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Liver-specific deletion of mechanistic target of rapamycin does not protect against acetaminophen-induced liver injury in mice

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Abstract

Background: Acetaminophen (APAP) overdose can cause liver injury and liver failure, which is one of the most common causes of drug-induced liver injury in the United States. Pharmacological activation of autophagy by inhibiting mechanistic target of rapamycin (mTOR) protects against APAP-induced liver injury likely via autophagic removal of APAP-adducts and damaged mitochondria. In the present study, we aimed to investigate the role of genetic ablation of mTOR pathways in mouse liver in APAP-induced liver injury and liver repair/regeneration.

Methods: Albumin-Cre (Alb-Cre) mice, $mTOR^{f/f}$ and $Raptor^{f/f}$ mice (C57BL/6J background) were crossbred to produce liver-specific mTOR knockout (L-mTOR KO, Alb Cre+/−, $mTOR^{f/f}$) and liver-specific Raptor KO (L-Raptor, Alb Cre+/−, Raptor $^{f/f}$) mice. Alb-Cre littermates were used as wild-type (WT) mice. These mice were treated with APAP for various time points for up to 48 h. Liver injury, cell proliferation, autophagy and mTOR activation were determined.

Results: We found that genetic deletion of neither Raptor, an important adaptor protein in mTOR complex 1, nor mTOR, in the mouse liver significantly protected against APAP-induced liver injury despite increased hepatic autophagic flux. Genetic deletion of Raptor or mTOR in mouse livers did not affect APAP metabolism and APAP-induced c-Jun N-terminal kinase (JNK) activation, but slightly improved mouse survival likely due to increased hepatocyte proliferation.

Conclusions: Our results indicate that genetic ablation of mTOR in mouse livers does not protect against APAP-induced liver injury but may slightly improve liver regeneration and mouse survival after APAP overdose.

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Authors' contributions

W.-X. Ding, H.-M. Ni, H. Wang and H. Jaeschke conceived the study and designed experiments. W.-X. Ding supervised the study. W.-X. Ding and H. Jaeschke wrote the manuscript. H. Sun, H.-M. Ni, S. Fulte, T. McKeen, J.M. McCracken, J.Y. Akakpo performed experiments and analyzed the data.

Declaration of competing interest

The authors declare that they have no conflict of interest.

Keywords

Acetaminophen (APAP); Autophagy; Hepatotoxicity; Liver injury; Liver regeneration; Mechanistic target of rapamycin (mTOR); Regulatory associated protein of mTOR complex (Raptor)

1. Introduction

Overdose of acetaminophen (APAP) can cause severe hepatotoxicity and is one of the top causes of acute liver failure in the United States.[1] Taken at therapeutic doses, APAP is safe, because the majority of APAP is glucuronidated and sulfated, which is then secreted into plasma and bile. Only a small portion of APAP is metabolized by cytochrome P450 enzymes such as cytochrome P450 family 2 subfamily E member 1 (CYP2E1) that generates N-acetyl-*p*-benzoquinone imine (NAPQI), a highly reactive metabolite.[2] NAPQI is further detoxified by conjugation with glutathione (GSH), which is relatively abundant in the liver. However, under APAP overdose conditions, excessive formation of NAPQI causes depletion of hepatic GSH levels. After the depletion of GSH, NAPQI binds to the cysteine residue of intracellular proteins to form APAP-adducts (APAP-ADs). Many of the APAP-ADs are formed on mitochondria resulting in mitochondrial damage and subsequent mitochondrial translocation of c-Jun N-terminal kinase (JNK) to amplify mitochondrial oxidative stress and eventual hepatocyte necrosis.[3,4] N-acetylcysteine, a precursor of GSH, remains the current standard of treatment for patients with APAP overdose, which is often more efficient for patients at the early phase of APAP injury but its efficiency declines significantly when patients are in the late phase of injury.[5] Therefore, there is still a need to search for new treatments to combat APAP-induced liver failure, in particular at the late phase of the injury.

Macroautophagy/autophagy is a lysosomal degradation pathway that delivers intracellular proteins and damaged/excess organelles including mitochondria to lysosomes via double membrane autophagosomes that fuse with lysosomes to form autolysosomes. Autophagy is generally considered a cellular adaptive and protective mechanism as it can provide nutrients such as amino acids and free fatty acids for cell survival by breaking down cellular components within the autolysosomes.[6] Autophagy plays a critical role in liver pathophysiology as deletion of several essential autophagy-related genes in the mouse liver leads to hepatomegaly, increased liver cell death, inflammation and the formation of hepatocellular adenomas.[7–11] Previous work from our group and others show that the activation of autophagy protects against APAP-induced liver injury by removing APAP-ADs and damaged mitochondria.[12–17]

Mechanistic target of rapamycin (mTOR) is a highly conserved serine-threonine protein kinase that serves as an important sensor for cellular nutrients and energy levels.[18] There are two functionally distinct mTOR complexes, mTOR complex 1 (mTORC1) and 2 (mTORC2). mTORC1 is sensitive whereas mTORC2 is relatively resistant to rapamycin-induced inhibition. The activities and substrate specificities of mTORC1 and mTORC2 are regulated by their complex co-factors. mTORC1 consists of mTOR, DEPTOR (DEP domain containing mTOR-interacting protein), Tti/Tel2, mLST8 (mammalian lethal with SEC13 protein 8), regulatory associated protein of mTOR complex (Raptor) and

PRAS40 (proline-rich AKT substrate of 40 kDa) whereas mTORC2 contains unique Rictor (rapamycin-insensitive companion of mammalian target of rapamycin) and mSin1 components in addition to the shared common mTOR, mLST8, DEPTOR and Tti/Tel2 components.[19] Activation of mTORC1 increases the anabolic process by enhancing protein and lipid synthesis and concurrently inhibits the catabolic process of autophagic degradation. mTORC2 mainly regulates protein kinase B (AKT) activation, cell survival, cell migration and actin remodeling.[19,20] Autophagy and cell proliferation have been implicated in APAP-induced liver injury and repair, and both autophagy and cell proliferation are regulated by mTOR.[18,21] However, whether genetic manipulation of mTOR in mice would affect APAP-induced liver injury and repair has not been investigated. In this study, we investigated the effect of genetic deletion of mTOR and Raptor in mouse livers in APAP-induced liver injury and repair. We further determined the changes of autophagy markers and JNK activation in liver-specific mTOR and Raptor knockout (KO) mice after APAP treatment.

