Fasting-Induced Protein Phosphatase 1 Regulatory Subunit Contributes to Postprandial Blood Glucose Homeostasis via Regulation of Hepatic Glycogenesis

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OBJECTIVE—Most animals experience fasting–feeding cycles throughout their lives. It is well known that the liver plays a central role in regulating glycogen metabolism. However, how hepatic glycogenesis is coordinated with the fasting–feeding cycle to control postprandial glucose homeostasis remains largely unknown. This study determines the molecular mechanism underlying the coupling of hepatic glycogenesis with the fasting–feeding cycle.

RESEARCH DESIGN AND METHODS—Through a series of molecular, cellular, and animal studies, we investigated how PPP1R3G, a glycogen-targeting regulatory subunit of protein phosphatase 1 (PP1), is implicated in regulating hepatic glycogenesis and glucose homeostasis in a manner tightly orchestrated with the fasting–feeding cycle.

RESULTS—PPP1R3G in the liver is upregulated during fasting and downregulated after feeding. PPP1R3G associates with glycogen pellet, interacts with the catalytic subunit of PP1, and regulates glycogen synthase (GS) activity. Fasting glucose level is reduced when PPP1R3G is overexpressed in the liver. Hepatic knockdown of PPP1R3G reduces postprandial elevation of GS activity, decreases postprandial accumulation of liver glycogen, and decelerates postprandial clearance of blood glucose. Other glycogen-targeting regulatory subunits of PP1, such as PPP1R3B, PPP1R3C, and PPP1R3D, are downregulated by fasting and increased by feeding in the liver.

CONCLUSIONS—We propose that the opposite expression pattern of PPP1R3G versus other PP1 regulatory subunits comprise an intricate regulatory machinery to control hepatic glycogenesis during the fasting–feeding cycle. Because of its unique expression pattern, PPP1R3G plays a major role to control postprandial glucose homeostasis during the fasting–feeding transition via its regulation on liver glycogenesis. ***Diabetes*** **60:1435–1445, 2011**

The blood glucose level fluctuates with the fasting–feeding cycle in most animals. On feeding, the increase in postprandial blood glucose is mainly reduced by increased glucose uptake in peripheral tissues, such as liver and skeletal muscles. This process is regulated by changes in the insulin/glucagon ratio, by portal signals, and by the blood glucose concentration itself (1–3). The liver, as a glucose sensor, actively contributes to the control of postprandial blood glucose homeostasis (4). In particular, the liver takes up approximately one-third of the oral glucose load in the animal (5). In the liver, glycogen metabolism is regulated in a complex manner to maintain postprandial blood glucose homeostasis (2,6–11). In brief, two critical enzymes are directly involved in glycogen metabolism, glycogen synthase (GS) for glycogenesis and glycogen phosphorylase (GP) for glycogenolysis. The activities of GS and GP are regulated by phosphorylation/dephosphorylation events, but in opposing directions. GS is inhibited by phosphorylation at multiple sites mediated by protein kinases, such as protein kinase A and glycogen synthase kinase 3 (GSK3), and activated by dephosphorylation via glycogen synthase phosphatase (GSP). On the other hand, GP is activated by phosphorylation at a single residue near the *N*-terminus by phosphorylase kinase and inhibited by dephosphorylation by protein phosphatase 1 (PP1). A postprandial increase in blood glucose results in an elevated intracellular concentration of glucose that binds activated GP (GPa) and promotes its dephosphorylation and inactivation, thus releasing the allosteric inhibitory effect of GPa on GSP (8). On the other hand, glucose-6-phosphate (G6P) produced from glucose is an allosteric activator of GS, and the potency of G6P as an activator increases as GS is dephosphorylated by GSP (7). In addition, the postprandial increase of insulin stimulates GS by reducing its phosphorylation and inactivation by GSK3, at least in muscle (12). Collectively, these events converge to activate GS and inhibit GP on feeding, resulting in accumulation of liver glycogen after a meal.

PP1 plays a critical role in glucose metabolism because of its regulatory effects on glycogen metabolizing enzymes, including GS, GP, and GP kinase (9,11). The PP1 holoenzyme is composed of a catalytic subunit (PP1c) and a regulatory subunit (11). In regulating glycogen metabolism, PP1c is anchored to the glycogen particles by a group of glycogen-targeting regulatory subunits (G subunits) that modulate the activities of the glycogen metabolizing enzymes through PP1-mediated dephosphorylation. According to the GenBank database, there are seven genes encoding G subunits (*PPP1R3A* to *PPP1R3G*), all of which possess a PP1-binding domain and a glycogen-binding domain (11,13). The intricate regulation of glycogen metabolism by G subunits has been established over the past 25 years by extensive and detailed analysis of two of these proteins, G_M/PPP1R3A and G_L/PPP1R3B, which are expressed relatively specifically in skeletal muscle and the liver, respectively, in rodents. The importance of these two proteins in regulating glycogen metabolism was firmly established by studies with mice that had a deletion of G_M/PPP1R3A (14,15), or mice with expression of a deregulated form of

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Received 30 November 2010 and accepted 25 February 2011.
DOI: 10.2337/db10-1663
This article contains Supplementary Data online at http://diabetes.diabetesjournals.org/lookup/suppl/doi:10.2337/db10-1663/-/DC1.
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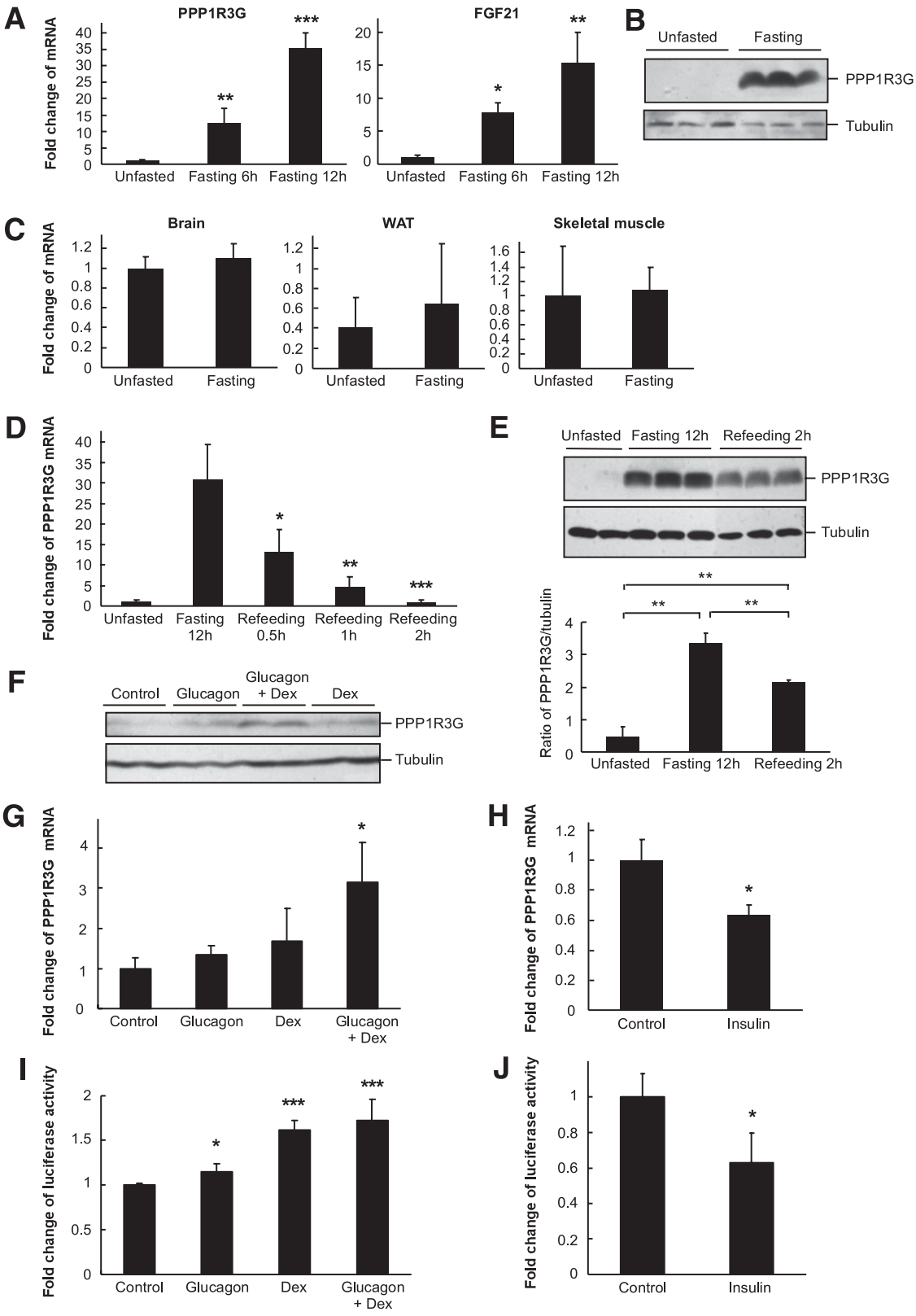

