PDK4-Deficiency Reprograms Intrahepatic Glucose and Lipid Metabolism to Facilitate Liver Regeneration in Mice

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Liver regeneration requires intrahepatic and extrahepatic metabolic reprogramming to meet the high hepatic bioenergy demand for liver cell repopulation. This study aims to elucidate how pyruvate dehydrogenase kinase 4 (PDK4), a critical regulator of glucose and lipid metabolism, coordinates metabolic regulation with efficient liver growth. We found that hepatic *Pdk4* expression was elevated after two-thirds partial hepatectomy (PHx). In *Pdk4*$^{-/-}$ PHx mice, the liver/body weight ratio was more rapidly restored, accompanied by more aggressive hepatic DNA replication; however, *Pdk4*$^{-/-}$ mice developed more severe hypoglycemia. In *Pdk4*$^{-/-}$ PHx livers, the pro-regenerative insulin signaling was potentiated, as demonstrated by early peaking of the phosphorylation of insulin receptor, more remarkable induction of the insulin receptor substrate proteins, IRS1 and IRS2, and more striking activation of Akt. The hepatic up-regulation of CD36 contributed to the enhanced transient regeneration-associated steatosis in *Pdk4*$^{-/-}$ PHx mice. Notably, CD36 overexpression in mice promoted the recovery of liver/body weight ratio and elevated intrahepatic adenosine triphosphate after PHx. CD36 expression was transcriptionally suppressed by FOXO1 (forkhead box protein O1), which was stabilized and translocated to the nucleus following AMPK (adenosine monophosphate–activated protein kinase) activation. PHx remarkably induced AMPK activation, which became incompetent to respond in *Pdk4*$^{-/-}$ livers. Moreover, we defined that PDK4-regulated AMPK activation directly depended on intracellular adenosine monophosphate *in vitro* and in regenerative livers. *Conclusion:* PDK4 inhibition reprograms glucose and lipid metabolism to promote liver regeneration by enhancing hepatic insulin/Akt signaling and activating an AMPK/FOXO1/CD36 regulatory axis of lipid. These findings may lead to potential therapeutic strategies to prevent hepatic insufficiency and liver failure. (*Hepatology Communications* 2020;4:504-517).

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Liver regeneration (LR) is a well-orchestrated and tightly regulated biological process integrating a complex network of cytokines, growth factors, and metabolic signals.[1] The mechanisms governing LR are still not fully defined. An important question is how liver cells coordinate metabolic functions with the regenerative response. Although it has been suggested that alterations in metabolism in response to experimentally induced hepatic insufficiency are functionally important for normal LR,[2] the molecular mediators that coordinate metabolism with regeneration have not been fully revealed and elucidated.

Abbreviations: Acaca, acetyl-CoA carboxylase α; Acadm, acyl-CoA dehydrogenase, medium chain; Acads, acyl-CoA dehydrogenase, short chain; Acadvl, acyl-CoA dehydrogenase, very long-chain; Acat1, acetyl-CoA acetyltransferase 1; Acetyl-CoA, acetyl-coA; Acox1, acyl-CoA oxidase 1, palmitoyl; Acox2, acyl-CoA oxidase 2, branched chain; AICAR, 5-aminoimidazole-4-carboxamide ribonucleotide; ALT, alanine aminotransferase; AMPK, adenosine monophosphate–activated protein kinase; AST, aspartate aminotransferase; Atgl (Pnpla2), adipose triglyceride lipase (patatin-like phospholipase domain containing 2); ATP, adenosine triphosphate; BrdU, bromodeoxyuridine; BW, body weight; Cd36, cluster of differentiation 36; Ces1d, carboxylesterase 1D; CHIP-seq, chromatin immunoprecipitation sequencing; CoA, coenzyme A; Cpt1α, Carnitine palmitoyl transferase 1α; Crat, carnitine O-acetyltransferase; Crot, carnitine O-octanoyltransferase; Dgat1/2, diacylglycerol O-acyltransferase 1/2; EGF, epidermal growth factor; ELISA, enzyme-linked immunosorbent assay; FA, fatty acid; Fabp1, fatty acid binding protein 1, liver; Fabp2, fatty acid binding protein 2, intestinal; Fabp3, fatty acid binding protein 3, muscle and heart; Fabp4, fatty acid binding protein 4, adipocyte; Fabp5, fatty acid binding protein 5, epidermal; Fasn, fatty acid synthase; Fatp1-5, fatty acid transport member 1-5; FOXO1, forkhead box protein O1; G6pc, glucose-6-phosphatase catalytic subunit; Gck, glucokinase; Gpat1 (Gpam), glycerol-3-phosphate acyltransferase, mitochondrial; GSK-3β, glycogen synthase kinase 3 beta; Hadha, hydroxyacyl-Coenzyme A dehydrogenase/3-ketoacyl-CoA thiolase/enoyl-CoA hydratase (trifunctional protein), alpha subunit; Hadhb, hydroxyacyl-Coenzyme A dehydrogenase/3-ketoacyl-Coenzyme A thiolase/enoyl-CoA hydratase (trifunctional protein), beta subunit; H&E, hematoxylin and eosin; HGF, hepatocyte growth factor; HNF, hepatocyte nuclear factor; Hsd17b4, hydroxysteroid 17-beta dehydrogenase 4;

Serving as a metabolic switch, pyruvate dehydrogenase kinase 4 (PDK4) inactivates the mitochondrial pyruvate dehydrogenase complex by phosphorylating the pyruvate dehydrogenase E1 alpha 1 subunit, thereby dictating the conversion of acetyl-coenzyme A (CoA) from pyruvate. Among the known isozymes, PDK4 is the most widely distributed and highly expressed in cardiac and skeletal muscles in mammals.[3] PDK4 coordinates glucose use and fat consumption to maintain normal blood glucose levels and is required for metabolic reprogramming.[4,5] Recent studies expand PDK4's roles from metabolic regulation to cell proliferation and cell cycle progression.[6,7] These intriguing findings indicate that PDK4 integrates metabolic regulation with proliferation mechanisms, implying a role of PDK4 in tissue regeneration.

Loss of PDKs confers insulin resistance in skeletal muscle.[8] However, genetic inactivation of *Pdk4* improves hyperglycemia and insulin resistance.[9] These contrary reports of PDK4 in insulin resistance raise the necessity to further define PDK4's role in insulin signaling. Among the downstream partners of insulin signaling, phosphoinositide 3-kinase (PI3K) has a major role in insulin function through the activation of Akt/protein kinase B. Activated Akt has pleiotropic effects. For instance, activated Akt induces glycogen synthesis through inhibitory phosphorylation of glycogen synthase kinase 3 (GSK-3) and promotes protein synthesis and cell growth through inhibition of TSC2 and indirect activation of mammalian target of rapamycin (mTOR) complex 1 (mTORC1).[10]

Most of the knowledge of LR comes from the useful paradigm of two-thirds partial hepatectomy (PHx). In mice, hepatic DNA replication peaks at 36-40 hours, and the liver mass increase has occurred within 3 days after partial hepatectomy (PH). Within 5-7 days, proliferation response subsides, and mass restoration is complete. To guide the progression of LR, growth factors along with some cytokines activate multiple signaling pathways, such as the PI3K/Akt signaling.[11]

Following the initiation of LR, hepatic and systemic metabolism is rapidly changed. For example, within

IF, immunofluorescence; IGF, insulin-like growth factor; IHC, immunohistochemistry; Insig1, insulin induced gene 1; IRS1/2, insulin receptor substrate 1/2; Klf15, Kruppel-like factor 15; KO, knockout; Lipa, lysosomal acid lipase A; Lipc, lipase, hepatic; Lipe, lipase, hormone sensitive; Lipg, lipase, endothelial; LR, liver regeneration; LXR, liver X receptor; Mgll, monoglyceride lipase; Mlxipl, MLX-interacting protein-like; mRNA, messenger RNA; mTOR, the mechanistic target of rapamycin; ORO, Oil Red O staining; Pck1/2, phosphoenolpyruvate carboxykinase 1/2; PCNA, proliferating cell nuclear antigen; PCR, polymerase chain reaction; PDC, pyruvate dehydrogenase complex; PDH, pyruvate dehydrogenase; PDK1–4, pyruvate dehydrogenase kinase 1-4; Pfkl, phosphofructokinase, liver type; Pgc1α, peroxisome proliferator-activated receptor gamma coactivator 1α; PHx, two-thirds partial hepatectomy; Pklr, pyruvate kinase L/R; Pkm, pyruvate kinase, muscle; PI3K, phosphoinositide 3-kinase; Pparg, peroxisome proliferator–activated receptor gamma; Pten, phosphatase and tensin homolog; PXR, pregnane X receptor; Scd1, stearoyl-CoA desaturase 1; TG, triglyceride; TRAS, transient regeneration–associated steatosis; WB, western blot; WT, wild type.