2. Materials and methods

2.1. Antibodies and other reagents

The rabbit anti-APAP-AD antibody was a kind gift from Dr. Lance Pohl (National Heart, Lung and Blood Institute, USA).[13] The other antibodies were: CYP2E1 (#ab28146) from Abcam (Cambridge, UK), p62 (#H00008878-M01) from Abnova (Taipei, China), phospho-S6 (#4858), total S6 (#2217), phospho-JNK (#4668), and GAPDH (#2118) from Cell Signaling Technology (Danvers, MA, USA); β-actin (#a5441) from Sigma-Aldrich (St. Louis, MO, USA), total JNK (#554285) from BD Pharmingen (Franklin Lakes, NJ, USA); proliferating cell nuclear antigen (PCNA) (#SC-56) from Santa Cruz Biotechnology (Dallas, TX, USA). The rabbit anti-microtubule-associated protein 1 light chain 3 (LC3) antibody was described previously.[22] The secondary antibodies were HRP-conjugated goat-anti-rabbit (#111-035-045), and HRP-conjugated goat-anti-mouse (#115-035-062) were from Jackson ImmunoResearch (West Glove, PA, USA). All other reagents were either from Sigma (USA) or Thermo Fisher Scientific (USA).

2.2. Animals and APAP treatment

Albumin-Cre (Alb-Cre) mice, mTOR$^{f/f}$ and Raptor$^{f/f}$ mice (C57BL/6J background) were purchased from Jackson Laboratory (Bar Harbor, ME, USA) and crossbred to produce liver-specific mTOR KO (L-mTOR, Alb Cre+/−, mTOR$^{f/f}$) and liver-specific Raptor KO (L-Raptor, Alb Cre+/−, Raptor $^{f/f}$) mice. Alb-Cre littermates were used as wild-type (WT) mice. Only male mice were used for the studies as female mice are known to be resistant to APAP-induced liver injury.

For animal experiments, APAP (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in saline and warmed to ensure its complete dissolution prior to injection. Mice were caged with free access to chow food and water in 12/12 light cycle. Male 8–12 weeks old L-mTOR KO, L-Raptor KO and their matched WT mice were injected with 500 mg/kg of APAP *i. p.* in the morning, and the mice were euthanized 6, 24 and 48 h after APAP injection. Matched volume of saline was injected as control.

2.3. Ethical approval

All procedures of animal breeding and APAP treatment were approved by the Institutional Animal Care and Use Committee of the University of Kansas Medical Center.

2.4. Western blot analysis

After the mice were euthanized, livers were harvested and snap-frozen in liquid nitrogen, and liver tissues were further homogenized in radioimmunoprecipitation assay (RIPA) buffer with a protease inhibitor cocktail (Biotool) to obtain total liver lysates. The liver lysates were further mixed with SDS loading buffer containing dithiothreitol and boiled at 95 °C for 5 min. Twenty-thirty μg of protein was loaded on SDS-PAGE gels for electrophoresis and transferred onto polyvinylidene difluoride membrane. The membranes were blocked in 5% milk in tris buffered saline with tween (TBST) for 1 h followed by incubating with primary then secondary antibodies prepared in 5% milk in TBST. The signals were detected with SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher, USA) and/or Immobilon Western HRP Substrate (Millipore, USA).

2.5. Histology analysis

Fresh liver tissues were kept in 10% formaldehyde overnight and transferred to 70% ethanol for at least 24 h. The tissues were dehydrated, embedded in paraffin, and cut into 5 μm slices. For general morphology, slides were stained with hematoxylin and eosin (H&E). For terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay, slides were stained with the *In Situ* Cell Death Detection Kit, AP (Roche Diagnostics, Risch-Rotkreuz, Switzerland) following the instruction manual. For immunohistochemistry staining of PCNA, paraffin-embedded slides were rehydrated, quenched of endogenous peroxidase and blocked. Then the tissues were incubated with PCNA primary antibody (1:1000) overnight at 4 °C, and then incubated with biotinylated secondary antibodies.

2.6. GSH measurement

Liver GSH levels were measured as previously described.[23] Frozen liver tissues were homogenized in 3% sulfosalicylic acid, centrifuged, and diluted in 0.01 N hydrochloric acid for GSH measurement with the modified Tietze assay.

2.7. Serum alanine aminotransferase (ALT) measurement

After the mice were euthanized, blood samples were collected from auxiliary artery. To collect the serum, the blood samples were allowed to sit for 30 min at room temperature, then centrifuged at 3000 rpm at 4 °C for 10 min to collect the supernatant. Serum ALT activities were measured using the ALT (SGPT) Reagent Set (POINTE Scientific, Canton, MI, USA) following the instruction manual at $\lambda = 340$ nm. Millipore water was used as blank control.

2.8. Statistical analysis

Data were presented as the mean ± standard error of the mean (SEM). Experimental data were subjected to Student's *t*-test when two groups were compared or one-way analysis of

variance (ANOVA) followed by the Newman-Keul's post-hoc test where appropriate. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Genetic deletion of mTOR or raptor in mouse livers does not affect liver injury after APAP overdose

To determine whether genetic ablation of mTOR pathway would affect APAP-induced liver injury, we treated L-mTOR KO and L-Raptor KO mice as well as matched WT littermates for 6, 24 and 48 h. The levels of serum ALT markedly increased after APAP treatment for 6 and 24 h in both L-mTOR and matched WT mice, indicating increased liver injury. The levels of serum ALT were comparable between L-mTOR KO and WT mice after APAP treatment for 6 and 24 h. However, after APAP treatment for 48 h, the levels of serum ALT decreased in both L-mTOR KO mice and WT mice compared with 24 h, suggesting a liver repair/regeneration process may occur after 24 h of APAP treatment. Notably, the levels of serum ALT in L-mTOR KO mice were much lower than matched WT mice after APAP treatment for 48 h although the difference did not reach statistical significance (Fig. 1A). We found that around 60% of WT mice survived whereas 80% and approximately 70% L-mTOR KO mice survived after 24 and 48 h treatment with APAP (Fig. 1B), but these differences did not reach statistical significance (Student's *t* test).

