Hepatic Protein Phosphatase 1 Regulatory Subunit 3B (Ppp1r3b) Promotes Hepatic Glycogen Synthesis And Thereby Regulates Fasting Energy Homeostasis

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Running title: Hepatic Ppp1r3b regulates fasting energy homeostasis

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ABSTRACT

Maintenance of whole-body glucose homeostasis is critical to glycemic function. Genetic variants mapping to chromosome 8p23.1 in genome-wide association studies have been linked to glycemic traits in humans. The gene of known function closest to the mapped region, PPP1R3B (protein phosphatase 1 regulatory subunit 3B), encodes a protein (G1) that regulates glycogen metabolism in the liver. We therefore sought to test the hypothesis that hepatic PPP1R3B is associated with glycemic traits. We generated mice with either liver-specific deletion (Ppp1r3bΔhep) or liver-specific overexpression of Ppp1r3b. The Ppp1r3b deletion significantly reduced glycogen synthase protein abundance, and the remaining protein was predominantly phosphorylated and inactive. As a consequence, glucose incorporation into hepatic glycogen was significantly impaired, total hepatic glycogen content was substantially decreased, and mice lacking hepatic Ppp1r3b had lower fasting plasma glucose than controls. The concomitant loss of liver glycogen impaired whole-body glucose homeostasis and increased hepatic expression of glycolytic enzymes in Ppp1r3bΔhep mice relative to controls in the postprandial state. Eight hours of fasting significantly increased the expression of two critical gluconeogenic enzymes, phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6PC), above the levels in control livers. Conversely, the liver-specific overexpression of Ppp1r3b enhanced hepatic glycogen storage above that of controls and, as a result, delayed the onset of fasting-induced hypoglycemia. Moreover, mice overexpressing hepatic Ppp1r3b upon long-term fasting (12–36 hrs) were protected from blood ketone-body accumulation unlike control and Ppp1r3bΔhep mice. These findings indicate a major role for Ppp1r3b in regulating hepatic glycogen stores and whole-body glucose/energy homeostasis.

INTRODUCTION

The Meta-Analysis of Glucose and Insulin related traits Consortium (MAGIC) study analyzed datasets from multiple cohorts that contained both genome-wide genotype data as well as quantitative clinical measurements of human plasma glycemic traits. Among the twelve loci identified in this study, one signal mapping to chromosome 8p23.1 was associated both with fasting plasma glucose and fasting plasma insulin levels. The lead variant, rs4841132, most strongly associated with fasting plasma glucose mapped to an intergenic region 1

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kilobase (kb) from the 5' end of \textit{LOC157273} gene, a long non-coding RNA transcript of unknown function, and 174kb from the protein-coding \textit{PPP1R3B} gene. Despite the 174kb separation from the \textit{PPP1R3B} gene, rs9987289 has been identified as an expression quantitative trait locus (eQTL) for \textit{PPP1R3B}: the minor allele is associated with higher levels of \textit{PPP1R3B} mRNA in the liver (and is also associated with higher fasting glucose and insulin levels). This observation prompted us to investigate the possibility that the genetic association with glycemic traits identified by these non-coding variants was due to their effects on the expression of \textit{PPP1R3B}.

\textbf{PPP1R3B} (Protein Phosphatase 1 Regulatory Subunit 3B; also historically known as \textit{GL}) is a regulator of liver glycogen metabolism. In the liver, sequestration of dietary glucose into glycogen is an important and tightly regulated component of whole-body glucose homeostasis, promoting maintenance of systemic homeostasis in blood glucose levels through glycogen storage during the postprandial period and its breakdown during fasting (1-4). Dysregulation of this pathway is associated with metabolic disorders including glycogen storage diseases and diabetes. Glycogen synthase (GS) and glycogen phosphorylase (GP) promote glycogen synthesis and glycogen catabolism respectively, and their activities are tightly controlled by endocrine signaling pathways that are in turn coupled to nutritional status. Glycogen-targeting subunits recruit the phosphatases and kinases that post-translationally regulate the activation of GS and inactivation of GP. Several proteins have been characterized that target protein phosphatase 1 (PP1) to glycogen (5,6). In liver, two major subunits, PPP1R3B (\textit{GL}) and PPP1R3C (PTG) are expressed at approximately equivalent levels, and together facilitate the storage of hepatic glycogen.

Previous studies of \textit{GL} and PTG in cell culture have revealed that overexpression increases glycogen content (7-9), likely due to a redistribution of PP1 and GS to glycogen particles, and a corresponding increase in GS activity through PP1-mediated dephosphorylation of GS and GP resulting in augmentation of glycogen synthesis, and inhibition of glycogenolysis, respectively. \textit{Ppp1r3c} knockout mice had reduced hepatic glycogen and were glucose intolerant (10); conversely, \textit{Ppp1r3c} transgenic mice had increased hepatic glycogen and improved glucose and insulin sensitivity (11). Surprisingly, while the glycogen phenotypes of the \textit{Ppp1r3c} knockout and transgenic overexpression mice were opposite in direction, both mouse models had reduced hepatic TG content compared to controls (11,12). In the case of the transgenic \textit{Ppp1r3c} mouse, hepatic fat remained lower than controls despite 12-24 weeks of high fat diet (45% Kcal fat) feeding. However, mice genetically modified to lack or overexpress \textit{Ppp1r3b} have not been reported to date.

\textit{GL} is unique among the glycogen targeting subunits, in that in addition to promoting the activation of glycogen synthase by PP1 dephosphorylation, \textit{GL} also targets PP1 to glycogen phosphorylase (GP), where the dephosphorylation inactivates GP. Thus, under conditions of abundant cellular glucose (as glucose-6-phosphate), the PP1-\textit{GL} complex simultaneously activates the glycogen synthetic pathway and inactivates glycogen catabolism. Mice in which this GP regulatory binding site has been mutated had improved hepatic glycogen storage capacity and systemic glucose disposal (13).

