Original Article

MOLECULAR METABOLISM

Purβ promotes hepatic glucose production by increasing Adcy6 transcription

Linna Jia [1,2], Yunfeng Jiang [2], Xinzhi Li [2], Zheng Chen [2,*]

ABSTRACT

Objective: Enhanced glucagon signaling and hepatic glucose production (HGP) can account for hyperglycemia in patients with obesity and type 2 diabetes. However, the detailed molecular mechanisms underlying the enhanced HGP in these patients are not fully understood. Here, we identify Purβ as a positive regulator of HGP and study its molecular mechanisms in the regulation of HGP both *in vivo* and *in vitro*.

Methods: Adenovirus-mediated knockdown or overexpression of Purβ was performed in either primary hepatocytes or the livers of db/db mice. Glucose metabolism, insulin sensitivity, and HGP were determined by glucose, insulin, and lactate tolerance tests, respectively. Purβ/ADCY6 protein levels, glucagon signaling (p-CREB/CREB), and insulin signaling (p-Akt/Akt) were measured by immunoblotting. Gene expression was measured by RNA-seq and real-time quantitative polymerase chain reaction. Luciferase reporter and chromatin immunoprecipitation assays were used to study the interaction between Purβ and the *Adcy6* promoter.

Results: Purβ was abnormally elevated in obese mice and was also increased under fasting conditions or via the glucagon signaling pathway, which promoted HGP by increasing *Adcy6* expression. Liver-specific knockdown of Purβ in db/db mice significantly ameliorated hyperglycemia and glucose intolerance by suppressing the glucagon/ADCY6/cAMP/PKA/CREB signaling pathway. Consistent with this observation, the knockdown of Purβ also inhibited glucose production in isolated primary hepatocytes by inhibiting the glucagon/ADCY6/cAMP/PKA/CREB signaling pathway, whereas the overexpression of Purβ promoted glucose production by activating this signaling pathway. Mechanistically, Purβ directly binds to the promoter of the *Adcy6* gene and thereby promotes its transcription.

Conclusions: Taken together, these results illustrate a new model in which Purβ functions to regulate the glucagon/ADCY6/cAMP/PKA/CREB signaling pathway to help maintain glucose homeostasis.

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Keywords Purβ; Liver; Gluconeogenesis; Glucagon signaling; ADCY6; Obesity

1. INTRODUCTION

The liver is the main organ producing glucose via glycogenolysis and gluconeogenesis in response to fasting. Under fasting conditions, hepatic glucose production (HGP) is increased in response to elevated blood glucagon levels [1,2]. Conversely, under fed conditions, HGP is suppressed by increased plasma insulin levels [1]. Thus, the HGP rate is determined by a balance between glucagon and insulin. Glucagon, via its G protein—coupled receptors, stimulates cAMP-mediated activation of protein kinase A (PKA), and PKA phosphorylates cAMP response element-binding protein (CREB) on Ser^{133} [3,4], which in turn activates the transcription of phosphoenolpyruvate carboxykinase (PEPCK) and glucose 6-phosphatase (G6Pase), both key gluconeogenic genes [3,4]. Insulin inhibits HGP by suppressing the expression of PEPCK and G6Pase [1,5]. In patients with obesity and type 2 diabetes, HGP is abnormally elevated, thus contributing to hyperglycemia and glucose intolerance [2,5]. Enhancement of the glucagon signaling pathway and hepatic insulin resistance are two major causes of enhanced HGP [2]. However, the detailed molecular mechanisms underlying this enhanced HGP in patients with obesity are not fully understood.

Purine-rich element binding proteins, such as Purα, Purβ, and Purγ, which form the evolutionarily conserved Pur family, are able to bind to purine-rich single- or double-stranded DNA or RNA [6]. Pur proteins play important roles in development, especially in the development of brain [7,8] and myeloid cells [8], and they are also involved in many human diseases, including cancer [9—11] and fragile-X mental retardation syndrome [7,8,12,13]. Recently, Purβ has been shown to bind to lncRNA (lnc-HOXA1) and mediate repression of Hoxa1 transcription [14]. In addition, Purβ binds to DNA and negatively or positively regulates gene expression in a cell type—specific manner. For example, Purβ serves as a repressor that inhibits the expression of smooth muscle α-actin in fibroblasts and vascular smooth muscles [15,16], whereas it also acts as a transcription factor that increases mTOR and SREBP1 expression in mammary epithelial cells [17]. However, whether Purβ regulates HGP is largely unknown.