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hours of surgery, mice subjected to PH develop significant hypoglycemia, which is likely due to the acute removal of part of the hepatic glycogen content and gluconeogenic capacity. However, glucose supplementation impairs LR in PHx-induced or hepatotoxin-induced LR, potentially because of suppressing lipid mobilization.[12] As with altered glycemia, liver triglyceride accumulates coincidentally with hepatic induction of an adipogenic transcriptional program, defined as transient regeneration–associated steatosis (TRAS), which is essential to LR.[13,14] It is proposed that the metabolic response itself serves as a pro-regenerative signal.[2]

Hepatic fatty acid (FA) uptake is primarily through the family of SLC27 fatty acid transport proteins and the scavenger receptor fatty acid translocase (cluster of differentiation 36 [CD36]). *Cd36* deletion in hepatocytes reduced high-fat diet (HFD)-induced hepatic steatosis, decreased hepatic FA uptake, and improved whole-body insulin sensitivity.[15] However, *Cd36* transgenic mice challenged with HFD also showed attenuation of hepatic steatosis and improved glucose tolerance and insulin sensitivity.[16] Thus, as a lipid sensor for energy balance, the intriguing pathophysiological role of CD36 needs to be further elucidated.

In this study, we tested our hypothesis that PDK4-mediated metabolic reprogramming energizes efficient liver growth. We elucidated the role of PDK4 in LR using the PHx model, defined PDK4 regulation of hepatic insulin signaling in regenerative livers, and unraveled PDK4 as a critical mediator of hepatic lipid metabolism through regulating an AMPK (adenosine monophosphate–activated protein kinase)/FOXO1 (forkhead box protein O1)/CD36 axis to promote LR efficiency.

Materials and Methods

ANIMALS

Wild-type (WT) and *Pkd4*$^{-/-}$ knockout (KO) mice on a C57BL/6J (inbred strain) genetic background were described previously and handled in accordance with a protocol approved by the Institutional Animal Care and Use Committee at the University of Connecticut.[7,17,18] Mice were maintained on a 12-hour light/dark cycle with free access to water and standard chow before and after the surgery. Breeding mice were fed with irradiated sterile Teklad S-2335 mouse breeder diet (7904; Envigo Bioproducts, Inc., Madison, WI). Experimental male mice (8 to 12 weeks old) were fed with irradiated Teklad Global 18% Protein (2918; Envigo Bioproducts, Inc.). Mice were cohoused in clean and ventilated cages bedded with Teklad aspen sani-chips (7090A; Envigo Bioproducts, Inc.).

TWO-THIRDS PARTIAL HEPATECTOMY

PHx has been described previously.[19] In brief, the median lobe and the left lateral lobe, which together make up about 70% of the liver, were separately tied and resected. Mice that were conscious and alert following surgery were used for further studies. The recovery rate of mice was greater than 95% for both the WT and *Pkd4*$^{-/-}$ mice. Sham operation was performed as a control surgery, which included abdomen incision, falciform ligament dissociation, liver lobes exposure, and wound suturing.

OTHER STANDARD METHODS

Cell culture, isolation of primary hepatocytes, transfection, PDK4 short hairpin RNA (shRNA) delivery, quantitative real-time polymerase chain reaction (PCR), western blot (WB), hematoxylin and eosin (H&E) staining, Oil Red O (ORO) staining, luciferase assay, immunohistochemistry (IHC), immunofluorescence (IF), preparation of nuclear and cytoplasmic extracts, CCl_4 treatment, and bromodeoxyuridine (BrdU) incorporation were described previously.[14,17] The antibodies and primers used in this study are listed in Supporting Tables S1 and S2, respectively. For additional experimental procedures and analysis, please refer to the Supporting Information.

Results

PDK4 WAS UP-REGULATED IN LR AND LOSS OF PDK4 ACCELERATED LIVER MASS RECOVERY

PHx is the widely used paradigm of studying LR in a "clean" setting.[20] The hepatic *Pdk4* messenger RNA (mRNA) was remarkably induced at days 2, 4, and 7 following PHx in WT mice (Fig. 1A,

