**PP2A α Positively Regulates Mice Liver Regeneration Termination
through AKT/GSK3 β /Cyclin D1 Pathway**

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Word count: 5929

Number of figures: 7

Number of tables: 0

List of abbreviations:

LR, liver regeneration; PH, partial hepatectomy; ALT, alanine aminotransferase; AST, aspartate aminotransferase; GSK3 β , Glycogen synthase kinase-3 β ; PP1, protein phosphatase1; NMR, nuclear magnetic resonance; NPCs, non-parenchymal cells.

Keywords: PP2A, liver injury, termination of liver regeneration, AKT, GSK3 β

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

Financial support: This work was supported by the Chinese National Science Foundation (30700794, 31171306, and 31371373), the Nature Science

Foundation of Jiangsu Province (BK2011568), National Basic Research Program of China (973 program, 2012CB524900), Jiangsu Province Funds for Distinguished Young Scientists (BK20140049), and also by China Postdoctoral Science Foundation (2013M541642).

Author's contributions:

Conceived and designed the work: B Xue, X Gao and CJ Li;

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Statistical analysis: J Liu, EZ Jiang and ZH Yu;

Funding support: P Cao, B Xue and CJ Li;

Technical/ material support: DC Yu, Xiao-Jun Xu;

Drafting of the manuscript: SS Lai;

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Abstract

Background & Aims: Liver injury triggers a highly organized and ordered liver regeneration (LR) process. Once regeneration is complete, a stop signal ensures that the regenerated liver is an appropriate functional size. The inhibitors and stop signals that regulate LR are unknown, and only limited information is available about these mechanisms.

Methods: A 70% partial hepatectomy (PH) was performed in hepatocyte-specific PP2A α -deleted (PP2A $\alpha^{-/-}$) and control (PP2A $\alpha^{+/+}$) mice. LR was estimated by liver weight, serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels and cell proliferation, and the related cellular signals were analyzed.

Results: We found that the catalytic subunit of PP2A was markedly up-regulated during the late stage of LR. PP2A $\alpha^{-/-}$ mice showed prolonged LR termination, an increased liver size compared to the original mass and lower levels of serum ALT and AST compared with control mice. In these mice, cyclin D1 protein levels, but not mRNA levels, were increased. Mechanistically, AKT activated by the loss of PP2A α inhibited glycogen synthase kinase 3 β (GSK3 β) activity, which led to the accumulation of cyclin D1 protein and accelerated hepatocyte proliferation at the termination stage. Treatment with the PI3K inhibitor wortmannin at the termination stage was sufficient to inhibit cyclin D1 accumulation and hepatocyte proliferation.

Conclusions: PP2A α plays an essential role in the proper termination of LR

via the AKT/GSK3 β /Cyclin D1 pathway. Our findings enrich the understanding of the molecular mechanism of LR termination control and provide a potential therapeutic target for treating liver injury.

Introduction

The Liver has a remarkable capacity to regain its size, architecture, and function in response to loss of mass caused by a variety of injuries. This regenerative capacity provides assurances for the clinical outcome of patients after a serious hepatic injury, cancer resection, or living donor liver transplantation. Therefore, understanding the mechanisms that govern LR will shed light on the etiology of chronic and acute liver diseases, as well as hepatocarcinogenesis.

After PH, hepatocytes exit their quiescent and highly differentiated state and rapidly re-enter the cell cycle, which causes enlargement of the residual lobe to compensate for the removed mass. The whole regenerative process includes initiation, proliferation and termination stages that involve the participation of numerous growth factors and cytokines, including HGF, EGF, TGF- β , and TNF- α [1]. After approximately two rounds of hepatocyte replication, the liver mass is reestablished, and the liver morphology is gradually rearranged and restored. Eventually, the regenerated liver mass is adjusted to the pre-hepatectomy size with a relatively high accuracy [2].