Inspired by the human genetics suggesting that variation in hepatic expression of \textit{PPP1R3B} is an important determinant of glycemic traits, we generated mice with liver-specific deletion of \textit{Ppp1r3b} (\textit{Ppp1r3b}\textsuperscript{\textit{hep}}) or with hepatic overexpression of \textit{Ppp1r3b} using adeno-associated virus (AAV). Floxed \textit{Ppp1r3b} was inactivated in liver either by a transgenic albumin promoter-driven Cre recombinase, or by injecting adeno-associated virus expressing Cre (AAV-Cre). Despite an almost complete lack of hepatic glycogen in the fed state as well as upon fasting, \textit{Ppp1r3b}\textsuperscript{\textit{hep}} mice are viable but are prone to hypoglycemia upon fasting and utilize non-carbohydrate precursors to maintain fasted blood glucose levels. Conversely, liver-specific overexpression of \textit{Ppp1r3b} enhanced hepatic glycogen storage, and as a consequence, AAV-\textit{Ppp1r3b} mice had a delayed decrease in plasma glucose and exhibited significantly delayed accumulation of blood ketone bodies in response to long term fasting. These observations are consistent with the fact that natural human genetic
variants associated with higher liver expression of PPP1R3B have higher fasting plasma glucose.

RESULTS

Hepatic Ppp1r3b deficiency leads to depletion of hepatic glycogen stores with significant reduction in incorporation of glucose into hepatic glycogen. To investigate the role of Ppp1r3b in the liver, we generated mice lacking this gene exclusively in the liver (see Experimental Procedures, Figure S1A). Liver-specific Ppp1r3b knockout mice (Ppp1r3b\(^{-}\)) were viable, born at the expected frequency for Mendelian inheritance, and showed normal fertility. In all subsequent experiments, mice without the Cre transgene (Ppp1r3b\(^{\text{flx/flx}}\)) were used as controls. In Ppp1r3b\(^{-}\) mice, transcript levels were negligible in the liver, with 97% deletion efficiency observed, and there was no compensatory increase in gene expression of other glycogen targeting subunits—G\(_{M}\) (Ppp1r3a) and PTG (Ppp1r3c) (Fig. 1A). Ppp1r3b\(^{-}\) mice showed normal growth rates on chow diet, with no differences observed in body weights between genotypes up to 50 weeks of age (Fig. S1B).

Hepatic glycogen content was significantly reduced in ad libitum fed state in Ppp1r3b\(^{-}\) mice, which was rapidly depleted upon short term four-hour fasting as measured biochemically (Fig. 1B). Periodic-acid Schiff (PAS) staining of liver sections showed positive staining for glycogen in control hepatocytes as indicated by the purple color under these two conditions (Fig. 1C). The weight of the liver of Ppp1r3b\(^{-}\) mice was also modestly decreased compared to controls (Fig. S1C), likely due to lack of glycogen stores, which with their associated water content account for 5% of liver mass (3). We used AAV-Cre in Ppp1r3b\(^{\text{flx/flx}}\) mice as a second approach to generate liver specific knockout of Ppp1r3b, and found comparable effects on hepatic glycogen and plasma glucose (Fig. S2) to those in the Alb-Cre mice. Alb-Cre mice were used for the studies presented here, unless otherwise specified. Glycogen content in skeletal muscle presented no substantial differences among genotypes under ad libitum fed and four-hour fasted conditions (Fig. S1D). To investigate the glycogen synthesis rate in these mice, we performed 2-deoxy-D-\(^3\)H]glucose incorporation into glycogen in glucose-responsive tissues: liver, skeletal muscle (gastrocnemius) and kidney, as a surrogate measurement of glycogen synthase (GS) activity. Radioactivity in the glycogen pellet from tissue lysates were measured at 30 and 60 minutes and quantified as percent of glucose specific activity of total radioactivity in plasma and of total protein content. In this experiment, Ppp1r3b\(^{-}\) mice exhibited significantly reduced glycogen synthase activity in the liver, while its activity was not different in skeletal muscle or kidney (Fig. 1D). Thus, loss of hepatic Ppp1r3b results in a smaller liver with reduced GS activity and glycogen content in ad libitum fed and four-hour fasted mice.

Deletion of Ppp1r3b in the liver causes significant reduction in total glycogen synthase protein and activity state. Ppp1r3b\(^{-}\) mice have significant reduction in glycogen synthase (GS) but not glycogen phosphorylase (GP) at the protein level, but no differences at the mRNA levels in both the liver and primary hepatocytes (Fig. 2). The ratio of GS phosphorylated at Serine position 641 to total GS, which determines the activity status of GS, is increased, suggesting that GS is phosphorylated and largely inactivated in Ppp1r3b\(^{-}\) mice (Fig. 2C). The near complete loss of dephosphorylated [activated] GS protein likely parallels the depletion of glycogen granules since overnight fasted control mice also reached a similar depletion of glycogen, and protein levels of active/dephosphorylated GS were similar in control and Ppp1r3b\(^{-}\) livers after an overnight fast (Fig. 2D). The molecular mechanism by which Ppp1r3b\(^{-}\) deletion leads to hyper-phosphorylation of GS, and marked reduction in total GS protein content even during the ad libitum fed state remains to be further explored. Thus, loss of hepatic Ppp1r3b results in a reduced glycogen synthase protein and activity state in the liver, consistent with the observation of reduced glucose incorporation of into liver glycogen and overall glycogen depletion.