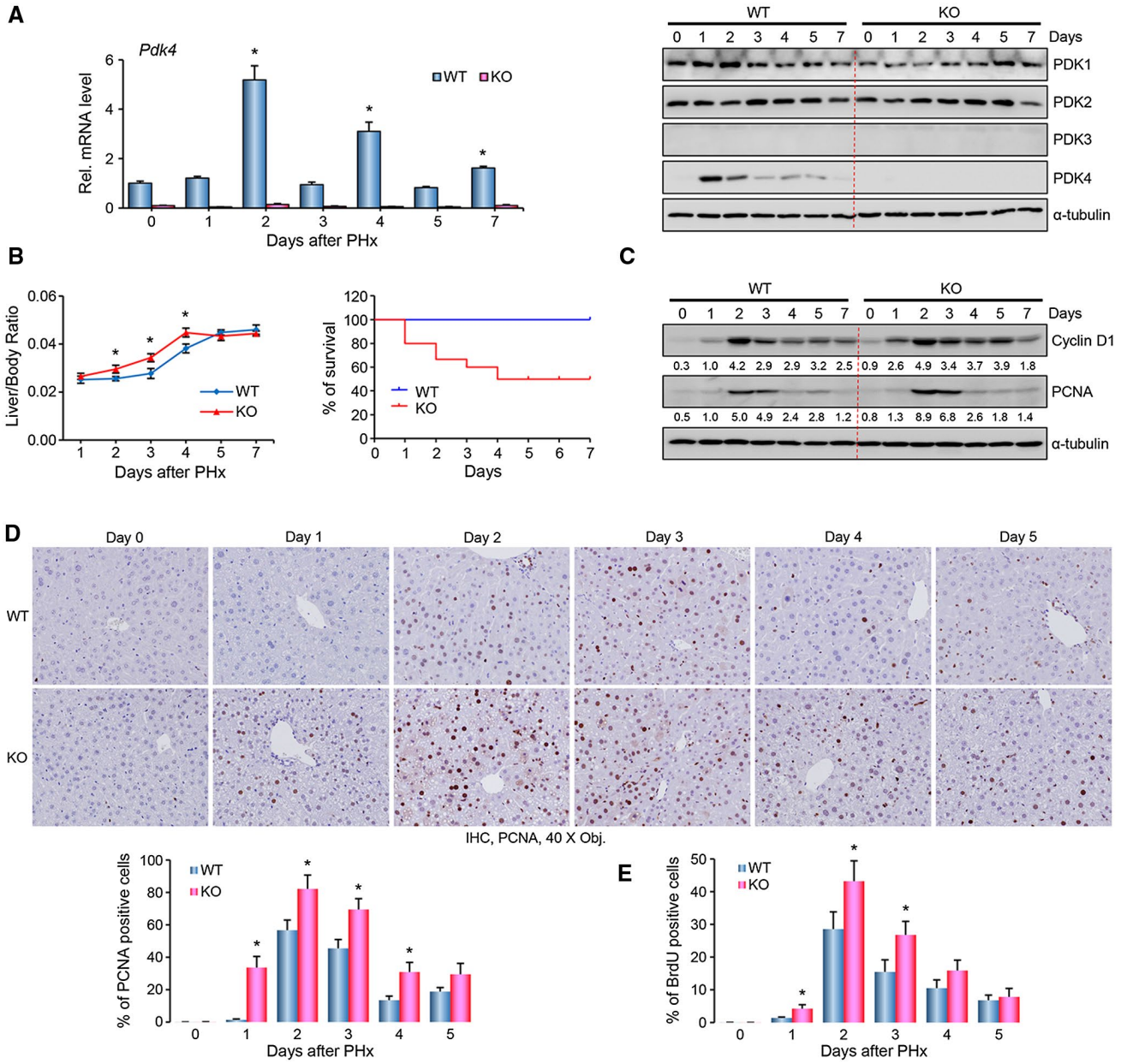

FIG. 1. Systemic deficiency of *Pdk4* accelerated liver mass recovery in mice after PHx. (A) Left: Quantitative real-time PCR of hepatic *Pdk4* mRNA in WT and *Pdk4*$^{-/-}$ KO mice before (day 0) and on different days after PHx. Right: WB of hepatic expression of PDKs. Samples were pooled from eight individual mice for each group. Data are shown as mean ± SEM; n = 8 mice/group; triplicate assays; **P* < 0.05 versus day 0. (B) Left: Liver to body weight ratio (n = 8/group). Right: Survival curve of WT and *Pdk4*$^{-/-}$ mice from PHx (n = 20/group). Data are represented as mean ± SEM; **P* < 0.05 versus WT. (C) WB of indicated proteins in pooled liver tissues (n = 8). The densitometry of each band relative to the loading control (α-tubulin) was quantified using ImageJ and denoted. Data are represented as mean ± SEM; **P* < 0.05 versus WT. (D) IHC of PCNA expression before (day 0) and after PHx. Representative IHC images and percentages of positive cells of PCNA staining in 24 randomly chosen fields from eight individual mice per group are shown (three fields per section per liver). Approximately the same portions of the right lateral lobes were used for histology. Slides were coded before examination, and cells were counted in a group-blinded manner. The sections were evaluated two times by two investigators, respectively. Data are represented as mean ± SEM; **P* < 0.05 versus WT. (E) BrdU incorporation. The percentages of BrdU-incorporated cells were quantitated as in (D). Data are represented as mean ± SEM; **P* < 0.05 versus WT.

left); *Pdk4* mRNA did not show induction in sham-operated WT livers (data not shown). Possibly due to the posttranscriptional regulation, PDK4 proteins were already increased at day 1, in comparison with an undetectable level at day 0, and then subsided at later time points. Compared with other PDKs, PDK3 was not detected in both WT and *Pdk4*$^{-/-}$ livers (Fig 1A, right). The mRNA levels of *Pdk1*, *Pdk2*, and *Pdk3* did not show significant induction following PHx in WT livers, except for an elevation of *Pdk3* (Supporting Fig. S1A). Notably, the liver/body weight ratio was much higher at day 2, day 3, and day 4 in *Pdk4*$^{-/-}$-PHx versus WT-PHx mice (Fig. 1B, left), indicating a permissive role of PDK4 inhibition in liver mass recovery. Unexpectedly, the survival of *Pdk4*$^{-/-}$-PHx mice decreased, although alanine aminotransferase was less induced at day 1 (Fig. 1B, right, and Supporting Fig. S1B).

DNA replication represents the proliferative response to liver resection. WB demonstrated that cyclin D1 protein was induced by PHx, which was to a moderately greater extent in *Pdk4*$^{-/-}$ livers. Impressively, proliferating cell nuclear antigen (PCNA) protein was more prominently induced at day 2 and day 3 following operation in *Pdk4*$^{-/-}$ versus WT livers (Fig. 1C), suggesting that loss of PDK4 potentiated DNA replication. IHC staining of PCNA showed that *Pdk4*$^{-/-}$ livers had more positive cells than WT livers from day 1 to day 4 after PHx, with an early peaking time at day 2 (Fig. 1D). PCNA staining on sham-operated mice following operation revealed no noticeable DNA replication in both WT and *Pdk4*$^{-/-}$ livers, similar to the pre-operation livers (data not shown). BrdU incorporation similarly showed that *Pdk4* deficiency potentiated proliferative response in LR (Fig. 1E). Both hepatocytes and nonparenchymal cells expanded during LR, but *Pdk4*$^{-/-}$-PHx livers had more proliferating hepatocytes (HNF4α^{+} [hepatocyte nuclear factor 4 α^{+}] and PCNA^{+}) and endothelial cells (CD31^{+} and PCNA^{+}), as revealed by the double IF staining (Supporting Fig. S2A).

LR involves complex liver conditions, which is often accompanied by pre-existing liver damage such as inflammation, fibrosis, cirrhosis, and necrosis. To further address how loss of PDK4 increases DNA replication and hepatic cell proliferation in regenerative livers, we injected CCl_4 in WT and *Pdk4*$^{-/-}$ mice. *Pdk4*$^{-/-}$ livers displayed more BrdU incorporation, suggesting augmented cell proliferation (Supporting Fig. S2B,C). Taken together, these results suggest that PDK4 serves as a critical regulator of LR.

Pdk4 DEFICIENCY DECREASED GLYCEMIA AND IMPROVED HEPATIC INSULIN SENSITIVITY IN LR

As expected, both WT and *Pdk4*$^{-/-}$ mice developed hypoglycemia, which was to a greater extent in *Pdk4*$^{-/-}$ mice (Fig. 2A). The plasma insulin level slightly increased at day 1 after PHx and then began to surge from day 4 or day 5, with *Pdk4*$^{-/-}$ mice showing higher plasma insulin levels than WT at day 1, day 4, and day 5 (Fig. 2B).

Quantitative real-time PCR of *Gck* (glucokinase) suggested that hepatic glycolysis had a compensatory enhancement (about 3 fold) in *Pdk4*$^{-/-}$ versus WT mice before PHx (day 0), which was consistent with the curbing effect of PDK4 in glycolysis; *Gck* mRNA expression immediately dropped down at day 1 following PHx and gradually recovered at later days in WT but not completely in *Pdk4*$^{-/-}$ livers (Fig. 2C, left). The glycolytic *Pklr* (pyruvate kinase L/R) mRNA expression in *Pdk4*$^{-/-}$ livers showed a similar trend as *Gck* during the regeneration course but almost recovered to presurgery level at day 5. The hepatic expression of other glycolytic genes, *Pkm* (pyruvate kinase, muscle), *Pfkl* (phosphofructokinase, liver type) and *Mlxipl* (MLX-interacting protein-like), did not show striking differences between WT and *Pdk4*$^{-/-}$ mice during LR (Supporting Fig. S3). Interestingly, *Pdk4*$^{-/-}$ livers also showed an elevation of the expression of the gluconeogenic gene *G6pc* (glucose-6-phosphatase catalytic subunit) before operation (day 0), compared with WT livers. During LR, *G6pc* was expressed at higher levels in *Pdk4*$^{-/-}$ versus WT livers from day 2 to day 5, which suggested that the remnant liver retained a higher gluconeogenic capacity in *Pdk4*-deficient conditions (Fig. 2C, right). Other gluconeogenic genes, *Pck1* (phosphoenolpyruvate carboxykinase 1), *Pck2* (phosphoenolpyruvate carboxykinase 2) and kruppel-like factor 15, showed negligible expression changes between *Pdk4*$^{-/-}$ and WT livers in LR (Supporting Fig. S3).

