GASTROINTESTINAL, HEPATOBILIARY, AND PANCREATIC PATHOLOGY

CDK5RAP3 Deficiency Restrains Liver Regeneration after Partial Hepatectomy Triggering Endoplasmic Reticulum Stress

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CDK5 regulatory subunit-associated protein 3 (CDK5RAP3) plays a crucial role in mammalian liver development and hepatic function by controlling hepatocyte proliferation and differentiation, glucose and lipid metabolism, UFMylation, and endoplasmic reticulum homeostasis. However, the role of CDK5RAP3 in liver regeneration remains unknown. A liver-specific *Cdk5rap3* knockout (CKO) mouse model was used to study the function of CDK5RAP3 during liver regeneration induced by standard two-thirds partial hepatectomy (PHx). Twenty-four hours after PHx, the liver-to-body weight ratio was markedly higher in CKO mice than in wild-type mice. However, this ratio did not increase significantly and gradually over time after PHx in CKO mice. Hepatocyte proliferation was significantly delayed in CKO mice compared with wild-type mice. Meanwhile, CDK5RAP3 deficiency increased lipid accumulation, impaired glycogen synthesis, and lowered blood glucose levels after PHx. Critically, the absence of CDK5RAP3 seemed to promote an inflammatory response and induce apoptosis at a late stage of liver regeneration. In addition, CDK5RAP3 deficiency disrupted UFMylation homeostasis and aggravated endoplasmic reticulum stress in hepatocytes after PHx. Taken together, these data suggest that CDK5RAP3 enhances liver regeneration, at least partially via controlling cell cycle and glucose and lipid metabolism. (*Am J Pathol* 2020, 190: 2403–2416; <https://doi.org/10.1016/j.ajpath.2020.08.011>)

The liver has a unique capacity to regenerate after various types of injuries.^{1,2} However, patients with acute liver failure or who undergo extensive liver resection frequently experience failed regeneration.^{3,4} Thus, it is critical to better elucidate the mechanisms underlying liver homeostasis and regeneration, and to identify new therapeutic targets that can regulate liver regeneration. This tightly modulated process involves priming of the hepatocytes, cell cycle entry, proliferation, and termination.^{5,6} Two-thirds partial hepatectomy (PHx) in rodents is a common model to investigate liver regeneration.⁷ After PHx, the residual hepatocytes replicate through both hypertrophic and hyperplastic mechanisms.^{8,9} Liver regeneration relies primarily on hepatocyte proliferation.¹⁰ Upon PHx, quiescent hepatocytes quickly reenter the cell cycle,¹¹ with cell proliferation peaking at day 2 or 3.^{12–14} High amounts of lipids and glycogen accumulate in hepatocytes immediately after two-thirds PHx.⁸ Transient liver steatosis on day 2 or 3 after PHx influences

liver regeneration.^{15,16} Lipid overload in mice with simple hepatic steatosis can delay DNA replication in hepatocytes during liver regeneration.¹⁷

CDK5RAP3 (or LZAP, C53) was first identified as a binding protein for cyclin-dependent kinase 5 activator.^{18,19} CDK5RAP3 is highly conserved in mammals and is expressed in multiple organs, especially in liver.^{18–20} CDK5RAP3 interacts with several proteins, including P35, Rel A, Chk1, TIP-1, and CBP, to regulate cell cycle, cell survival, cell adherence/invasion, tumorigenesis, and metastasis.^{21–25} In addition, CDK5RAP3 participates in maintenance of protein homeostasis by regulating endoplasmic

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reticulum (ER) homeostasis and UFMylation, the latest ubiquitylation-like modification identified.^{20,26,27}

Ubiquitin-fold modifier 1 (UFM1) is the most recently identified ubiquitin-like protein.²⁸ The UFM1 conjugation system comprises UFM1, UBA5 (E1 activating enzyme), UFC1 (E2 conjugating enzyme), UFL1 (E3 ligase), and UFSP1 and UFSP2 (UFM1-specific proteases).^{29–31} CDK5RAP3 has been identified as a substrate for UFM1³² and can interact with UFC1 and UFL1.^{27,33} A growing body of evidence implicates the UFMylation system in many biological processes, including hematopoiesis, brain and liver development, ER stress, and fatty acid metabolism.^{20,34} However, the function of the UFMylation system in liver regeneration remains largely unknown and warrants further investigation.