As Raptor is the specific adaptor protein for mTORC1 to specifically investigate the role of mTORC1 in APAP-induced liver injury, we treated L-Raptor KO mice for 6, 24 and 48 h. We did not observe a significant difference in serum ALT levels between L-Raptor KO and their matched WT mice after APAP treatment for 6, 24 and 48 h (Fig. 1C). However, all the L-Raptor KO mice survived whereas only 80% of the WT mice survived after APAP treatment for 24 and 48 h (Fig. 1D).

Liver H&E staining and TUNEL staining at 6 and 24 h showed severe necrosis and increased TUNEL-positive cells in the pericentral vein areas in L-mTOR KO, L-Raptor KO and their matched WT mice (Fig. 2). No necrosis and TUNEL positive staining cells were observed in saline-treated L-mTOR and L-Raptor KO mice, suggesting genetic ablation of mTORC1 alone in the liver does not cause liver injury (Fig. 2). Collectively, these data suggest that genetic deletion of mTOR or Raptor in mouse livers does not have significant effects on APAP-induced liver injury but may improve survival after APAP overdose in mice.

3.2. Genetic deletion of mTOR or Raptor in mouse livers does not affect APAP metabolism

In the liver, CYP2E1 metabolizes and bioactivates APAP resulting in the production of the highly reactive metabolite NAPQI, which can bind to various intracellular proteins to form APAP-ADs. We found that the levels of hepatic CYP2E1 were comparable between L-mTOR KO and their matched WT mice, or between L-Raptor KO and their matched WT mice at both basal levels and after APAP treatment (Fig. 3A and B). The level of APAP-ADs increased after APAP treatment for 6 and 24 h but no differences were observed between

L-mTOR KO and WT mice (Fig. 3A). Similarly, no differences of APAP-ADs were found between L-Raptor KO and their matched WT mice after APAP treatment for 6 h (Fig. 3B). However, we found some variation of levels of APAP-ADs in L-Raptor KO mice after 24 h treatment (Fig. 3B), which is likely due to recovery of some of the L-Raptor KO mice after APAP treatment.

After APAP treatment for 6 h, hepatic GSH levels were depleted to the same levels regardless of the genotypes of the mice. However, after APAP treatment for 24 h, the levels of hepatic GSH recovered to almost basal levels in all four groups (Fig. 3C and D). Together, these data indicate that genetic deletion of mTOR or Raptor in mouse livers does not affect APAP metabolism.

3.3. Autophagic flux increases in APAP-treated L-mTOR and L-Raptor KO mouse livers

We found that APAP treatment for 6 h increased LC3-II levels and decreased p62 levels in L-mTOR WT mouse livers (Fig. 4A), suggesting increased formation of LC3-II positive autophagic vacuoles and autophagy-mediated p62 degradation. Interestingly, after APAP treatment for 24 h, levels of hepatic p62 markedly increased, which is likely due to either increased p62 transcription or impaired autophagic degradation. The levels of phosphorylated S6, a substrate protein that is phosphorylated by mTOR, did not change significantly at 6 h but slightly increased at 24 h after APAP treatment (Fig. 4A and B). The levels of phosphorylated S6 decreased in L-mOTR KO mouse liver regardless of APAP treatment in both 6 and 24 h time points. Inhibition of mTOR generally leads to increased autophagy. Indeed, we found that the levels of LC3-II and p62 in APAP-treated L-mTOR KO mouse livers were lower than that of WT mice, likely due to increased autophagic degradation of both LC3-II and p62 (Fig. 4A and B). Similar to L-mTOR KO mice, the levels of phosphorylated S6 as well as the hepatic levels of p62 and LC3-II decreased in L-Raptor KO mice after APAP treatment in both 6 and 24 h compared with L-Raptor WT mice (Fig. 4C and D). Together, these results suggest that genetic deletion of mTOR or Raptor in mouse livers may increase autophagic flux.

3.4. Genetic deletion of mTOR or raptor in mouse livers does not affect APAP-induced JNK activation

JNK activation through increased JNK phosphorylation can potentiate APAP-induced mitochondrial damage and necrosis.[24] APAP treatment markedly increased levels of phosphorylated JNK in APAP-treated WT mouse livers in both 6 and 24 h time points (Fig. 5A and B). No obvious differences of JNK phosphorylation were found after APAP treatment for 6 h among L-mTOR KO, L-Raptor KO and their matched WT mice (Fig. 5A and C). However, after APAP treatment for 24 h, we observed variations of phosphorylated JNK in L-mTOR and L-Raptor KO mice, with some mice having higher levels and other mice having lower levels of phosphorylated JNK (Fig. 5B and D). Overall, genetic deletion of mTOR or Raptor in mouse livers does not affect JNK activation in the early phase of APAP-induced liver injury.

3.5. Genetic deletion of mTOR in mouse livers increases liver regeneration in the late phase of APAP-induced liver injury

Liver has the capacity to regenerate and recover from APAP-induced injury via hepatocyte proliferation and liver regeneration if the injury is not too severe. We found the number of PCNA positive hepatocytes increased in the pericentral vein areas adjacent to the necrosis areas after APAP treatment for 48 h in both WT and L-mTOR KO mice (Fig. 6A, white arrows). Interestingly, there are many PCNA positive cells that are non-parenchymal cells, likely immune cells or progenitor cells (Fig. 6A, red arrows). Consistent with the immunohistochemistry of PCNA staining, Western blot analysis also revealed increased levels of PCNA in L-mTOR KO mice after APAP treatment for 48 h compared with WT mice (Fig. 6B). Since mTOR can also regulate AKT phosphorylation and activation, we next checked the levels of phosphorylated GSK3β (glycogen synthase kinase 3β), a substrate of AKT. While APAP treatment markedly increased the levels of phosphorylated GSK3β, no difference was found between the WT and L-mTOR KO mice. These data suggest that loss of mTOR in mouse liver may promote liver regeneration in the late phase of APAP-induced liver injury.