FIG. 1. PPP1R3G is regulated in the fasting–feeding cycle. *A*: Male C57BL/6 J mice at 8 weeks of age were killed in the constant feeding state or after 6-h (from 0:00 to 6:00 A.M.) or 12-h fasting (from 6:00 P.M. to 6:00 A.M. the next day). Liver PPP1R3G and FGF21 mRNA levels were measured by RT-qPCR (n = 4 mice/group). Fold change of each gene compared with actin is shown as mean ± SD. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ as compared with the feeding group. *B*: Liver protein samples in *A* were used in immunoblotting to detect protein levels of PPP1R3G and tubulin. *C*: PPP1R3G mRNA levels in brain, white adipose tissue, and skeletal muscle in feeding or after 12-h fasting. *D*: Male C57BL/6 J mice at 8 weeks of age were killed in the feeding state, after 12-h fasting, or after refeeding for the time as indicated. Liver PPP1R3G mRNA level was measured by RT-qPCR (n = 4 mice/group). Statistical analysis is done by comparing the refeeding group with the fasting group. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ as compared with the group of feeding for 12 h. *E*: Liver samples in *D* were used in immunoblotting with the antibodies as indicated (*top*). Quantitation of the data is shown (*bottom*). **$P < 0.01$ between the groups as indicated. *F*: Primary hepatocytes isolated from overnight-fasted

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G_L/PPP1R3B (16). Mice with heterozygous deletion of PTG/R5/PPP1R3C had reduced glycogen levels in several tissues and became glucose intolerant and insulin resistant as they aged (17). In contrast, little is known about the physiologic function of other G subunits and the reason for the plethora of genes encoding this group of proteins. Furthermore, how G subunits are coordinated with the fasting–feeding cycle to control postprandial glucose homeostasis is largely unknown. In this study, we demonstrate that PPP1R3G, a previously uncharacterized glycogen-targeting regulatory subunit of PP1, is actively involved in the control of blood glucose homeostasis by regulating hepatic glycogenesis in a manner closely coordinated with the fasting–feeding cycle.

RESEARCH DESIGN AND METHODS

Descriptions of the detailed protocols used in many assays of this study are provided in the Supplementary Data.

Animal studies. All animal protocols were approved by the Institutional Animal Care and Use Committee of the Institute for Nutritional Sciences, Chinese Academy of Sciences. Male C57BL/6 J mice were from Shanghai Laboratory Animal Co. Ltd.

Plasmids, cell culture, and transfection. The full-length mouse cDNA of PPP1R3C, PPP1R3G, and PP1c was cloned from mouse liver by RT-PCR and confirmed by DNA sequencing. PPP1R3C, PPP1R3G, and PPP1R3G with deletion of putative glycogen binding domain PPP1R3G(ΔGB) (deletion of the amino acid residues 225 to 247) were subcloned into the mammalian expression vector pRc/CMV with a Flag epitope tag added to the *N*-termini. PP1c was cloned into the mammalian expression vector pCS2+MT with six Myc tags at the N terminus. PPP1R3G was also cloned into the pGEX-4T1 vector to generate glutathione-S-transferase (GST)-fused protein. PP1c was also cloned into the pET-30a vector to generate His-fused protein. Cell culture and transfection were as previously described (18).

Antibodies, immunoblotting, and coimmunoprecipitation. The antibodies were purchased as follows: monoclonal anti-Flag antibody from Sigma-Aldrich (St. Louis, MO); antibodies against Myc, tubulin from Santa Cruz Biotechnology (Santa Cruz, CA); antibodies against PP1c and GS from Cell Signaling Technology (Boston, MA); antibody against phosphorylated GS at Ser641 from Abcam (Boston, MA); Alexa Fluor 488 donkey anti-mouse IgG, Alexa Fluor 546 goat anti-mouse IgG, and Hoechst 33342 from Molecular Probes (Eugene, OR). The polyclonal PPP1R3G antibody was generated by mouse immunization with a GST-fused PPP1R3G protein. Immunoblotting and coimmunoprecipitation assays were previously described (18).

Isolation of primary hepatocytes and adenovirus experiments. Isolation of primary hepatocytes and adenovirus infection was previously described (19). Flag-tagged PPP1R3G adenovirus was generated using the AdEasy Adenoviral Vector System (Stratagene, La Jolla, CA) according to the manufacturer's instructions. Adenoviruses with either scrambled sequence or short hairpin (sh) RNA sequence specific for mouse PPP1R3G were generated using the BLOCK-iT Adenoviral RNAi Expression System (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions.

Measurement of glycogen content. Cell glycogen content measurement was performed as previously described (20). Liver glycogen content measurement was described previously (21).

Analysis of newly synthesized glycogen using D-[3-^{3}H]glucose tracer. Mice were fasted overnight and injected intraperitoneally with glucose (2 g/kg body wt) containing a trace amount of D-[3-^{3}H]glucose (Perkin Elmer Life and Analytical Sciences, Waltham, MA; specific activity 740 GBq/mmol) at 0.5 μCi/g body wt. One hour later, the mice were killed and liver glycogen was isolated using an ethanol-based method (21). The glycogen precipitate was dissolved in H_2O, and an aliquot was used in scintillation counting.

GS activity and GP assays. GS activity assay was performed as previously described (14,22). GP activity assay was previously described (14,23).

Statistical analysis. All data were analyzed by two-tailed Student *t* test.

RESULTS

PPP1R3G is regulated by the fasting–feeding cycle. To gain a global view of genes regulated by fasting in the liver, we examined genes of the whole mouse genome by mRNA microarray (Supplementary Table 1). Unexpectedly, *PPP1R3G*, a predicted glycogen-targeting regulatory subunit of PP1 (13), was found to be highly upregulated by fasting. By RT-PCR analysis, *PPP1R3G* was expressed in many mouse tissues with a relatively high level in liver, brain, lung, white adipose, and adrenal gland (Supplementary Fig. 1*A*). As confirmed by real-time RT-PCR, *PPP1R3G* mRNA level was increased to ~12-fold and 35-fold in the liver after fasting for 6 and 12 h, respectively (Fig. 1*A*). As a positive control, *FGF21*, which was reported to be highly induced by fasting (24), was also elevated in our experiment (Fig. 1*A*). The protein level of PPP1R3G was upregulated consistently by fasting in the mouse liver (Fig. 1*B*) using an antibody generated in our laboratory (Supplementary Fig. 1*B* and *C*). However, none of the other three glycogen-related tissues, including brain, white adipose, and skeletal muscle, had an induction of PPP1R3G on fasting at the mRNA and protein levels (Fig. 1*C*, Supplementary Fig. 1*D*). PPP1R3G mRNA level was quickly reduced after refeeding, reaching the prefasting level after 2-h feeding (Fig. 1*D*). PPP1R3G protein was also significantly downregulated after refeeding (Fig. 1*E*). Collectively, these data demonstrate that *PPP1R3G* is a cyclic gene that changes along with the fasting–feeding cycle in the mouse liver.

