotes liver regeneration following APAP overdose. To test my hypothesis, we determined activation of YAP and induction of its target genes: AmotL2 and CTGF following treatment of 300 mg/kg APAP (APAP 300) in mice.

Specific Aim 2: To Evaluate the effect of hepatocyte specific deletion of YAP on liver injury and regeneration after APAP overdose.

To confirm the role of YAP, we generated hepatocyte specific YAP KO mice by treating two-three months old male YAP fl/fl mice with AAV8.**TBG**.PI.**Cre**.rBG virus (hYAP-KO) and AAV8.**TBG**.PI.**eGFP**.WPRE.bGH (Control). We then tested the effect of virus on the liver and effect of YAP deletion on the components of APAP metabolism pathway. We also observed liver injury, APAP metabolism pathway and liver regeneration in the hYAP-KO and control group following 300 mg/kg APAP administration.

Materials and Methods

Animals, Treatments, and Tissue Collection

Two to three month old male C57BL/6J mice were purchased from Jackson Laboratories (Bar Harbor, ME). YAP floxed/floxed (YAP fl/fl) mice were generated by the NIH supported Knockout Mouse Project (KOMP) at the University of California Davis and were on the C57BL/6J background. All animals were housed in an Association for Assessment and Accreditation of Laboratory Animal Care-accredited facilities at the University of Kansas Medical Center (KUMC) under a standard 12 hour light-dark cycle with access to chow and water *ad libitum*. All studies were approved by the Institutional Animal Care and Use Committee at KUMC.

Generation of Hepatocyte specific YAP KO mice (hYAP-KO): hYAP-KO mice were generated using Cre-LoxP technology. Two to three month old male YAP fl/fl mice (n= 3-5) were treated with either AAV8.**TBG**.PI.**Cre**.rBG virus (hYAP-KO) or the AAV8.**TBG**.PI.**eGFP**.WPRE.bGH (Control mice) purchased from University of Pennsylvania Vector Core. The AAV8.TBG.PI.Cre.rBG ($2.5 * 10^{11}$ viral particles) and AAV8.TBG.PI.eGFP.WPRE.bGH ($2.5 * 10^{10}$ viral particles) were diluted in 200µl of sterile 1X PBS and injected into YAP fl/fl mice intraperitoneally as described previously[52]. All mice were euthanized one week after AAV8 injection to determine gene deletion.

Acetaminophen (APAP) overdose experiments: Mice (n= 3-5) were fasted 12 hours before APAP administration. APAP was dissolved in warm 0.9% saline, and mice were treated with 300 mg/kg APAP intraperitoneally. Food was returned to the mice after one hour of APAP treatment. Mice were sacrificed at 0, 3, 6, 12, 24, 48, 72 and 96 hours after APAP administration by cervical dislocation under isoflurane anesthesia[22]. The YAP fl/fl mice were treated with 300 mg/kg APAP after 7 days of virus treatment and were sacrificed at 0, 1, 12, 24 and 48 hours after APAP treatment. Blood and livers were collected. Serum samples were obtained by centrifuging blood at 5000 rpm for 10 minutes at 4°C and used for analysis of alanine aminotransferase (ALT) activity. The liver tissue was frozen in liquid N_2 and stored at −80°C until it was used to isolate RNA and to prepare total cell extracts.

Protein Isolation and Western Blot Analysis

Total protein isolated from liver tissues using RIPA buffer [1% SDS, 20mM Tris-HCl (pH 7.5), 150mM NaCl, 0.5% NP-40, 1% Triton X-100, and 0.25% sodium deoxycholate]. Cocktail of protease and phosphatase inhibitors (Halt Protease and Phosphatase Inhibitor Cocktail with EDTA: Thermo Fisher Scientific) was added at a concentration of 1:100 to the RIPA buffer before use.

Protein concentration of the lysates was determined using the bicinchoninic acid (BCA) protein assay reagent (Thermo Fisher Scientific). Total proteins (50 µg) were separated by electrophoresis on 4-12% NuPage Bis-Tris gels with MOPS buffer (Invitrogen,

Carlsbad, CA), then transferred to Immobilon-P membranes (Millipore) in NuPAGE transfer buffer containing 20% methanol. Membranes were stained with Ponceau S to verify loading and transfer efficiency. Membranes were then probed with primary and secondary antibodies in Tris-buffered saline with Tween 20 containing either 5% nonfat milk or 5% bovine serum albumin depending upon the antibody used. The signal was visualized by incubating the blots in SuperSignal West Pico Chemiluminescence Substrate (Thermo Fisher Scientific) and exposing to X-ray film (MidSci, St. Louis, MO).

All antibodies used for Western analysis are listed in Table 1.

Histology and immunohistochemistry (IHC)

Paraffin-embedded liver sections (4 μm thick) were used. Slides were stained with hematoxylin and eosin (H &E) to observe necrosis in liver sections using an autostainer (model CV 5030, Leica Microsystems, Buffalo Grove, IL).

Paraffin sections were also stained with proliferating cell nuclear antigen (PCNA) (Cell Signaling, Catalog# 2586) to detect cell proliferation and YAP antibody (1:25 dilution) (Cell Signaling, Catalog# 4912). Antigen retrieval was achieved by the citrate buffer method. Slides were placed in boiling citrate buffer for 5 mins followed by incubation at sub-boiling temperature for 10 mins. The tissues were then blocked with 5% normal goat serum for 1 hr. Then the tissues were incubated with the primary antibody overnight at 4°C. The next day, the primary antibody was linked to a biotinylated secondary antibody followed by amplification using the routine avidin-biotin complex method (ABC Vectastain kit: Vector laboratories, Burlingame, CA). Diaminobenzidine

(DAB) was used as the chromogen, which resulted in a brown reaction product which precipitated on to the tissue surface.