Here, the function of CDK5RAP3 in liver regeneration was explored in the most commonly used two-thirds PHx mice model.³⁵ We found that CDK5RAP3 deficiency does not completely abolish but dramatically impairs liver regeneration, as indicated by delays in hepatocyte proliferation, lipid accumulation, and impaired glycogen synthesis in remnant livers. In regenerating livers, CDK5RAP3 deficiency perturbs UFMylation homeostasis and aggravates ER stress. Taken together, these data indicate that CDK5RAP3 plays a significant role in liver regeneration by regulating hepatocyte proliferation and glucolipid metabolism.

Materials and Methods

Mice

Alb-Cre induced liver-specific *Cdk5rap3* knock-out (CKO) mice were generated, as previously described.²⁰ The mice were kept with an alternating 12-hour light/dark cycle and provided with standard chow and water *ad libitum*. The Institutional Animal Care and Use Committee at Peking Union Medical College & Chinese Academy of Medical Sciences approved all mice procedures. All mice were fed in specific pathogen-free facilities.

PHx Model

The two-thirds PHx was performed on 2- to 3-month-old C57BL6/J mice as previously reported.^{36,37} Bromodeoxyuridine (BrdU) (50 mg/kg body weight) was injected intraperitoneally into mice 2 hours before euthanasia at the indicated time points.

Immunohistochemistry and Western Blot

Paraffin-embedded liver tissues were cut into sections for hematoxylin and eosin (H&E) and

immunohistochemistry staining. Optimal cutting temperature—embedded blocks were cut into sections for Oil Red O staining. Whole liver protein extracts were obtained by homogenizing liver samples in a radioimmunoprecipitation assay buffer (Beyotime, Beijing, China) and protease/phosphatase inhibitors. H&E staining, immunohistochemistry staining, periodic acid-Schiff staining, Oil Red O staining, and Western blot were performed as previously reported.²⁰

Antibodies

Primary antibodies were used against β -catenin (Santa Cruz Biotechnology, Dallas, TX), matrix metalloproteinase 2 (Santa Cruz Biotechnology), P21 (Abcam, Cambridge, UK), β -actin (Santa Cruz Biotechnology), BrdU (Abcam), CDK5RAP3 (Abcam, Cambridge, UK), Chop (Abcam), glyceraldehyde-3-phosphate dehydrogenase (Santa Cruz Biotechnology), proliferation cell nuclear antigen (PCNA) (Santa Cruz Biotechnology), pHH3 (Abcam), p-PERK (Cell Signaling Technology, Boston, MA), activating transcription factor 6 (Cell Signaling Technology), XBP1 (Abcam), IRE1 α (Santa Cruz Biotechnology), activating transcription factor 4 (Abcam), p-eIF2 α (Cell Signaling Technology), CyclinB1 (Santa Cruz Biotechnology), CyclinD1 (Abcam), Bip (Cell Signaling Technology), Ufm1 (Abcam), and active caspase-3 (R&D system, Minneapolis, MN). All horseradish peroxidase—linked secondary antibodies were purchased from Cell Signaling Technology.

RNA Extraction and Real-Time Quantitative PCR

Total RNA was extracted from liver tissues in various time points after PHx using Trizol reagent (Thermo Fisher Scientific, Waltham, MA). Reverse transcription and real-time quantitative PCR were performed as previously described.²⁰ β -actin expression was used to normalize gene expression. The sequences of primers are given in Table 1.

Serum Biochemical Analysis

After at least 30 minutes of coagulation at room temperature and centrifugation, serum was separated and stored at -80°C . Hitachi 7100 Automatic Analyzer (Hitachi, Tokyo, Japan) was used to determine serum levels of cholesterol, triglycerides, total bilirubin, alanine aminotransferase, and aspartate aminotransferase. The OneTouch Ultra System Kit (BD Biosciences, San Jose, CA) was used to measure the serum level of glucose.