Hepatic loss of Ppp1r3b promotes dysregulated glycogen metabolism and is associated with impaired glucose homeostasis and glucose mobilization. Postprandial glucose levels in Ppp1r3b\(^{-}\) mice are modestly lower and Ppp1r3b\(^{-}\) mice become mildly hypoglycemic after a four-hour fast (Fig. 3A). Within four to eight hours of fasting, blood glucose in
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$Ppp1r3b^{Δhep}$ mice dropped to a significantly lower value than control mice, which reached similarly low blood glucose only after longer term fasting of up-to 24 hrs. (Fig. 3B). We hypothesized that alternative metabolites compensate for the lack of hepatic glycogen stores to substitute for the maintenance of blood glucose as an energy source to peripheral tissues and the central nervous system. In the post-prandial state, plasma lactate levels were increased in $Ppp1r3b^{Δhep}$ mice compared to controls (Fig. 3C). Upon prolonged fasting (12-hour), total plasma ketone bodies were significantly increased, and were progressively elevated at 24-hour and 36-hour fasting periods in $Ppp1r3b^{Δhep}$ mice, compared to controls (Fig. 3D). Moreover, after an overnight fast, gluconeogenesis was increased in $Ppp1r3b^{Δhep}$ mice, as evidenced by a challenge with intraperitoneal injection of sodium pyruvate in which these mice were more efficiently able to synthesize glucose from an exogenous pyruvate load, compared to controls (Fig. 3E). Taken together, these data suggest that while the $Ppp1r3b^{Δhep}$ mice are more prone to enter into a quasi-fasted state following short-term food deprivation, they are nonetheless able to maintain stable long-term fasting plasma glucose levels by compensatory upregulation of the gluconeogenic pathway from alternative precursors.

Hepatic deletion of Ppp1r3b causes upregulation of glycolytic genes in the postprandial state and upregulation of gluconeogenic genes within 8-hours of fasting. In the $Ppp1r3b$-deficient livers, under ad lib fed conditions, genes that encode enzymes critical in the glycolytic pathway upregulated compared to control livers (Fig. 4A); these included aldolase (Aldob), triose phosphate isomerase (Tpi), glyceraldehyde phosphate dehydrogenase (Gapdh), phosphoglycerate kinase (Pgk1), phosphoglycerate mutase (Pgaml), enolase (Enol), and L-pyruvate kinase (Pklr). Upon an 8-hour fasting period, there was an upregulation of gluconeogenic genes ($Pepck$ and $G6pc$) in $Ppp1r3b^{Δhep}$ mice (Fig. 4B), suggesting that de-novo synthesis of glucose is initiated much earlier on in these mice compared with controls. Collectively, these data suggest that with $Ppp1r3b$ deficiency there is a significant perturbation in nutrient balance which favors shunting of glucose into the glycolysis pathway in the fed state, and an early upregulation of the gluconeogenic pathway in response to fasting.

Overexpression of Ppp1r3b in the liver enhances hepatic glycogen storage. We generated a reciprocal mouse model to express $Ppp1r3b$ at higher than wild-type levels, combining the tropism of AAV serotype 8 and the liver-specificity of the TBG/Serpina7 promoter, which has been previously shown to promote efficient, specific, and persistent gene expression in the liver (14), to overexpress $Ppp1r3b$ in hepatocytes in vivo. $Ppp1r3b$ was approximately 4-fold higher compared to control mice receiving a null AAV vector (Fig. 5A). There were no compensatory changes in the mRNA expression of other glycogen targeting subunits ($Ppp1r3a$ and $Ppp1r3c$), as well as rate limiting enzymes in glycogen metabolism: glycogen synthase ($Gys2$), or glycogen phosphorylase ($Pgfl$), in mice overexpressing $Ppp1r3b$. Following a short term four-hour fasting period, mice overexpressing $Ppp1r3b$ had ~4-fold higher levels of hepatic glycogen content than controls, and this was maintained at eight hours of fasting (Fig. 5B, 5C). Levels of total GS protein were also higher in livers from mice overexpressing $Ppp1r3b$, and the ratio of phospho-GS to total GS protein was decreased, consistent with increased activation status at four and eight hour fasting period (Fig. 5D). Additionally, total GP or PP1 protein content was not affected (Fig. 5D). Blood glucose levels were not different between the two groups at ad libitum and 12-hour fasting conditions, indicating that despite the larger reservoir of available glycogen, glucose levels are maintained, without exceeding the normal physiological range (Fig. 5E). Between 24 and 48 hours of fasting, AAV-$Ppp1r3b$ mice maintained persistently higher (by ~15mg/dl) plasma glucose levels than the control group (Fig. 5E). Upon long term fasting, AAV-$Ppp1r3b$ were protected from the onset of ketogenesis (Fig. 5F), suggesting that the enhanced glycogen storage buffered the onset of the fasting-induced metabolic shift to β-oxidation. Taken together, these data suggest that enhancement of hepatic glycogen reservoir in mice overexpressing $Ppp1r3b$ improves the capacity for maintenance of plasma glucose homeostasis and minimizes the dependence on other energy substrates under longer term fasting conditions.
DISCUSSION

Variants at chromosomal locus 8p23.1, in an intergenic region 174kb from the \textit{PPPIR3B} gene, have recently been associated with human glycemic traits including fasting plasma insulin and glucose in a large-scale meta-analysis of genome wide association studies (15). Rs4841132 has two alleles (G or A) in the 1000 Genomes database (Phase 3), of which the minor A allele has an overall frequency of 0.093. The rs4841132 A allele is associated with increases in both fasting plasma glucose and insulin (15). Rs4841132 is in high linkage disequilibrium with another SNP, rs9987289 (linkage disequilibrium R-squared=1; physical distance, 238 base pairs). The minor allele at rs9987289 was associated with increased plasma lactate levels in individuals of European ancestry, and this effect was found to be strongest in diabetics taking metformin, a drug known to limit excessive hepatic glucose production (HGP) (16). The locus was also identified with other plasma lipid traits (total cholesterol, and both high and low-density lipoprotein cholesterol) (17).

Lastly, several subsequent independent GWAS studies have associated the locus with plasma lactate levels (16), and with hepatic steatosis (18,19).