We also examined the activation of hepatic insulin signaling. The phosphorylation of insulin receptor accumulated and peaked at day 4 in WT livers, which was shifted to an earlier time of day 3 in *Pdk4*$^{-/-}$ livers. The insulin receptor substrate (IRS) proteins,

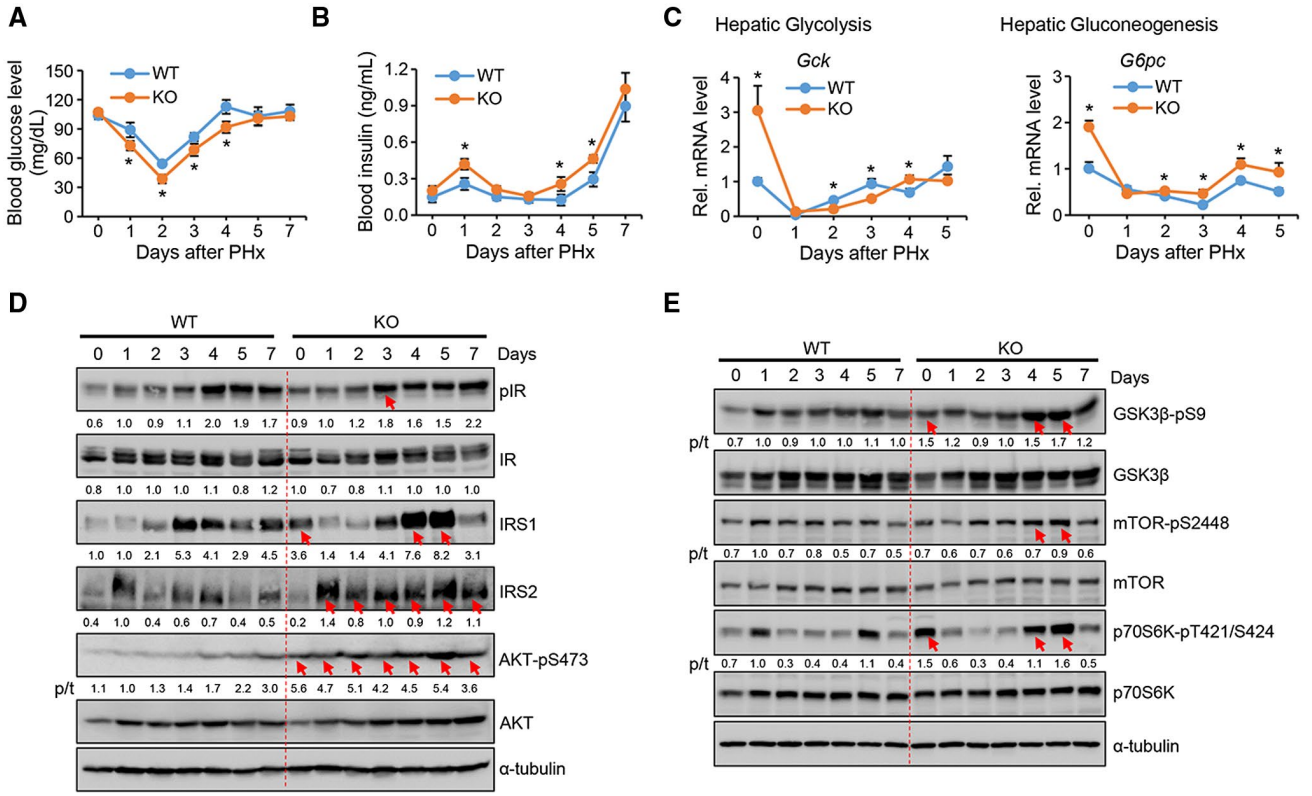

FIG. 2. *Pdk4*-deficiency-deteriorated glycemia and increased hepatic insulin sensitivity after PHx in mice. Blood glucose (A) and plasma insulin (B) levels before (day 0) and on different days after PHx (n = 8 mice/group). Data are shown as mean ± SEM; **P* < 0.05 versus WT. (C) Quantitative real-time PCR of hepatic gene expression. Data are shown as mean ± SEM of triplicate assays; n = 8 mice/group; **P* < 0.05 versus WT. (D,E) WB of hepatic expression of insulin signaling and AKT pathway mediators. Samples were pooled from eight individual mice for each group. Red arrows indicate protein induction in comparison with WT. Bands were quantified using ImageJ and denoted under each blot. Abbreviations: IR, insulin receptor; pIR, phosphorylation of insulin receptor; p/t, densitometric ratio of phosphorylated to total proteins.

IRS1 and IRS2, act as adaptor proteins, transmitting signals from the insulin and receptors to elicit cellular responses.[21] These proteins were markedly induced in *Pdk4*$^{-/-}$-PHx versus WT-PHx livers; notably, Akt activation (phosphorylation of S473) was remarkably higher in *Pdk4*$^{-/-}$ versus WT livers before and after PHx (Fig. 2D). Partially consistent with Akt activation, WB revealed that downstream of Akt, GSK-3 activation (GSK-3α/β S21/9 phosphorylation) and S6K activation (p70S6K T421/424 phosphorylation) were more pronounced in *Pdk4*$^{-/-}$ livers both before (day 0) and after PHx (day 4 and day 5), compared with WT livers; mTORC1 activation (mTOR S2448 phosphorylation) was also apparently enhanced at day 4 and day 5 (Fig. 2E).

Taken together, these results indicate that PDK4 regulates glycemia and insulin sensitivity during LR, and Akt activation may contribute to the enhanced hepatic proliferation.

Pdk4 DEFICIENCY FACILITATED TRAS BY PROMOTING FA UPTAKE

The TRAS in hepatocytes is a characteristic manifestation in regenerative livers before the major wave of parenchymal growth in the PHx model. H&E and ORO staining revealed that the content and duration of hepatic lipid accumulation were potentiated under *Pdk4*-deficient conditions; the TRAS rapidly disappeared at day 2 after surgery in WT-PHx livers but lasted for 3 days in *Pdk4*$^{-/-}$-PHx livers (Fig. 3A,B). Similar to histological observation, the *Pdk4*$^{-/-}$-PHx livers were enriched with more triglyceride (TG) and for a longer duration, compared with WT-PHx livers

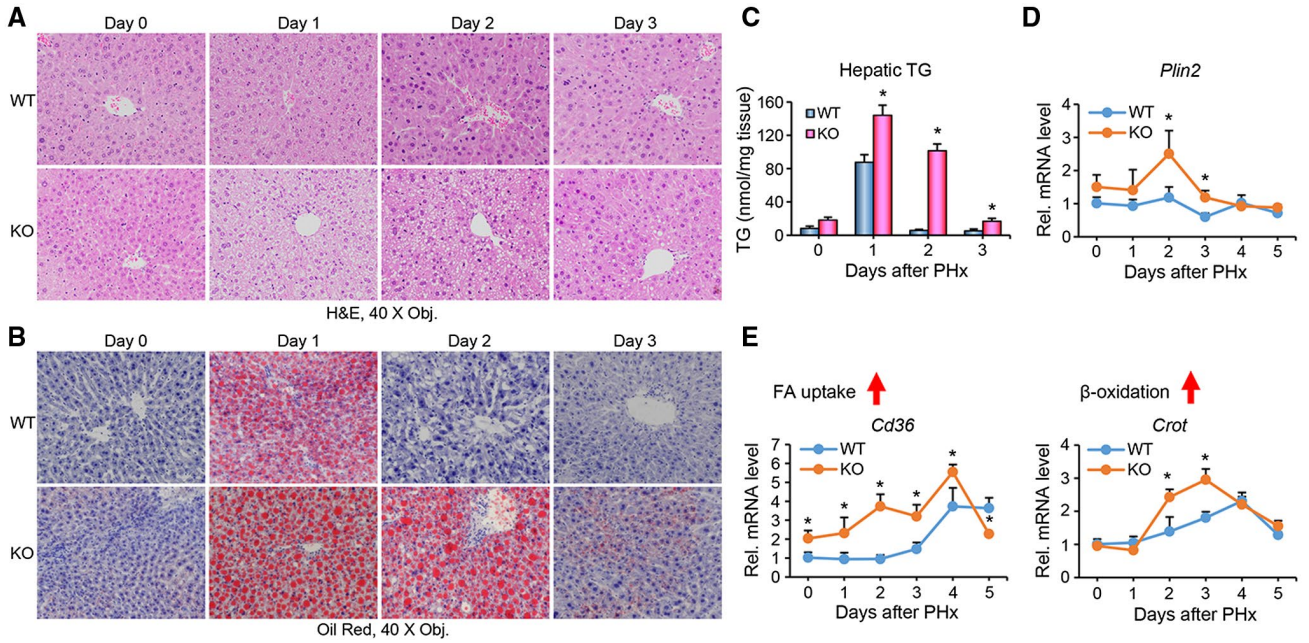

FIG. 3. *Pdk4* deficiency promoted TRAS to meet the high energy demand in regenerative livers. Representative H&E (A) and ORO staining (B) showing hepatic lipid accumulation in WT and *Pdk4*$^{-/-}$ mouse livers before (day 0) and on different days after PHx (n = 8). (C) Hepatic TG. Data are shown as mean ± SEM; n = 8; **P* < 0.05 versus WT. (D,E) Quantitative real-time PCR of hepatic expression of lipid droplet formation gene *Plin2*, FA uptake gene *Cd36*, and β-oxidation gene *Crot*. Samples were from eight individual mice for each group. Data are represented as mean ± SEM; **P* < 0.05 versus WT.