Here we identify Purβ as a positive regulator of HGP. Purβ, induced by fasting or glucagon, promotes HGP by increasing *Adcy6* transcription, leading to cAMP accumulation, increased PKA activity, CREB activation, and increased transcription of *PEPCK* and *G6Pase*, both key gluconeogenic genes. Purβ is abnormally elevated in mice with

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obesity, and liver-specific knockdown of Purβ in db/db mice significantly ameliorates hyperglycemia and glucose intolerance by suppressing the glucagon/ADCY6/cAMP/PKA/CREB signaling pathway. Consistent with this observation, in isolated primary hepatocytes, knockdown of Purβ also inhibits glucose production by inhibiting this signaling pathway. Moreover, Purβ binds to the promoter of the *Adcy6* gene, promoting its expression and activating the cAMP/PKA/CREB signaling pathway. These results support a new model in which Purβ regulates the glucagon/ADCY6/cAMP/PKA/CREB signaling pathway to help maintain glucose homeostasis, indicating that Purβ/ADCY6 may serve as a promising drug target for the treatment of hyperglycemia in patients with obesity.

2. MATERIALS AND METHODS

2.1. Animals

C57BL/6 and db/db mice were purchased from GemPharmatech (Nanjing, China). Mice were housed on a 12-h light/12-h dark cycle and fed either a normal chow or a high-fat diet with free access to water. All animal procedures described in this study were performed in adherence with the *Guide for the Care and Use of Laboratory Animals* (National Institutes of Health, Bethesda, MD, USA) and with the approval by the Institutional Animal Care and Use Committee of Harbin Institute of Technology.

Liver-specific Purβ knockdown db/db mice were generated via tail vain injection of a purified adenovirus expressing sh*PURB*1 for 8—12 days, and the experiments were conducted in conscious mice. In glucose tolerance tests, insulin tolerance tests, glucagon tolerance tests, and lactate tolerance tests, mice were fasted 6 h (8:00 AM—2:00 PM) and intraperitoneally injected with glucose (0.5 g/kg body weight), insulin (4 U/kg body weight), glucagon (6 μg/kg body weight), and sodium lactate (0.5 g/kg body weight), respectively. Blood glucose was monitored after injection. Blood samples were collected from tail veins, and blood glucose was measured as described previously [18,19]. Plasma insulin was measured using insulin enzyme-linked immunosorbent assay (ELISA) kits (MS100, EZassay). Liver triacylglycerol (TAG) levels were measured using Free Glycerol Reagent (Sigma).

2.2. Primary hepatocyte cell cultures, adenoviral infection, and HGP assays

Primary hepatocytes were isolated from C57BL/6 mice by liver perfusion with type II collagenase (Worthington Biochem, Lakewood, NJ) and cultured at 37 °C and 5% CO_2 in DMEM medium supplemented with 5% fetal bovine serum. For Purβ overexpression experiments, primary hepatocytes were infected with βGal or Purβ adenoviruses overnight. For knockdown of Purβ, two target sequences (sh*PURB*1: GTCGGTATGCAGATGAAATGA and sh*PURB*2: GATGAAATGAAAGAGATCCAG) were selected. For Purβ knockdown experiments, primary hepatocytes were infected with Scramble, sh*PURB*1, or sh*PURB*2 adenoviruses for 30 h. These hepatocytes were used for immunoblotting, quantitative polymerase chain reaction, RNA sequencing, cAMP production, PKA activity, and HGP assays. For HGP assays, these hepatocytes were incubated in Hank's Balanced Salt Solution supplemented with 10 mM lactate and 1 mM pyruvate in the presence or absence of glucagon (100 nM) for 4 h. Glucose in the medium was measured and normalized to total protein levels.