Table 1 Primer Sequences of Real-Time Quantitative PCR Used in This Study

Name	Sequence
Cd36-F	5'-ATGGGCTGTGATCGGAACGTG-3'
Cd36-R	5'-TTTGCCACGTCATCTGGGTTT-3'
Cpt1 α -F	5'-TGGCATCATCACTGGTGTGT-3'
Cpt1 α -R	5'-GTCTAGGGTCCGATTGATCTTTG-3'
Hadha-F	5'-TGCATTTGCCGAGCTTTAC-3'
Hadha-R	5'-GTTGGCCAGATTTCGTTCA-3'
Hadhb-F	5'-TCGGGTTTGTTCATCGGA-3'
Hadhb-R	5'-GGCCAGAAGCTATCAGACCAA-3'
Scd1-F	5'-TTCTTGCGATACACTCTGGTGC-3'
Scd1-R	5'-CGGGATTGAATGTTCTTGTCGT-3'
Acaca-F	5'-CTCCCGATTTCATAATTGGGTCTG-3'
Acaca-R	5'-TCGACCTTGTCTTACTAGGTGC-3'
Fasn-F	5'-GGAGTGGTGATAGCCGGTAT-3'
Fasn-R	5'-TGGGTAATCCATAGAGCCAG-3'
Cdk5rap3-F	5'-CAACACTGCCATCCAGGACAT-3'
Cdk5rap3-R	5'-GCCTCGGTGCCTTTAAGAAG-3'
β -actin-F	5'-ACCAACTGGGACGACATGGAGAAG-3'
β -actin-R	5'-TACGACCAGAGGCATACAGGGACA-3'

F, forward; R, reverse.

Statistical Analysis

Results are presented as means $\pm$ SD. Data were assessed by using a *t*-test. Differences were considered statistically significant at $P < 0.05$. Prism 7.0 (GraphPad Software, La Jolla, CA) was used to perform statistical analyses.

Results

Cdk5rap3 Deletion Causes Aberrant Liver Regeneration after PHx

CDK5RAP3 reportedly regulates cell proliferation and differentiation^{23–25} and plays a crucial role in liver development.²⁰ However, the function of CDK5RAP3 in liver regeneration remains unknown. To investigate the kinetics of CDK5RAP3 expression during liver regeneration, wild-type (WT) mice underwent two-thirds PHx or sham surgery, and were euthanized at different time points after surgery. CDK5RAP3 expression level did not vary significantly during this process (Figure 1A). Cyclin D1 expression was dramatically increased at 24, 36, and 48 hours after PHx (Figure 1A). Meanwhile, the liver-to-body weight (LW/BW) ratio increased significantly from 24 hours after PHx onward (Supplemental Figure S1A). The number of PCNA-positive hepatocytes also dramatically increased at 24, 36, and 48 hours after PHx (Supplemental Figure S1B).

To further investigate the role of CDK5RAP3 in liver regeneration, CKO mice were studied. In addition, the *Cre*-negative littermates were used as WT controls. CKO mice displayed no obvious defects and survived well into

adulthood, possibly because of incomplete deletion of *Cdk5rap3* in hepatocytes at early developmental stages.²⁰ CKO mice had an LW/BW ratio comparable to that of WT mice, although there was high efficiency of *Cdk5rap3* deletion in adult mice (Figure 1, B–D, and Supplemental Figure S1C). H&E staining showed that the hepatocytes in CKO mice were slightly larger than in WT mice (Figure 1B). Meanwhile, lipid accumulation measured by using Oil Red O staining was slightly increased in CKO mice (Figure 1B). There were still a few CDK5RAP3-positive hepatocytes in CKO due to leaky *Cre* (Figure 1B).

Next, CKO and WT adult mice underwent two-thirds PHx. The LW/BW ratio was markedly increased at 24 hours after PHx in both WT and CKO mice (Figure 1D). Interestingly, the LW/BW ratio at 24 hours after PHx was significantly higher in CKO mice than in WT mice (Figure 1D). After PHx, the LW/BW ratio of WT mice increased gradually and significantly over time, contrary to the LW/BW ratio in CKO mice (Figure 1D). Sham surgery did not affect survival of CKO mice, and the LW/BW ratio did not statistically differ between WT and KO mice subjected to sham operation at the time points studied (Supplemental Figure S1D). In addition, the level of serum alanine aminotransferase, indicative of the extent of liver damage, was elevated more in CKO mice than in WT mice (Figure 1E).

To further assess the consequence of liver CDK5RAP3 deficiency on proliferation, cell cycle, and inflammation, the expression of the proteins β -catenin, cyclin D1, matrix metalloproteinase 2, and P21 was measured after PHx. The expression of β -catenin and cyclin D1 was significantly decreased in CKO mice compared with WT mice, whereas matrix metalloproteinase 2 and P21 levels were up-regulated in CKO mice (Figure 1, F and G). Collectively, these results suggest that *Cdk5rap3* deletion leads to aberrant liver regeneration after PHx.