To identify the molecular mechanism that underlies the regulation of *PPP1R3G* expression, we analyzed the effects of three major signals/hormones—glucagon, dexamethasone, and insulin—that have been shown by others to regulate other fasting-related gene expression (25). In primary mouse hepatocytes, treatment of glucagon or dexamethasone alone could not significantly elevate PPP1R3G at both protein and mRNA levels (Fig. 1*F* and *G*). However, when the cells were treated with these two factors together, PPP1R3G was significantly elevated at both protein and mRNA levels (Fig. 1*F* and *G*). We next analyzed the activity of a putative *PPP1R3G* promoter that contained a 2-kb fragment in the 5′ region upstream of *PPP1R3G* coding region. In concert with the change at the protein and mRNA levels, the *PPP1R3G* promoter activity was also significantly induced by dexamethasone and glucagon (Fig. 1*I*). We found that PPP1R3G mRNA level and PPP1R3G promoter activity were both reduced by insulin (Fig. 1*H* and *J*), indicating that insulin signaling likely plays a role to turn off PPP1R3G expression during refeeding.

Interaction of PPP1R3G with the catalytic subunit of PP1. To confirm whether PPP1R3G is indeed a glycogen-related regulatory subunit of PP1, we first investigated whether PPP1R3G and PP1c were colocalized in the cell. When the catalytic subunit of PP1 (the α isoform of PP1c, the same for other experiments used in Fig. 2) was expressed alone, it was localized in the cytoplasm and nuclei (Fig. 2*A*), consistent with a previous report (26). However, when coexpressed with PPP1R3G, the cytoplasmic

mice were serum-starved for 10 h and then treated with 100 nmol/L glucagon or 1 μmol/L dexamethasone (Dex) for 12-h reagents as indicated for 12 h. The cell lysate was used in immunoblotting with the antibodies as indicated. *G* and *H*: Effect of glucagon, Dex, and insulin on PPP1R3G mRNA level. Primary hepatocytes isolated from overnight-fasted mice were serum-starved for 10 h, treated with 100 nmol/L glucagon, 1 μmol/L Dex, or 100 nmol/L insulin as indicated for 12 h, followed by RT-qPCR. The fold change of PPP1R3G mRNA in the control group is set to 1. Data are shown as mean ± SD. *$P < 0.05$ in comparison with control group. *I* and *J*: Effect of glucagon, Dex, and insulin on PPP1R3G promoter activity. HepG2 cells were transiently transfected with a PPP1R3G promoter luciferase reporter, serum-starved for 24 h, and then treated the reagents as in *G* for 12 h, followed by luciferase assay. A renilla luciferase was cotransfected and used a reference control. Data are shown as mean ± SD. *$P < 0.05$ and ***$P < 0.001$ compared with the untreated control. WAT, white adipose tissue.

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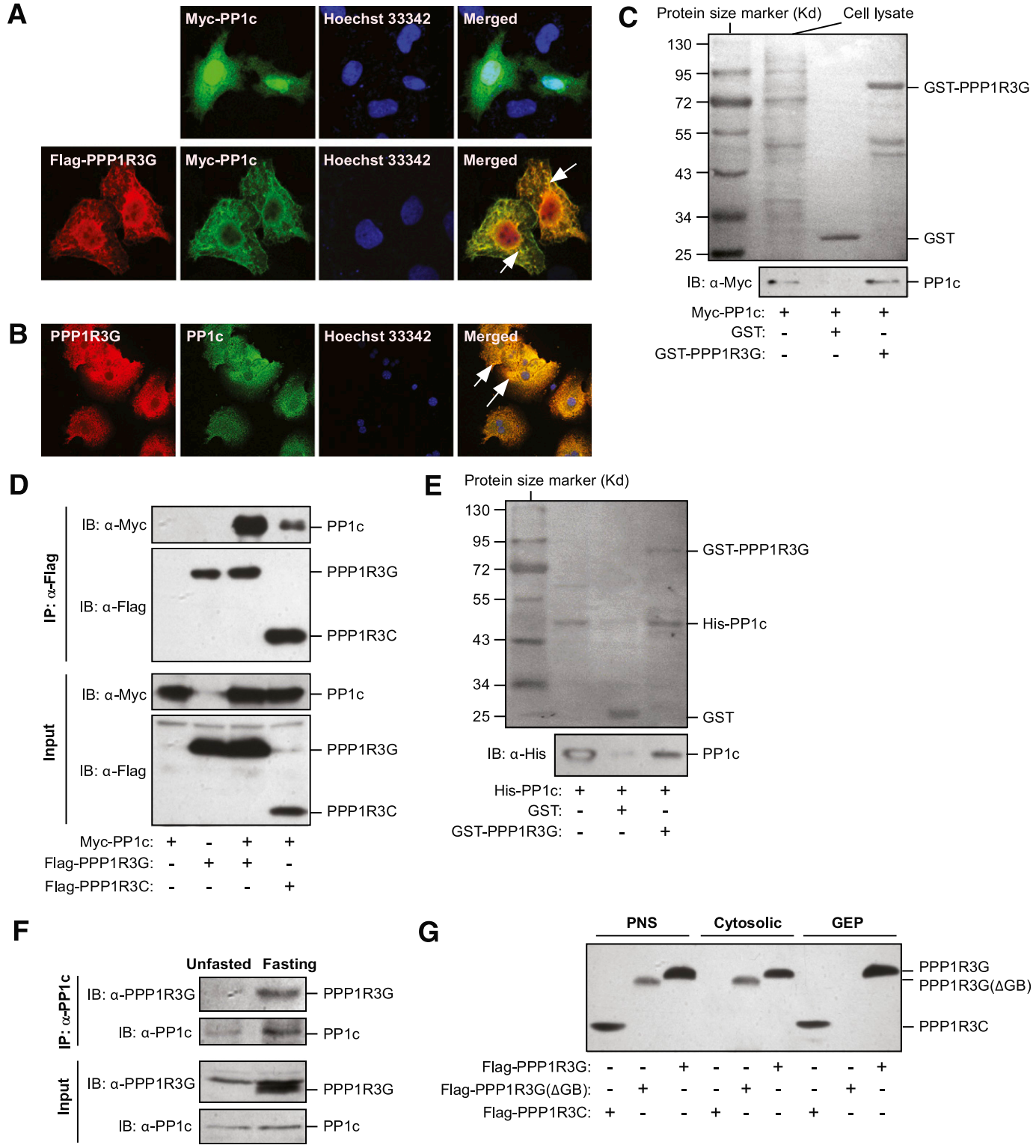

FIG. 2. Interaction of PPP1R3G with PP1c. ***A*****: PPP1R3G colocalizes with PP1c in the cytoplasm. Hela cells were transfected with Myc-PP1c alone (*top*) or together with Flag-tagged PPP1R3G (*bottom*), followed by immunostaining and confocal analysis. Arrows indicate apparent colocalization of PPP1R3G with PP1c.** ***B*****: Endogenous PPP1R3G colocalizes with endogenous PP1c in the cytoplasm. Primary hepatocytes isolated from overnight-fasted mice were used in immunostaining with the antibodies as indicated. Arrows indicate apparent colocalization of PPP1R3G with PP1c.** ***C*****: Interaction of PPP1R3G with PP1c as analyzed by GST pull-down assay. HEK293T cells were transiently transfected with Myc-tagged PP1c. Twenty-four hours after transfection, cell lysate was incubated with GST proteins as indicated (*top*, Coomassie-stained gel), and the GST pull-down product was used in immunoblotting (*bottom*).** ***D*****: In vivo interaction of overexpressed PPP1R3G with PP1c. HEK293T cells were transiently transfected with Flag-tagged PPP1R3G, Flag-tagged PPP1R3C, and Myc-tagged PP1c as indicated. Twenty-four hours after transfection, cell lysate was used in immunoprecipitation (IP) and immunoblotting (IB) with the antibodies as indicated.** ***E*****: In vitro interaction of PPP1R3G with PP1c. Purified GST, GST-PPP1R3G, and His-PP1c (*top*, Coomassie-stained gel) were incubated as indicated, followed by GST pull-down and immunoblotting (*bottom*).** ***F*****: Interaction of endogenous PPP1R3G with PP1c. Male C57BL/6 J mice at 8 weeks of age were killed in the feeding state or after 12 h fasting. Liver protein was used in IB and IP.** ***G*****: PPP1R3G is localized at the GEPs. HepG2 cells were transfected using the plasmids as indicated. Twenty-four hours after transfection, the cells were homogenized and post-nuclear supernatant (PNS) was used in fractionation and IB with an anti-Flag antibody. (A high-quality digital representation of this figure is available in the online issue.)**

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localization of PP1c in the cytoplasm was significantly increased with a profound colocalization with PPP1R3G (Fig. 2*A*). Endogenous PP1c and endogenous PPP1R3G also had a profound cytoplasmic colocalization in primary hepatocytes isolated from overnight-fasted mouse (Fig. 2*B*).