Real- Time PCR

RNA was isolated from frozen liver tissues using the Trizol method according to the manufacturer's protocol (Sigma, St. Louis, MO). mRNA for various genes were quantified using SYBR Green method on a real-time PCR system (model 7300, Applied Biosystems, Foster City, CA). RNA concentration was measured by spectrophotometry and samples were diluted to 0.1 µg/µl. cDNA was made using 1 µg of RNA per sample using M-MuLV Reverse transcriptase enzyme (Invitrogen, Carlsbad, CA) in a reverse transcription (RT)-mastermix containing random primers, 5× RT buffer, dNTP mix, RiboLock, and M-MuLV Reverse transcriptase enzyme. Analysis of cDNA was done using SYBR Green technology on a real-time PCR system (Applied Biosystems). 18S gene was used as an internal control. The Ct value of the 18S and the gene of interest obtained from the PCR system were used to calculate dCt value. ddCt values for each time points were then calculated subtracting mean dCt value of 0 hr of the respective group from that time point dCt value. Fold change was then measured using the formula 2^{-ddCt}. All samples were used in triplicate. All primers are obtained from Integrated DNA Technologies (IDT) and listed in Table 2.

Table 1: List of antibodies used for Western blot analysis

Antibodies	Catalog No.	Company
YAP	4912	Cell Signaling
p-YAP	4911	Cell Signaling
CYP2E1	19140	Abcam
RIP1	3493	Cell Signaling
RIP3	2283	ProSci Inc.
Total JNK	9252	Cell Signaling
p-JNK	4668	Cell Signaling
PCNA	2586	Cell Signaling
Cyclin D1	2978	Cell Signaling
p-Rb	8516	Cell Signaling
CDK4	2906	Cell Signaling
GAPDH	2118	Cell Signaling

Table 2: List of primer sequences used for real-time PCR analysis

Gene Name	Forward Primer	Reverse Primer
18S	TTGACGGAAGGGCACCACCAG	GCACCACCACCCACGGAATCG
AmotL2	GAGAGAGATTGGAATCGGCA	GCTTCTCCTGTTCCTGTTGC
CTGF	GGGCCTCTTCTGCGATTTC	ATCCAGGCAAGTGCATTGGTA

APAP protein adducts

APAP protein adducts were measured in Dr. Hartmut Jaeschke's laboratory using high pressure liquid chromatography with electrochemical detection as previously described[53].

ALT and Glutathione measurement

ALT in serum was measured by using ALT (GPT) kit (ThermoFisher Scientific, Pittsburg, PA) and total glutathione in the liver tissues were measured by using Glutathione Assay Kit (Sigma Life Science).

Statistical Analysis

All results were expressed as mean ± SE. Comparison between multiple groups were performed using one-way ANOVA and between two groups were performed using ANOVA with Tukey's post hoc adjustment. $P<0.05$ was considered significant.

Results

Activation of YAP in APAP 300

After APAP 300, there is an increase in liver injury until 12 hours, after which there is regression of injury and complete recovery by 72 and 96 hours (Fig. 3A). The hepatic cells enter the cell cycle and divide in response to liver injury, which was studied using PCNA analysis (Fig. 3B)[22]. We first examined the activation of YAP over the time course following treatment of 300 mg/kg APAP. The activation of YAP was studied using the Western blot analysis of total YAP and p-YAP. Additionally, immunohistochemical staining of YAP on liver sections was performed. A ratio of densitometric analysis of Western blot data on YAP to p-YAP was used as a measure of YAP activity. Western blot analysis indicated that YAP activity was unchanged during the initial time points and the change was seen at 72 hours after APAP treatment (Fig. 3, C and D). In IHC staining, YAP was mostly localized in the nucleus of biliary cells at 0 hr. At 3, 6 and 12 hours, YAP was localized in the nucleus of both biliary and hepatic cells suggesting increased activation of YAP at these time points. The nuclear localization of YAP decreased while cytoplasmic localization increased at 24,48 and 72 hours. These data suggest YAP activation was reduced at these time points after APAP-induced liver injury. When the hepatic cells are completely recovered at 96 hours, YAP was localized in the biliary compartments, similar to the 0 hour time point (Fig. 4). Next, we looked at mRNA expression of the target genes of YAP: AmotL2 and CTGF. The mRNA levels of both genes were moderately higher at early time points compared to later when there is activation of YAP (Fig. 3, E and F). Taking the IHC and target gene expression data together, we concluded that YAP activity increases during initial time points up to 12 hr

after APAP overdose followed by a significant decrease. Because of the known role of YAP in proliferation and regulation of organ size as demonstrated by previous studies[35, 38, 51], we hypothesized that YAP activation would occur at later time points when the hepatic cells are proliferating and recovering. By contrast, YAP activation was seen when there is progression of injury.