Although studies on LR have been conducted for many years, most have focused on the initiation stage, whereas few have concentrated on the termination stage. Until now, only limited information has been available about the inhibitory factors and stop signals, such as TGF- β 1, that are involved in hepatic regeneration. Inhibiting type II TGF- β receptor activity with adenovirus

or gene knockout markedly increases DNA synthesis in murine hepatocytes [3, 4]. Other studies have shown that up-regulation of SnoN, an inhibitor of TGF- β 1, can also delay the termination of LR [5]. However, intact TGF- β 1 signaling is not required for termination of LR, unless the Activin receptor has been inactivated [6]. In addition, Apte's group demonstrated that the ablation of integrin-linked kinase leads to enhanced liver proliferation, ultimately resulting in a liver that is 1.3-fold larger than the original [2]. A recent report suggested that cooperation among C/EBP family proteins and chromatin remodeling proteins is essential for LR termination [7].

Reversible phosphorylation regulated by phosphokinases and phosphatases is one of the most important mechanisms for regulating proliferation, migration, and tissue regeneration. PP2A makes up 1% of all cellular proteins and, together with protein phosphatase 1 (PP1), accounts for over 90% of serine/threonine phosphatase activity in cells [8]. The PP2A holoenzyme complex consists of three distinct functional components: the catalytic subunit (PP2Ac), the structural subunit (PR65 or A subunit) and the regulatory subunit (B subunit). PP2A regulates many cellular processes, including signal transduction, cell cycle progression, DNA replication, gene transcription and protein translation [9-11]. PP2A has been proposed as a negative regulator of cellular growth. Inhibiting PP2A triggers premature meiosis and mitosis and the increased expression of several proto-oncogenes [9, 12]. Although a number of studies have demonstrated that PP2A is critical for cell cycle control, the role

of PP2A α in regulating the mitogenic capacity of the regenerated liver remains unexplored. Herein, we performed PH on wild-type and PP2A α ^{-/-} mice and analyzed the rate of LR and the activity of PP2A-regulated signaling pathways. We demonstrated that the induction of PP2A α expression following PH is responsible for normal termination of regeneration. Mice with liver-specific ablation of PP2A α exhibited impaired LR termination, resulting in a liver that was 30% larger than the pre-PH mass. The sustained cell proliferation and resulting abnormal regeneration size might be mediated by the AKT/GSK3 β /Cyclin D1 pathway. To the best of our knowledge, this is the first time that PP2A α has been characterized as a novel positive regulator of LR termination.

Materials and Methods

Partial Hepatectomy

All the animals used in the study were 8- to 10-week-old male mice on a mixed genetic background (129SV and C57/BL6). PH was performed between 8 and 12 am, and the mortality rate was <5%. Mice were sacrificed at the indicated time points after PH.

Histology

Liver tissues were fixed in 4% paraformaldehyde and embedded in paraffin. 5 μ m paraffin liver sections were stained with hematoxylin and eosin for morphological examination and hepatocyte mitotic figures or used for routine immunofluorescent staining.

Protein Extraction and Western Blot

Whole liver protein was extracted from mice using RIPA buffer containing 1% SDS. 50 μ g of total lysates was separated by 10~15% SDS-PAGE gel and transferred onto PVDF membranes, and blotted with appropriate antibodies.

Statistical Analysis

Results are presented as means $\pm$ standard deviation. Statistical analyses were performed with Students' *t*-test, and *p* values less than 0.05 were considered significant.

Detailed Materials and Methods are provided in Supplementary Information.

Results

PH induces a gradual and significant elevation of PP2A α mRNA and protein in regenerated liver.

To explore the function of PP2A α in LR, we first examined whether PH could

induce PP2A α in regenerated liver. At the early stage of LR, there was no obvious change in the mRNA levels of PP2A α (a small decrease on days 2 and 3) (Fig. 1A). PP2A α expression gradually increased as regeneration advanced, increasing by approximately 3.1-fold and 5.6-fold on days 5 and 7 post-PH, respectively. However, there was a sharp decrease thereafter. PP2A α protein levels also increased as regeneration progressed and reached a peak on day 5 (Fig. 1B). According to Mohammed's schematic of the start and stop signals during LR [13], we postulated that the increase in PP2A α expression after PH is specific to the late stage and might contribute to the termination of LR.

Generation of PP2A α conditional knockout mice.