We used GST pull-down and coimmunoprecipitation assays to analyze the interaction between the two proteins. We found that the purified PPP1R3G GST fusion protein could pull down PP1c in vitro (Fig. 2*C*). At the in vivo level, these two proteins could also interact with each other in a coimmunoprecipitation assay when they were coexpressed in HEK293T cells (Fig. 2*D*). As a positive control, a known PP1 regulatory subunit, PPP1R3C (27), could also interact with PP1c in the assay (Fig. 2*D*). Next, we explored whether the interaction is direct. We found that the purified His-tagged PP1c protein was able to interact with the purified PPP1R3G GST fusion protein in vitro (Fig. 2*E*). At the animal level, the interaction of endogenous PPP1R3G with endogenous PP1c in the liver of fasted mice was confirmed (Fig. 2*F*). In addition, the β isoform of PP1c was also able to colocalize with PPP1R3G (Supplementary Fig. 2*A*) and interact with PPP1R3G (Supplementary Fig. 2*B* and *C*).

To determine whether PPP1R3G is localized in the glycogen-enriched pellet (GEP) fraction within the cell, we fractionated PPP1R3G-transfected HepG2 cells by ultracentrifugation to obtain cytosolic and GEP fractions from post-nuclear supernatant (28). PPP1R3G could be found in both post-nuclear supernatant and GEP fractions (Fig. 2*G*), and deletion of the predicted glycogen-binding motif led to complete loss of GEP localization of PPP1R3G (Fig. 2*G*). Furthermore, PPP1R3G could be detected in the supernatant fraction after GEP fractions were treated with α-amylase and sedimentation, further indicating glycogen association of PPP1R3G (Supplementary Fig. 2*D*).

PPP1R3G stimulates glycogen synthesis in hepatocytes. We next investigated the functional role of PPP1R3G in glycogen synthesis. We constructed an adenovirus that could overexpress PPP1R3G in mouse hepatocytes (Fig. 3*A*). In vivo staining of glycogen in the primary hepatocytes and HepG2 cells revealed that the cells with high expression of PPP1R3G were associated with an increase of glycogen content (Supplementary Fig. 3*A* and *B*). Direct measurement of glycogen also demonstrated that PPP1R3G dose-dependently increased the glycogen content in primary hepatocytes (Fig. 3*B*). However, when the predicted glycogen binding motif of PPP1R3G was deleted, the glycogen-stimulating effect was completely abrogated (Supplementary Fig. 3*B* and *C*).

Although overexpression of PPP1R3G itself was able to elevate glycogen content in the absence of glucose in the cultured cells (Fig. 3*C*), PPP1R3G-induced elevation of glycogen synthesis was more effective with increasing the level of glucose than the cells without PPP1R3G overexpression (Fig. 3*C*), indicating that PPP1R3G-regulated glycogenesis is dependent on available glucose substrate. On the other hand, it has been proposed that glycogen-targeting subunits of PP1 are involved in insulin regulation of glycogen synthesis (29,30). We found that insulin could increase glycogenesis in primary hepatocytes with or without PPP1R3G overexpression (Fig. 3*D*). Meanwhile, forskolin could reduce glycogen content in the presence of overexpressed PPP1R3G (Fig. 3*E*). To further confirm that PPP1R3G is able to elevate glycogenesis, we used small interfering RNA strategy to silence the expression of endogenous PPP1R3G. Two of three shRNA sequences could significantly downregulate PPP1R3G expression at both the mRNA level (Fig. 3*F*) and protein level (Fig. 3*G*). Consistently, these two shRNA sequences reduced glycogen content in primary hepatocytes (Fig. 3*H*). We also analyzed glucose dependence of PPP1R3G shRNA (#962) on glycogenesis and found that PPP1R3G knockdown could reduce glycogen content at various glucose concentrations (Fig. 3*I*). Collectively, our data indicate that PPP1R3G is a regulatory subunit of PP1 to facilitate stimulation of glycogen synthesis in hepatocytes.

In vivo function of PPP1R3G on glycogen synthesis and blood glucose homeostasis. We next analyzed the in vivo function of PPP1R3G on the regulation of glycogen synthesis and blood glucose homeostasis. We first used recombinant adenovirus to deliver PPP1R3G-expressing plasmid to the mouse liver. Previous studies have shown that intravenous administration of adenoviral vectors in mice almost exclusively targets the transgene to the liver (31). C57BL/6 J mice were injected with the control or PPP1R3G-expressing adenovirus. Evaluation of aminotransferases in the serum indicates that the PPP1R3G-expressing adenovirus did not cause apparent functional damage to the liver (Supplementary Fig. 4*A*).

By immunoblotting assay and real-time RT-PCR, we confirmed that the PPP1R3G-expressing adenovirus was able to drive abundant expression of PPP1R3G in the mouse liver (Fig. 4*A* and *B*). The PPP1R3G mRNA level was elevated by the adenovirus to ~12-fold (Fig. 4*B*), comparable to the level induced by fasting (Fig. 1*A*). Consistent with the observation with cultured hepatocytes, the liver glycogen content of mice infected with PPP1R3G-expressing adenovirus was significantly elevated, reaching to approximately threefold higher than in control animals (Fig. 4*C*). We next performed glucose tolerance tests with the animals. PPP1R3G overexpression led to a decrease in fasting blood glucose level and an increase in glucose clearance rate (Fig. 4*D*), with the area under curve (AUC) of glucose tolerance test reducing by ~20% (Fig. 4*E*). However, insulin tolerance test revealed no difference between the two groups of mice (Fig. 4*F*), indicating that insulin sensitivity is not altered by PPP1R3G overexpression in vivo. Collectively, these data suggest that overexpressed PPP1R3G could increase clearance of blood glucose likely by increasing conversion of blood glucose into liver glycogen.

To support our hypothesis that fasting-induced PPP1R3G is involved in postprandial blood glucose homeostasis, we injected the mice with recombinant adenovirus that contains shRNA specific for PPP1R3G, and the adenovirus did not cause apparent functional damage to the liver (Supplementary Fig. 4). When PPP1R3G was silenced in the liver, the fasting-induced PPP1R3G expression was largely abrogated (Fig. 5*A*). After 12-h fasting, the mice infected with PPP1R3G shRNA adenovirus had less hepatic glycogen content than the control mice (Fig. 5*B*). Refeeding for 1 h increased the hepatic glycogen content, and such increase was significantly attenuated when PPP1R3G was downregulated (Fig. 5*B*). Most important, the postprandial blood glucose clearance rate was altered when PPP1R3G expression was silenced. The glucose tolerance test revealed that the glucose clearance rate was significantly decreased in PPP1R3G-downregulated mice in comparison with the control animals (Fig. 5*C*), with AUC increasing by ~50% (Fig. 5*D*). However, insulin sensitivity did not seem to be affected by downregulation of PPP1R3G (Fig. 5*E*).