2.3. Immunoblotting

For insulin signaling assays *in vivo*, mice were fasted for 20—24 h and administrated insulin (4 units/kg body weight) via the inferior vena cava for 5 min. For insulin signaling assays *in vitro*, hepatocytes were treated with insulin at different doses for 5 min. For glucagon signaling assays, hepatocytes were treated with glucagon for 10 min. Total proteins were extracted from livers or hepatocytes in a lysis buffer (50 mM Tris HCl, pH 7.5, 1.0% NP-40, 150 mM NaCl, 2 mM EGTA, 1 mM Na_3VO_4, 100 mM NaF, 10 mM $Na_4P_2O_7$, and 1 mM phenylmethylsulfonyl fluoride [PMSF]) as described previously [20,21]. Extracts were then immunoblotted with antibodies against phospho-Akt ($pSer^{473}$ and Thr^{308} from CST), total Akt (CST), phospho-CREB ($pSer^{133}$) (CST), total CREB (Proteintech), Purβ (Proteintech), ADCY6 (Proteintech), Flag (Sigma), or Tubulin (Santa Cruz).

2.4. Quantitative real-time PCR (RT-qPCR) analysis

qPCR analysis was performed as previously described [18,19,22]. Briefly, total RNAs were extracted from indicated hepatocytes or livers using TriPure Isolation Reagent (Roche, Mannheim, Germany). The first-strand cDNAs were synthesized using random primers and M-MLV reverse transcriptase (Promega, Madison, WI). RNA abundance was measured using the Roche LightCycler 480 real-time PCR system (Roche, Mannheim, Germany). The expression of individual genes was normalized to the expression of 36B4, a housekeeping gene. Primers for real-time qPCR are listed below: PEPCK-F: 5′-ATCATCTTTGGTGGCCGTAG-3′, PEPCK-R: 5′-ATCTTGCCCTTGTGTTCTGC-3′; G6Pase-F: 5′-CCGGTGTTTGAACGTCATCT-3′, G6Pase-R: 5′-CAATGCCTGACAAGACTCCA-3′; 36B4—F: 5′-AAGCGCGTCCTGGCATTGTCT-3′, 36B4-R: 5′-CCGCAGGGGCAGCAGTGGT-3′; PURB-F: 5′-TGCAACAAGTACGGGGTGTT-3′, PURB-R: 5′-TCAATCCTCA TCCACTTCCT C-3′; Adcy6-F: 5′-GCATCCTGTTTGCGGACATT-3′, Adcy6-R: 5′-ACAGTGATTCTCCCTCACCG-3′; IL1β-F: 5′-GCCTTGGGCCTCAAAGGAAAGAATC-3′, IL1β-R: 5′-GGAAGACACGGATTCCATGGTGAAG-3′; IL6-F: 5′-AGCCAGAGTCCTTCAGA-3′, IL6-R: 5′-GGTCCTTAGCCACTCCT-3′; TNFα-F: 5′-CATCTTCTCAAAATTCGAGTGACAA-3′, TNFα-R: 5′-TGGGAGTAGACAAGGTACAACCC-3′.

2.5. Nuclear run-on RT-qPCR

Nascent mRNA levels were measured by nuclear run-on RT-qPCR following a published protocol [23]. Briefly, primary hepatocytes were infected with βGal or Purβ adenoviruses overnight. Nuclei were extracted in NP-40 lysis buffer (10 mM Tris HCl pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, and 0.5% NP-40) and then incubated with BrUTP and unlabeled ribonucleotides in a transcription reaction buffer supplemented with 100U RNase OUT, 0.5 mM BrUTP, 1 mM ATP, 1 mM GTP, 1 mM CTP, and 0.5 mM UTP at 30 °C for 30 min. Nuclear RNA was extracted using TriPure Isolation Reagent. Labeled and unlabeled nuclear RNA samples were immunoprecipitated with anti-BrdU antibodies. Nascent *Adcy6* mRNA levels were then measured by RT-qPCR and normalized by 36B4.

2.6. cAMP and PKA activity assays

Mice were fasted for 20—24 h, and livers were harvested for cAMP and PKA activity assays. Primary hepatocytes were infected with indicated adenoviruses and then treated with 100 nM glucagon for 10 min. cAMP was measured using an ELISA kit (H164-1-2, Nanjing Jiancheng).

For PKA activity assays, livers and hepatocytes were lysed in buffer containing 20 mM MOPS, 50 mM β-glycerolphosphate, 50 mM sodium fluoride, 1 mM DTT, 1 mM benzamidine, 1 mM PMSF, 10 μg/ml leupeptin and aprotinin. PKA activity assays were performed following the manufacturer's protocol (ab9435, Abcam).