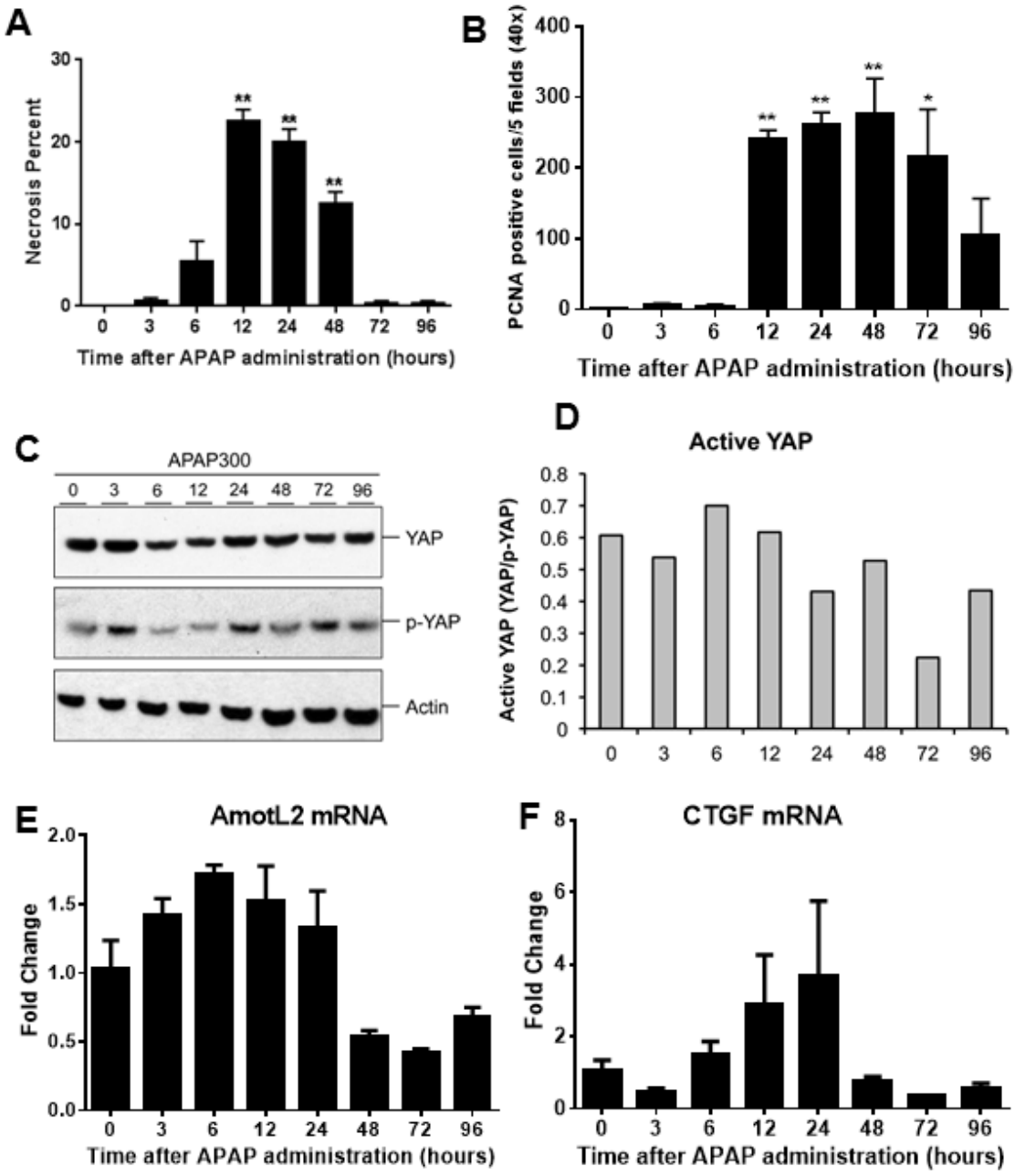

Figure 3: Activation of YAP in APAP 300

A-B: Bar graphs show % necrosis area based on H&E stained liver sections (**A**), total number of PCNA positive cells per five high power fields (**B**). **C:** Western blot analysis of total YAP and p-YAP using total liver extract. **D:** Densitometric analysis shows active YAP, which is the ratio of total YAP to p-YAP. **E-F:** Bar graphs show mRNA expression of YAP target genes: AmotL2 (**E**) and CTGF (**F**) in liver. *P<0.05 and **P<0.001 between different time points with respect to 0 hour.

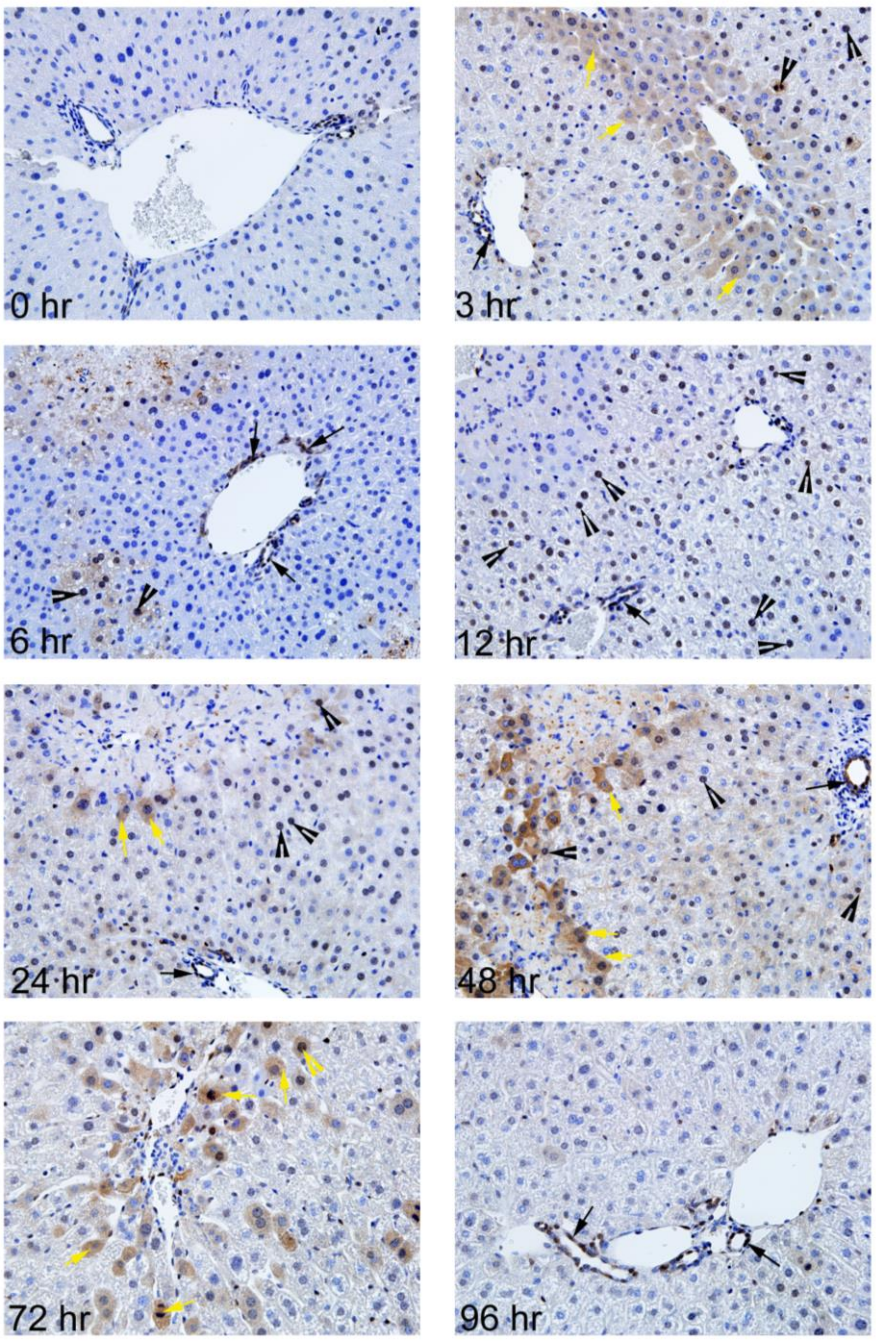

Figure 4: YAP staining in APAP 300

A: Representative photomicrographs of YAP-stained liver sections per five high power (40x) fields from mice treated with 300 mg/kg APAP. **Arrowheads** represent YAP positive cells (black: nuclear staining and yellow: cytoplasmic staining).