To provide further evidence that PPP1R3G is directly involved in postprandial hepatic glycogenesis, we analyzed

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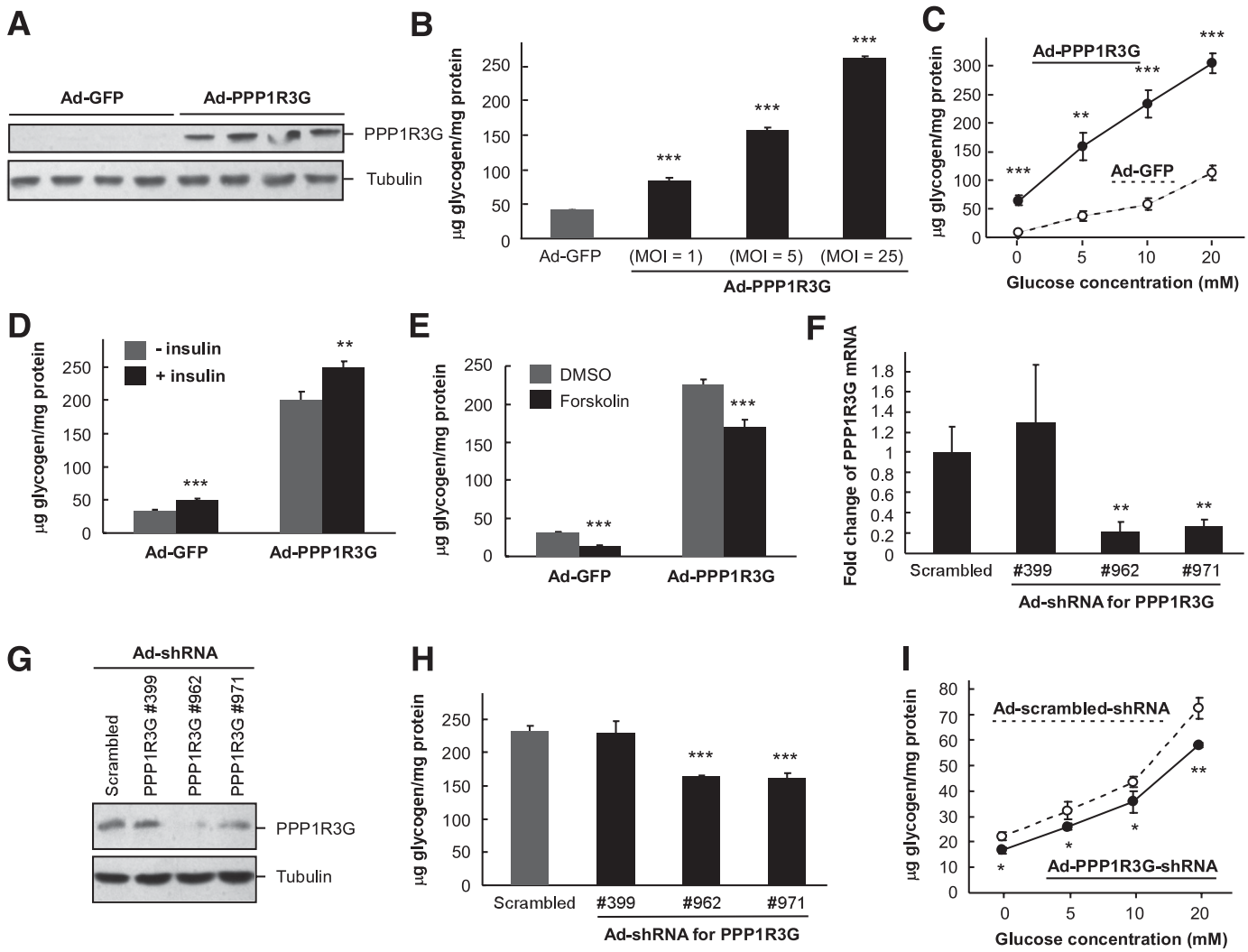

FIG. 3. PPP1R3G stimulates glycogen synthesis in hepatocytes. *A*: Primary hepatocytes isolated from overnight-fasted mice were cultured overnight and then treated with 25 multiplicity of infection (MOI) of Ad-GFP or Ad-PPP1R3G with a Flag tag at the *N*-terminus. Twenty-four hours later, the cells were harvested for immunoblotting with antibodies as indicated. *B*: Primary hepatocytes isolated from overnight fasted mice were infected with Ad-GFP or Ad-PPP1R3G at different MOI, followed by glycogen measurement. ***$P < 0.001$ in comparison with the Ad-GFP group. *C*: The cells as in *A* were treated for 24 h with various glucose concentrations, followed by measurement of glycogen content. Data are shown as mean ± SD. **$P < 0.01$ as comparison between the two groups. *D* and *E*: The cells as in *A* were treated with 100 nmol/L insulin (for *D*) or 25 μmol/L forskolin (for *E*) for 24 h, followed by measurement of glycogen content. Data are shown as mean ± SD. **$P < 0.01$ and ***$P < 0.001$ as comparison between the treated and untreated groups. *F*: Hepatocytes isolated from overnight-fasted mice were cultured overnight and then treated with 25 MOI of Ad-shRNA as indicated. Cells were harvested 72 h later and analyzed by RT-qPCR. The fold change of PPP1R3G mRNA in Ad-Scrambled shRNA group is set to 1. **$P < 0.01$ in comparison with the Ad-Scrambled shRNA group. *G*: Cell lysate from hepatocytes of *G* was used in immunoblotting with the antibodies as indicated. *H*: Cells as in *G* were used to measure glycogen content. ***$P < 0.001$ as compared with the scrambled shRNA group. *I*: Primary hepatocytes isolated from overnight-fasted mice were cultured overnight and then treated with 25 MOI of adenovirus as indicated. Forty-eight hours later, the cells were treated at various glucose concentrations for 24 h, followed by measurement of glycogen content. Data are shown as mean ± SD. *$P < 0.05$ and **$P < 0.01$ compared between the two groups.

the amount of newly synthesized glycogen in the mouse by using ^{3}H-labeled glucose as a tracer. Mice injected with control or PPP1R3G-shRNA adenovirus were fasted for 12 h, followed by intraperitoneal injection of glucose containing a trace amount of ^{3}H -glucose. The mice were killed in 1 h, and the isolated liver glycogen was subjected to radioactivity measurement. As shown in Fig. 5*F*, the amount of newly synthesized glycogen in the liver was reduced to ~50% by PPP1R3G knockdown. These data collectively indicate that PPP1R3G is directly involved in postprandial regulation of hepatic glycogenesis.

PPP1R3G modulates the activity of GS in coordination with the fasting–refeeding cycle. Because GS and GP are two key enzymes modulated by PP1-mediated dephosphorylation, we investigated whether PPP1R3G could influence the activities of these two enzymes. When PPP1R3G was overexpressed by adenovirus in the liver in the fed state, GS activity was markedly elevated (Fig. 6*A*), whereas GP activity was not affected (Fig. 6*B*). Because our results indicate that PPP1R3G mainly acts on GS instead of GP to regulate glycogenesis in the liver, we next focused on analyzing the effect of PPP1R3G on GS activity during the fasting–feeding cycle (Fig. 6*C*). In the fasting–feeding cycle, GS activity deceased ~50% after fasting for 6 h and slightly elevated to the unfasted level in 24 h. However, GS activity was robustly increased on refeeding for 1 to 2 h and subsequently declined to a very low level in 12 h. This phenomenon is consistent with previous reports (32–34). We