The centrality of the liver in the maintenance of whole-body glucose homeostasis in both fed, and fasted states has been well established, and glycogen metabolism is in turn a critical mediator of this function, serving as an immediate source of glucose that can be rapidly mobilized to buffer falling plasma glucose levels, and conversely, as a reservoir to capture excess glucose. Glycogen Synthase (GS) is a rate-limiting glycogen synthetic enzyme that is allosterically regulated by glucose-6-phosphate (G6P). Binding of G6P induces a conformational shift that promotes recruitment of protein phosphatase 1 (PP1) that dephosphorylates and activates GS (4,20). Dephosphorylation of serine at position 641 (S641) inactivates GS. PP1 promotes dephosphorylation of GS at S641 by juxtaposing PP1 to GS through glycogen targeting subunits (21). Moorehead and colleagues demonstrated that heterodimeric complexes consisting of PP1-catalytic subunit (\textit{α} and \textit{β} isoforms) and Ppp1r3b/\textit{G}\textsubscript{L} (then termed PP1G) purified from rat liver, bound both glycogen, and glycogen phosphorylase-\textit{α} (GP \textit{α}) with high affinity. The G\textsubscript{L}-GP\textit{α} interaction is thought to antagonize PP1G mediated GS activation in an allosteric fashion (22). Later, Printen et.al showed that FLAG-Ppp1r3c/PTG fusion protein simultaneously bound PP1-catalytic subunit (PP1C) and glycogen. Independent of insulin stimulation, PTG could form stable complexes with PP1C, GS, and phosphorylase kinase, an activator of glycogen phosphorylase, but not with other phosphatases or kinases (9). Ppp1r3a/G\textsubscript{M}/RGL is the most abundant glycogen-targeting subunit in rodent skeletal muscle, which upon phosphorylation dissociates from PP1 and regulates GS activation in a similar fashion to that of PTG (23). Lastly, Gasa, et.al characterized different glycogenic capacities of three major subunits (Ppp1r3b (G\textsubscript{L}), Ppp1r3a (G\textsubscript{M}), and Ppp1r3c (PTG) in rat primary hepatocytes with respect to their regulatory role in activation and deactivation of Glycogen synthase (GS) and Glycogen phosphorylase (GP) (8). The study concluded that: 1) Ppp1r3b (G\textsubscript{L}) had the highest capacity to maintain increased GS activity ratio, independent of insulin stimulation; 2) Ppp1r3c (PTG) maintained the glycogen synthesis capacity induced by insulin in a dose-dependent manner; 3) G\textsubscript{L} and G\textsubscript{M} are responsive to forskolin-mediated glycogenolysis, however PTG remains unresponsive due to lack of inhibition by Glycogen Phosphorylase (GP). In summary, it is well established that Ppp1r3a, Ppp1r3b, and Ppp1r3c directly control the phosphorylation of GS.

Although mechanistic studies demonstrate that G\textsubscript{L} mediates the intracellular scaffolding of PP1 to glycogen in the liver, the \textit{in vivo} physiology of G\textsubscript{L} has been largely unexplored prior to this study, in contrast to PTG, for which both whole body knockout, and liver specific overexpression mouse models have been described (11,12).

To elucidate the role of Ppp1r3b \textit{in vivo}, we generated liver-specific knockout mice, and first investigated the hepatic glycogen metabolism and whole-body glucose homeostasis. Liver-specific genetic ablation of \textit{Ppp1r3b} gene yielded viable mice in which liver glycogen storage and glycogen synthase activity were severely impaired. Despite the absence of \textit{Ppp1r3b}, there were no compensatory changes in liver mRNA expression of \textit{Ppp1r3a} and \textit{Ppp1r3c}, and muscle glycogen content and incorporation of plasma glucose into
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muscle glycogen were unaffected. It has been previously suggested that liver glycogen is essential for neonatal survival until gluconeogenesis is fully established in the newborn liver (24). The Alb-Cre has been shown to drive incomplete deletion at birth, with progressively complete ablation over the first six weeks of life (25,26), which is presumably what permitted the survival of the $Ppp1r3b^{\Delta \text{hep}}$ pups. The residual signal in livers from $Ppp1r3b^{\Delta \text{hep}}$ mice in biochemical assays and histological stains for glycogen, likely is due to the combination of the presence of rare hepatocytes that escaped Cre-deletion of $Ppp1r3b$, as well as simple glucose polymers in hepatocytes throughout the lobule.

We sought to study the importance of $Ppp1r3b$ in regulation of systemic plasma glucose homeostasis. In many regards, the $Ppp1r3b^{\Delta \text{hep}}$ phenotype resembles that of mice with liver specific knockout of glycogen synthase (27) and patients with liver specific glycogen storage disease 0 (GSD0) due to mutation of the GYS2 gene (28,29). All three cases are characterized by elevated lactate in the postprandial state, fasting hypoglycemia, and elevated blood ketone bodies. Interestingly, while no mutations in $PPP1R3B$ have been reported in GSD0 patients to date, our findings suggest that human genetic deficiency of $PPP1R3B$ would likely manifest with GSD0. Indeed, the phenotypic similarity of $Ppp1r3b^{\Delta \text{hep}}$ mice to glycogen synthase liver specific knockout mice is consistent with the unexpected and novel finding that loss of hepatic Ppp1r3b function impacts not only the phosphorylation state, but also the total abundance of GS. We are actively investigating potential mechanisms to explain the effect of Ppp1r3b on GS protein abundance.

*Ad libitum* fed $Ppp1r3b^{\Delta \text{hep}}$ mice have modestly reduced blood glucose levels, compared with controls. In euglycemia, glucose in the liver can be either stored as glycogen or converted to pyruvate and lactate by glycolysis. In $Ppp1r3b^{\Delta \text{hep}}$ mice, synthesis of glycogen is blocked, and this is associated with a reduced ability to dispose of glucose after feeding. Under these conditions, increased lactate in postprandial state is consistent with an increase in glucose flux through glycolysis. Since the expression of $Ppp1r3b$ is restricted mainly to liver in adult mice, the contribution of plasma lactate should presumably be derived mainly from the liver. The fact that human $PPP1R3B$ is also expressed in skeletal muscle (30), may explain the lack of observed $PPP1R3B$ GSD0 patients: the combination of deficiency in both liver and muscle may be incompatible with life.