(Fig. 3C). The adipose differentiation-related gene perilipin 2, which promotes hepatocyte-adipocyte transdifferentiation and is required for lipid droplet formation, was strikingly induced in *Pdk4*$^{-/-}$ livers at day 2 following PHx but had negligible induction in WT livers (Fig. 3D). The serum TG levels were elevated at day 1, day 2, day 4, and day 5 in *Pdk4*$^{-/-}$-PHx versus WT-PHx livers (Supporting Fig. S4). The TRAS is essential to LR, and FA uptake is an important element for TG storage.[2,13,14,22] Hepatic FA metabolism is a tightly controlled process that is subjected to regulation at levels of uptake, oxidation, *de novo* synthesis, intracellular transport, and export to circulation. Quantitative real-time PCR showed that the hepatic expression of the FA translocase *Cd36* was enhanced in *Pdk4*$^{-/-}$-PHx versus WT-PHx livers (days 1 to 4); even before PHx, *Cd36* was about 2-fold higher in *Pdk4*$^{-/-}$ livers than WT livers (Fig. 3E, left). In primary hepatocytes, oleic acid or palmitic acid treatment increased intracellular TG levels and *Cd36* mRNA expression, which were augmented by *Pdk4* deficiency (Supporting Fig. S5A,B). Liver X receptor (LXR), pregnane X receptor (PXR), and peroxisome proliferator–activated receptor gamma (PPARγ) are transcriptional factors known to regulate *Cd36* expression.[23] The hepatic messenger RNA expression of LXR and PXR was not increased, but decreased, by *Pdk4* deficiency at day 1 and/or day 2, whereas PPARγ mRNA was only increased at day 4 (Supporting Fig. S5C). The genes *Fatp1-5* (fatty acid transport member 1-5) coding the FA transport proteins exhibited almost similar expression profiles between the two genotypes (Supporting Fig. S6A). These results suggest that the up-regulation of *Cd36* contributes to the enhanced TRAS in *Pdk4*$^{-/-}$ livers.

As glycolysis decreased (day 2 and day 3, Fig. 2C), the TRAS and FA oxidation might meet the high energy demand for the rapid cell division in LR. Indeed, β-oxidation of FA significantly increased in *Pdk4*$^{-/-}$-PHx versus WT-PHx livers at day 2 and day 3, as demonstrated by the quantitative real-time PCR of *Crot* (carnitine O-octanoyltransferase) expression, the protein of which catalyzes the transfer of fatty acyl groups between coenzyme A (CoA)

and carnitine, a crucial step in medium-length acyl chain breakdown (Fig 3E, right). Consistently, *Acadm* (acyl-CoA dehydrogenase, medium chain) expression also increased, which encodes acyl-CoA dehydrogenase for breaking down medium-chain FAs. The expression of other FA oxidation-related genes, including *Cpt1α* (carnitine palmitoyl transferase 1α), *Acat1* (acetyl-CoA acetyltransferase 1), *Pgc1α* (peroxisome proliferator–activated receptor gamma coactivator 1α), *Slc25a20* (solute carrier family 25 member 20), *Hsd17b4* (hydroxysteroid 17-beta dehydrogenase 4), *Crat* (carnitine O-acetyltransferase), *Acads* (acyl-CoA dehydrogenase, short chain), *Acadvl* (acyl-CoA dehydrogenase, very long chain), Hadha (hydroxyacyl-CoA dehydrogenase/3-ketoacyl-CoA thiolase/enoyl-CoA hydratase [trifunctional protein], alpha subunit), *Hadhb* (hydroxyacyl-CoA dehydrogenase/3-ketoacyl-CoA thiolase/enoyl-CoA hydratase [trifunctional protein], beta subunit), *Acox1* (acyl-CoA oxidase 1, palmitoyl), and *Acox2* (acyl-CoA oxidase 2, branched chain), displayed negligible or slight elevation in *Pdk4*$^{-/-}$-PHx livers at day 2 and/or day 3 (Supporting Fig. S6B). In addition, at day 2 and/or day 3 in *Pdk4*$^{-/-}$-PHx livers, the expression of lipolytic genes *Lipa* (lysosomal acid lipase A) and *Mgll* (monoglyceride lipase), but not *Lipc* (lipase, hepatic), *Lipe* (lipase, hormone sensitive) and *Lipg* (lipase, endothelial), increased (Supporting Fig. S6C); the expression of FA intracellular transportation genes *Fabp1* (fatty acid binding protein 1, liver), *Fabp2* (fatty acid binding protein 2, intestinal), *Fabp4* (fatty acid binding protein 4, adipocyte), and *Fabp5* (fatty acid binding protein 5, epidermal), but not *Fabp3* (fatty acid binding protein 3, muscle and heart), increased (Supporting Fig. S6D); the expression of FA esterification genes *Dgat1* (diacylglycerol O-acyltransferase 1), *Dgat2* (diacylglycerol O-acyltransferase 2), and *Gpat1* (glycerol-3-phosphate acyltransferase, mitochondrial) did not change (Supporting Fig. S6E); and the expression of lipogenic genes *Fasn* (fatty acid synthase), *Scd1* (stearoyl-CoA desaturase 1), *Acaca* (acetyl-CoA carboxylase α), *Insig1* (insulin-induced gene 1), but not *Pten* (phosphatase and tensin homolog), decreased (Supporting Fig. S6F), compared with WT-PHx livers. In contrast to the higher levels at day 0, *Pdk4*$^{-/-}$-PHx livers decreased FASN and SCD1 protein expression at day 3 and then increased their expression at day 4 and day 5. As their transcriptional activator, both the active form (cleaved) and precursor (full-length) of SREBP1 (sterol regulatory element-binding protein 1) were markedly induced at day 4 and day 5 (Supporting Fig. S6G). Importantly, hepatic adenosine triphosphate (ATP) levels were higher in *Pdk4*$^{-/-}$-PHx than WT-PHx livers at day 2 and day 3 (Supporting Fig. S7A). These results demonstrate that *Pdk4* deficiency induces intrahepatic metabolic reprogramming of lipid to provide a higher energetic basis for efficient LR.

CD36 PROMOTED LR IN PHx MICE

To define the role of CD36 up-regulation in PHx-induced LR, we overexpressed CD36 in mice and found that CD36 overexpression promotes the recovery of liver/body weight ratio in WT mice after PHx at day 3 (Fig. 4A), along with an increased ATP production (Fig. 4B), suggesting that CD36 facilitated LR through increasing intrahepatic energy. Intrahepatic TG levels were also potentiated by CD36 overexpression (Supporting Fig. S7B), indicating that CD36 contributed to the TRAS.

To understand how CD36 expression is regulated in the liver, we predicted the binding of transcription factors (using the online tool PROMO 3.0) and cloned mouse *Cd36* gene promoter (−1133 to +141 bp) into a luciferase reporter vector (Supporting Fig. S7C,D). In addition to the known C/EBP family members (C/EBPα/β/γ) and nuclear receptors that regulate CD36 expression,[23,24] multiple FOX proteins and HNFs were suggested to potentially regulate CD36 expression. We found that FOXO1 repressed, but the pioneer factor FoxA1 (also known as HNF3A [hepatocyte nuclear factor 3α]) activated, mouse *Cd36* gene promoter, whereas HNF4α did not affect it (Fig. 4C).