Characterization of hYAP-KO mice

To further investigate the role of YAP in liver injury and regeneration following APAP overdose, we did additional studies in hYAP-KO model. Deletion of YAP was confirmed by Western blot analysis (Fig. 5A). The liver-to-body-weight ratio was not affected by YAP deletion (Fig. 5B). We measured serum ALT levels to determine whether deletion of YAP resulted in liver injury, which was normal in both the groups (Fig. 5C). Furthermore, hepatic CYP2E1 protein expression and total glutathione levels, both of which are the major components in APAP bioactivation process, were not changed between the groups (Fig. 5, D and E). These results suggest that the AAV8 virus is not causing liver injury and not is changing the components required for APAP bioactivation. Hence, the virus is experimentally good to use for hepatocyte specific YAP deletion.

Kinetics of APAP-induced liver injury in hYAP-KO mice

Next, we compared liver injury in two groups over the time course of 0-48 hours by determining the serum ALT activity level and histopathological analysis of liver sections (Fig. 6, A, B and C). The injury developed similarly in both groups until 12 hours after APAP treatment. However, liver injury in hYAP-KO mice decreased by 24 hours leading to almost complete recovery by 48 hours. The injury in control mice further progressed and increased at 24 hr. At 48 hr, liver injury declined in control mice as well but was significantly higher than the liver injury in hYAP-KO mice. These data indicate that

hepatocyte-specific YAP deletion leads to rapid regression of liver injury after APAP overdose.

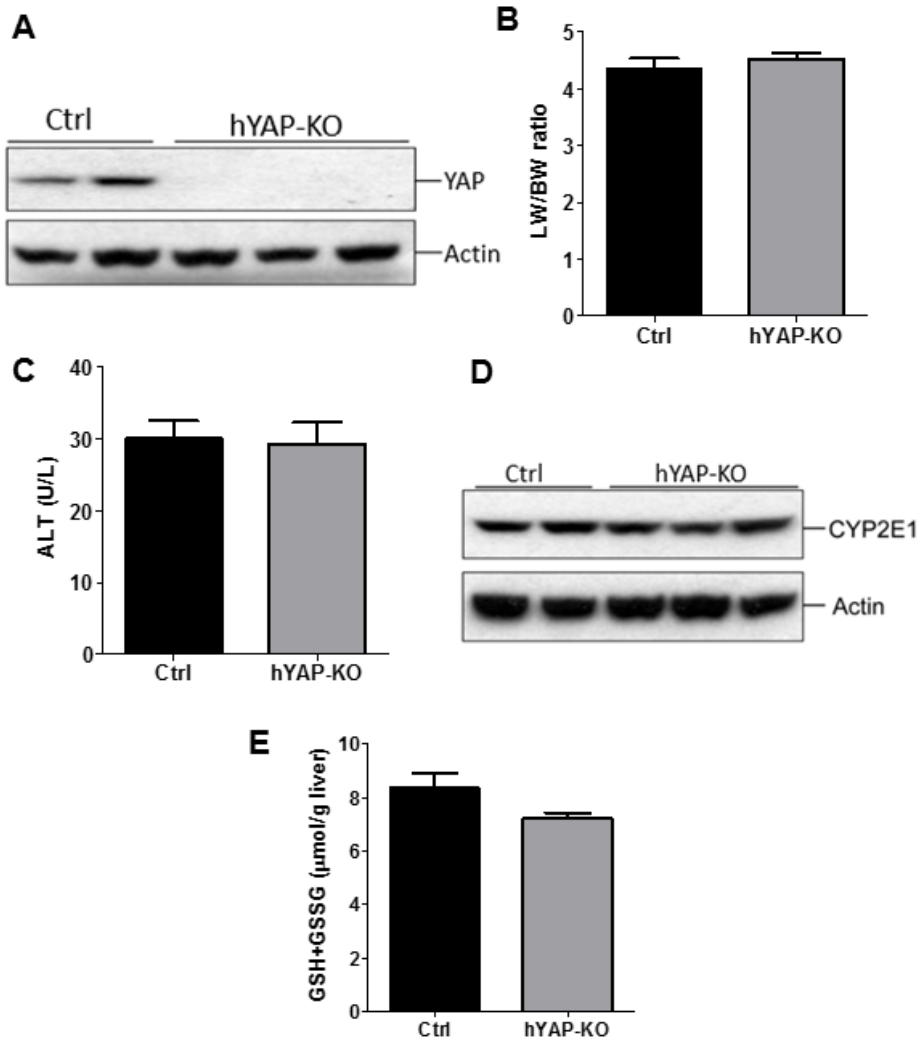

Figure 5: Characterization of hYAP-KO mice

A: Western blot analysis of YAP in Control (Ctrl) and YAP knockout (hYAP-KO) mice. **B-C:** Bar graphs show liver to body weight ratio (**B**) and Serum ALT level (**C**). **D:** Western blot analysis of CYP2E1 in Ctrl and hYAP-KO mice. **E:** Bar graph shows total glutathione (GSH+ GSSG) concentration in Ctrl and hYAP-KO mice.