In the fasted state, when plasma glucose levels are lower, the $Ppp1r3b^{\Delta \text{hep}}$ mice are incapable of liver glycogenolysis and they rely on gluconeogenesis. Our understanding, based on the metabolic status of $Ppp1r3b^{\Delta \text{hep}}$ mice, is that even when fed, they are already conditioned toward fasting physiology, which they attain more rapidly than control animals. Blood glucose, decreases more rapidly upon fasting, but is nonetheless maintained through increased gluconeogenesis, as evidenced by a greater and earlier upregulation of key gluconeogenic enzymes. Further supporting this interpretation, our results from the pyruvate tolerance test also show that $Ppp1r3b^{\Delta \text{hep}}$ mice depend on utilization of pyruvate as one of the non-carbohydrate precursors to maintain plasma glucose levels. Decreased levels of plasma lactate by four hours of fasting suggest that liver begins to utilize non-carbohydrate precursors at an earlier onset of fasting in these mice. By 12 hours of fasting, blood ketone bodies produced by the liver are already elevated and are sustained compared with control animals.

In reciprocal experiments to determine the effects of activating the glycogen synthesis pathway, we overexpressed mouse $Ppp1r3b$ in liver in WT mice. Many of the phenotypes we observe in the overexpressing mice are reciprocal to mice lacking hepatic expression of the $Ppp1r3b$ gene. Both the protein abundance and activation state of GS were increased, mirroring the effects of the $Ppp1r3b^{\Delta \text{hep}}$. Additionally, with respect to blood glucose, there were no significant differences between AAV-$Ppp1r3b$ mice and controls under fed and short-term fasted conditions, but the overexpressing mice were resistant to reduced plasma glucose upon 36 hours of fasting. Reflecting this, the rise in plasma total ketone bodies was significantly delayed in AAV-$Ppp1r3b$ mice, suggesting that the requirement for the liver to shift to utilization of fat and protein for fuel sources was obviated by the larger glycogen reservoir and likely efficient glycogenolysis.

Hepatic overexpression of another glycogen targeting protein, PTG, impaired activation of glycogenolysis by forskolin and
glucagon, resulting in a failure to mobilize glycogen stores even during longer term fasting (31). In contrast, mice overexpressing hepatic Ppp1r3b remained responsive to various nutritional states, and have increased capacity for maintenance of plasma glucose under long term fasting conditions, perhaps because $G_L$ is allosterically regulated by binding to Glycogen Phosphorylase $\alpha$ (32), rather than by post-translational modification. Moreover, mice expressing a Ppp1r3b transgene with a mutated allosteric binding site for glycogen phosphorylase $\alpha$, had improved hepatic glycogen synthesis capacity under fed conditions, but impaired glycochenolysis led to hypoglycemia and weight loss upon longer term (36hr) fasting (13). Further mechanistic studies will be required to specifically test the hypothesis that increasing hepatic Ppp1r3b might provide the benefit of improved glucose disposal, without inappropriate sequestration of glycogen during fasting.

In summary, when combined with the human genetics pointing to the PPP1R3B locus as compellingly associated with glycemic traits in humans, our studies in mice genetically deficient in or overexpressing hepatic Ppp1r3b indicate the central role of this protein in regulating hepatic glycogen stores and energy homeostasis in the fasting state.

**EXPERIMENTAL PROCEDURES**

**Generation of Liver-Specific Ppp1r3b Knockout Mice.** Ppp1r3bflox/flox mice were provided by Merck and were produced for Merck by Taconic. Full details of the design of the Ppp1r3bflox/flox mice may be found at [http://www.taconic.com/10482](http://www.taconic.com/10482). Briefly, in the conditional allele, two loxP sites were introduced flanking Ppp1r3b exon 2, which contains the entire protein coding sequence of the gene (Figure S1B). Ppp1r3b liver specific knockout mice (Ppp1r3b<sup>Δhep</sup>) were generated by crossing Ppp1r3bflox/flox mice on a C57BL/6J background with transgenic mice expressing Cre recombinase under the expression of the hepatocyte specific albumin promoter (C57BL/6-Cg-Tg (Alb-Cre) 21Mgn/J; Jackson Laboratories). The resultant Ppp1r3b<sup>Δhep; Alb-Cre+</sup> progeny were crossed with Ppp1r3bflox/flox mice to obtain tissue-specific knockout mice (Ppp1r3b<sup>Δhep; Alb-Cre+</sup>), termed liver-specific Ppp1r3b knockout mice (Ppp1r3b<sup>Δhep</sup>). Mice without the Cre transgene (Ppp1r3b<sup>fl/</sup><sup>fl</sup>) were used as control mice. Genotyping was confirmed for the expression of Cre transgene and Ppp1r3b<sup>fl/</sup><sup>fl</sup> alleles prior to the onset of studies. We also developed a liver specific knockout of Ppp1r3b utilizing AAV-TBG-Cre approach in Ppp1r3bflox/flox mice. Animals were housed under controlled temperature (23°C) and lighting (12-hour light/dark cycle) with free access to water and standard mouse chow diet (LabDiet, 5010).

For 36-hour fasting experiments, indicated mice were initiated on a fasting protocol in the morning (8:00AM), which was considered as *ad libitum* fed state. Blood glucose measurements were taken at time points indicated. Mice were continued to fast for 36 hours until the second day evening (8:00PM) at which point food was returned back to their cages. All Animal experiments were reviewed and approved by Institutional Animal Care and Use Committees of the University of Pennsylvania, Philadelphia, USA.

