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 regula-
tory subunit 3B), encodes a protein (Gπ) that regulates glycogen metabolism in the liver. We therefore sought to test the hypoth-
thesis that hepatic PPP1R3B is associated with glycemic traits. We generated mice with either liver-specific deletion (Ppp1r3b<sup>Δ<sup> heg</sup></sup>) or liver-specific overexpression of Ppp1r3b. The Ppp1r3b dele-
tion significantly reduced glycogen synthase protein abundance, and the remaining protein was predominantly phosphor-
ylated and inactive. As a consequence, glucose incorporation
into hepatic glycogen was significantly impaired, total hepatic
glycogen content was substantially decreased, and mice lacking
this protein were protected from blood ketone-body accumulation, unlike control
hepatic Ppp1r3b<sup>Δ<sup> heg</sup></sup> mice relative to controls in the
postprandial state. Eight hours of fasting significantly increased
the expression of two critical gluconeogenic enzymes, phos-
phoenolpyruvate carboxykinase and glucose-6-phosphatase,
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 fast-
ing-induced hypoglycemia. Moreover, mice overexpressing
hepatic Ppp1r3b upon long-term fasting (12–36 h) were pro-
tected from blood ketone-body accumulation, unlike control and
Ppp1r3b<sup>Δ<sup> heg</sup></sup> mice. These findings indicate a major role for
Ppp1r3b in regulating hepatic glycogen stores and whole-body
glucose/energy homeostasis.

The Meta-Analysis of Glucose and Insulin related traits Con-
sortium (MAGIC) study analyzed data sets from multiple
cohorts that contained both genome-wide genotype data and
quantitative clinical measurements of human plasma glycemic
traits. Among the 12 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 kb from the 5’
end of LOC157273 gene, a long non-coding RNA transcript of
unknown function, and 174 kb from the protein-coding
PPP1R3B gene. Despite the 174-kb separation from the
PPP1R3B gene, rs9987289 has been identified as an expression
quantitative trait locus for PPP1R3B: the minor allele is associ-
ated with higher levels of 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 PPP1R3B.

PPP1R3B (protein phosphatase 1 regulatory subunit 3B; also
historically known as Gπ) is a regulator of liver glycogen metab-
olism. In the liver, sequestration of dietary glucose into glyco-
gen 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 fast-
ing (1–4). Dysregulation of this pathway is associated with met-
abolic disorders including glycogen storage diseases and dia-
tes. Glycogen synthase (GS)3 and glycogen phosphorylase (GP)
promote glycogen synthesis and glycogen catabolism, respec-
tively, and their activities are tightly controlled by endocrine
signaling pathways that are in turn coupled to nutritional sta-
tus. Glycogen-targeting subunits recruit the phosphatases and
kinases that post-translationally regulate the activation of GS
and inactivation of GP. Several proteins have been character-
ized that target protein phosphatase 1 (PP1) to glycogen (5, 6).
In liver, two major subunits, PPP1R3B (Gπ) and PPP1R3C

<sup>1</sup> The abbreviations used are: GS, glycogen synthase; GP, glycogen phos-
phorylase; PP1, protein phosphatase 1; AAV, adeno-associated virus; PAS, pe-
riodic acid-Schiff; GSD0, glycogen storage disease 0; PTT, pyruvate toler-
ance test.

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Hepatic Ppp1r3b regulates fasting energy homeostasis

Ppp1r3b knock-out mice (Ppp1r3b\(^{\Delta lqep}\)) were viable, born at the expected frequency for Mendelian inheritance, and showed normal fertility. In all subsequent experiments, mice without the Cre transgene (Ppp1r3b\(^{flox/flox}\)) were used as controls. In Ppp1r3b\(^{\Delta lqep}\) 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\(_S\) (Ppp1r3a) and PTG (Ppp1r3c) (Fig. 1A). Ppp1r3b\(^{\Delta lqep}\) mice showed normal growth rates on chow diet, with no differences observed in body weights between genotypes up to 50 weeks of age (supplemental Fig. S1B).