In vitro studies demonstrated that FOXO1 overexpression in both Huh7 and Hep3B cells inhibited CD36 mRNA expression (Fig. 4D); however, we could not detect CD36 protein expression in both cell lines using WB, which was consistent with a previous finding that CD36 protein expression is low/undetectable but inducible in the liver (data not shown).[15] However, Chromatin immunoprecipitation sequencing (ChIP-seq) data showed that FOXO1 indeed interacted with *Cd36* gene promoter (Fig. 4E), and the first intron region was also found to have extensive FOXO1 association. Taken together,

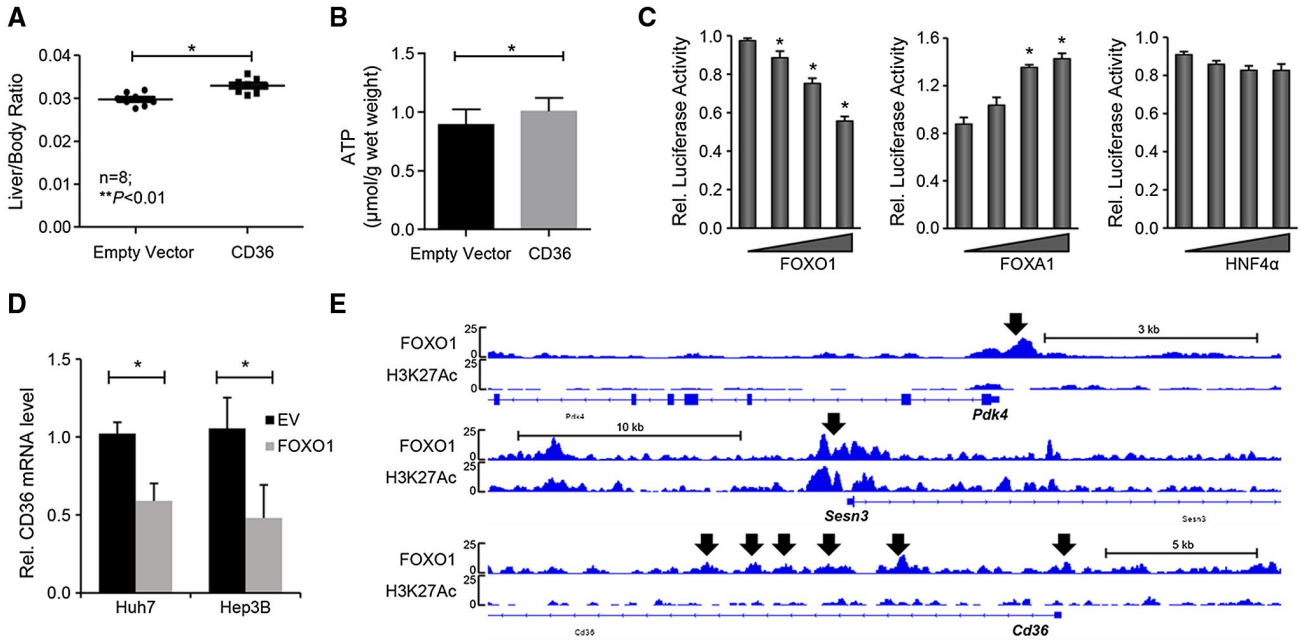

FIG. 4. CD36 facilitated liver mass recovery after PHx, and its expression was repressed by FOXO1. (A) Liver to body weight ratio. Empty vector or CD36 plasmid were intravenously injected into WT mice using *in vivo* transfection reagent. PHx was performed 24 hours after transfection, and regenerative livers were collected on day 3 following PHx. (B) Intrahepatic ATP levels. Data are represented as mean ± SEM; n = 8; **P* < 0.05 versus empty vector. (C) Luciferase reporter assays. The *Cd36* promoter reporter was transfected in 293T cells along with increasing amounts of the indicated plasmids (0 ng, 100 ng, 200 ng, and 400 ng). Data are shown as mean ± SEM of triplicate assays; **P* < 0.05. (D) Quantitative real-time PCR of *Cd36* expression. An empty vector or a FOXO1-expressing plasmid was transfected into hepatic Huh7 or Hep3B cells. CD36 mRNA expression was determined 48 hours after transfection. Data are shown as mean ± SEM of triplicate assays; **P* < 0.05. (E) Integrative Genomics Viewer display of ChIP-seq data for FOXO1 (GSM3831422) and H3K27Ac occupancy (GSM3831423) on the indicated gene promoters. Abbreviation: EV, empty vector.

these results suggest that FOXO1 is a suppressive transcription factor of *Cd36* expression.

AMPK ACTED UPSTREAM OF FOXO1 TO INHIBIT CD36 EXPRESSION

How the expression and function of FOXO1 are regulated in the liver is still not well understood.[25] Although AMPK directly phosphorylates FOX proteins,[26] it remains unclear how AMPK functionally regulates FOXO1. The well-defined mechanism of AMPK activation is the phosphorylation of T172 on its α-subunit.[27] We titrated the working concentration of AICAR (5-aminoimidazole-4-carboxamide ribonucleotide), an AMPK agonist, in Huh7 cells (Fig. 5A). IF staining revealed that FOXO1 displayed a more intensive enrichment in the nucleus following AMPK activation by AICAR, in contrast to control cells, in which large amounts of FOXO1 was distributed in the cytoplasm (Fig. 5B, left and right top). The AMPK-promoted nuclear translocation of endogenous FOXO1 has also been revealed in rat cardiomyocytes.[28] In primary hepatocytes, AICAR also facilitated FOXO1 nuclear translocation (Fig. 5B, right bottom). In consonance with a previous finding that AICAR treatment increases endogenous FoxO1 protein levels in Chang cells,[29] the same reinforcement was detected in hepatic cells (Fig. 5C). Meanwhile, CD36 mRNA expression was decreased by AICAR (Fig. 5D), demonstrating that AMPK activation inhibited *Cd36* expression.

In regenerative livers, FOXO1 protein levels did not show striking differences between WT and *Pdk4*$^{-/-}$ mice following PHx, except a less prominent induction at day 1 and day 2 in *Pdk4*$^{-/-}$ mice, compared with WT mice (Fig. 5E). Immunoblot revealed that FOXO1 was less abundantly distributed in the