**Adeno-Associated (AAV) Viral Vector Preparation.** Adeno-associated viral (AAV) vectors, serotype 8, containing either an empty expression cassette (AAV-Null), Cre recombinase (AAV-Cre), or mouse Ppp1r3b gene (AAV-Ppp1r3b) were generated by the University of Pennsylvania Vector Core (Philadelphia, PA, USA). The transgene is selectively expressed in hepatocytes these vectors from the Thyroxine-binding globulin (TBG/SerpinA7) promoter (Wang et al., 2010). For overexpression experiments utilizing AAV-Ppp1r3b, C57BL/6J wild type mice were injected with AAV vectors at a dose of 1x10<sup>12</sup> genome copies (GC) via intraperitoneal (i.p.) injection and examined at the indicated time points. For experiments utilizing AAV-TBG-Cre, Ppp1r3b<sup>fl/</sup><sup>fl</sup> mice were injected with AAV vectors at a dose of 1.5x10<sup>11</sup> genome copies (GC) via i.p. injection and examined at the stated time points.

**Western Blotting Analysis.** Tissues were isolated from mice both in fed and fasted conditions at different ages and then immediately frozen in liquid nitrogen. Tissue homogenates were prepared with RIPA buffer containing 50mM Tris-HCl (pH 7.4), 150mM NaCl, 1mM EDTA, 1% Triton-X-100, 1% Sodium deoxycholate and 0.1% SDS with 1X protease and phosphatase
inhibitor cocktail (Cat#: 5872, Cell Signaling Technology) added. Homogenates were quantified for protein by the BCA method. Thirty to fifty micrograms of total protein was resolved on 4-12% SDS-PAGE gels and subjected to Western Blotting. Immunoblots were performed using the antibodies against the following proteins: GS, P-GS (S641) (CST-3886, CST-3891S; Cell Signaling Technology), PP1, GP (AB-53315, AB-198268; Abcam) and β-actin (SC-81178; Santa Cruz Biotechnology) was used as a loading control.

**RNA Isolation and Quantitative RT-PCR.** RNA was isolated from approximately 50mg flash frozen liver tissue using Trizol solution (Invitrogen) according to the manufacturer’s instructions. cDNA was synthesized from 2.0-2.5 µg total RNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer’s protocol. Real-time PCR (qPCR) was performed using SYBR Green Mix (Applied Biosystems) on an AB17900 RT-PCR machine. The relative quantity of each mRNA was calculated using the delta CT method with β-actin as the housekeeping gene. The sequences for the primer pairs used in the studies are listed in Table S1.

**Intraperitoneal Pyruvate Tolerance Tests.** For pyruvate challenge test (PTT), mice were overnight fasted for 14-16 hours, and then administered 2g/kg of body weight of sodium pyruvate (Sigma-Aldrich) by i.p. injection. Blood glucose concentrations were measured using a One-Touch Ultra glucometer (LifeScan, Inc.) at the indicated time points.

**Metabolic Measurements.** Blood glucose, lactate and ketone bodies were measured using a One-Touch Ultra glucometer (LifeScan, Inc.), Lactate Plus lactate meter (Nova Biomedical) and Nova Max Plus Ketone meter respectively at indicated nutritional conditions.

**Measurement of Tissue Glycogen Levels.** Non-fasting and fasting male mice were sacrificed and tissues were flash frozen in liquid nitrogen. Frozen tissue samples (~50µg) were homogenized in 300µL PBS. Homogenates were quantified for protein by the BCA method (Thermo Scientific). Tissue samples (4µg of total protein for liver and 50ug total protein for skeletal muscle) were used for detection of glycogen levels by colorimetric assay protocol according to manufacturer’s instructions (Biovision). Glycogen levels in samples were determined from a standard-curve method generated by the assay.

**Incorporation of 2-deoxy-D-[3H] glucose into Liver Glycogen**

Incorporation of glucose into glycogen was accessed as described previously (10). As a tracer, 2-deoxy-D-[3H] glucose (PerkinElmer) was combined with 20% (0.2g/ml) glucose and then administered 2g/kg of body weight at 1mCi/mouse by intraperitoneal injection into 6hr-fasted mice. Tissues (liver, skeletal muscle, and kidney) were harvested and flash frozen in liquid nitrogen at 30 minutes and 60 minutes after glucose and tracer injection. Plasma glucose concentrations were measured at these indicated time points. Frozen tissue samples (100mg) were digested in 750µL of 30% KOH at 100°C for 10 minutes. Upon digestion, a sample aliquot was used for measurement of protein concentration by the BCA method (ThermoScientific). Samples were then neutralized with 200µL 20% Na2SO4 and macromolecules were precipitated with 1mL 100% ethanol overnight at -20°C. Macromolecules were pelleted by centrifugation at 10,000 x g for 15minutes and washed twice with 70% ethanol. Sample pellets were air-dried and then glycogen was digested by heat and acid with 500µL 4N H2SO4 for 10 minutes at 100°C. Pellets were then neutralized with an equal volume of 4N NaOH and radioactivity was determined by liquid scintillation counting. 2-deoxy-D-[3H] glucose incorporation into glycogen was determined by dividing radioactivity of digested samples by glucose specific activity in plasma (total radioactivity in plasma) over time course and by sample protein content using protein BCA method.

**Primary Hepatocytes Isolation.** Primary hepatocytes were isolated from ad libitum fed male WT and Ppp1r3bΔhep mice by collagenase perfusion, as described previously (33). Briefly, cells were suspended in Dulbecco’s modified Eagle’s medium (DMEM, Life technologies, Inc.), supplemented with 10% fetal bovine serum, 25mM glucose, and antibiotics (2 units/mL penicillin, 2ug/mL streptomycin) and seeded onto collagen-coated plates at a cell density of 6x10^5 cells per well for a standard 6-well plate. After attachment (4hours at 37°C), hepatocytes were washed and harvested for isolation of protein immune-blotting.
Tissue Staining. The liver sections were prepared from mice at indicated ages, fixed in 4% paraformaldehyde and embedded in paraffin. To detect polysaccharide content, slices of 5-10µm were de-paraffinized, oxidized with 0.5% periodic acid for 5 minutes, stained with Schifff reagent for 15 minutes, and then counterstained with hematoxylin and eosin for 15 minutes (34).