Hepatic glycogen content was significantly reduced in ad libitum fed state in Ppp1r3b\(^{\Delta lqep}\) mice, which was rapidly depleted upon short-term, 4-h 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\(^{\Delta lqep}\) mice was also modestly decreased compared with controls (supplemental Fig. S1C), likely because of a lack of glycogen stores, which with their associated water content account for 5% of liver mass (3). We used AAV-Cre in Ppp1r3b\(^{flox/flox}\) mice as a second approach to generate liver-specific knock-out of Ppp1r3b and found effects on hepatic glycogen and plasma glucose (supplemental Fig. S2) comparable 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 4-h fasted conditions (supplemental Fig. S1D). To investigate the glycogen synthesis rate in these mice, we performed 2-deoxy-d-[\(^{13}\)C]glucose incorporation into glycogen in glucose-responsive tissues: liver, skeletal muscle (gastrocnemius), and kidney, as a surrogate measurement of GS activity. Radioactivity in the glycogen pellet from tissue lysates was measured at 30 and 60 min and quantified as a percentage of glucose-specific activity of total radioactivity in plasma and of total protein content. In this experiment, Ppp1r3b\(^{\Delta lqep}\) mice exhibited significantly reduced glycogen synthase activity in the liver, whereas its activity was not different in skeletal muscle or kidney (Fig. 1D). Thus, the loss of hepatic Ppp1r3b results in a smaller liver with reduced GS activity and glycogen content in ad libitum fed and 4-h fasted mice.

Deletion of Ppp1r3b in the liver causes significant reduction in total glycogen synthase protein and activity state

Ppp1r3b\(^{\Delta lqep}\) mice have significant reduction in GS but not 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\(^{\Delta lqep}\) mice (Fig. 2C). The nearly complete loss of dephosphorylated [activated] GS protein likely parallels the depletion of glycogen granules because overnight fasted control mice also reached a similar depletion of glycogen, and protein levels of active/dephosphorylated GS were similar in control and Ppp1r3b\(^{\Delta lqep}\) livers after an overnight fast (Fig. 2D). The molecular mechanism by which Ppp1r3b\(^{\Delta lqep}\) deletion leads to hyperphosphorylation of

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” and supplemental Fig. S1A). Liver-specific
GS and marked reduction in total GS protein content even during the ad libitum fed state remains to be further explored. Thus, a 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 Ppp1r3bΔhep mice are modestly lower, and Ppp1r3bΔhep mice become mildly hypoglycemic after a 4-h fast (Fig. 3A). Within 4–8 h of fasting, blood glucose
in Ppp1r3bΔhep 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 h. (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 postprandial state, plasma lactate levels were increased in Ppp1r3bΔhep mice compared with controls (Fig. 3C). Upon prolonged fasting (12 h), total plasma ketone bodies were significantly increased and were progressively elevated by 24- and 36-h fasting periods in Ppp1r3bΔhep mice, compared with controls (Fig. 3D). Moreover, after an overnight fast, gluconeogenesis was increased in Ppp1r3bΔhep 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 with controls (Fig. 3E). Taken together, these data suggest that although the Ppp1r3bΔhep mice are more prone to enter into a quasifasted state following short-term food deprivation, they are nonetheless able to maintain stable long-term fasting plasma glucose levels by compensatory up-regulation of the gluconeogenic pathway from alternative precursors.