Because the lack of the α isoform of the catalytic subunit results in loss of catalytic activity of the PP2A holoenzyme, we generated liver-specific PP2A α -knockout mice by breeding PP2A α -floxed mice [14] with albumin-Cre transgenic (Alb-Cre) mice (provided by Model Animal Research Center, Nanjing), which express Cre recombinase specifically in the liver under the control of the albumin promoter (Fig. 2A). We crossed PP2A $\alpha^{\text{flox/flox}}$ mice with Alb-Cre mice to generate Alb-Cre-PP2A $\alpha^{\text{flox/+}}$ mice (PP2A $\alpha^{+/-}$); PP2A $\alpha^{+/-}$ mice were crossed to PP2A $\alpha^{\text{flox/flox}}$ mice, yielding Alb-Cre-PP2A $\alpha^{\text{flox/flox}}$ mice (hereafter termed PP2A $\alpha^{-/-}$). PP2A $\alpha^{\text{flox/flox}}$ (PP2A $\alpha^{+/+}$) mice obtained from the same breeding were used as controls. As

expected, PP2A α ^{-/-} mice exhibited an 85% reduction in PP2A α protein expression in the liver (Fig. 2B). Because the Alb promoter is active mainly in hepatocytes, it is likely that PP2A α is expressed in other liver cells. Therefore, there was still significant expression of PP2A α in PP2A α ^{-/-} mice. PP2A α ^{-/-} mice showed a similar liver structure and morphology to control mice (Fig. 2C). Although the body weight of PP2A α ^{-/-} mice was not different from that of control mice, the liver weight and the liver/body weight ratio were slightly increased (Fig. 2D). Next, we compared the proliferation status and hepatocyte cell size in the livers of 2-month-old PP2A α ^{-/-} and control mice. Surprisingly, neither the proliferation ratio nor the hepatocyte cell size was higher in the mutant mice (Fig. 2E and F). Because there is a wave of hepatocyte proliferation between 2 and 30 weeks of age [15], PP2A α ^{-/-} and control mice were sacrificed, and their livers were harvested 3 weeks after birth. As shown in Figure 2G, hepatocyte proliferation was markedly increased after PP2A α deletion. Therefore, we speculate that the sustained cell proliferation in the livers of young PP2A α ^{-/-} mice leads to the increased liver weight in adult mice.

PP2A α deficiency delays LR termination and protects against liver damage induced by PH.

To further investigate the function of PP2A α in LR, we performed PH on PP2A α ^{-/-} and control mice. Before PH, the liver mass of PP2A α ^{-/-} mice was slightly higher than that of control mice, but their liver morphology was normal

(Fig. 3A). The liver weight of control mice recovered to the pre-PH value by day 5 post-PH, whereas that of PP2A α ^{-/-} mice significantly surpassed the pre-PH value at the same time point (Fig. 3A, lower panel, and 3B). To normalize the differences in pre-PH liver weight and body weight, the liver-to-body weight ratio at each time point post-PH was normalized to the corresponding value prior to hepatectomy (Fig. 3C and D). There was no significant difference in absolute regeneration between control and knockout mice for the first 4 days. Control mice exhibited the typical pattern of LR termination, as their livers immediately stopped growing when the liver/body weight ratio reached the pre-PH value. However, PP2A α ^{-/-} mice exhibited a termination defect; their livers continued to grow even after the liver/body weight ratio reached the pre-PH value, ultimately resulting in livers that were 30% larger than the originals by day 10 (Fig. 3C). The liver mass and liver weight/body weight ratio returned to baseline 30 days after PH in control and PP2A α ^{-/-} mice (Fig. 3B and C). Animals were subjected to nuclear magnetic resonance (NMR) scan analysis 2 months after surgery; the regenerated livers from both control and PP2A α ^{-/-} mice exhibited normal structure (Supplementary Fig. 1).

We also analyzed the serum activity of ALT and AST, two indicators of liver damage after PH. The serum levels of ALT and AST increased immediately after surgery and then decreased gradually as the liver mass and physiological structures recovered. However, the serum levels of ALT and AST recovered to normal more quickly in PP2A α ^{-/-} mice than in control mice (Fig. 3E and F).

These data suggest that PP2A α deficiency accelerates liver recovery after PH.