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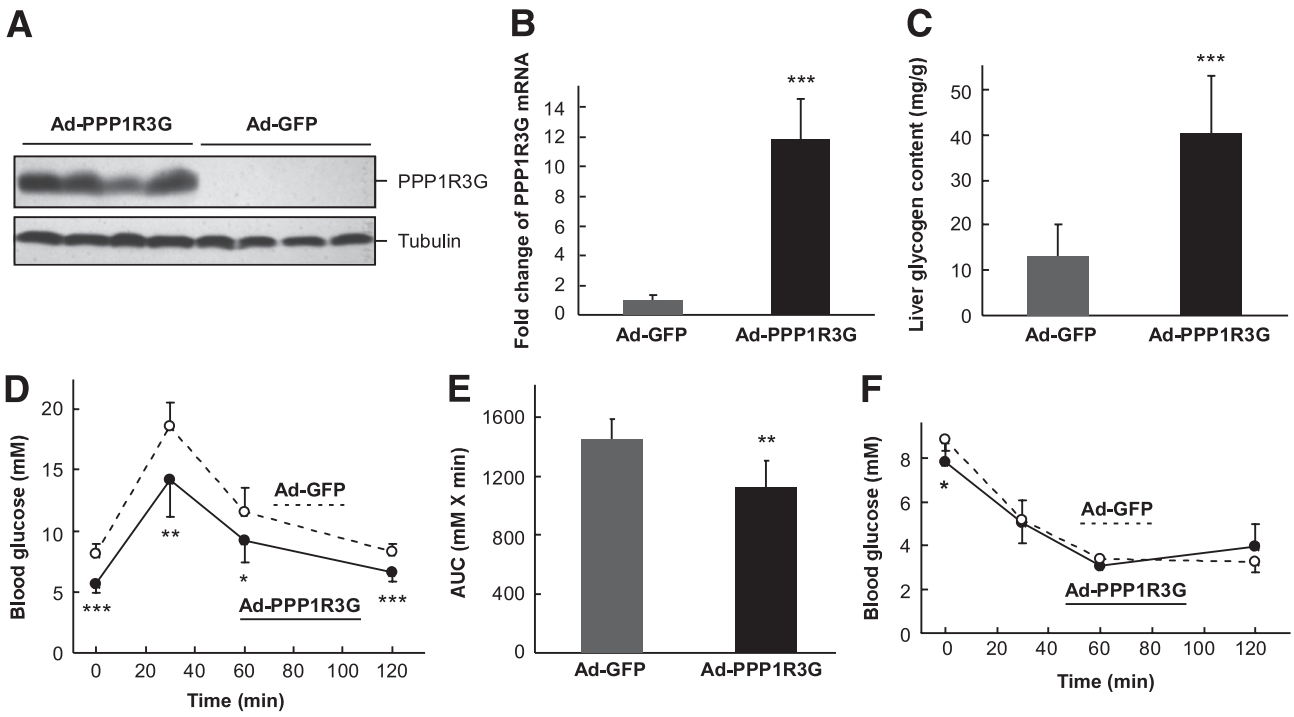

FIG. 4. PPP1R3G overexpression accelerates postprandial blood glucose clearance in the mouse. *A*: Overexpression of PPP1R3G in the mouse liver. Male C57BL/6 J mice at 8 weeks of age were infected with adenovirus Ad-GFP or Ad-PPP1R3G (containing a Flag tag at *N*-terminus) via tail-vein injection. At 9 days post-infection, animals were killed in the fed state. The livers were used in immunoblotting with antibodies as indicated. *B*: PPP1R3G mRNA level in the liver was measured by RT-qPCR (n = 6 mice/group) from the samples in *A*. Data are shown as mean ± SD. ***$P < 0.001$ between the two groups. *C*: Liver glycogen content from samples in *A* were measured (n = 8 for Ad-GFP and n = 9 for Ad-PPP1R3G). ***$P < 0.001$ as compared between the two groups. *D–F*: Glucose tolerance test (for *D*) and insulin tolerance test (for *F*) were performed at 4 days post-infection (n = 7 for Ad-GFP and n = 8 for Ad-PPP1R3G). Mice were fasted for 4 h before glucose injection or insulin injection. Bar graph (*E*) represents the AUC calculated from the glucose tolerance test. Data are shown as mean ± SD. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ between the two groups of mice.

next investigated how PPP1R3G knockdown could affect GS activity during the fasting–feeding cycle (Fig. 6*C*). We found that the major effect of PPP1R3G knockdown is to reduce GS activity around the fasting–refeeding transition. During constant feeding and at early fasting or refeeding for 12 h, PPP1R3G knockdown only slightly reduced GS activity. However, the GS activity was decreased to >40% in the liver at fasting for 24 h and at refeeding for 1 to 2 h when PPP1R3G was silenced. Accordingly, we found that the phosphorylation level of GS at Ser641 in mouse livers was very high during fasting and robustly reduced by refeeding for 1 to 2 h (Supplementary Fig. 5), consistent with the observation that GS activity was robustly stimulated by refeeding (Fig. 6*C*). Furthermore, GS phosphorylation at Ser641 was elevated by PPP1R3G knockdown during refeeding (Supplementary Fig. 5), consistent with the finding that GS activity was reduced by PPP1R3G knockdown (Fig. 6*C*). Collectively, these data indicate that PPP1R3G mainly functions during the period of fasting–refeeding transition to regulate GS activity.

It is noteworthy that PPP1R3G is not the only glycogen-targeting regulatory subunit of PP1 to regulate hepatic glycogenesis. In addition to PPP1R3G, other glycogen-targeting regulatory subunits of PP1, such as PPP1R3B, 3C, 3D, and 3E, are also expressed in the liver (13,27,35,36). In the fed state, G_L/PPP1R3B accounts for ~60% of GS phosphatase activity, and the PPP1R3C, 3D, and 3E account for the remaining activity (13,37). To investigate how these glycogen-targeting regulatory subunits orchestrate to control hepatic glycogenesis, we analyzed the expression patterns of PPP1R3B, 3C, 3D, 3E, and 3G at different times during fasting and refeeding (Fig. 6*D*). The mRNA of PPP1R3A and PPP1R3F was hardly detectible in the liver in our experiment (data not shown). Fasting for 24 h could reduce the mRNA levels of PPP1R3B, 3C, and 3D, whereas refeeding for 1 h significantly elevated the mRNA levels of these three subunits. The mRNA level of PPP1R3E seemed to be unaffected by fasting and refeeding. In contrast, the expression of PPP1R3G was markedly stimulated by fasting and rapidly reduced by refeeding. Taken together, these data indicate that the expression pattern of PPP1R3G differs from other glycogen-targeting subunits during the fasting–feeding cycle.

DISCUSSION

So far there are seven glycogen regulatory subunits (G subunits) of PP1 in mammals, PPP1R3A to PPP1R3G (11,13). Phylogenetic tree analysis reveals that although all seven human subunits and their rodent orthologs possess known or putative PP1-interacting and glycogen-binding domains, none of the subunits shares more than 40% amino acid identity, suggesting that each subunit may serve a nonredundant function in mammals (13). In this study, we demonstrate that PPP1R3G is indeed a glycogen-targeting regulatory subunit of PP1. At the cellular level, PPP1R3G is associated with glycogen pellet, interacts with the catalytic subunit of PP1, and regulates GS activity. At the animal level, PPP1R3G is able to regulate glycogen synthesis in the liver and modulate blood glucose homeostasis. Fasting glucose level is reduced when PPP1R3G is overexpressed in the liver. On the other hand, hepatic silencing of PPP1R3G