CDK5RAP3 Deficiency Impairs Hepatocyte Proliferation after PHx

To assess whether liver-specific *Cdk5rap3* knockout suppressed hepatocyte proliferation after PHx, PCNA expression and BrdU incorporation were measured. Unlike in WT mice, PCNA protein levels did not increase markedly at 24 hours after PHx in CKO livers (Figure 2A). In CKO mice, expression of PCNA was not up-regulated until 48 hours after PHx (Figure 2A). The number of PCNA- and BrdU-positive hepatocytes was also decreased in CKO mice compared with control mice (Figure 2, B and C). Considering previous reports showing that *Cdk5rap3* deficiency led to G2/M arrest,²⁰ cell cycle progression was evaluated by measuring the level of pHH3. At 36 and 48 hours after PHx, the

Figure 1 CDK5 regulatory subunit-associated protein 3 (CDK5RAP3) deletion impairs liver regeneration after partial hepatectomy (PHx). **A**: Expression of CDK5RAP3 and cyclin D1 in wild-type (WT) livers was determined by using Western blot analysis, 0 to 48 hours after PHx and 1 to 3 hours after sham surgery. **B**: Representative images of immunohistochemistry staining for CDK5RAP3, and hematoxylin and eosin (H&E) staining and Oil Red O staining of WT and liver-specific *Cdk5rap3* knockout (CKO) liver sections. **C**: Expression of CDK5RAP3 in WT and CKO livers assessed by using Western blot analysis. **D**: Liver-to-body weight ratio (LW/BW) of CKO and WT mice at 0, 24, 36, and 48 hours after PHx. **E**: Serum alanine aminotransferase (ALT) levels in CKO and WT mice at 24, 36, and 48 hours after PHx. **F** and **G**: Expression of β -catenin, cyclin D1, matrix metalloproteinase 2 (MMP2), and P21 in WT and CKO livers assessed by using Western blot analysis at 24, 36, and 48 hours after PHx. Data are expressed as means $\pm$ SD (**D** and **E**). Representative results from three experiments (**A**, **C**, **F**, and **G**). $n \geq 4$ in each group (**D** and **E**). * P < 0.05, *** P < 0.0005. Scale bars = 100 μ m. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

number of pHH3-labeled cells was significantly decreased in CKO mice (Figure 2D). Moreover, at 48 hours after PHx, PCNA- and BrdU-labeled cell numbers in CKO mice reached levels comparable to that found

at 24 hours after PHx in WT control mice (Figure 2, B and C). Taken together, these results suggest that PHx-induced liver regeneration is significantly delayed in CKO mice.

Figure 2 The disruption of CDK5 regulatory subunit-associated protein 3 (CDK5RAP3) in hepatocytes inhibits cell proliferation after partial hepatectomy (PHx). **A:** Western blot analysis of proliferation cell nuclear antigen (PCNA) protein expression in liver-specific *Cdk5rap3* knockout (CKO) and wild-type (WT) livers at 0, 24, 36, and 48 hours after PHx. Quantification of Western blot results is normalized to β -actin level. **B–D:** Immunohistochemistry with PCNA (**B**) and bromodeoxyuridine (BrdU) incorporation (**C**), and pHH3 (**D**) at 24, 36, and 48 hours after PHx in WT and CKO liver sections. The numbers of PCNA-positive (**B**), BrdU-positive (**C**), and pHH3-positive (**D**) hepatocytes are quantified from CKO and WT liver sections at 24, 36, and 48 hours after PHx. Data are expressed as means $\pm$ SD. $n = 9$ in each group (**B–D**). * $P < 0.05$, *** $P < 0.0005$. Scale bars = 50 μ m (**B–D**).

Figure 3 Hepatic CDK5 regulatory subunit-associated protein 3 (CDK5RAP3) deficiency promotes hepatic lipid accumulation and decreases glycogen synthesis during liver regeneration. **A:** Representative images of hematoxylin and eosin staining from wild-type (WT) and liver-specific *Cdk5rap3* knockout (CKO) liver sections at 0, 24, 36, and 48 hours after partial hepatectomy (PHx). **B:** The number of mitotic hepatocytes was quantified in CKO and WT mice at 24, 36, and 48 hours after PHx. **C:** Representative images of Oil Red O staining of liver tissues from CKO and WT mice at 0, 24, 36, and 48 hours after PHx. **D:** Hepatic triglycerides (TG) level in CKO and WT liver tissues at 24, 36, and 48 hours after PHx. **E:** Hepatic gene expression for *Cd36* (lipid import), *Cpt1a*, *Hadha*, *Hadhb* (β -oxidation), and *Scd1*, *Acaca*, and *Fasn* (lipogenesis). β -actin expression was used to normalize gene expression. **F:** Representative images of periodic acid-Schiff staining in WT and CKO livers at 0, 24, 36, and 48 hours after PHx. **G:** Serum glucose (Glu) levels of adult CKO and WT mice. Data are expressed as means $\pm$ SD (**B** and **D–F**). $n = 5$ in each group (**B**); $n = 3$ to 6 (**D**); $n = 3$ to 4 (**G**). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Scale bars: 50 μ m (**A** and **F**); 100 μ m (**C**).