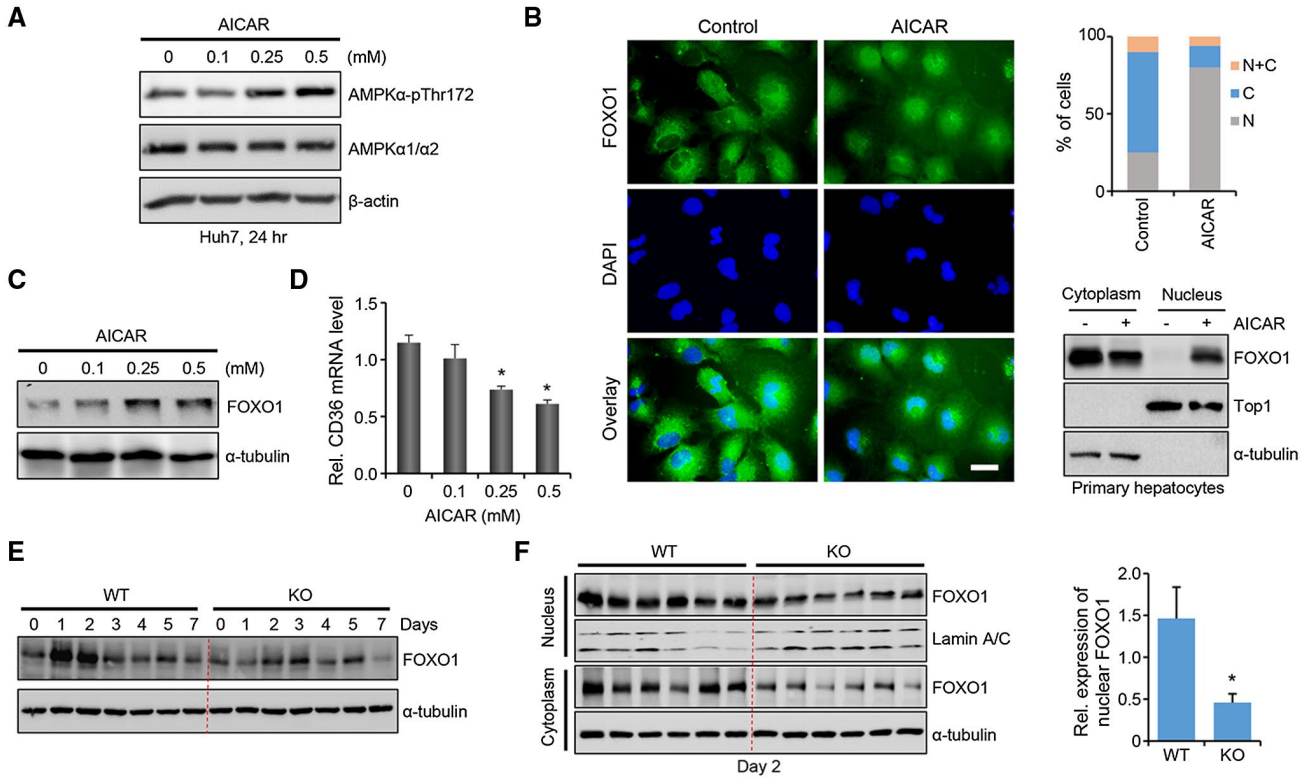

FIG. 5. AMPK promoted FOXO1 nuclear translocation to inhibit CD36 expression. (A) WB for titrating the working concentration of AICAR in Huh7 cells. (B) Left: IF staining of nuclear and cytoplasmic localization of FOXO1 in Huh7 cells treated with AICAR (0.5 mM, 24 hours). Scale bar, 10 μm. Right top: Quantification of a representative IF experiment. Right bottom: WB of FOXO1 nuclear and cytoplasmic localization in primary hepatocytes treated as in Huh7 cells. WB of FOXO1 (C) and quantitative real-time PCR of *Cd36* mRNA (D) expression in Huh7 cells treated with AICAR. (E) WB of FOXO1 expression. Samples were pooled from eight individual mice for each group. (F) WB of FOXO1 expression in the nuclear and cytoplasmic extracts from individual WT and *Pdk4*$^{-/-}$ regenerative livers (n = 6). The bar graph shows the densitometric quantification. $^{*}P < 0.05$ versus WT. Abbreviations: C, cytoplasmic; DAPI, 4′,6-diamidino-2-phenylindole; N, nuclear.

nuclear extract of *Pdk4*$^{-/-}$ livers, compared with WT livers (Fig. 5F), which corresponded to the enhanced *Cd36* expression (Fig. 3E). These results demonstrate that *Cd36* expression depends on FOXO1 nuclear exclusion, and the latter can be mediated by AMPK during LR.

PDK4 REGULATED AMPK ACTIVITY IN LR BY CONTROLLING THE INTRACELLULAR LEVEL OF ADENOSINE-5-MONOPHOSPHATE

AMPK is a heterotrimeric protein complex comprising of α, β, and γ subunits, senses the changes of intracellular adenosine-5-monophosphate (AMP)/ATP or adenosine diphosphate (ADP)/ATP ratio, and is allosterically regulated by ATP, ADP, and AMP binding on its γ-subunit.[27] AMPK activity was remarkably induced after PHx in WT mice; however, *Pdk4* deficiency prevented the induction of AMPK activation (Fig. 6A). When overexpressing PDK4 *in vitro*, PDK4 dose-dependently activated AMPK in both hepatic Huh7 and Hepa1 cells (Fig. 6B). In an *in vitro* analysis to dissect PDK4's role in regulating intracellular metabolites, among the 34 metabolites that were significantly changed, AMP was one of the most strongly decreased metabolites after PDK4 inhibition by shRNA in Huh7 cells (Fig. 6C). Further, AMPK's incompetence to respond to PHx in *Pdk4*-deficient livers corresponded to the finding that AMP levels declined in *Pdk4*$^{-/-}$-PHx versus WT-PHx livers (Fig. 6D). In PDK4-overexpression primary hepatocytes, intracellular AMP significantly increased

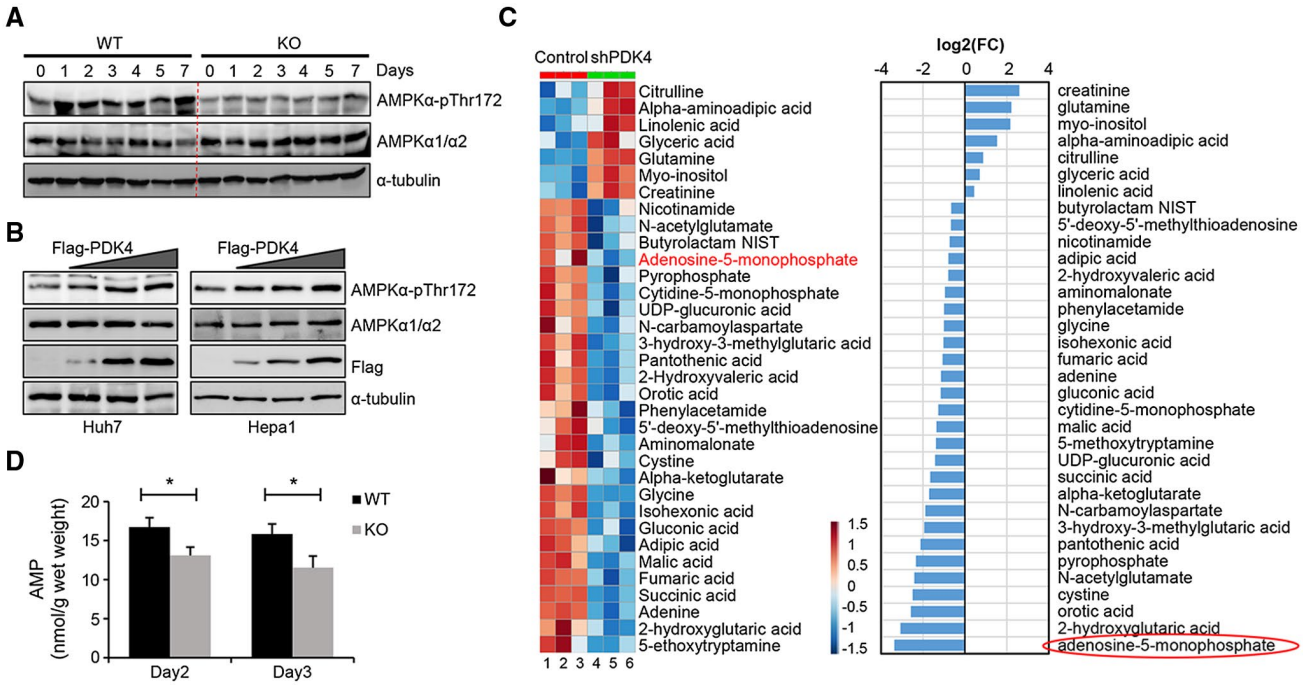

FIG. 6. *Pdk4-d*eficiency impaired hepatic AMPK activation by reducing intracellular AMP. (A) WB of the indicated proteins in regenerative livers from WT and KO mice before (day 0) and on different days after PHx. Samples were pooled from eight individual mice for each group. (B) WB of the indicated proteins in Huh7 and Hepa1 cells transfected with increasing amounts of FLAG-PDK4. (C) Metabolomics analysis of primary metabolites. The heatmap shows 34 metabolites that were significantly changed among 179 metabolites detected by mass spectrometry in control and shPDK4 Huh7 cells. The experiments were performed in triplicate from independent samples. The histogram shows the log2 value of fold change of the 34 metabolites in shPDK4 Huh7 cells versus control. (D) Intrahepatic AMP levels in PHx livers. Data are represented as mean ± SEM; n = 8; **P* < 0.05 versus WT. Abbreviation: FC, fold change.