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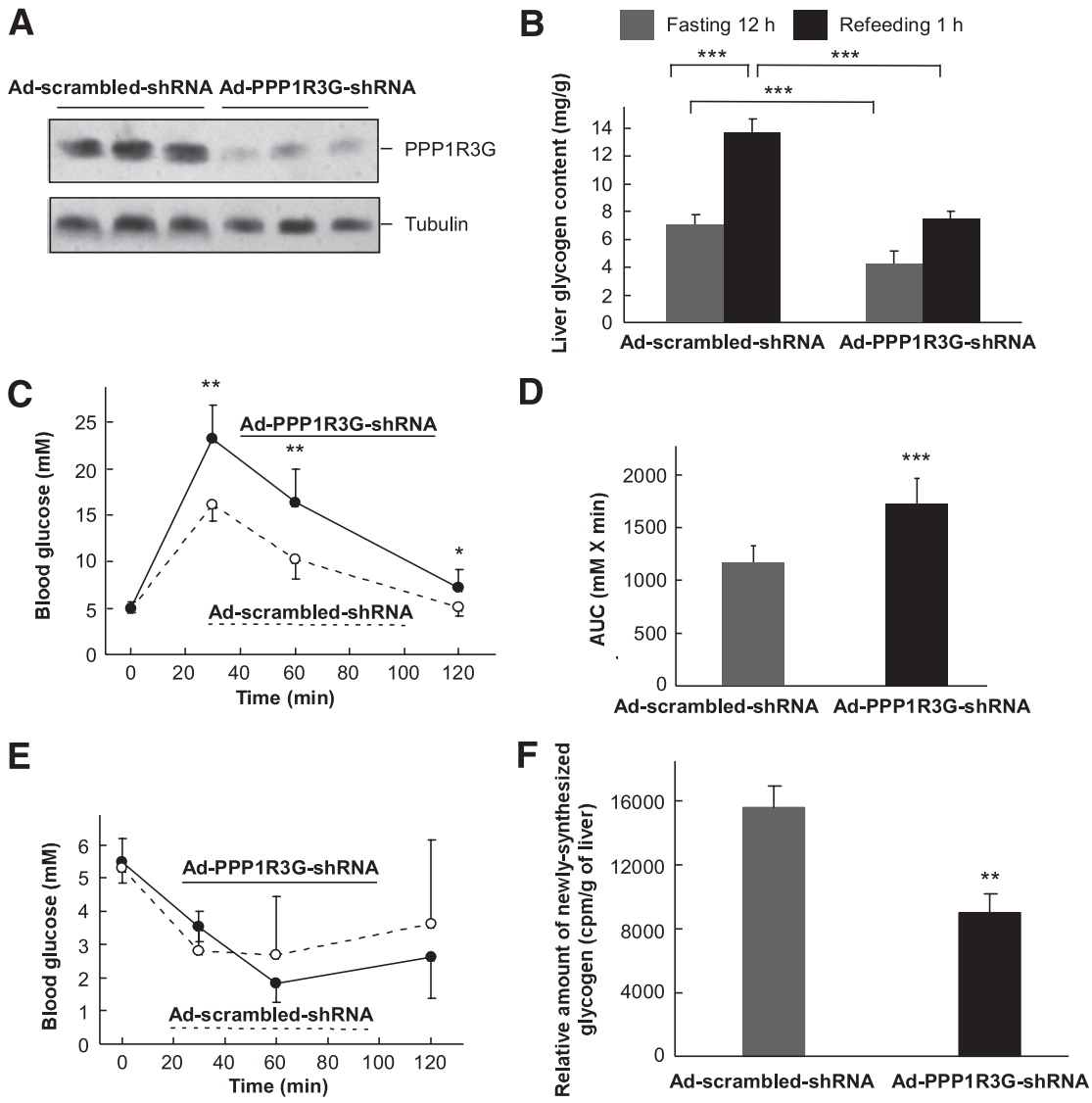

FIG. 5. PPP1R3G knockdown slows down postprandial blood glucose clearance in the mouse. *A*: Knockdown of PPP1R3G in the mouse liver. Male C57BL/6 J mice at 8 weeks of age were infected with shRNA adenovirus as indicated via tail-vein injection. At 15 days post-infection, animals were killed in the fasted state (overnight fasting). Liver proteins were used in immunoblotting with the antibodies as indicated. *B*: Liver glycogen content was measured from mice as in *A* after fasting and feeding for the time as indicated. Data are shown as mean ± SD. ***$P < 0.001$ between groups as indicated. *C–E*: Glucose tolerance test (for *C*) and insulin tolerance test (for *E*) were performed at 4 days post-infection ($n = 6$ for Ad-Scrambled-shRNA and $n = 8$ for Ad-PPP1R3G-shRNA). Mice were fasted for 12 h before the test. AUC (for *D*) was calculated from the glucose tolerance test. Data are shown as mean ± SD. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ between the two groups. *F*: Postprandial newly synthesized liver glycogen using D-[3-^{3}H]glucose as a tracer was measured with mice at 7 days post-infection ($n = 8$ for both Ad-Scrambled-shRNA and Ad-PPP1R3G-shRNA groups). Mice were fasted for 12 h and intraperitoneally injected with glucose (2 g/kg body wt) containing trace amount of D-[3-^{3}H]glucose. Mice were killed in 1 h, and liver glycogen was isolated and used in scintillation counting. Data are shown as mean ± SE. **$P < 0.01$ between the two groups.

reduces postprandial elevation of GS activity and slows down postprandial clearance of blood glucose. Collectively, our data reveal for the first time that PPP1R3G is a functional regulatory subunit of PP1 and plays a role in regulating GS activity, hepatic glycogenesis, and postprandial blood glucose homeostasis.

One of the most intriguing findings of this study is that PPP1R3G is involved in the regulation of hepatic glycogenesis in a manner coupled to the fasting–feeding cycle and distinct from other G subunits, especially G_L/PPP1R3B, a major protein that regulates liver glycogen metabolism. It was originally reported in the early 1970s that liver GSP is inhibited by GPa (38), explaining why GS becomes inhibited while GP is activated. It was later found that glucose binds to GPa and promotes its dephosphorylation and inactivation, thereby terminating the inhibition on GSP by GPa so that glycogen can be resynthesized when blood glucose is high after a meal (8). In the mid-1980s it became clear that a form of PP1 was the major hepatic GSP in the fed state and that this enzyme was inhibited allosterically by GPa (39–41). The liver GSP in the fed state was later purified and shown to be a complex of GL/PPP1R3B with PP1 (42). The allosteric binding site for GPa was found to be located on the G_L/PPPR3B subunit, and not the PP1 catalytic subunit (42), and the GPa-binding site was located at the extreme C-terminus of G_L/PPPR3B (43,44). Mice that expressed G_L/PPP1R3B(Y284F) mutant that could not by inhibited by GPa consistently showed enhanced activation of hepatic GS and conversion of blood glucose into hepatic glycogen (16). This information has been exploited to

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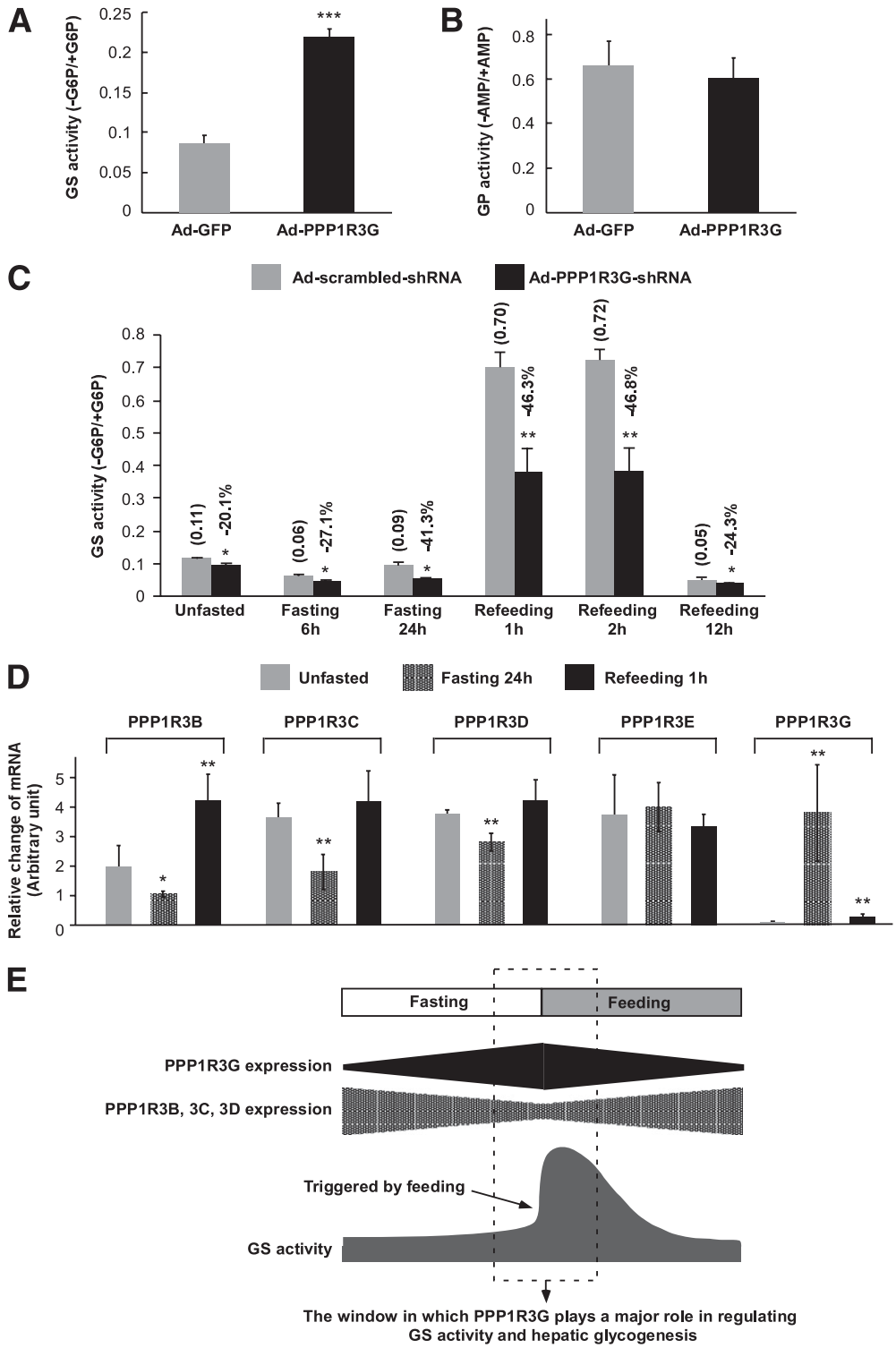