Figure 4 Hepatic CDK5 regulatory subunit-associated protein 3 (CDK5RAP3) deficiency leads to exacerbation of inflammatory response and hepatocyte apoptosis during liver regeneration. Representative images of hematoxylin and eosin (H&E) staining, Oil Red O staining, immunohistochemistry for F4/80, periodic acid-Schiff (PAS) staining, immunohistochemistry for active caspase-3, and CDK5RAP3 of wild-type (WT) and liver-specific *Cdk5rap3* knockout (CKO) liver sections at 7 (**A**) and 14 (**C**) days after partial hepatectomy (PHx). Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), glucose (Glu), triglycerides (TG), and cholesterol (CHO) levels in CKO and control mice at 7 (**B**) and 14 (**D**) days after PHx. Data are expressed as means $\pm$ SD. $n = 3$ (**A–D**). * $P < 0.05$, ** $P < 0.005$. Scale bars = 50 μ m.

Figure 5 Absence of CDK5 regulatory subunit-associated protein 3 (CDK5RAP3) alters UFMylated substrates in regenerating liver. Ubiquitin-fold modifier 1 (UFM1) conjugates in adult liver-specific *Cdk5rap3* knockout (CKO) and control mice were detected by Western blot using anti-UFM1 antibodies at 24 (A), 36 (B), and 48 (C) hours after partial hepatectomy (PHx). **Triangles** indicate the bands with significant differences. Representative results of three experiments. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

CDK5RAP3 Deficiency Causes Hepatic Lipid Overload and Impairs Glucose Metabolism during Liver Regeneration

To evaluate the histologic changes occurring during liver regeneration, H&E staining on liver sections taken from CKO and WT mice was performed at various times after PHx. The organization of the hepatocytes was disrupted in CKO mice compared with WT mice (Figure 3A). Moreover, hepatocyte replication was evaluated by quantifying mitotic figures. After PHx, fewer hepatocytes underwent mitosis in CKO mice than in WT mice (Figure 3B). There were no significant histologic changes in WT and CKO mice before and after the sham operation (Supplemental Figure S1E). The size of the hepatocytes increased after two-thirds PHx, peaked at 24 hours, and then gradually decreased.³⁸ Nevertheless, it was noted that at 36 and 48 hours after PHx, the hepatocytes of CKO mice were bigger and with larger cytoplasm than those of WT mice (Figure 3A). Based on these results, an onset of steatosis was suspected in CKO mice after PHx. Oil Red O staining was used to visualize hepatic lipid accumulation. Expectedly, more lipid droplets were found in CKO mice at 24, 36, and 48 hours after PHx (Figure 3C). Consistent with this finding, the amount of liver triglycerides was markedly higher in CKO mice (Figure 3D). Moreover, lipid metabolism was monitored at different time points after PHx. The expression of the lipid import gene *Cd36* was markedly increased in CKO mice at 24, 36, and 48 hours after PHx. By contrast, *Cdk5rap3* deletion caused decreased expression of β -oxidation genes (*Cpt1 α* and *Hadha/b*) and lipogenic genes (*Scad1*, *Acaca*, and *Fasn*) after PHx (Figure 3E). In addition to lipid oxidation, glucose metabolism provides energy for liver regeneration.^{39,40} Liver glycogen accumulation and blood glucose levels were therefore measured after PHx. Periodic acid-Schiff staining showed that glycogen storage was strongly disrupted in CKO livers compared with WT livers (Figure 3F). Glucose levels in the blood of CKO mice were significantly lower than in the control mice (Figure 3G). These data suggest that CDK5RAP3 is involved in energy supply for liver regeneration by regulating lipid accumulation and glucose metabolism.