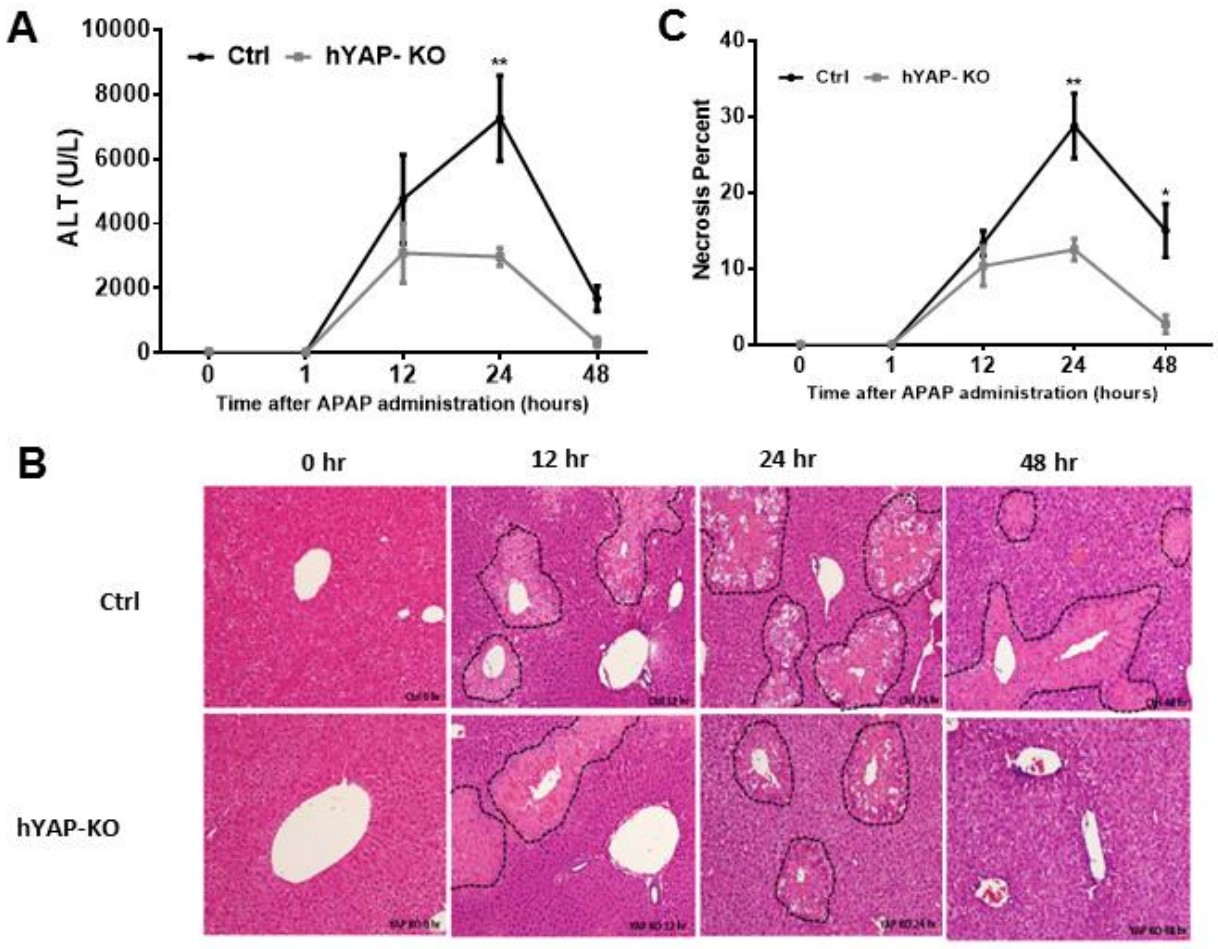

Figure 6: Kinetics of APAP-induced liver injury in hYAP-KO mice

A: Line graph show serum ALT level in Ctrl (black line) and hYAP-KO (gray line) mice treated with 300 mg/kg APAP. **B:** Representative photomicrograph of H&E stained liver sections from Ctrl and hYAP-KO mice treated with 300 mg/kg APAP with necrotic areas outlined.

C: Line graph shows % necrosis area based on H&E stained liver sections. *P<0.05 and **P<0.001 between Ctrl and hYAP-KO group.

Mechanism of decreased liver injury after APAP overdose in hYAP-KO mice

We then looked into the components of APAP metabolism and injury pathway to understand the mechanism of the differences in liver injury in control and hYAP-KO mice. There was no significant difference in APAP-protein adducts between the groups at 1 and 12 hours after APAP treatment, suggesting that there is no difference in metabolic activation of APAP (Fig. 7A). The depletion of hepatic glutathione level from 0 to 1 hour also indicated that there was no difference in APAP metabolism between the two groups. However, the recovery of glutathione was faster in hYAP-KO mice compared to control mice. GSH levels increased in both control and hYAP-KO mice but were significantly higher in hYAP-KO mice at 12 and 24 hours after APAP treatment (Fig. 7B). These data indicate that the early GSH replenishment in hYAP-KO mice could be the reason for the faster regression of injury in those mice. Previous studies have shown that RIP1 and RIP3 mediate hepatic injury after APAP overdose[54, 55]. In our studies, RIP1 level was similar in both control and hYAP-KO mice, while RIP3 level was higher in hYAP-KO mice at 1, 12 and 24 hours after APAP treatment (Fig. 7C). The activation of c-jun N-terminal kinase (JNK), as indicated by its phosphorylated form (p-JNK), started at 1 hour in both groups. JNK activation remained higher until 12 hours after APAP treatment in hYAP-KO but was observed until 24 hr in the control mice (Fig. 7D). These data indicate that YAP may be attenuating GSH recovery and also mediating JNK phosphorylation, which could be the reasons for decreased liver injury in hYAP-KO mice after APAP overdose.