2.7. RNA sequencing

Total RNAs were extracted from hepatocytes using TriPure Isolation Reagent (Roche, Mannheim, Germany). RNA-seq was performed by

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using the Illumina NovaSeq 6000 platform. Paired-end clean reads were aligned to the mouse reference genome (Ensemble_GRCm38.96) with TopHat (version 2.0.12), and the aligned reads were used to quantify mRNA expression by using HTSeq-count (version 0.6.1) as described previously [19]. RNA-seq data that support the findings of this study have been deposited in GEO under accession code GSE136728.

2.8. Luciferase reporter assays

Mouse *Adcy6* promoter (−2001 to −1 or −1001 to −1) luciferase reporter plasmids and β-galactosidase reporter plasmids were transiently cotransfected with Purβ expression plasmids into HEK293T cells using polyethylenimine reagents. Cells were collected 24 h after transfection, and luciferase activity was measured using a luciferase assay system (Promega Corporation). Luciferase activity was normalized to β-galactosidase levels.

2.9. Chromatin immunoprecipitation (ChIP) assays

Primary hepatocytes were isolated from C57BL/6 mice and infected with βGal or Flag-Purβ adenoviruses overnight. These hepatocytes were washed with cold phosphate-buffered saline and fixed with 1% formaldehyde for 10 min at 37 °C. Their nuclei were isolated and subjected to sonication (M220 Focused-ultrasonicator; Covaris) to break genomic DNA into 500- to 1000-bp fragments using a chromatin shearing kit (520127 truChIP Chromatin Shearing Kit, Covaris). The samples were immunoprecipitated with Flag beads (A2220, Sigma). DNA was extracted and used for qPCR analysis. Primers for qPCR were as follows: Adcy6 promoter −74 to −147: 5′-TCATGACATTTCTCT TCCGCCT-3′ (forward) and 5′-AGTGGTAGTGGTGGCGAGAT-3′ (reverse); Adcy6 promoter −288 to −387: 5′-GACTCCCCAAGGGGA-TAACT-3′ (forward) and 5′-GGAGCCCTGTGAGTCCTTTAG-3′ (reverse); Adcy6 promoter −572 to −798: 5′-ATACAACCAGCTCCCACAACC-3′ (forward) and 5′-TCATTTTGCCAACAAGGGCA-3′ (reverse); Adcy6 promoter −1060 to −1211: 5′-GGGAGACACAGGTACCGAAAG-3′ (forward) and 5′-CAATGCCTACTTCCCCAAGGC-3′ (reverse); Adcy6 promoter −1366 to −1543: 5′-TCTGGGCAAGCCTGAAAACT-3′ (forward) and 5′-CAGCGGAGTCCCAAGAGTTG-3′ (reverse); Adcy6 promoter −1558 to −1850: 5′-GATCCCCCACGCTTACCTG-3′ (forward) and 5′-ACAAAAGGAGCTTGTGCCT-3′ (reverse).

2.10. Statistical analysis

Data were presented as means ± SEM. Differences between groups were analyzed by two-tailed Student's *t* tests. $P < 0.05$ was considered statistically significant.

3. RESULTS

3.1. Identification of Purβ as a positive regulator of HGP

HGP is increased during fasting via enhanced glucagon/cAMP/CREB signaling. Thus, fasting-induced genes may regulate HGP. We first measured the levels of the Purβ mRNA and protein in the livers of fasted mice via qPCR and immunoblotting analyses, respectively. As shown in Figure 1A,B, fasting increased the levels of both Purβ mRNA and protein. As fasting increases glucagon secretion and its signaling pathway in the liver, these observations raise the possibility that glucagon may increase the levels of hepatic Purβ in primary hepatocytes. To test this hypothesis, primary hepatocytes were isolated from C57BL/6 mice and treated with glucagon. As shown in Figure 1C,D, glucagon treatment increased both the Purβ mRNA and protein levels. To determine whether Purβ was essential for HGP, primary hepatocytes from C57BL/6 mice were infected with an Ad-sh*PURB*1 or Ad-sh*PURB*2 adenovirus to knockdown Purβ expression, and HGP assays were then performed. Adenovirus-mediated expression of sh*PURB*1 or sh*PURB*2 in primary hepatocytes led to a significant reduction in Purβ (Figure 1E), which resulted in a decrease in HGP under both basal and glucagon-treated conditions (Figure 1F).