CDK5RAP3 Deletion Intensifies Inflammation and Induces Apoptosis at Late-Stage Liver Regeneration

In the two-thirds PHx rodent model, the remaining liver enlarges until the original liver mass is restored, approximately 1 week after surgery, after which the regenerative process stops.¹⁴ The effect of CDK5RAP3 deficiency was thus further examined on late-stage liver regeneration. As indicated by their heterogeneous size visualized by H&E staining, the arrangement of hepatocytes was strongly disorganized in CKO mice at 7 and 14 days after PHx, compared with WT mice (Figure 4, A and C). Next, the inflammatory cell infiltrates in WT and CKO livers were

Figure 6 CDK5 regulatory subunit-associated protein 3 (CDK5RAP3) deficiency induces endoplasmic reticulum stress. **A–C:** Expressions of BiP, p-PERK, p-eIF2α, activating transcription factor (ATF) 4, CCAAT-enhancer-binding protein homologous protein (CHOP), IRE1α, unspliced XBP-1 [XBP-1(u)] and its spliced forms [XBP-1(s)], and ATF6 were analyzed by Western blot in 2-month-old liver-specific *Cdk5rap3* knockout (CKO) and control livers at 24 (**A**), 36 (**B**), and 48 (**C**) hours after partial hepatectomy (PHx). **Black arrows** indicate the P-eIF2α target proteins. **D–F:** Expression of endoplasmic reticulum stress-related proteins was measured according to gray scale values from the Western blot bands presented in **Figure 6**. Quantification of Western blot results is all normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) levels. Data are expressed as means ± SD. * $P < 0.05$, ** $P < 0.005$, *** $P < 0.0005$.

Figure 7 Model for the function of *Cdk5rap3* during liver regeneration. In wild-type (WT) mice, CDK5 regulatory subunit-associated protein 3 (CDK5RAP3) controls the substrate specificity of UFMylation to maintain endoplasmic reticulum (ER) homeostasis, sustain normal lipid metabolism, and liver regeneration. In liver-specific *Cdk5rap3* knockout (CKO) mice, CDK5RAP3 deficiency leads to altered substrate UFMylation, ER stress induction, impairment of hepatocyte proliferation, aberrant lipid accumulation, and even hepatocyte inflammation and apoptosis. UBA5, E1 activating enzyme; UFC1, E2 conjugating enzyme; UFL1, E3 ligase; UFM1, Ubiquitin-fold modifier 1; UFS1, and UFS2, UFM1-specific proteases.

detected (Figure 4, A and C). Consistently, at 7 and 14 days after PHx, there were more F4/80-positive cells in CKO livers than in WT livers (Figure 4, A and C). In addition, evaluation of hepatocytes apoptosis by immunohistochemistry staining for active caspase-3 showed that CDK5RAP3 deficiency induced liver cell apoptosis at 7 and 14 days after PHx (Figure 4, A and C). Day 7 after PHx, lipid accumulation was higher in CKO livers than in WT livers, but no significant difference was visible at day 14 (Figure 4, A and C). Periodic acid-Schiff staining showed that glycogen synthesis was severely disrupted in CKO livers compared with WT livers at 7 and 14 days after PHx.

Serum alanine aminotransferase and aspartate transaminase levels were elevated at 7 and 14 days after PHx; lower serum glucose and higher serum triglycerides were noted in CKO livers compared with WT livers at 14 days after PHx (Figure 4, B and D). The data indicate abnormal CKO liver function at late-stage liver regeneration.

CDK5RAP3 Deficiency Alters UFMylation Profiles in Regenerating Liver

CDK5RAP3 reportedly interacts with several components of a novel ubiquitin-like modification system, UFMylation.^{27,32,33,41,42} In our previous study, CDK5RAP3 had been recognized as a novel substrate adaptor for

UFMylation.²⁰ Whether the aberrant liver regeneration in CKO was accompanied by an alteration of UFMylated substrates has been explored. Intriguingly, the level of UFMylated substrates appeared down-regulated in WT mice after PHx, compared with the homeostatic state (Supplemental Figure S1F). However, CKO mice exhibited the opposite change of UFMylation level after PHx (Supplemental Figure S1F). At the same time, the UFMylation profile in regenerating liver was dramatically altered in CKO mice compared with control mice at 24, 36, and 48 hours after PHx (Figure 5). UFMylated proteins with molecular weights around 100 KDa, 55 KDa, and 40 KDa were especially up-regulated in CKO mice (Figure 5). In addition, no UFMylated proteins with a molecular weight ranging from 35 KDa to 40 KDa were detected in those CKO mice with higher *Cdk5rap3* knockout efficiency (Figure 5). Together, these results indicate that CDK5RAP3-mediated UFMylation may also play important roles in mammalian liver regeneration.