PP2A α deficiency results in enhanced proliferation at the late stage of LR.

We next assessed cell proliferation in the regenerating liver by Ki67 labeling. In control mice, hepatocyte proliferation reached a maximum by day 2 and then began to decrease. Only 3.8% of hepatocytes were still proliferating at day 7 post-PH (Fig. 4A and B). Hepatocyte proliferation was the same in PP2A α ^{-/-} livers as in control livers for the first 3 days, but the proliferation index remained high in PP2A α ^{-/-} livers on days 4, 5 and 7. As a result, 6.9% of the hepatocytes were still proliferating at day 7 in PP2A α ^{-/-} livers, which represents an approximately 1.8-fold increase compared to control livers (Fig. 4A and B). The proliferation indexes in control and PP2A α ^{-/-} livers returned to the levels observed in quiescent livers by 14 days post-PH (Fig. 4B). Consistent with the Ki67 labeling results, mitotic figures were abundant in PP2A α ^{-/-} livers on days 4, 5 and 7 post-PH, whereas few mitotic figures were found in control livers (Fig. 4C). Furthermore, we used BrdU labeling to evaluate hepatocyte DNA synthesis after PH and found that PP2A α knockout affected DNA replication only at the termination stage of LR (Fig. 4D).

We also measured hepatocyte cell size to ascertain whether hyperplasia participates in increasing the liver size at the end of LR. The number of

hepatocytes per visual field was lower on day 7 post-PH than on day 0 in PP2A α ^{-/-} and control mice. However, there was no significant difference between the two groups (Fig. 4E). These results confirm that PP2A α deficiency results in increased hepatocyte proliferation specifically at the late stage of LR.

PP2A α deficiency enhances the induction of early cell cycle genes during LR.

To further confirm whether PP2A α deficiency prolongs the cell cycle, we compared the kinetics of cyclin protein expression in control and PP2A α ^{-/-} mice. Protein levels of cyclin D1 and E were elevated in PP2A α ^{-/-} mice, which was consistent with the enhanced cell proliferation in PP2A α ^{-/-} mice during LR (Fig. 5A). Because cyclin D1 is a key regulator of the cell cycle, we determined the mRNA expression of cyclin D1. The mRNA levels of cyclin D1 in PP2A α ^{-/-} mice were similar to those in control mice for the first 5 days post-PH, but subsequently, they were significantly lower than those in control mice (Fig. 5B). PCNA expression is cell cycle dependent and is commonly used as a simple, accurate and reproducible marker of LR [16]. Consistent with the induced hepatocyte proliferation, PCNA levels sharply increased on day 2 after PH and then declined on day 4 in control mice. Protein levels of PCNA in PP2A α ^{-/-} mice showed a delayed decline (Fig. 5A). As a result, PP2A α ^{-/-} mice showed a significant induction of PCNA on days 4, 5 and 7 after PH. Additionally, we

investigated the expression of p21Cip1 (p21) and p27Kip1 (p27), known cell cycle inhibitors (Fig. 5C). The expression of p27 markedly increased over 3 days post-PH, but p27 levels were much lower in PP2A $\alpha^{-/-}$ mice on days 4, 5 and 7. However, no significant changes in p21 protein levels were observed between the control and PP2A $\alpha^{-/-}$ mice.

PP2A α affects LR termination through the AKT/GSK3 β pathway.

Cyclin D1 is an important regulator of the G0/G1 transition in the cell cycle and participates in the development of various types of cancer. We speculated that increased cyclin D1 expression caused the prolonged cell cycle in PP2A $\alpha^{-/-}$ mice. Here, we observed changes in the RAS/MAPK and PI3K/AKT/GSK3 β pathways, which have been reported to control the synthesis and degradation of cyclin D1, respectively [17]. There was no significant difference in the activation of MAPK between control and PP2A $\alpha^{-/-}$ mice during LR (Fig. 6A). In control mice, phosphorylation of AKT at S473 gradually decreased as regeneration progressed, reaching a minimum at day 4, and slightly increased thereafter. AKT phosphorylation in PP2A $\alpha^{-/-}$ mice showed the same trend for the first 4 days, although the basal level was much higher than that in the control mice. However, thereafter, PP2A $\alpha^{-/-}$ mice showed substantially higher phosphorylated AKT levels (Fig. 6A). Furthermore, we investigated the expression and activation of GSK3 β . The amount of GSK3 β gradually