Image manipulation. All images for the histological sections presented were captured using NIS-Elements-D Version 4.12.01, in a single session under identical settings with a Nikon Digital Sight DS-U3 camera, attached to a Nikon Eclipse 80i microscope, using a Plan Fluor 10x objective [0.30 DIC L/N1; 0.17 WD: 16.0]. A 500 µm scale bar was digitally added using NIS-Elements-D. Each set of images was incorporated into a single Adobe Photoshop file; the image flattened, and the white balance calibrated for the entire field using the lumen of a vein as a reference. A 1mm scale bar was superimposed in Adobe Illustrator.

Statistical Analyses. All results are presented as mean ±S.E. Results were analyzed by the unpaired two-tailed Student’s t-test using Microsoft Excel or GraphPad Prism Software (Version 7.0, GraphPad Software, Inc.) as appropriate. Statistical significance was defined as *p<0.05, **p<0.005, ***p<0.0001.

Acknowledgements
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Conflict of Interest
The authors declare that they have no conflicts of interest with the contents of this article.

Author Contributions
MBM designed and performed experiments, analyzed and interpreted the data, and wrote the manuscript; SVS analyzed and interpreted the data, and wrote and edited the manuscript; RS performed experiments; JSM performed experiments, and analyzed and interpreted the data; NJH supervised experiments, analyzed and interpreted the data, and wrote and edited the manuscript. DJR conceived the study, oversaw all its aspects, and wrote and edited the manuscript.

References
Hepatic Ppp1r3b regulates fasting energy homeostasis


FIGURE LEGENDS:

Figure 1: Liver-specific deletion of Ppp1r3b results in depletion of hepatic glycogen content. (A) Hepatic mRNA levels of Ppp1r3a, Ppp1r3b, and Ppp1r3c measured by SYBR-Green RT-qPCR in control and Ppp1r3bΔhep [Alb-Cre] mice (n=10-14/genotype). (B) Hepatic glycogen content in ad libitum chow-fed and four-hour fasted control and Ppp1r3bΔhep mice (n=6/genotype). (C) PAS staining of paraffin-embedded liver sections from ad lib fed, and four-hour fasted Ppp1r3bΔhep and control mice shows depletion of glycogen (purple stain). Note that the 4 hour fasted sections were not counterstained with hematoxylin. Scale bar 1mm. (D) 2-deoxy-D-[3H] glucose (2-DOG) incorporation into glycogen was measured after 6-hour of fasting in control and Ppp1r3bΔhep mice (n=3-4/genotype). Label incorporation was measured as counts per minute (CPM) from precipitated glycogen from tissue lysates and normalized to plasma CPM/mg protein. Results were replicated in at least two independent experiments. All mice were adults (2-8 months) and were age-matched within experiments. The data are expressed as the mean ± S.E. Significance was determined in all panels by unpaired Student’s t-test (*p<0.05, **p<0.005, ***p<0.0001). (n.s), not significant.

Figure 2: Ppp1r3b deficient livers have reduced total GS protein but higher relative phospho-GS. (A) Four-hour fasted liver lysates were used for measurement of transcript levels of Gys2 and Pygl (n=6-9/genotype). (B) Protein expression of total and p-GS GS in primary hepatocytes isolated from ad libitum chow-fed control and Ppp1r3bΔhep mice. (C) Protein expression of total GS, P-GS (S641), total GP, and PP1 in liver lysates from four-hour fasted control and Ppp1r3bΔhep mice. Ratio of total GS to β-actin is decreased and P-GS to total GS is increased Ppp1r3bΔhep compared to control mice (n=6/genotype). (D) Liver GS protein expression and PAS staining in ad libitum chow-fed and overnight fasted Ppp1r3bΔhep mice compared to control mice (n=4-6/genotype). Scale bar 1mm. All mice were adults (2-8 months) and were age-matched within experiments. Results were replicated in at least two independent experiments. The data are expressed as the means ± S.E. Significance was determined in all panels by unpaired Student’s t-test (*p<0.05, **p<0.005, ***p<0.0001). (n.s), not significant.

Figure 3: Plasma glucose homeostasis is impaired in Ppp1r3bΔhep mice. (A) Blood glucose levels of ad libitum chow-fed, and four-hour fasted mice (n=10-14/genotype). (B) Blood glucose levels during a 36-hour fasting period in Ppp1r3bΔhep mice compared to control mice (n=6/genotype). (C) Blood lactate levels in ad libitum chow-fed and in four-hour fasted control and Ppp1r3bΔhep mice (n=10-14/genotype). (D) Total ketone bodies in blood measured during ad libitum ad fed, 12, 24 and 36-hour fasting conditions in Ppp1r3bΔhep mice compared to control mice (n=6-8/genotype). (E) Pyruvate tolerance test (PTT) in control and Ppp1r3bΔhep mice (n=6/genotype). PTT was performed in mice by administering 2g/kg body weight sodium pyruvate by intraperitoneal injection after overnight (14-16hr) fasting. Values are reported as percentage of basal glucose levels; area under the curve (AUC) is expressed as arbitrary units. All mice were adults (2-8 months) and were age-matched within experiments). Results were replicated in at least two independent experiments. The data are expressed as the means ± S.E. Significance was determined in all panels by unpaired Student’s t-test (*p<0.05, **p<0.005, ***p<0.0001). (n.s), not significant.