(Supporting Fig. S7E). Taken together, these results demonstrate that PDK4 controls intracellular AMP levels to regulate AMPK activity. Therefore, we propose that the PDK4/AMPK/FOXO1/CD36 axis is an important signaling pathway in regulating PHx-induced LR.

Discussion

LR is an energy-demanding process and depends on intracellular metabolic reprogramming. In this study, *Pdk4* deficiency increases insulin-signaling sensitivity and AKT activation to drive the proliferative response in regenerative livers. On the other hand, *Pdk4*-deficient livers initiate an AMPK/FOXO1/CD36 cascade to enhance FA oxidation and intracellular ATP production to meet the high energy demand, which is triggered by intracellular AMP reduction (Fig. 7). These findings point to a critical role of PDK4 in the PHx-induced LR, suggesting that PDK4 might be a target to manipulate LR and combat liver failure.

We find that PDK4 governs insulin signaling–mediated hepatocytes proliferation and liver mass restoration, which is consistent with previous findings that PDK4 expression negatively correlates with insulin sensitivity,[30,31] that *Pdk4* deficiency leads to accelerated hepatocyte proliferation,[7] and that the insulin signaling pathway plays an important role in LR.[11,32,33] It is unknown as to how PDK4 enhances insulin secretion during PHx. IRS1 and IRS2 are major insulin receptor adaptors and serve complementarily.[34] How PDK4 regulates the expression of IRS1 and IRS2 is yet to be determined. Interestingly, PDK4 mRNA is rhythmically up-regulated during LR in mice (Fig. 1A), implying that PDK4 might be able to restrict the overreaction of LR. How PDK4 regulates LR under an overexpression condition needs further investigation. In contrast to the unchanged mRNA

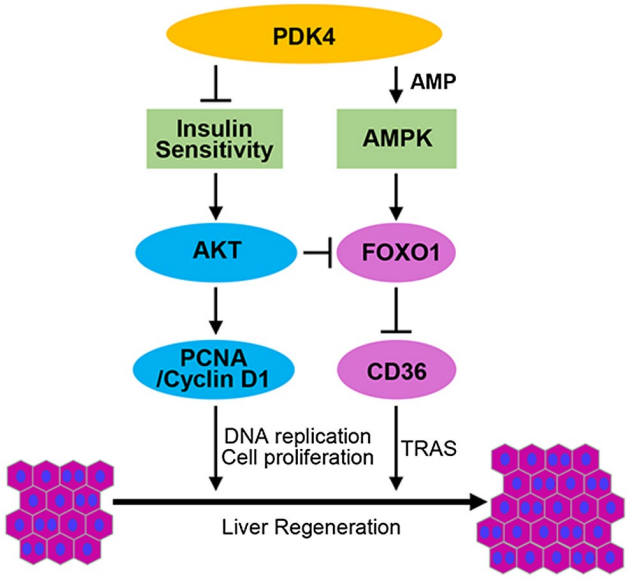

FIG. 7. Schematic of the role of PDK4 in PHx-induced LR. PDK4 coordinates hepatic proliferation and metabolism and is critical to resection-induced LR. In regenerative mouse livers, PDK4 withholds hepatic insulin signaling sensitivity and the downstream AKT-mediated proliferative response. PDK4 also restricts the pro-regenerative TRAS through an AMPK/FOXO1/CD36 lipid metabolism axis.

level, hepatic PDK4 protein level is strikingly induced at day 1 following PHx (Fig. 1A), which suggests that the translation efficiency and/or protein stability of PDK4 are presumably increased in response to PHx.

Proliferation and hypertrophy almost equally contribute to regeneration after PHx.[35] It is unknown whether PDK4 affects hepatocyte hypertrophy. *Pdk4* deficiency decreases glycemia after PHx (Fig. 2A), which is consistent with previous findings in diet-induced obesity mice.[31] *Pdk4* deficiency also increases the mortality of PHx; however, it remains to be determined as to whether it is due to severe hypoglycemia or other systemic changes. If the survival can be rescued through monitoring and rectifying glycemia, it would be straightforward to target PDK4 to facilitate resection-induced LR.

The main molecules involved in the proliferative phase of resection-induced LR are varieties of mitogens.[33] Determining how PDK4 regulates levels of these factors in the liver and serum will allow us to further understand how loss of *Pdk4* accelerates LR. In the priming phase of PHx, the Kupffer cell–derived tumor necrosis factor (TNF) primes hepatocytes to re-enter into the cell cycle.[11,33] *Pdk4* deficiency facilitates TNF expression.[17] It will be interesting to determine the role of PDK4 in the priming phase after PHx as well as the hepatic activation of other signaling pathways during LR under *Pdk4*-deficient conditions.

Mild steatosis and TRAS are beneficial to LR[13,14,22,36]; however, severe and chronic lipid overload may cause successive inflammation and impair LR.[37] *De novo* hepatic lipogenesis is not required for the development of TRAS, and β-oxidation of FAs serves as the predominant source of new ATP production in the regenerative liver.[22,38,39] Indeed, β-oxidation rather than lipogenesis increases in $Pdk4^{-/-}$-PHx livers (Fig. 3E and Supporting Fig. S6B,F). A recent study showed that PTEN coordinates liver growth with its energy demands and emphasizes the requisition of lipids for LR.[22] We identify PDK4 as a key molecule coordinating hepatic lipid homeostasis with liver growth, but the role of PDK4 in the metabolism of extrahepatic tissues during LR needs to be identified. Moreover, we cannot exclude that more lipid accumulation has a certain contribution to the higher liver/body weight ratio in $Pdk4^{-/-}$-PHx mice, compared with WT-PHx mice. In this study, we use whole-body KO mice and cannot exclude the capacity of other tissues in handling lipids also affected by PDK4 deficiency during LR. The increased serum TG levels in KO mice could be caused by lipid redistribution between organs (Supporting Fig. S4). For day 4 and day 5, it could also be caused by increased intrahepatic lipogenesis. (Supporting Fig. S6F,G shows how the lipogenic genes *Fsn*, *Scd1*, and *Acaca* increased in $Pdk4^{-/-}$ livers.) It is also possible that the increased serum TG is enhanced by the exportation from the robustly loaded lipid hepatocytes; thus, the livers display the decreased ORO staining in hepatocytes.

In this study, we experimentally show that PDK4 regulates intracellular AMP and thereby AMPK activation. AMPK induces phosphorylation of p53, and this phosphorylation is required to initiate AMPK-dependent cell-cycle arrest,[40] suggesting a negative role of AMPK activation in LR. Our finding aligns with this point of view.

It is possible that PDK4 directly regulates FOXO1 and that AMPK can directly inhibit the cell membrane location of CD36 to decrease FA uptake. It is known that AKT directly phosphorylates FOXO1 and promotes its shuttling to cytoplasm for degradation;

we suppose that both AKT activation and AMPK inactivation contribute to FOXO1 nuclear exclusion. Other FOX proteins and transcription factors downstream of AMPK might also regulate the expression of CD36. It is noted that PDK4 is a direct target of FOXO1 (Fig. 4E) and that there are feedbacks between CD36 and AMPK,[41] and among CD36, PDK4, and FOXO1.[42]

The function of PDK4 in LR needs to be further elucidated under pathological settings, such as in hepatotoxic models, given the irreconciliation that loss of PDK4 sensitizes mouse livers to apoptotic injury and that PDK4 activation may be essential for rapid LR in acetaminophen-induced liver injury.[4,17] Whether CD36 directly influences cell proliferation needs to be determined. FOXO1 suppresses endothelial CD36 transcription through nuclear association with histone deacetylase 7.[43] We find that FOXO1 associates with *Cd36* promoter and inhibits CD36 expression. The deletion of FOXO1 rescues loss-of-AKT-impaired LR,[44] which is consistent with our finding that FOXO1 has a suppressive role in LR by inhibiting CD36 expression. The transcription repression function of FOXO1 on CD36 might depend on co-repressors.[45,46] In summary, the PDK4/AMPK/FOXO1/CD36 axis is critical to resection-induced LR, and pharmacological inhibition of PDK4 might improve LR efficiency.

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Supporting Information

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