Hepatic deletion of Ppp1r3b causes up-regulation of glycolytic genes in the postprandial state and up-regulation of gluconeogenic genes within 8 h of fasting

In the Ppp1r3b-deficient livers, under ad lib fed conditions, genes that encode enzymes are critical in the glycolytic pathway up-regulated compared with control livers (Fig. 4A); these included aldolase (Aldob), triose phosphate isomerase (Tpi), glyceraldehyde phosphate dehydrogenase (Gapdh), phosphoglycerate kinase (Pgk1), phosphoglycerate mutase (Pgaml), enolase (Eno1), and l-pyruvate kinase (Pklr). Upon an 8-h fasting period, there was an up-regulation of gluconeogenic genes (Pepck and G6pc) in Ppp1r3bΔhep 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 that favors shunting of glucose into the glycolysis pathway in the fed state and an early up-regulation 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/Serpin7 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 ~4-fold higher compared with 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 (Pygl), in mice overexpressing Ppp1r3b. Following a short-term 4-h fasting period, mice overexpressing Ppp1r3b had ~4-fold higher levels of hepatic glycogen content than controls, and this was maintained at 8 h of fasting (Fig. 5, B and C). 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 after 4- and 8-h fasting periods (Fig. 5D). Additionally, total GP or PP1 protein content was not affected (Fig. 5D).

Figure 3. Plasma glucose homeostasis is impaired in Ppp1r3bΔhep mice. A, blood glucose levels of ad libitum chow-fed and 4-h fasted mice (n = 10–14/genotype). B, blood glucose levels during a 36-h fasting period in Ppp1r3bΔhep mice compared with control mice (n = 6/genotype). C, blood lactate levels in ad libitum chow-fed and in 4-h fasted control and Ppp1r3bΔhep mice (n = 10–14/genotype). D, total ketone bodies in blood measured during ad libitum ad fed and 12-, 24-, and 36-h fasting conditions in Ppp1r3bΔhep mice compared with control mice (n = 6–8/genotype). E, PTT in control and Ppp1r3bΔhep mice (n = 6/genotype). PTT was performed in mice by administering 2 g/kg body weight sodium pyruvate by intraperitoneal injection after overnight (14–16 h) fasting. The values are reported as percentages of basal glucose levels; the area under the curve (AUC) is expressed as arbitrary units. All mice were adults (2–8 months) and were age-matched within experiments. The 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.
Hepatic Ppp1r3b regulates fasting energy homeostasis

Blood glucose levels were not different between the two groups with ad libitum and 12-h 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 h of fasting, AAV-∗Ppp1r3b mice maintained persistently higher (by ~15 mg/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 174 kb from the PPI1R3B gene, have recently been associated with human glycomic 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^2 = 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 (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. GS is a rate-limiting glycogen synthetic enzyme that is allosterically regulated by glucose-6-phosphate. Binding of glucose-6-phosphate induces a conformational shift that promotes recruitment of PP1 that dephosphorylates and activates GS (4, 20). Phosphorylation of Ser-641 inactivates GS. PP1 promotes dephosphorylation of GS at Ser-641 by juxtaposing PP1G onto GS (4, 20). Phosphorylation of Ser-641 inactivates GS. PP1 promotes dephosphorylation of GS at Ser-641 by juxtaposing PP1G onto GS through glycogen-targeting subunits (21). Moorhead et al. (22) demonstrated that heterodimeric complexes consisting of PP1-catalytic subunit (α and β isofoms) and Ppp1r3b/GL (then termed PPI1G) purified from rat liver bound both glycogen and glycogen phosphorylase-a (GP α) with high affinity. The G_α-GPα interaction is thought to antagonize PP1G-mediated GS activation in an allosteric fashion. Later, Printen et al. (9) showed that FLAG-Ppp1r3c/PTG fusion protein simultane-

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, Gpi, Pkl1, Aldob, Tpi1, Gapdh, Pgk1, Pgam1, Enol1, Pklr, Pepck, and G6pc (n = 6–9/genotype). B, 8-h fasted liver lysates were used for measurement of transcript levels of the above glycolytic and gluconeogenic genes (n = 5/genotype). The 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.
Hepatic Ppp1r3b regulates fasting energy homeostasis