To determine whether Purβ promotes HGP, primary hepatocyte cultures were prepared from C57BL/6 mice and infected with Purβ or βGal adenoviruses. The results indicated that recombinant Purβ was dramatically increased in Purβ adenovirus-infected hepatocytes (Figure 1G). These infected hepatocytes were then treated with or without glucagon and subjected to HGP assays. The results demonstrated that Purβ overexpression could significantly enhance HGP under both basal and glucagon-treated conditions (Figure 1H). These results demonstrated that Purβ could function to promote HGP.

3.2. Liver-specific knockdown of Purβ protects against hyperglycemia and glucose intolerance in obesity

Enhanced HGP contributes to hyperglycemia in both patients and rodents with obesity. To determine whether Purβ expression is abnormally increased under such conditions, both Purβ mRNA and protein levels were measured using qPCR and western blot analysis, respectively, in two obese mouse models (high-fat-diet—induced obesity and leptin receptor deficiency [db/db] mice). Both Purβ mRNA and protein levels were significantly increased in the livers of these models (Figure 2A,B), and these data indicate that Purβ might function to regulate hepatic glucose metabolism in both patients and rodents with obesity.

To determine whether Purβ could regulate hepatic glucose metabolism in obese mice, we generated liver-specific Purβ knockdown db/db mice via tail-vain injection of a purified adenovirus expressing sh*PURB*1 and measured the blood glucose and glucose tolerance in these mice. The expression of Purβ was significantly decreased in the liver of Purβ-KD db/db mice compared with control db/db mice (Figure 2C), but body weight was similar between Purβ-KD and control db/db mice (Figure 2D). However, fasting blood glucose was 42.8% lower in Purβ-KD than in the control db/db mice (Figure 2E), and blood glucose levels were also reduced by 36.8% in the former mice group under fed conditions (Figure 2F). Plasma insulin levels were also significantly decreased in Purβ-KD db/db mice (Figure 2G). Glucose tolerance in Purβ-KD db/db mice was significantly improved (Figure 2H). The area under the curve (AUC) was decreased by 62.9% in Purβ-KD db/db mice (Figure 2I). To further assess insulin sensitivity in these mice, insulin tolerance tests were performed. Surprisingly, exogenous insulin reduced blood glucose in these two groups to a similar extent (Figure 2J). These data suggest that the knockdown of Purβ can ameliorate hyperglycemia and glucose intolerance in db/db mice independent of insulin sensitivity.

3.3. Purβ does not regulate hepatic insulin signaling, steatosis, or inflammation

To further determine whether Purβ regulates glucose metabolism independent of insulin signaling in obesity, we measured insulin-induced phosphorylation of Akt in Purβ-KD db/db mice. The phosphorylation (Ser 473 and Thr 308) of Akt was similar in Purβ-KD and control db/db mice (Figure 3A). Insulin sensitivity was also associated with hepatic steatosis and inflammation. As shown in Figure 3B,C, liver-specific knockdown of Purβ did not alter liver TAG levels or the expression of inflammatory genes such as *IL1β*, *IL6*, and *TNFα*. Furthermore, insulin-induced phosphorylation (Ser 473 and Thr 308) of Akt was not altered by either knockdown or overexpression of Purβ in primary hepatocytes (Figure 3D,E). These data demonstrated that Purβ could