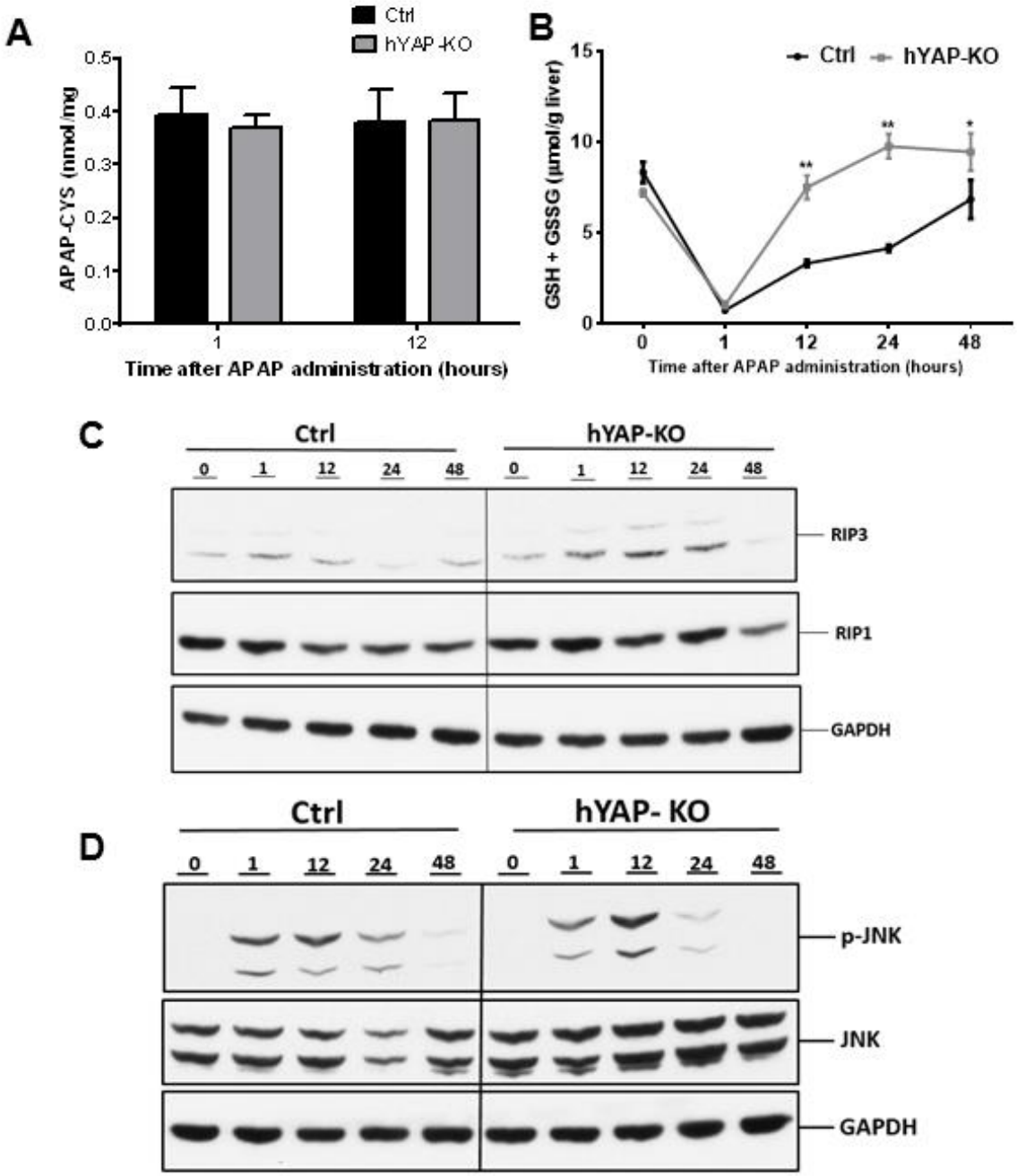

Figure 7: Mechanism of decreased liver injury after APAP overdose in hYAP-KO mice

A: Bar graph shows APAP adduct in Ctrl (black bar) and hYAP-KO (gray bar) mice at 1 and 12 hours after APAP 300 treatment. **B:** Line graph shows total glutathione (GSH+ GSSG) in Ctrl

(black line) and hYAP-KO (gray line) mice treated with 300 mg/kg APAP. **C-D:** Western blot analysis of RIP3 and RIP1 (**C**) and JNK and p-JNK (**D**) in Ctrl and hYAP-KO mice treated with 300 mg/kg APAP. *P<0.05 and **P<0.001 between Ctrl and hYAP-KO group.

Early induction of regenerative markers in hYAP-KO mice

Next, we studied liver regeneration in control and hYAP-KO mice using PCNA analysis. The hepatic cells in hYAP-KO showed more proliferation at 24 hours after APAP treatment than the control mice, as indicated by PCNA positive cells around the necrotic areas. However, there was more proliferation in control mice than hYAP-KO mice at 48 hours of APAP treatment (Fig. 8A). These data associated with the lower injury in hYAP-KO mice at 24 hours following APAP treatment (Fig. 6, A-B). We further studied expression of cell cycle proteins: Cyclin D1, CDK4 and p-Rb in the two groups. Cyclin D1 is the marker for cell cycle (G1 phase) progression. Cyclin D1 and CDK4 complexes phosphorylate Rb to overcome the late restriction checkpoint in the cell cycle[56]. Similar to the PCNA data, induction of Cyclin D1, CDK4 and p-Rb were higher in hYAP-KO mice than control mice at 24 hours, while these proteins were expressed more in control mice than hYAP-KO mice at 48 hours after APAP treatment (Fig. 8, B-D). These data suggest that hYAP-KO mice show an earlier onset of regeneration than control mice after APAP treatment.