Previously bound PP1-catalytic subunit and glycogen. Independent of insulin stimulation, PTG could form stable complexes with the PP1-catalytic subunit, GS, and phosphorylase kinase, an activator of glycogen phosphorylase, but not with other phosphatases or kinases. Ppp1r3a/GM/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. (8) characterized different glycogenic capacities of three major subunits (Ppp1r3b (GL), Ppp1r3a (GM), and Ppp1r3c (PTG) in rat primary hepatocytes with respect to their regulatory role in activation and deactivation of GS and GP. The study

Figure 5. Overexpression of Ppp1r3b in liver enhances hepatic glycogen storage and delayed responses to fasting. A, 4-h 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 4- and 8-h 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 4- and 8-h 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 (Ser-641), total GP, and PP1 in liver lysates from 4-h fasted from C57BL/6J mice injected with AAV-null and AAV-Ppp1r3b (n = 4/group). The ratio of total GS to β-actin is increased and P-GS to total GS is decreased in AAV-Ppp1r3b-overexpressing mice compared with the Null group (n = 4/group). E, blood glucose levels during a 36-h 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 and 12-, 24-, and 36-h fasting conditions in C57BL/6J mice injected with AAV-null and AAV-Ppp1r3b (n = 7–8/group). The mice were injected at 9 weeks of age, and liver lysates were analyzed at 11–12 weeks post injection. The 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.
concluded that: 1) Ppp1r3b (Gt) 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; and 3) Gt and Gs are responsive to forskolin-mediated glycogenolysis; however, PTG remains unresponsive because of a lack of inhibition by GP. In summary, it is well established that Ppp1r3a, Ppp1r3b, and Ppp1r3c directly control the phosphorylation of GS.

Although mechanistic studies demonstrate that Gt mediates the intracellular scaffolding of PP1 to glycogen in the liver, the in vivo physiology of Gt has been largely unexplored prior to this study, in contrast to PTG, for which both whole body knock-out and liver-specific overexpression mouse models have been described (11, 12). To elucidate the role of Ppp1r3b in vivo, we generated liver-specific knock-out mice and first investigated the hepatic glycogen metabolism and whole-body glucose homeostasis. Liver-specific genetic ablation of the Ppp1r3b gene yielded viable mice in which liver glycogen storage and glycogen synthase activity were severely impaired. Despite the absence of Ppp1r3b, there were no compensatory changes in liver mRNA expression of Ppp1r3a and Ppp1r3c, and muscle glycogen content and incorporation of plasma glucose into 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 6 weeks of life (25, 26), which is presumably what permitted the survival of the Ppp1r3b−/− pups. The residual signal in livers from Ppp1r3b−/− 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−/− phenotype resembles that of mice with liver-specific knock-out of glycogen synthase (27) and patients with liver-specific glycogen storage disease 0 (GSD0) because of 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, although 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−/− mice to glycogen synthase liver-specific knock-out 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−/− 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−/− 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. Because 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−/− mice are incapable of liver glycogenolysis and rely on gluconeogenesis. Our understanding, based on the metabolic status of Ppp1r3b−/− 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 up-regulation of key gluconeogenic enzymes. Further supporting this interpretation, our results from the pyruvate tolerance test also show that Ppp1r3b−/− mice depend on utilization of pyruvate as one of the non-carbohydrate precursors to maintain plasma glucose levels. Decreased levels of plasma lactate by 4 h of fasting suggest that liver begins to utilize non-carbohydrate precursors at earlier onset of fasting in these mice. By 12 h 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 Ppp1r3b−/−. 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 h 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 Gt is allosterically regulated by binding to glycogen phosphorylase α (32) rather than by post-translational modification. Moreover, mice expressing a Ppp1r3b transgene with a mutated allosteric binding site for glycogen phosphorylase α had improved hepatic glycogen synthesis capacity under fed conditions, but impaired glycogenolysis led to hypoglycemia and weight loss upon longer-term (36 h) fasting (13). Further mechanistic studies will be required to specifically test the
Hepatic Ppp1r3b regulates fasting energy homeostasis

A 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 PPR13B 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 knock-out mice

The Ppp1r3b<sup>flx/flox</sup> mice were provided by Merck and were produced for Merck by Taconic. Full details of the design of the Ppr13b<sup>flx/flx</sup> mice may be found on the Taconic website (Model 10482). Briefly, in the conditional allele, two loxP sites were introduced flanking Ppr13b exon 2, which contains the entire protein coding sequence of the gene (supplemental S1B).