4. Discussion

Owing to decades of research on APAP-induced liver injury, we now have a much enriched understanding on the mechanisms and temporal changes of liver pathogenesis after APAP overdose. APAP hepatotoxicity can be divided into four phases based on the temporal changes of the liver pathogenesis after APAP overdose. Two early phases including the metabolism/detoxification of APAP and cell death, and two late phases including innate immune response for the resolution of liver injury and liver repair/regeneration phase. In the metabolism phase, the majority of APAP is metabolized by phase-II conjugation mainly via UDP-glucuronosyltransferase and sulfotransferase and secretion into bile and urine. A small portion (5–10%) of APAP can be further metabolized to NAPQI via CYP2E1, which is further removed by conjugating with GSH. Once hepatic GSH is depleted, the excess NAPQI forms APAP-ADs. In the second phase of these early events, some of the APAP-ADs are formed on mitochondria that trigger mitochondrial dysfunction and increase the production of reactive oxygen species (ROS) as well as JNK mitochondrial translocation and activation resulting in hepatocyte necrosis.[25–27] Concurrently, hepatocytes can also trigger adaptive protective mechanisms such as autophagy to remove APAP-ADs and damaged mitochondria to protect against APAP-induced necrosis.[12,13,15,28] In the third phase, necrotic cells then release damage associated molecular patterns such as mitochondrial DNA and high mobility group box 1 protein (HMGB1) to trigger the innate immune response and blood coagulation events as adaptive responses, which may either exacerbate or suppress APAP-induced liver injury.[29–31] Infiltrated macrophages from the blood circulation promotes the resolution of the damage likely via clearance of dead cells and induction of neutrophil apoptosis, which is dependent on C–C chemokine receptor 2 (CCR2).[32] In addition, the platelet-adhesive protein von Willebrand factor (VWF) recruits and induces platelet aggregation, which inhibits liver damage repair and halts liver regeneration.[33]

The liver has a great capacity for regeneration and repair, which is critical for the late phase of recovery from experimental APAP-induced liver injury in mice or liver failure from overdosed patients. Many factors and signaling pathways have been identified that are involved in liver regeneration after APAP overdose, which include growth factors such as epidermal growth factor receptor (EGFR) ligands and hepatocyte growth factor (HGF), cytokines such as tumor necrosis factor-alpha and IL-6, and members of the Wnt/β-catenin signaling pathways.[34–36]

In addition to the above-mentioned factors, mTOR signaling pathway has also been implicated in liver regeneration.[37,38] After partial hepatectomy (PHx), phosphatidylinositol 3-kinase (PI3K)/AKT/mTOR signaling pathway is activated, and the ablation of PI3K/AKT results in impaired liver regeneration in mice.[39,40] Pharmacological inhibition of mTORC1 by rapamycin leads to decreased hepatocyte proliferation and liver regeneration after PHx.[41] It is conceivable that impaired liver regeneration after mTORC1 inhibition may result from decreased protein and lipid synthesis, which are building blocks for cell proliferation. In addition, decreased mTORC1 activity also inhibits the phosphorylation of its down-stream substrate p70 ribosomal protein S6 kinase (S6K) that decreases cyclin D1 expression resulting in decreased hepatocyte proliferation.[42] In addition to mTORC1, a recent study reported that ablation of mTORC2 function by genetic deletion of its adaptor protein Rictor in the mouse liver also leads to impaired liver regeneration after PHx likely via impaired AKT activation.[38]

In our present study, we found no difference of hepatic PCNA levels between L-mTOR KO and WT mice, suggesting genetic ablation of mTOR alone in the liver does not affect the basal hepatocyte proliferation. In contrast to the impaired liver regeneration after PHx, we did not observe decreased liver regeneration after APAP treatment in L-mTOR KO mice. Instead, we observed a mild increase in hepatocyte proliferation based on the levels of hepatic PCNA. It is well known that mTORC1 can inhibit AKT activation via the phosphorylation of insulin receptor substrate 1 (IRS-1), whereas mTORC2 promotes AKT activation via direct phosphorylation of serine 473 of AKT. Therefore, deletion of mTOR that compromises the functions of both mTORC1 and mTORC2 may counteract its negative and positive effects on AKT activation. Indeed, we did not observe differences in levels of phosphorylated AKT between L-mTOR KO and WT mice after APAP treatment (data not shown). AKT activation can increase the levels of phosphorylated GSK3β. We found that APAP treatment increased the levels of phosphorylated GSK3β to almost the same levels in both L-mTOR and WT mice, which further support the notion that genetic ablation of mTOR may not affect AKT activation after APAP treatment.

It has been reported that autophagy is activated likely to remove damaged mitochondria and increase fatty acid beta oxidation to provide energy for liver regeneration after PHx in mice. Liver-specific deletion of Atg5 in mice increases hepatocyte senescence resulting in impaired liver regeneration after PHx.[43] Pharmacological activation of autophagy by amiodarone also improves liver regeneration in mice after PHx.[44] Therefore, it is likely that the mild increase in liver regeneration and improved survival after APAP treatment in L-mTOR and L-Raptor KO mice could be due to increased hepatic autophagy. However, these beneficial effects seem to be compromised by impaired protein and lipid synthesis due

to the inhibition of mTOR. We previously reported that chronic deletion of Parkin using Alb-Cre *vs.* acute deletion of Parkin using adenovirus-mediated delivery of shRNA has different outcomes for APAP-induced liver injury in mice.[15] Therefore, it is also likely that many other compensatory effects may occur in the L-mTOR KO mice due to the chronic deletion of mTOR in mouse livers. These positive and negative effects on the regulation of liver regeneration after genetic ablation of the mTOR pathway as well as other unknown compensatory effects likely results in minimal beneficial effects against APAP-induced liver injury.