increased after PH in control and PP2A α ^{-/-} mice. However, the inhibitory phosphorylation of GSK3 β at S9 was higher in PP2A α ^{-/-} mice than in control mice throughout the whole process (Fig. 6A). GSK3 β can regulate the phosphorylation and subsequent degradation of cyclin D1 [18]. Cyclin D1 was immunoprecipitated from whole extracts of control and PP2A α ^{-/-} livers on day 5 after PH, and the corresponding blots were incubated with a p-Thr antibody. As expected, the phosphorylation of cyclin D1 was reduced in PP2A α ^{-/-} livers (Fig. 6B).

To further analyze signaling pathways during the time of prolonged proliferation in PP2A α ^{-/-} mice, we investigated the levels of c-Myc. C-Myc and cyclin D1 play distinct roles in cell cycle progression through G1. There was no significant difference between control and knockout mice, with only a slight decrease on day 4 (Fig. 6A). These observations suggest that increased cyclin D1 expression in PP2A α ^{-/-} mice may result from enhanced AKT activity and decreased GSK3 β activity.

Previously, it has been reported that both AKT and GSK3 β are directly regulated by PP2A through de-phosphorylation of certain important active sites [19, 20]. It has also been reported that GSK3 β activity is regulated by AKT activity [21]. To further examine whether PP2A α affects cyclin D1 expression through GSK3 β and/or through the AKT/GSK3 β pathway during LR termination, we treated mice with wortmannin, a PI3K/AKT inhibitor, to inhibit AKT activity at the termination stage. This experiment was designed according

to Jackson' method [22] : control and PP2A α ^{-/-} mice were subjected to PH and then subdivided to receive either vehicle (5% ethanol ip bid) or wortmannin (0.75 mg/kg in 5% ethanol ip bid), which was administered at 60 h post-PH and every 12 h thereafter to ascertain the function of AKT at the termination stage. The livers were harvested 7 days post-PH (Fig. 6C). Wortmannin drastically repressed AKT activation at the termination stage without affecting ERK activation (Supplementary Fig. 2). Both control and PP2A α ^{-/-} mice that were treated with wortmannin showed a slight decrease in body weight and a marked decrease in regenerated liver weight (Fig. 6D). There was no obvious change in the liver/body weight ratio in control mice after wortmannin treatment, but the ratio significantly decreased in wortmannin-treated PP2A α ^{-/-} mice. Consistently, the number of Ki67-positive nuclei in PP2A α ^{-/-} mice decreased from 6.5% to 0.7% (Fig. 6E). Furthermore, we investigated the downstream signaling pathway and found that PCNA, cyclin D1 and p-GSK3 β levels were decreased after wortmannin treatment (Fig. 6F). These results suggest that PP2A α can affect cyclin D1 expression levels by directly inhibiting the AKT/GSK3 β pathway.

□

PP2A α deficiency results in enhanced HGF expression at the termination stage.

HGF is a key factor for liver growth and function. Several studies have shown

that the plasma concentrations of HGF are substantially increased in surgical patients after PH [23]. In rat, the serum concentrations of HGF increase more than 20-fold within 1 h after PH and then decrease slowly, but they remain higher than normal for more than 72 hrs [24]. The gene expression of HGF was higher on days 4 and 5 post-PH in PP2A α ^{-/-} mice than in control mice (Fig. 7A). We also observed a significant increase in HGF protein levels on day 4 (Fig. 7B). Elevated HGF expression is a reflection of the higher regeneration capacity in PP2A α ^{-/-} mice. Moreover, we observed earlier activation of hepatic stellate cells, as indicated by α -SMA, and an increase in the number of non-parenchymal cells (NPCs) in PP2A α ^{-/-} mice (Fig. 7B and C). HGF is secreted by stellate cells and endothelial cells in the liver [25]. We speculate that PP2A α deficiency results in increased proliferation of NPCs, which stimulate LR by secreting HGF.