CDK5RAP3 Loss Disturbs ER Homeostasis in Regenerating Liver

ER stress is associated with liver diseases involving hepatic lipid and glucose metabolism, including deficient regeneration.^{17,43} In recent years, ER homeostasis has been

associated with steatosis and DNA replication occurring after PHx.^{17,44} Therefore, whether CDK5RAP3 loss disturbed ER homeostasis was immediately investigated in regenerating liver. The expression of proteins involved in three unfolded protein response pathways was analyzed by using Western blot. From the PERK signaling pathway, the phosphorylation level of both PERK and CCAAT-enhancer-binding protein homologous protein was up-regulated at 24, 36, and 48 hours after PHx (Figure 6), and the phosphorylated form of eIF2 α was only slightly increased at 36 hours after PHx (Figure 6). Activating transcription factor 4 expression showed no change in CKO mice (Figure 6). From the IRE1 α signaling pathway, up-regulation of spliced XBP1 was detected in CKO mice after PHx (Figure 6). Levels of IRE1 α and unspliced XBP1 remained unchanged (Figure 6). In the same way, changes in activating transcription factor 6 expression were not detected in CKO mice after PHx (Figure 6). In summary, the up-regulation of p-PERK, CCAAT-enhancer-binding protein homologous protein, and spliced XBP1 at different time points after PHx shows that CDK5RAP3 loss activated PERK and IRE1 α signaling pathways, thereby increasing ER stress (Figure 6).

Based on our data, a mechanism involving CDK5RAP3 in liver regeneration was proposed (Figure 7). In WT mice, CDK5RAP3 controls the substrate specificity of UFMylation to maintain ER homeostasis, sustain normal lipid metabolism, and promote liver regeneration. Although CDK5RAP3 deficiency does not influence the priming of liver regeneration, it leads to altered substrate UFMylation, ER stress induction, impairment of hepatocyte proliferation, aberrant lipid accumulation, and even hepatocyte inflammation and apoptosis.

Discussion

PHx is increasingly used to manage diverse malignant and nonmalignant lesions.⁴⁵ The regenerative capacity of the liver is crucial for survival of patients undergoing partial resection and after acute and chronic liver injuries secondary to toxins, infections, immune dysfunction, or metabolic diseases.^{5,12} Two-thirds PHx is widely used to assess liver regeneration capacity in rodent models.⁴⁶ This study showed that CDK5RAP3 deficiency impairs liver regeneration through inhibiting hepatocyte proliferation and affecting lipid and glucose metabolism. CDK5RAP3 deletion induced inflammatory response and even liver apoptosis at a late stage of liver regeneration. Alteration of UFMylation profile and induction of ER stress response occurring upon CDK5RAP3 deficiency likely explain the impaired liver regeneration. Collectively, these results indicate that CDK5RAP3 plays a significant role in liver regeneration.

Lipid oxidation and glucose metabolism are reported as the primary energy source early after hepatectomy.^{15,47} Lipids are expended as energy for hepatocyte DNA