5. Conclusion

We report that genetic ablation of mTOR and Raptor in mouse liver does not protect against APAP-induced acute liver injury but slightly improves liver regeneration and mouse survival after APAP treatment.

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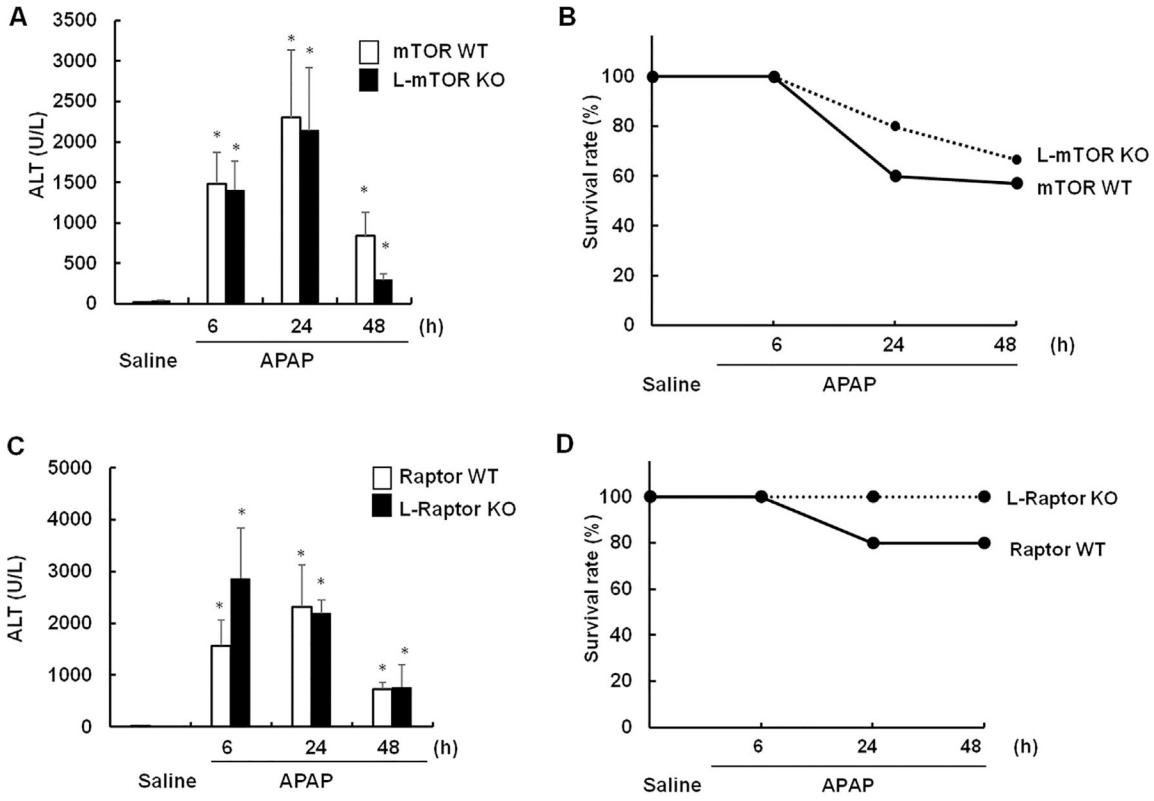

Fig. 1. Genetic deletion of mTOR or Raptor in mouse livers does not affect liver injury but improves mouse survival after APAP overdose.

(A) L-mTOR KO and WT mice were injected intraperitoneally (*i.p.*) with APAP at 500 mg/kg. Serum and livers were collected at 6, 24 and 48 h, respectively. Serum ALT was measured at different time points ($n = 5–9$). **(B)** Mouse survival rate was calculated ($n = 5–9$). **(C)** L-Raptor KO and WT mice were injected *i. p.* with APAP at 500 mg/kg. Serum and livers were collected at 6, 24 and 48 h, respectively. Serum ALT was measured at different time points ($n = 3–10$). **(D)** Mouse survival rate was calculated ($n = 5$). Results are presented as the mean ± SEM. One-way ANOVA analysis, $*P < 0.05$ *vs.* saline group. Abbreviations: APAP, acetaminophen; ALT, alanine aminotransferase; ANOVA, analysis of variance; KO, knockout; mTOR, mechanistic target of rapamycin; Raptor, regulatory associated protein of mTOR complex; SEM, standard error of the mean; WT, wild-type.