Discussion

The liver is highly susceptible to damage by physical and chemical factors. LR is a protective mechanism by which the liver recovers from such damage. However, the exact cellular and molecular mechanisms involved in LR remain unclear. In this study, we demonstrated that the expression of PP2A α significantly increases at the termination stage of LR after PH. Deletion of PP2A α resulted in enhanced proliferation and increased expression of cyclins at the late stage of LR. Furthermore, we showed that knockout of PP2A α

delayed LR termination by inhibiting the degradation of cyclin D1 through direct regulation of GSK3 β or through the AKT/GSK3 β pathway (Fig. 7D). Our findings support the hypothesis that PP2A α is not required for the initiation and proliferation stages of LR but is important for the termination stage. Here, we describe a new molecular mechanism for the regulation of LR termination, which will enrich the understanding of the molecular mechanism of liver regeneration termination control. As PP2A appears to be critically involved in cellular growth control and is potentially involved in the development of cancer, our research will provide a valuable reference for the study of PP2A in tumorigenesis as well as in tumor therapy.

PP2A is one of the most abundant cellular phosphatases and is involved in a variety of cellular processes by regulating the phosphorylation status of its substrates. In 1975, Ballal found that the incorporation of ^{32}P by proteins increased by 20-fold 48 hrs post-PH, indicating that protein phosphorylation is important for LR [26]. However, Kakinoki analyzed the gene expression and activity of protein phosphatases in regenerating livers in rat and found that PP2A mRNA levels had two peaks, at 6 and 10-12 h, but its activity remained constant [27]. PP2A consists of three subunits: A, B and C. The A and B subunits exert regulatory functions, and C is the catalytic subunit. Kakinoki determined the mRNA expression of PP2AA, but not of PP2AC; however, changes in PP2AC expression directly determine the activity of the holoenzyme. According to our results, PP2A α functions specifically at the late

stage of LR, and PP2A α deletion results in overgrowth of the regenerated liver.

It has been reported that PI3K/AKT is markedly activated after PH. Total or selective PI3K inhibition attenuates the proliferative response of the liver following PH, which results in impaired initiation of LR [22, 28]. We determined that AKT was markedly activated at the late stage of LR, which was consistent with Ugi's and Padmanabhan's reports that PP2A modulates AKT phosphorylation [29, 30]. Furthermore, we inhibited AKT activity with wortmannin specifically at the termination stage of LR and found that blocking AKT promoted the termination of LR in PP2A α ^{-/-} mice. We and Jackson used wortmannin to study the function of AKT in LR. However, they injected it before PH, while we injected it specifically at the termination stage of LR. This difference ultimately led to different experimental results. Taken together, we conclude that AKT regulates not only the initiation of LR but also the termination of LR. In our present work, the decreased expression of cyclin D1 mRNA in PP2A α ^{-/-} mice on days 7 and 10 post-PH seemed to conflict with the protein results. The levels of cyclin D1 are controlled primarily through post-translational mechanisms, and the phosphorylation of cyclin D1 at Thr 286 induces degradation through a ubiquitin-dependent mechanism [31]. We speculated that the discrepancy of high cyclin D1 protein and low mRNA expression in PP2A α ^{-/-} mice on days 7 and 10 post-PH might be due to attenuated protein degradation by post-transcriptional modification. Chen

demonstrated that AKT induces the inhibitory phosphorylation of GSK3 β , which then triggers phosphorylation on Thr 286 of cyclin D1, eventually leading to degradation of cyclin D1 in cancer cells [21]. Here, our findings showed that the inhibitory phosphorylation of GSK3 β is increased in PP2A α -deficient livers, and wortmannin is sufficient to reduce the phosphorylation of GSK3 β , which leads to decreased cyclin D1 protein levels. Moreover, we found that the phosphorylation of Akt and GSK3 β was constitutively activated in PP2A α ^{-/-} mice and that hepatocyte proliferation was markedly increased in PP2A α -deleted mice 3 weeks after birth. We speculate that PP2A α regulates liver regeneration and postnatal hepatocyte proliferation by similar mechanisms. There is a small wave of hepatocyte apoptosis at the end of LR that helps to control cell numbers in the liver [32]. We also examined hepatocyte apoptosis at the termination stage of LR. The overall number of apoptotic cells was very low and was similar in PP2A α mutants and controls (Supplementary Fig. 3).