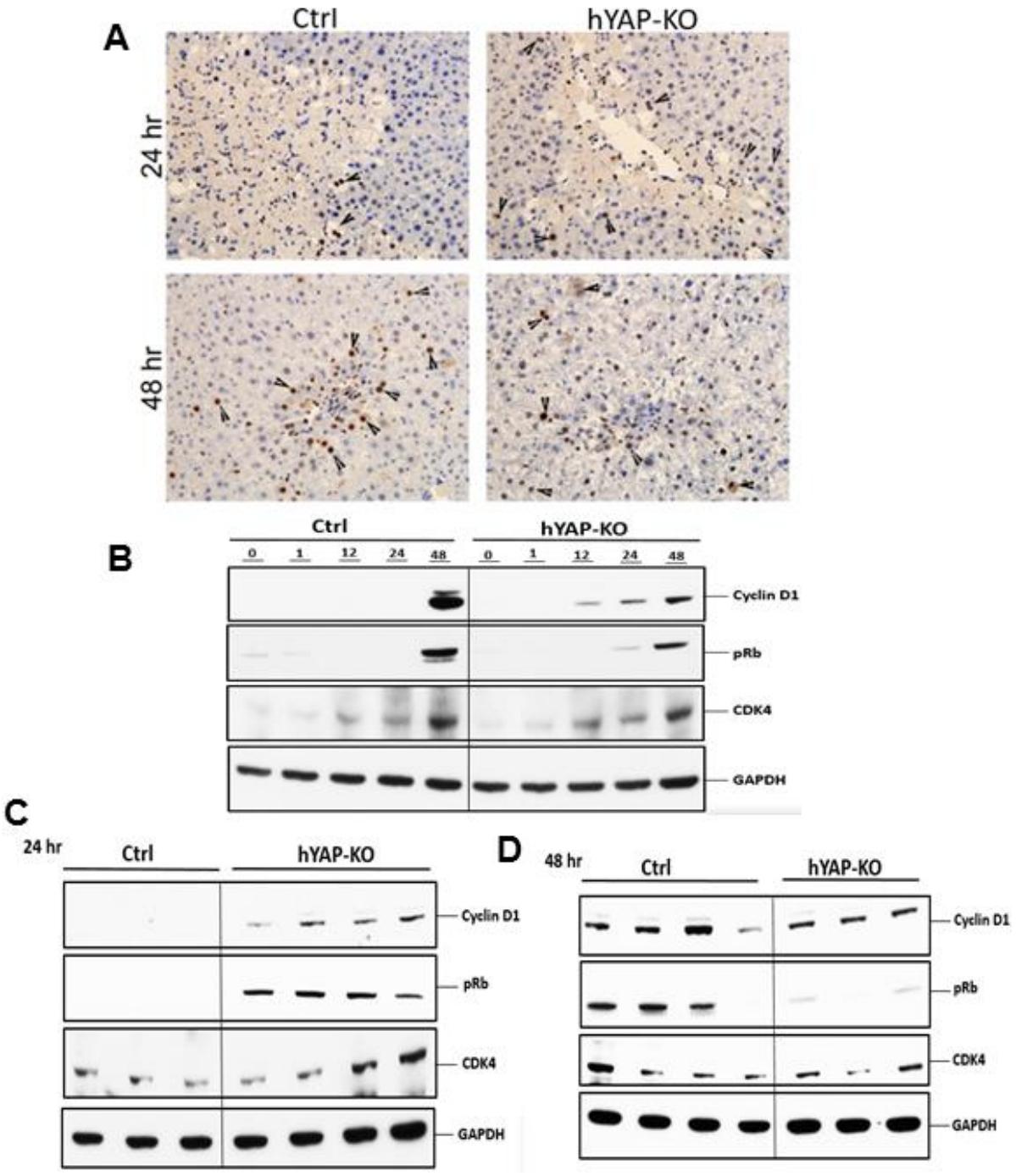
A
Ctrl
hYAP-KO
24 hr
48 hr
B
Ctrl
hYAP-KO
0
1
12
24
48
Cyclin D1
pRb
CDK4
GAPDH
C
24 hr
Ctrl
hYAP-KO
Cyclin D1
pRb
CDK4
GAPDH
D
48 hr
Ctrl
hYAP-KO
Cyclin D1
pRb
CDK4
GAPDH

Figure 8: Early induction of regenerative markers in hYAP-KO mice

A: Representative photomicrograph of PCNA stained liver sections from Ctrl and hYAP- KO mice at 24 and 48 hours after APAP treatment. **B-D:** Western blot analysis of regenerative markers Cyclin D1, pRb and CDK4 in Ctrl and hYAP-KO mice during the time course (**B**), at 24 hours (**C**) and at 48 hours (**D**) after APAP 300 treatment. **Arrowhead** represents PCNA positive cells.

Discussion

Acetaminophen overdose is the most common cause of acute liver failure in the US[2, 3]. Treatment options for this drug-induced ALF are limited[5-7]. A number of studies on chemicals and drugs that induce liver injury have shown that compensatory liver regeneration is the critical determinant of overall survival following APAP overdose[19, 57, 58]. Several studies using animal models have shown that stimulating liver regeneration can be a potential treatment option for the APAP induced ALF[21, 22]. Herein, we studied the role of YAP, the downstream effector of the Hippo Kinase pathway, in liver injury and regeneration after APAP overdose.

YAP is involved in the regulation of organ size, hepatic differentiation and proliferation[31, 35, 38, 51]. Based on the known role of YAP, we hypothesized that activation of YAP would increase at the time points when the hepatic cells are proliferating and regenerating following APAP-induced acute liver injury. Thus, we investigated YAP activation in male C57BL/6J mice following 300 mg/kg of APAP. Unexpectedly, the activation of YAP was seen at earlier time points when there is progression of injury in hepatic cells while a decrease in YAP activation was observed during the regenerative phase post 24 hr. To further evaluate the role of YAP after APAP overdose, we did additional studies on hepatocyte-specific YAP-KO mice (hYAP-KO).

First, we looked at the injury level caused by APAP overdose in control and hYAP-KO mice. There was faster regression of injury in hYAP-KO mice. To understand the mechanism behind this faster recovery in hYAP-KO mice, we looked into several components of APAP metabolism pathway. There was no change in the APAP-protein adduct levels and the depletion of glutathione during the metabolism of APAP was

similar. This indicates that there is no difference in the bioactivation of APAP to its reactive metabolite NAPQI. However, the total hepatic glutathione level (GSH+GSSG) recovered faster in hYAP-KO mice. Glutathione is the major anti-oxidant that protects the liver from free radical injury. Increased oxidative stress in mitochondria during APAP toxicity is due to increased levels of peroxides and peroxynitrite. The superoxide formed during APAP toxicity is converted to hydrogen peroxide increasing oxidative stress. Glutathione is the cofactor for glutathione peroxidase, which detoxifies peroxides. Additionally, nitric oxide reacts with superoxide to form peroxynitrite, amplifying oxidative stress. Peroxynitrite is also detoxified by glutathione[59]. The higher glutathione level detoxifies the reactive oxygen species and the peroxynitrite radicals leading to reduced oxidative stress and lower injury. Hence, the faster recovery of glutathione in hYAP-KO mice could be the reason for the faster regression of injury in those mice.