Ppr13b liver-specific knock-out mice (Ppr13b<sup>Alb-Cre</sup>) were generated by crossing Ppr13b<sup>flx/flox</sup> mice on a C57BL/6J background with transgenic mice expressing Cre recombinase-expressing under the expression of the hepatocyte-specific albumin promoter (C57BL/6-Cg-Tg (Alb-Cre) 21Mgn/J; Jackson Laboratories). The resultant Ppr13b<sup>flx/+;Alb-Cre</sup> progeny were crossed with Ppr13b<sup>flx/flox</sup> mice to obtain tissue-specific knock-out mice (Ppr13b<sup>flx/flox, Alb-Cre+</sup>), termed liver-specific Ppr13b<sup>Cre</sup>-knock-out mice (Ppr13b<sup>Cre</sup>-lox<sup>+/</sup>). Mice without the Cre transgene (Ppr13b<sup>flx/flox</sup>) were used as control mice. Genotyping was confirmed for the expression of Cre transgene and Ppr13b<sup>flx/flox</sup> alleles prior to the onset of studies.

We also developed a liver-specific knock-out of Ppr13b utilizing an AAV-TBG-Cre approach in Ppr13b<sup>flx/flox</sup> mice. The animals were housed under controlled temperature (23 °C) and lighting (12-h light/dark cycle) with free access to water and standard mouse chow diet (LabDiet, 5010).

For 36-h fasting experiments, the indicated mice were initiated on a fasting protocol in the morning (8:00 a.m.), which was considered as the ad libitum fed state. Blood glucose measurements were taken at the time points indicated. The mice were continually fasted for 36 h until the second day evening (8:00 p.m.) 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, PA).

AAV vector preparation

AAV vectors, serotype 8, containing either an empty expression cassette (AAV-null), Cre recombinase (AAV-Cre), or mouse Ppr13b gene (AAV-Ppr13b), were generated by the University of Pennsylvania Vector Core (Philadelphia, PA). The transgene is selectively expressed in hepatocytes these vectors from the thyroxine-binding globulin (TBG/SerpinA7) promoter (14). For overexpression experiments utilizing AAV-Ppr13b, C57BL/6J wild-type mice were injected with AAV vectors at a dose of 1 × 10<sup>12</sup> genome copies via i.p. injection and examined at the indicated time points. For experiments utilizing AAV-TBG-Cre, Ppr13b<sup>flx/flox</sup> mice were injected with AAV vectors at a dose of 1.5 × 10<sup>12</sup> genome copies via i.p. injection and examined at the stated time points.

Western blotting analysis

The 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 radioimmune precipitation assay buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% Triton-X-100, 1% sodium deoxycholate, and 0.1% SDS with 1× protease and phosphatase inhibitor mixture (catalog no. 5872; Cell Signaling Technology) added. Homogenates were quantified for protein by the BCA method. 30–50 μg 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 (Ser-641) (CST-3886 and CST-3891S, Cell Signaling Technology), PPI, and GP (AB-53315 and 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 ~50 mg of flash-frozen liver tissue using TRIzol solution (Invitrogen) according to the manufacturer’s instructions. cDNA was synthesized from 2.0–2.5 μg of total RNA using the high-capacity cDNA reverse transcription kit (Applied Biosystems) according to the manufacturer’s protocol. Real-time quantitative PCR was performed using SYBR Green Mix (Applied Biosystems) on an ABI7900 RT-PCR machine. The relative quantity of each mRNA was calculated using the ΔCT method with β-actin as the housekeeping gene. The sequences for the primer pairs used in the studies are listed in supplemental Table S1.