HGF has been reported to be a key regulator of LR. Injection of HGF in the portal vein of normal rats and mice causes hepatocyte proliferation and liver enlargement [33, 34]. In the present work, we found increased expression of HGF in PP2A α ^{-/-} mice on days 4 and 5 post-PH. This suggests a greater capacity for mitosis in PP2A α ^{-/-} mice at the termination stage of LR.

Finally, we analyzed the TGF- β 1/smad and Hippo/YAP pathways in our animal model, which are considered responsible for regulating LR termination. We

found increased synthesis of TGF- β 1 and phosphorylation of smad2/3 in PP2A $\alpha^{-/-}$ mice, which suggested that the termination of LR in PP2A $\alpha^{-/-}$ mice was independent of the TGF- β 1/smad pathway (Supplementary Fig. 4). YAP plays a fundamental and widely conserved role in regulating tissue growth and organ size. It has been reported that YAP activation is controlled by ILK signaling during LR. ILK-KO livers show increased YAP protein levels and activation, which may partly account for the enhanced LR after ILK knockout [2]. In the present work we found an increase in total YAP and a decrease in the phospho-YAP/total YAP ratio in PP2A α -knockout mice at day 5 post-PH, which was consistent with the increased liver mass at this time point (Supplementary Fig. 5A). However, the expression of YAP was comparable in control and knockout mice at day 7 post- PH (Supplementary Fig. 5B). This result suggest us that the up-regulated YAP at day 5 may participates in PP2A α induced LR termination. Because it was previously reported that YAP can be phosphorylated by AKT/PKB at S127 [35], we further checked the expression and phosphorylation of YAP in wortmannin-treated livers. We found wortmannin neither decrease YAP S127 phosphorylation nor alter YAP expression in the liver (Supplementary Fig. 5C). These results indicate that YAP may participate in PP2A α -induced LR termination independent of AKT/GSK3 β .

In conclusion, PP2A α plays an important role in the termination of LR by regulating the AKT/GSK3 β /Cyclin D1 pathway. As a critical factor for the

termination of LR, PP2A α may provide guidance for the treatment of chronic liver diseases, liver transplantation, and liver malignancy.

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Figure legends

Fig. 1. PP2A α expression pattern during LR progression. (A)

Quantification of hepatic PP2A α expression at the indicated time points after PH. **, $p < 0.01$. (B) Protein expression of PP2A α during LR progression was analyzed via western blot.

Fig. 2. Liver-specific deletion of PP2A α in mice. (A) *Pp2a*^{flox/flox} mice were

crossed with mice harboring Cre recombinase under control of the albumin promoter to specifically delete PP2A α in hepatocytes. (B) Knockout efficiency in PP2A α ^{-/-} mice. (C) Histology of knockout and control mice at 8 weeks of age (Magnification: 200X; Scale bar: 200 μ m). (D) Body weight, liver weight and liver-to-body weight of mice at 8 weeks of age. *, $p < 0.05$; **, $p < 0.01$. (E) A typical image of BrdU staining of livers from quiescent 2-month-old mice. BrdU-positive nuclei are indicated by white arrowheads (Magnification: 200X; Scale bar: 200 μ m). The bar graph shows the percent of BrdU-positive hepatocytes. (F) Cell size was visualized by staining the hepatocyte cell membrane with a β -catenin antibody. The bar graph shows the number of hepatocytes per field under 200X magnification (Scale bar: 200 μ m). (G) A typical image of BrdU and Ki67 staining of livers from WT and PP2A α ^{-/-} mice 3 weeks after birth (Magnification: 200X; Scale bar: 200 μ m). The number of BrdU-positive and Ki67-positive hepatocytes in livers from the indicated genotypes 3 weeks after birth. *, $p < 0.05$; **, $p < 0.01$.