replication and as materials for phospholipid synthesis.^{48,49} The liver has a robust and unique regenerative capacity in response to damage,¹⁴ but this capacity is suppressed in hepatic steatosis.⁴⁶ Impaired fatty liver regeneration is a leading cause of postoperative complications after hepatectomy.^{50–52} Fatty liver has been recognized as a significant risk factor for defective liver regeneration in humans and animal models.⁴⁰ However, a transient hepatocellular lipid accumulation characteristic of early regeneration after PHx in rodents is virtually required for physiological liver regeneration.⁴⁰ Transient liver steatosis at 12 to 24 hours after PHx is crucial for normal liver regeneration, but excess hepatic steatosis and disturbance of hepatic lipid accumulation before PHx suppress liver regeneration in mice.⁵³ Posthepatectomy recovery is impaired in fatty liver, characterized by both a decrease in hepatocyte proliferation and an increase in cell death.⁵⁴ In this study, CKO mice displayed no obvious defects and survived well into adulthood. However, homeostatic lipid storage in adult CKO mice is up-regulated, indicating that CKO mice may exhibit abnormal lipid metabolism. In addition, CDK5RAP3 deficiency in liver resulted in excessive hepatic lipid accumulation and reduced hepatocyte proliferation after PHx, which may contribute to impaired liver regeneration. Through expression analysis of genes involved in lipid metabolism, lipid import was significantly up-regulated in CKO mice. However, the β -oxidation and lipogenesis were impaired in the absence of CDK5RAP3. Hence, we speculated that CDK5RAP3 deletion did not affect initiation of transient liver steatosis but impaired the lipid consumption. Apart from lipids, a large amount of stored glycogen provides an energy reserve useable for hepatocytes' DNA replication in case of PHx.⁸ In this study, the lower glycogen synthesis observed in *Cdk5rap3* mutants might be the cause of proliferation delay. Blood glucose level reflects the liver mass, which may act as a sensor after PHx.⁸ Hypoglycemia and increased circulating insulin immediately follow PHx.^{15,55} The shift from glucose to lipid consumption is significant for the regeneration of phosphatase and tensin homolog–deficient livers after PHx.⁴⁷ In this study, blood glucose levels were decreased in CKO mice. Whether this difference affects steatosis and hepatocyte proliferation is worthy of concern. From our study, we propose that some liver functions, such as regulation of glucolipid metabolism and damage response, may be defective in CKO mice. Part of this hypothesis can be addressed by feeding CKO mice a high-fat diet after liver regeneration to verify the function of the regenerative liver.

Proteins involved in ubiquitination and proteasomal degradation were highly up-regulated after PHx versus sham surgery.⁵⁶ The E3 ubiquitin-protein ligase UHRF1 increases during liver regeneration independently of BIRC5.⁵⁷ Another ubiquitin ligase, Nedd4-1, was reported as a crucial regulator of liver regeneration.⁵⁶ Deletion of *HOIP*, which is the catalytic subunit of the linear ubiquitin chain assembly complex, promotes liver regeneration and DNA

damage.⁵⁸ However, thus far, the role of UFMylation, a novel ubiquitin-like modification system, in mammalian liver regeneration was unknown. Previous studies showed the interactions between CDK5RAP3 and multiple UFMylation-related factors.^{32,41,59} Moreover, we have identified CDK5RAP3 as a novel substrate adaptor for this ubiquitin-like modification.²⁰ This study showed that CDK5RAP3 deficiency also influences the UFMylation profile in liver after PHx. Meanwhile, fluctuation of UFMylation level was detected in some proteins at different time points after PHx. To our knowledge, this report is the first regarding the function of UFMylation in liver regeneration.

Liver regeneration after PHx enhances the unfolded protein burden at the ER of remnant hepatocytes, which in turn triggers the unfolded protein response.⁴⁵ ER stress plays an important role in cell fate control, including cell cycle, metabolism, and lipid synthesis.⁶⁰ In this study, depletion of CDK5RAP3 triggered ER stress and activated unfolded protein response after PHx, through the up-regulation of phosphorylation of PERK, spliced XBP-1, and CCAAT-enhancer-binding protein homologous protein. XBP-1 is induced by PHx and modulates unfolded protein, acute-phase, and DNA damage responses during mouse liver regeneration.⁴⁵ Thus, XBP-1 may mediate the effect of CDK5RAP3 defect on impaired liver regeneration. However, many components of the UFM1 system regulate ER homeostasis.^{31,61} The disturbance of UFMylation mediated by CDK5RAP3 may result in exacerbated ER stress after PHx.

Nonalcoholic fatty liver disease is a liver metabolic syndrome associated with insulin resistance and dyslipidemia.⁶² Fatty liver has been identified as a major risk factor for defective liver regeneration.¹⁷ ER stress is involved in hepatic lipid and glucose metabolism in liver diseases, including deficient regeneration.⁴³ In the current study, we found that *Cdk5rap3* deficiency not only increases homeostatic lipid accumulation but also prominently intensifies steatosis after PHx, at least partially through exacerbation of ER stress. In conclusion, CDK5RAP3 constitutes a potential therapeutic target in the treatment of patients with nonalcoholic fatty liver disease and aberrant regeneration.

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Author Contributions

Y.H., Y.J., and S.Y. wrote the original draft; Y.H., Y.J., S.Y., and R.Y. reviewed and edited the manuscript; Y.H. and Y.J. supervised the work; Y.H. and Y.J. provided administration for the project; Y.H. and Y.J. acquired

funding; all authors contributed to investigation and data curation.

Supplemental Data

Supplemental material for this article can be found at <http://doi.org/10.1016/j.ajpath.2020.08.011>.

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