FIG. 6. PPP1R3G modulates the activity of GS in coordination with the fasting–refeeding cycle. *A* and *B*: Liver GS and GP activities were measured from the samples as in Fig. 4*A*. Data are shown as mean ± SD. ***$P < 0.001$ between the two groups. *C*: Male C57BL/6 J mice at 8 weeks of age were infected with shRNA adenovirus as indicated via tail-vein injection. Seven days after infection, animals were killed in constant feeding state, after fasting, or after refeeding for the time as indicated ($n = 4$ or 5 mice/group). Liver GS activity is shown as mean ± SE. *$P < 0.05$ and **$P < 0.01$. The numbers above the bars of Ad-scrambled-shRNA group mark the actual GS activity. The numbers above the bars of Ad-PPP1R3G group represent the percentage decrease of GS activity in this group in comparison with the control group, respectively. *D*: Expression patterns of PPP1R3G and other glycogen-targeting regulatory subunits of PP1 in the liver during fasting and refeeding. The liver samples of mice ($n = 4$ mice/group) with fasting and refeeding for different amounts of time were used to determine the mRNA levels of PPP1R3B, 3C, 3D, 3E, and 3G by RT-qPCR. Data are shown as mean ± SD. *$P < 0.05$ and **$P < 0.01$ compared with the unfasted group. *E*: Simplified model to depict the expression patterns of glycogen-targeting regulatory subunits of PP1 in the liver during the fasting–feeding cycle and their roles in regulating GS activity and hepatic glycogenesis in mouse. Fasting increases PPP1R3G expression but decreases the expression of other glycogen-targeting regulatory subunits of PP1, including PPP1R3B, 3C, and 3D, whereas refeeding has an opposite effect. The combined effects of PPP1R3G together with other glycogen-targeting regulatory subunits of PP1 determine how these subunits contribute to GS activity and liver glycogenesis at different phases of the fasting–feeding cycle. Because of its unique expression pattern, PPP1R3G plays a major role in regulating hepatic glycogenesis during the fasting–feeding transition. For simplicity purposes, the expression pattern of PPP1R3E is not included because its expression level is not changed during the fasting–feeding cycle in the liver.

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develop small molecule inhibitors to disrupt the interaction between GPa and G_L/PPP1R3B to enhance the conversion of blood glucose to hepatic glycogen (45). It is noteworthy that inhibition by GPa is the unique feature of G_L/PPP1R3B not shared by any other G subunit, including PPP1R3G. We propose that the lack of a GPa binding site in PPP1R3G, and therefore presumably the lack of allosteric inhibition of the PP1-PPP1R3G complex by GPa, comprises a crucial difference from the PP1-PPP1R3B complex. Such a difference may explain why PPP1R3G is needed at the fasting–feeding transition. During starvation, when the glucagon/insulin ratio is high, GP would be expected to be largely in the active form. At the early stage of the fasting–feeding transition when GPa has not been inactivated, one would not want GPa to inhibit GSP activity; otherwise, GS activity would not be activated efficiently and the glucose could not be rapidly used to replenish hepatic glycogen after a meal. At the early stages of the fasting–feeding transition, the expression of PPP1R3G reaches its maximum while G_L/PPP1R3B expression is minimal (Fig. 6*D*). Thus, PP1-PPP1R3G complex may function as the major GSP at this time. In the normally fed state, the PP1-PPP1R3B complex may replace the PP1-PPP1R3G complex as the major GSP so that the important allosteric regulation by GPa can be introduced. Consistent with our model, it was found that G_L/PPP1R3B accounts for ~60% of GSP activity in the fed state (13,37). Because of the functional and expressional difference between PPP1R3G and other G subunits, especially G_L/PPP1R3B in the liver, it is expected that the major physiologic mission of PPP1R3G is to ensure rapid activation of GS and rapid glycogen synthesis in the liver shortly after a meal, subsequently contributing to postprandial glucose clearance (Fig. 6*E*).

When feeding triggers stimulation of GS activity via different means, such as a rapid increase of blood glucose and insulin levels, insulin-mediated phosphorylation and inactivation of GSK-3, translocation of GS to the cellular periphery, conversion of intracellular glucose to G6P, and portal signals (2,5,10,11), the PPP1R3G-mediated GS activity would rapidly lead to hepatic glycogenesis and removal of postprandial blood glucose. In a simplistic way, and as judged by the increase of AUC in the glucose tolerance test in PPP1R3G knockdown mice (Fig. 5*D*), the reduction of postprandial newly synthesized liver glycogen (Fig. 5*F*), and the decrease of GS activity by these mice shortly after refeeding (Fig. 6*C*), it can be estimated that at least 50% of the postprandial hepatic glycogen synthesis and the reduction in blood glucose are mediated by a PPP1R3G-mediated mechanism. In humans, hepatic glycogenesis is reduced in diabetic patients, and genetic variations of genes involved in glycogen metabolism have been found in diabetic patients (46–48). In mice, although Suzuki et al. (14) reported that deletion of G_M/PPP1R3A had no obvious defects, other reports indicate that deletion of G_M/PPP1R3A leads to increased weight gain, obesity, glucose intolerance, and insulin-resistance (15,49). Hepatic expression of a C-terminus–truncated form of G_M/PPP1R3A in streptozotocin-induced diabetic rats can reverse hyperglycemia and hyperphagia (50). Mice that expressed the G_L/PPP1R3B(Y284F) mutant that could not be inhibited by GPa had an enhanced activation of hepatic GS activity and improved glucose tolerance (16). In addition, heterozygous deletion of PTG/PPP1R3C in mice led to glucose intolerance, hyperinsulinemia, and insulin resistance with aging (17). Therefore, the next challenge will be to determine whether alteration of PPP1R3G is associated with insulin resistance and type 2 diabetes and whether modulation of PPP1R3G can serve as a new strategy to improve glucose metabolism.

ACKNOWLEDGMENTS

This work was supported by research grants from the Chinese Academy of Sciences (KSCX2-EW-R-08), the National Natural Science Foundation of China (30830037 and 81021002), and the Ministry of Science and Technology of China (2007CB947100) to Y.C. The work was also supported by the Ministry of Science and Technology of China (2010CB529506 to Y.P. and Z.W.) and the National Natural Science Foundation of China (30971660 to Y.P.).

No potential conflicts of interest relevant to this article were reported.

X.L. performed the experiments, analyzed data, and wrote the article. Y.Z., X.R., X.J., L.Z., X.W., and Q.D. performed the experiments. W.L., Y.P., and Z.W. contributed the reagents, material, and analysis tools. Y.C. analyzed data, wrote the article, and conceived and designed the experiments.

The authors thank Drs. Peter J. Roach and Anna A. DePaoli-Roach at the Indiana University School of Medicine for valuable suggestions and discussion; Qiong Wang and Lei Jiang at the Institute for Nutritional Sciences (INS) for technical assistance with adenovirus purification; Shanshan Pang and Haiqing Tang at the INS for assistance with tail-vein injection; Ting Mao and Mengle Shao at the INS for assistance with hepatocyte isolation; and Dr. Yong Liu at the INS for providing recombinant GFP adenovirus.

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