Intraperitoneal pyruvate tolerance tests

For the pyruvate tolerance test (PTT) challenge, the mice were overnight fasted for 14–16 h and then administered 2 g/kg body weight of sodium pyruvate (Sigma-Aldrich) by i.p. injection. Blood glucose concentrations were measured using a OneTouch 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.), a Lactate Plus lactate meter (Nova Biomedical), and a Nova Max Plus Ketone meter, respectively, at the 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 (~50 μg) were homogenized in 300 μl of PBS. Homogenates were quantified for protein by the BCA method (Thermo Scientific). Tissue samples (4 μg of total protein for liver and 50 μg of 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 assessed as described previously (10). As a tracer, 2-deoxy-D-[3H] glucose (PerkenElmer Life Sciences) was combined with 20% (0.2 g/ml) glucose and then administered 2 g/kg body weight at 1 mCi/mouse by intraperitoneal injection into 6-hasted mice. Tissues (liver, skeletal muscle, and kidney) were harvested and flash-frozen in liquid nitrogen at 30 and 60 min after glucose and tracer injection. Plasma glucose concentrations were measured at these indicated time points. Frozen tissue samples (100 mg) were digested in 750 μl of 30% KOH at 100 °C for 10 min. Upon digestion, a sample aliquot was used for measurement of protein concentration by the BCA method (ThermoScientific). The samples were then neutralized with 200 μl of 20% Na2SO4 and macromolecules were precipitated with 1 ml of 100% ethanol overnight at −20 °C. Macromolecules were pelleted by centrifugation at 10,000 × g for 15 min and washed twice with 70% ethanol. Sample pellets were air-dried, and then glycogen was digested by heat and acid with 500 μl of 4 N H2SO4 for 10 min at 100 °C. The pellets were then neutralized with an equal volume of 4 N NaOH and radioactivity was determined by liquid scintillation counting. 2-deoxy-D-[3H]glucose incorporation into glycogen was determined by dividing the radioactivity of digested samples by glucose specific activity in plasma (total radioactivity in plasma) over time course and by sample protein content using the protein BCA method.

**Primary hepatocyte isolation**

Primary hepatocytes were isolated from *ad libitum* fed male WT and *Ppp1r3b*Δhep mice by collagenase perfusion, as described previously (33). Briefly, the cells were suspended in DMEM (Life Technologies, Inc.), supplemented with 10% fetal bovine serum, 25 mM glucose, and antibiotics (2 units/ml penicillin and 2 μg/ml streptomycin), and seeded onto collagen-coated plates at a cell density of 6 × 105 cells/well for a standard 6-well plate. After attachment (4 h at 37 °C), hepatocytes were washed and harvested for isolation of protein immunoblotting.

**Tissue staining**

The liver sections were prepared from mice at the indicated ages, fixed in 4% paraformaldehyde, and embedded in paraffin. To detect polysaccharide content, slices of 5–10 μm were deparaffinized, oxidized with 0.5% periodic acid for 5 min, stained with Schiff reagent for 15 min, and then counterstained with hematoxylin and eosin for 15 min (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 10× 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 was flattened, and the white balance was calibrated for the entire field using the lumen of a vein as a reference. A 1-mm scale bar was superimposed in Adobe Illustrator.

**Statistical analyses**

All results are presented as means ± S.E. The 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 follows: *, p < 0.05; **, p < 0.005; and ***, p < 0.0001.

**Author contributions**—M. B. M. designed and performed experiments, analyzed and interpreted the data, and wrote the manuscript; S. V. S. analyzed and interpreted the data and wrote and edited the manuscript; R. N. S. performed experiments and analyzed and interpreted the data; N. J. H. supervised experiments, analyzed and interpreted the data, and wrote and edited the manuscript; and D. J. R. conceived the study, oversaw all its aspects, and wrote and edited the manuscript.

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