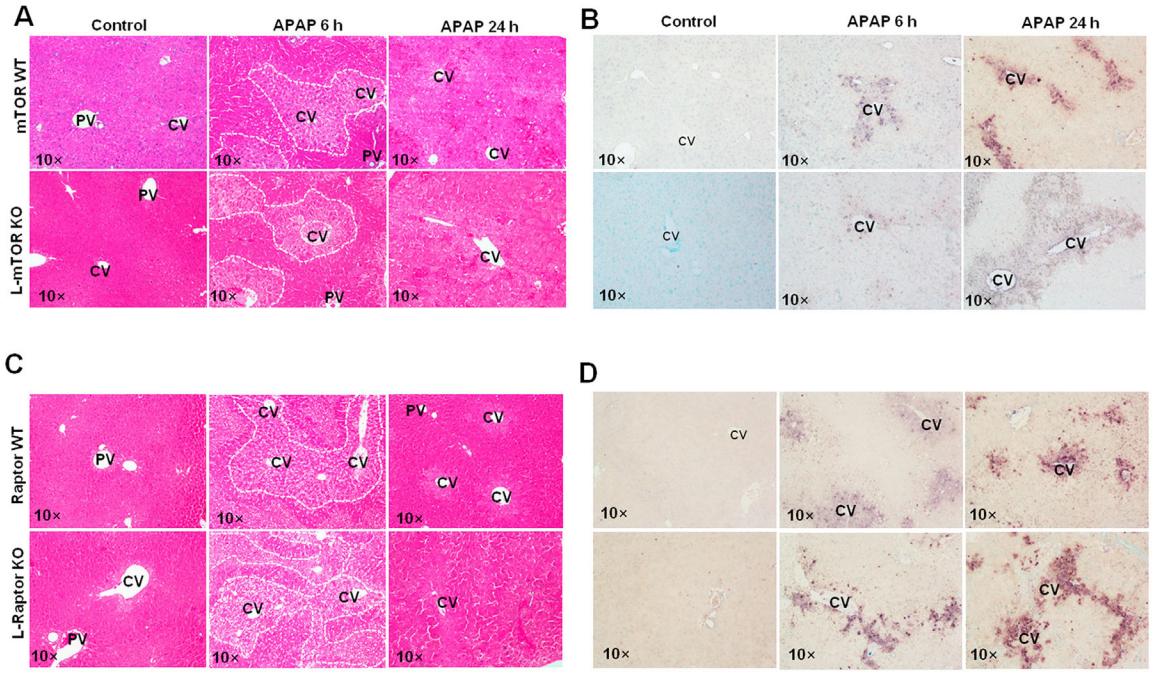

Fig. 2. Genetic deletion of mTOR or Raptor in mouse livers does not affect APAP-induced liver injury.

Mice were treated with APAP as described in Fig. 1. **(A)** Representative images (10 ×) of liver H&E staining of L-mTOR KO and WT mice at 6 and 24 h after treatment. Dashed line encloses necrotic area. **(B)** Representative images (10 ×) of liver TUNEL staining of L-mTOR KO and WT mice at 6 and 24 h after treatment. **(C)** Representative images (10 ×) of liver H&E staining of L-Raptor KO and WT mice at 6 and 24 h after treatment. Dashed line encloses necrotic area. **(D)** Representative images (10 ×) of liver TUNEL staining of L-Raptor KO and WT mice at 6 and 24 h after treatment. Abbreviations: APAP, acetaminophen; CV, central vein; H&E, hematoxylin and eosin; KO, knockout; mTOR, mechanistic target of rapamycin; PV, portal vein; Raptor, regulatory associated protein of mTOR complex; WT, wild-type.

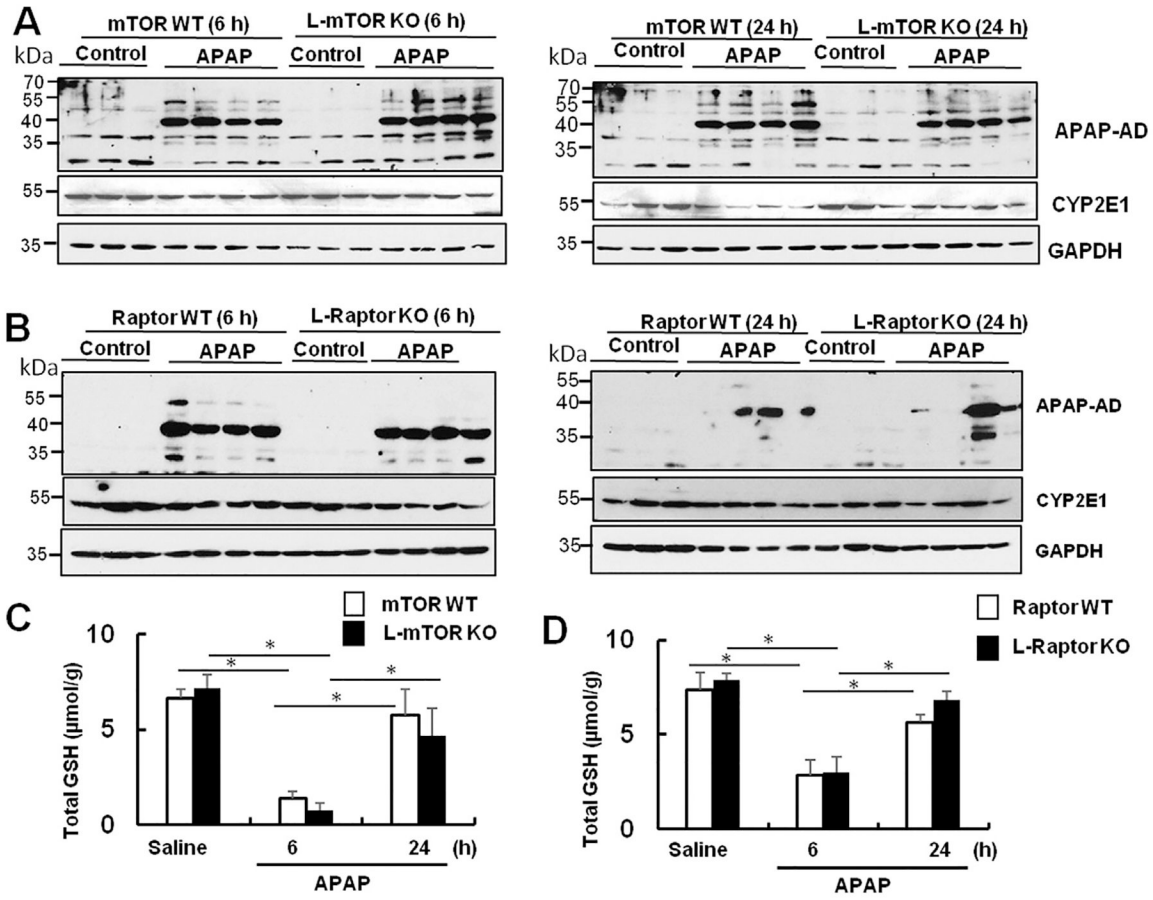

Fig. 3. Genetic deletion of mTOR or Raptor in mouse livers does not affect APAP metabolism.