Fig. 3. Enhanced liver growth and reduced liver injury in PP2A α -knockout mice after PH. (A) Representative livers from mice before and 5 days after PH indicating the difference in liver size between knockout and wild-type mice. (B) Recovery of liver weight in PP2A α ^{-/-} and wild-type mice. *, $p<0.05$; **, $p<0.01$. (C) Liver weight relative to body weight at the indicated time points after PH and normalized to the pre-PH ratios. *, $p<0.05$; **, $p<0.01$. (D) Liver weight relative to body weight before and after PH in both PP2A α ^{-/-} and wild-type mice. **, $p<0.01$; ^{##}, $p<0.01$. (E, F) Serum AST and ALT levels in PP2A α ^{-/-} and wild-type mice pre- and post-PH. *, $p<0.05$; **, $p<0.01$.

Fig. 4. Delayed liver regeneration termination in PP2A α -knockout mice after PH. (A) Representative sections from PP2A α ^{-/-} and wild-type mice at the indicated time points after PH (Magnification: 200X; Scale bar: 200 μ m). The sections were subjected to immunostaining for Ki67 (red) and counterstaining with DAPI (blue). (B) The percent of Ki67-positive cells was calculated as the ratio of Ki67-positive nuclei to total nuclei. *, $p<0.05$; **, $p<0.01$. (C) Kinetics of hepatocyte mitosis during LR. *, $p<0.05$; **, $p<0.01$. (D) The number of BrdU-positive hepatocytes on days 2 and 7 after PH. Representative images of BrdU staining on day 7 after PH (Magnification: 200X; Scale bar: 200 μ m). *, $p<0.05$. (E) IF of β -catenin in livers from PP2A α ^{-/-} and wild-type mice 7 days

after PH. The bar graph shows the number of hepatocytes per field under 200X magnification.

Fig. 5. Expression of cell cycle-related genes in regenerating livers. (A) Western blot analyses were performed on livers harvested at different time points after PH using the indicated antibodies. GAPDH was used as the loading control. (B) Quantification of hepatic cyclin D1 expression at the indicated time points after PH. Expression was normalized to 18s RNA expression and calculated as the fold change compared to that in wild-type mice. *, $p<0.05$; **, $p<0.01$. (C) Western blot analysis of p21 and p27.

Fig. 6. Increased AKT/GSK3 β /Cyclin D1 signal transduction in PP2A α -knockout mice after PH. (A) Western blot analysis of MAPK/ERK, AKT, GSK3 β , and c-Myc at the indicated time points after PH. (B) Phosphorylation of cyclin D1 in PP2A α ^{-/-} and wild-type mice 5 days after PH. Cyclin D1 was immunoprecipitated with a cyclin D1 antibody and then analyzed by western blot using a p-Thr antibody. (C) Wortmannin treatment in PP2A α ^{-/-} and wild-type mice after PH. (D) Liver weight relative to body weight at 7 days post-PH in mice with or without wortmannin treatment. **, $p<0.01$ vs WT mice; #, $p<0.05$; ##, $p<0.01$ vs vehicle. (E) Hepatocyte proliferation was examined by Ki67 immunofluorescence in livers from PP2A α ^{-/-} and wild-type mice 7 days after vehicle or wortmannin treatment (Magnification: 200X; Scale

bar: 200 μ m). *, $p < 0.05$ vs WT mice; ##, $p < 0.01$ vs vehicle. (F) Expression of proteins in the AKT/GSK3 β /Cyclin D1 pathway and PCNA was analyzed 7 days post-PH in wortmannin- or vehicle-treated liver samples.

Fig. 7. Enhanced HGF synthesis and increased number of NPCs in PP2A α ^{-/-} mice after PH. (A) Quantification of hepatic HGF expression at the indicated time points after PH. **, $p < 0.01$. (B) Western blot analysis of HGF and α -SMA. (C) Percentage of non-parenchymal cells on day 5 after PH. **, $p < 0.01$. (D) A model for the regulation of LR termination by PP2A α . At the termination stage of LR, PP2A α activates GSK3 β directly or indirectly. Activated GSK3 β promotes the degradation of cyclin D1 through phosphorylation, which arrests proliferating hepatocytes at G1. In addition, PP2A may affect the release of certain regeneration-related growth factors by NPCs through cross-talk between hepatocyte and NPCs.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7