RIP1 and RIP3 proteins are involved in hepatic injury after APAP overdose[54, 55]. The RIP1 protein level was similar in the two groups, while the RIP3 level was higher in hYAP-KO mice where there is protection from injury. RIP3 regulates JNK activation and the mitochondrial oxidant stress[54]. Adduct formation is unchanged, while downstream mitochondrial dysfunction and oxidant stress is reduced in RIP3-deficient mice[54]. RIP3 expression was higher in hYAP-KO mice in our study. However, the activation of JNK was higher and sustained longer in control mice as compared to the YAP KO mice. This shows that JNK activation is not regulated by RIP3 expression in hYAP-KO mice following APAP overdose.

The activation of JNK amplifies mitochondrial oxidant stress and causes the formation of mitochondrial membrane permeability transition (MPT) pores, which are

responsible for the disruption of mitochondrial membrane permeability and cessation of ATP synthesis, ultimately leading to hepatic cell necrosis[15]. Thus, higher activation of JNK leads to higher injury. In our study, we observed a decrease in progression of injury in hYAP-KO mice, which was accompanied by an early decrease in JNK activation. However, the mechanism of how YAP regulates JNK remains to be elucidated. Interestingly, a previous study shows JNK1 and JNK2 act as YAP kinases, phosphorylating multiple sites on YAP[60]. The p-YAP is stabilized by binding to pro-apoptotic transcription factor resulting in apoptosis[60]. Our study shows that YAP may be involved in liver injury following APAP overdose, which is a novel function of YAP. This seemingly contradictory role of YAP has been previously studied. Besides regulating growth, YAP also promotes apoptosis and is often used as chemotherapeutic target to induce cell-death in tumor-derived cell lines and as tumor suppressor in certain breast cancers[61, 62]. The contradictory function may be explained by YAP's ability to bind to transcription factors other than TEAD, which can result in the activation of a different set of YAP target genes when compared to the ones we studied here. For example, the YAP- TEAD interaction promotes growth, while YAP binding to TAp73 signals apoptosis[60]. Whether or not this process occurs in our model, where there is necrotic cell death, remains to be studied.

Next, we studied liver regeneration in control and hYAP-KO mice following APAP overdose. A previous study has shown involvement of YAP in liver regeneration after PH[35]. However, in our study the hYAP-KO mice showed an earlier onset of regeneration than the control. The differences in the results may be due to inherent differences in models used to study liver regeneration. The APAP overdose has significant

mechanistic differences from the PH model. APAP overdose results in significant necrotic cell death and subsequent inflammation, both of which are minimal in PH[63, 64]. Additionally, liver regeneration is synchronous and involves proliferation of all the remaining hepatocytes in case of PH[64]. However, mostly the cells around the necrotic zones proliferate after APAP overdose[22]. Faster regeneration in hYAP-KO mice was most likely due to lower injury in those mice. There was an early induction of cyclin D1 (which governs entry into cell cycle) in hYAP-KO mice. Cyclin D1 is one of the target genes of Wnt/β-catenin pathway; YAP inactivates β-catenin[65]. Similarly, the NF-κB pathway is also activated during liver regeneration following APAP overdose, when the hepatic cells are less stressed[22]. Additionally, increased GSH levels is observed during proliferation of rat hepatocytes after PH. Increased GSH levels stimulates hepatocytes to shift from G_0 to G_1 phase of cell cycle[66]. In our study, we have observed increased levels of GSH in hYAP-KO mice. Thus, the early regeneration in hYAP-KO mice could be the result of increased activation of β-catenin and NF-κB pathway or increased GSH levels. The increased proliferation in the control mice was observed at 48 hr because these mice are still recovering as shown by presence of necrotic foci, while the hYAP-KO mice have almost completely recovered by this time.

Early regeneration in hYAP-KO mice may be due to decreased progression of injury or due to proliferative advantage of YAP-KO hepatocytes. To study this, we could perform an *in vitro* growth assay. Primary mouse hepatocytes could be isolated from control and hYAP-KO mice, without APAP treatment, and cultured in media with growth factors. Expression of several proliferative markers like PCNA, pRb, CDK4 and cyclins could be studied in the two groups at different time points (2, 4, and 6 days). If these

proliferative markers are induced early and higher in hYAP-KO mice, then it demonstrates that deleting YAP in hepatocytes helps the hepatocytes to enter the cell cycle and initiate proliferation.

To further evaluate the role of YAP in liver injury and regeneration, C57BL/6J male mice could be treated with 300 mg/kg APAP with or without exposure to the YAP inhibitor, verteporfin. As shown earlier, regeneration starts in these mice at 12 hours after APAP administration (Fig. 3B). Thus, these mice are then treated with verteporfin, the chemical that disrupts the interaction between YAP and TEAD, around 10 hours following APAP administration and regeneration is studied. If proliferation is higher in verteporfin treated group, then it shows that disrupting YAP activity promotes proliferation of hepatocytes. Thus, if we observe higher proliferation after knocking out the YAP gene or disrupting YAP activity, then we could say that the early regeneration in hYAP-KO mice is due to the proliferative advantage of YAP-KO hepatocytes. However, if the proliferation is higher in the control group in both of the studies mentioned above, then the reason for early regeneration could be the decreased progression of injury in hYAP-KO mice where the hepatic cells are less stressed. In such case, other signaling pathways which are involved in proliferation may be activated in hYAP-KO mice.

In summary, our findings indicate that deletion of YAP is associated with the protection of liver injury after APAP overdose. Faster regression of injury was observed after YAP deletion. This was followed by early regeneration in hYAP-KO mice as shown by an early induction of regenerative markers. Hence, YAP may serve as a potential therapeutic target for the treatment of APAP- induced ALF.

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