WT, L-mTOR KO and L-Raptor KO mice were treated with APAP (500 mg/kg) or saline, and livers were collected at 6 and 24 h, respectively. **(A)** Representative immunoblot of APAP-AD and CYP2E1 in the liver collected from WT and L-mTOR KO mice at 6 and 24 h after treatment. **(B)** Representative immunoblot of APAP-AD and CYP2E1 in liver collected from WT and L-Raptor KO mice at 6 and 24 h after treatment. GAPDH was used as loading control. Total GSH from L-mTOR KO and WT mice **(C)** and L-Raptor KO and WT mice **(D)** were measured. Results are presented as the mean ± SEM (n = 3–4). One-way ANOVA analysis, $*P < 0.05$. Abbreviations: APAP, acetaminophen; APAP-AD, APAP-adduct; ANOVA, analysis of variance; CYP2E1, cytochrome P450 family 2 subfamily E member 1; GSH, glutathione; KO, knockout; mTOR, mechanistic target of rapamycin; Raptor, regulatory associated protein of mTOR complex; SEM, standard error of the mean; WT, wild-type.

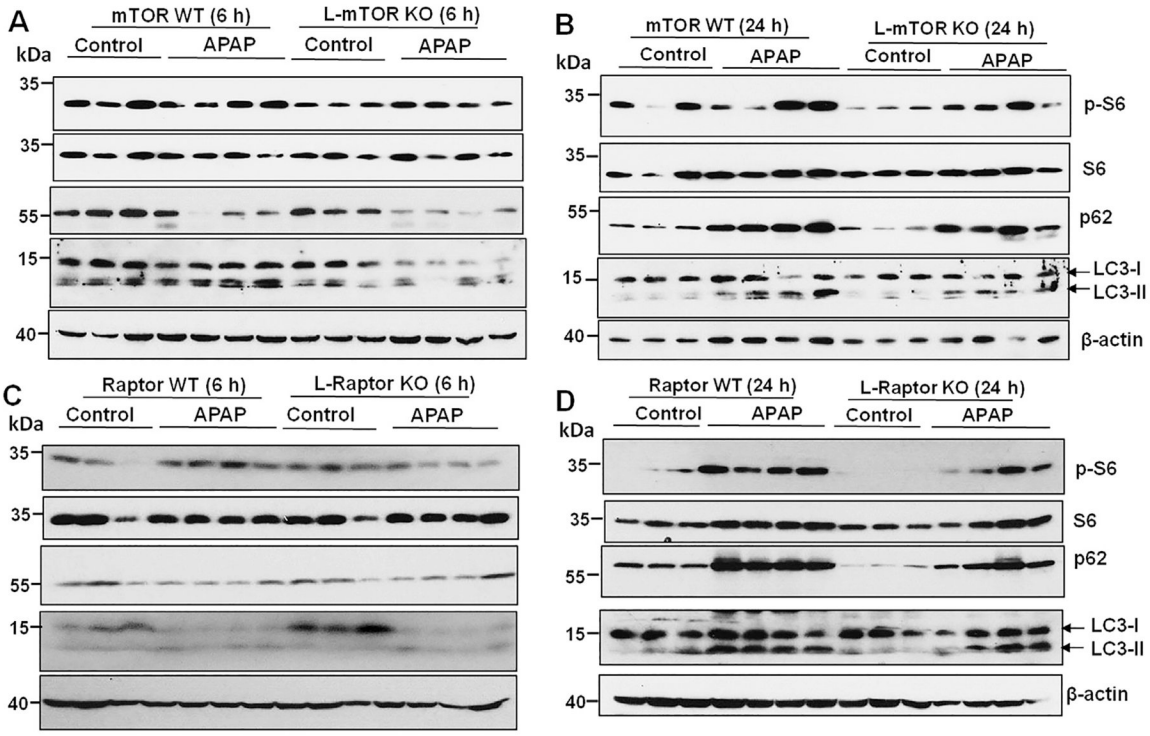

Fig. 4. Decreased mTOR activity and increased autophagic flux in APAP-treated L-mTOR KO and L-Raptor KO mice.

WT, L-mTOR KO and L-Raptor KO mice were treated with APAP (500 mg/kg) or saline, and livers were collected at 6 and 24 h, respectively. Representative immunoblot of liver p-S6, total S6, p62 and LC3 from L-mTOR KO and WT mice for 6 h treatment **(A)** and 24 h treatment **(B)**. Representative immunoblot of liver p-S6, total S6, p62 and LC3 from L-Raptor KO and WT mice for 6 h treatment **(C)** and 24 h treatment **(D)**. β-actin was used as loading control. Abbreviations: APAP, acetaminophen; KO, knockout; LC3, microtubule-associated protein 1 light chain 3; mTOR, mechanistic target of rapamycin; p-S6, phosphorylated S6; Raptor, regulatory associated protein of mTOR complex; WT, wild-type.