Figure 4: Ppp1r3b deficient livers undergo rapid switching from glycolysis to gluconeogenesis. (A) Ad libitum chow-fed liver lysates were used for measurement of transcript levels of glycolytic and gluconeogenic genes (Gck, Gpi1, Pfk1, Aldob, Tpi1, Gapdh, Pgk1, Pgam1, Enol, Pklr, Pepck, and Gapdh (n=6-9/genotype). (B) Eight-hour fasted liver lysates were used for measurement of transcript levels of the above glycolytic and gluconeogenic genes (n=5/genotype). Results were replicated in at least two independent experiments. The data are expressed as the means ± S.E. Significance was determined in all panels by unpaired Student’s t-test (*p<0.05, **p<0.005, ***p<0.0001). (n.s), not significant.

Figure 5: Overexpression of Ppp1r3b in liver enhances hepatic glycogen storage and delayed responses to fasting. (A) Four-hour fasted liver lysates were used for measurement of transcript levels of Ppp1r3b, Ppp1r3c (PTG), Ppp1r3a (Gm), Gys2, and Pygl from C57BL/6J mice injected with AAV-Null
and AAV-Ppp1r3b (n=4/group). (B) Hepatic glycogen content in four-hour and eight-hour fasted liver lysates from C57BL/6J mice injected with AAV-Null and AAV-Ppp1r3b (n=3-4/group). (C) PAS staining of paraffin-embedded liver sections from four-hour and eight-hour fasted C57BL/6J mice injected with AAV-Null and AAV-Ppp1r3b, showing increased amount of glycogen (purple stain) in AAV-Ppp1r3b overexpressing mice. (D) Protein expression of total GS, P-GS (S641), total GP, and PP1 in liver lysates from four-hour fasted from C57BL/6J mice injected with AAV-Null and AAV-Ppp1r3b (n=4/group). Ratio of total GS to β-actin is increased and P-GS to total GS is decreased in AAV-Ppp1r3b overexpressing mice compared to the Null group (n=4/genotype). (E) Blood glucose levels during a 36-hour fasting period in C57BL/6J mice injected with AAV-Null and AAV-Ppp1r3b (n=7-8/group). (F) Total ketone bodies in blood measured during ad libitum fed, 12, 24 and 36-hour fasting conditions in C57BL/6J mice injected with AAV-Null and AAV-Ppp1r3b (n=7-8/group). Mice were injected at 9 weeks of age and liver lysates were analyzed at 11-12 weeks post injection. Results were replicated in at least two independent experiments. The data are expressed as the means ± S.E. Significance was determined in all panels by unpaired Student’s t-test (*p<0.05, **p<0.005, ***p<0.0001). (n.s), not significant.
Figure 1

A

Relative mRNA Expression

- control
- Ppp1r3b<sup>Δ</sup>hep

- Ppp1r3b (GL)
- Ppp1r3c (PTG)
- Ppp1r3a (GM)

B

Liver glycogen

- Ad lib Fed
- 4-hr Fasted

C

Liver PAS

- Ad lib Fed
- 4-hr Fasted

control  
Ppp1r3b<sup>Δ</sup>hep

D

[% Plasma CPM/mg Protein]

- Liver
- Gastrocnemius
- Kidney

30 minutes

- ns

60 minutes

- ***
Figure 2

A

Relative mRNA Expression

Gys2 (GS)  Pygl (GP)

control  Ppp1r3bΔhep  n.s  n.s

B

Primary Hepatocytes

control  Ppp1r3bΔhep

GS

p-GS (S641)

β-actin

C

4-hour Fasted Liver

control  Ppp1r3bΔhep

GS

p-GS (S641)

GP

PP1

β-Actin

D

Ad Libitum Fed

control  Ppp1r3bΔhep

GS

Liver PAS

Overnight Fasted

control  Ppp1r3bΔhep
Hepatic Ppp1r3b regulates fasting energy homeostasis

Figure 3

A

B

C

D

E

Pyruvate Tolerance Test

% Basal Blood Glucose

AUC IP-PTT (a.u.)

Time post injection (minutes)
Figure 4

A  **Ad libitum Fed**

<table>
<thead>
<tr>
<th>Glycolytic Genes</th>
<th>Gluconeogenic Genes</th>
</tr>
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<td><strong>Gpi1</strong></td>
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<tr>
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<td>control</td>
</tr>
<tr>
<td><strong>Abc1b</strong></td>
<td>control</td>
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<td><strong>Tpi1</strong></td>
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<td><strong>Pkg1</strong></td>
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<td><strong>Pgam1</strong></td>
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<tr>
<td><strong>Eno1</strong></td>
<td>control</td>
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<tr>
<td><strong>Pnfi</strong></td>
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B  **8-hour Fasted**

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Hepatic Ppp1r3b regulates fasting energy homeostasis
Figure 5

A

Relative mRNA Expression

<table>
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<tr>
<th>Gene</th>
<th>AAV-Null</th>
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<td>Ppp1r3a (GM)</td>
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<td>Ppp1r3c (PTG)</td>
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<tr>
<td>Gys2 (GS)</td>
<td><img src="image7" alt="Bar Chart" /></td>
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<td>Pygl (GP)</td>
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B

Liver Glycogen (mg/g wet weight)

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<thead>
<tr>
<th>Time</th>
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<tr>
<td>4-hr Fasted</td>
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<td>8-hr Fasted</td>
<td><img src="image13" alt="Bar Chart" /></td>
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C

4-hour Fasted Liver

<table>
<thead>
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<th>Protein</th>
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<td>β-actin</td>
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D

Total GS/β-actin

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</thead>
<tbody>
<tr>
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E

Blood Glucose (mg/dL)

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<th>24-hr Fasted</th>
<th>36-hr Fasted</th>
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<td>12-hr Fasted</td>
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<td>24-hr Fasted</td>
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F

Blood Total Ketone Bodies (mmol/L)

<table>
<thead>
<tr>
<th>Group</th>
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Hepatic protein phosphatase 1 regulatory subunit 3B (Ppp1r3b) promotes hepatic glycogen synthesis and thereby regulates fasting energy homeostasis
Minal B. Mehta, Swapnil V. Shewale, Raymond N. Sequeira, John S. Millar, Nicholas J. Hand and Daniel J. Rader

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