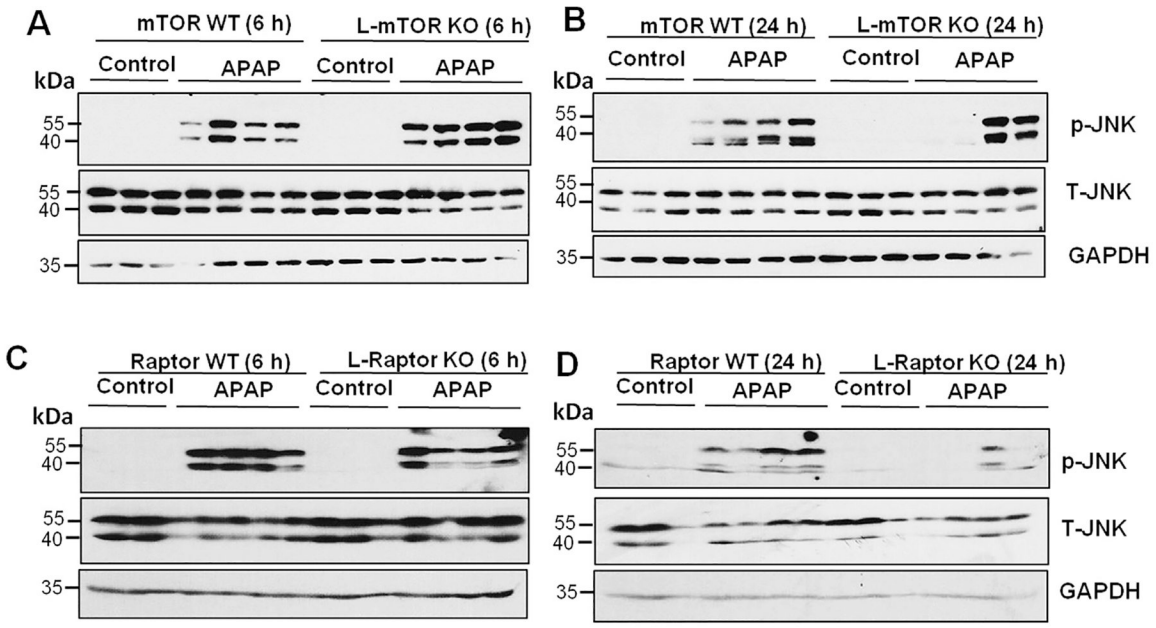

Fig. 5. Genetic deletion of mTOR or Raptor in mouse livers does not affect APAP metabolism.

WT, L-mTOR KO and L-Raptor KO mice were treated with APAP (500 mg/kg) or saline, and livers were collected at 6 and 24 h, respectively. Representative immunoblot of p-JNK and total JNK from L-mTOR KO and WT mice after 6 h treatment **(A)** and 24 h treatment **(B)**. Representative immunoblot of liver p-JNK and total JNK from L-Raptor KO and WT mice after 6 h treatment **(C)** and 24 h treatment **(D)**. GAPDH was used as loading control. Abbreviations: APAP, acetaminophen; KO, knockout; mTOR, mechanistic target of rapamycin; *p*-JNK, phosphorylated c-Jun N-terminal kinase; Raptor, regulatory associated protein of mTOR complex; T-JNK, total c-Jun N-terminal kinase; WT, wild-type.

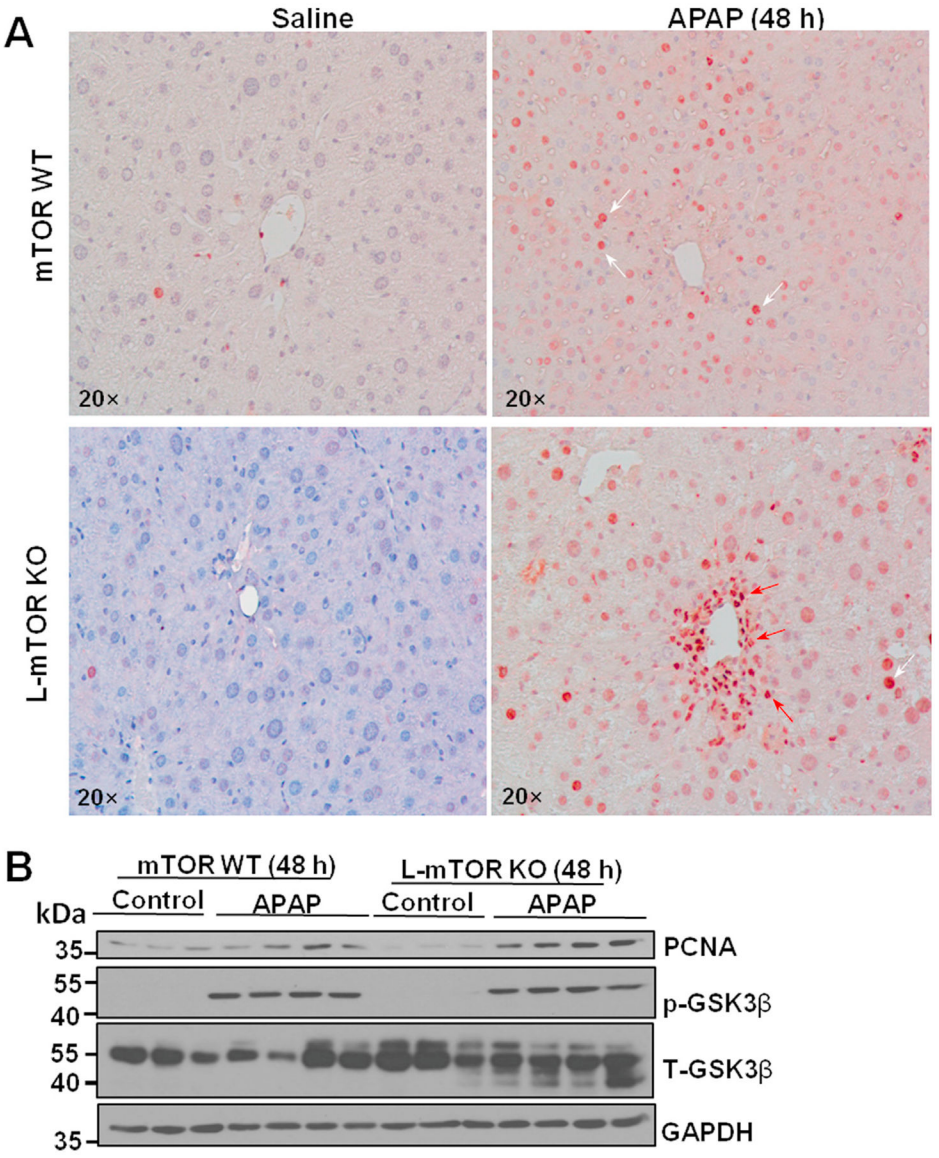

Fig. 6. Genetic deletion of mTOR in mouse livers increases liver regeneration in the late phase of APAP-induced liver injury.

WT and L-mTOR KO mice were treated with APAP (500 mg/kg) or saline, and livers were collected at 48 h. **(A)** Representative immunohistochemistry of PCNA staining images (20 ×) are shown. **(B)** Immunoblot of PCNA, *p*-GSK3β and T-GSK3β from total liver lysates. GAPDH was used as loading control. Abbreviations: APAP, acetaminophen; KO, knockout; mTOR, mechanistic target of rapamycin; PCNA, proliferating cell nuclear antigen; WT, wild-type.