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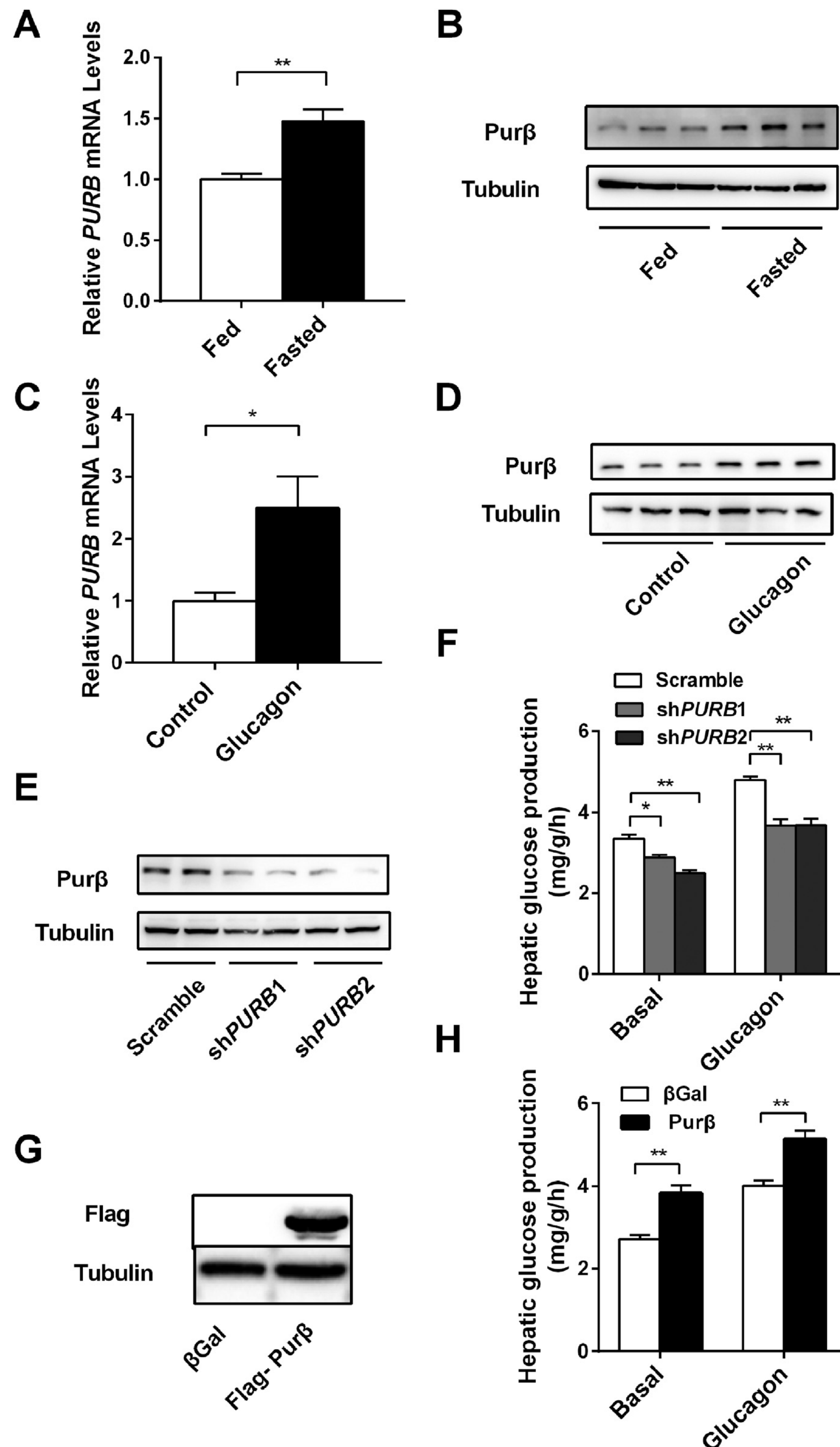

Figure 1: Identification of Purβ as a positive regulator of hepatic glucose production. (A) C57BL/6 male mice (8 weeks) were fasted for 24 h. Liver Purβ mRNA abundance was measured by qPCR and normalized to 36B4 expression. Randomly fed: n = 6, fasted: n = 6. B. C57BL/6 male mice were fasted as described above. Liver extracts were immunoblotted with antibodies against Purβ or Tubulin. (C–D) Primary hepatocytes were prepared from C57BL/6 males (8–10 weeks), grown overnight, and then treated with 0 or 100 nM glucagon for 4 h. Purβ mRNA and protein levels were measured by qPCR and immunoblotting analysis (n = 4). (E–F) Primary hepatocytes were infected with Scramble, sh*PURB*1, or sh*PURB*2 adenovirus for 30 h, then Purβ protein levels were measured by immunoblotting assays (E), and HGP assays were performed (F) (n = 4). (G–H) Primary hepatocytes were infected with βGal or Purβ adenovirus overnight, and Flag-Purβ protein levels were then measured by immunoblotting assays (G) and HGP assays were also performed (H) (n = 4). *$p < 0.05$. **$p < 0.01$.

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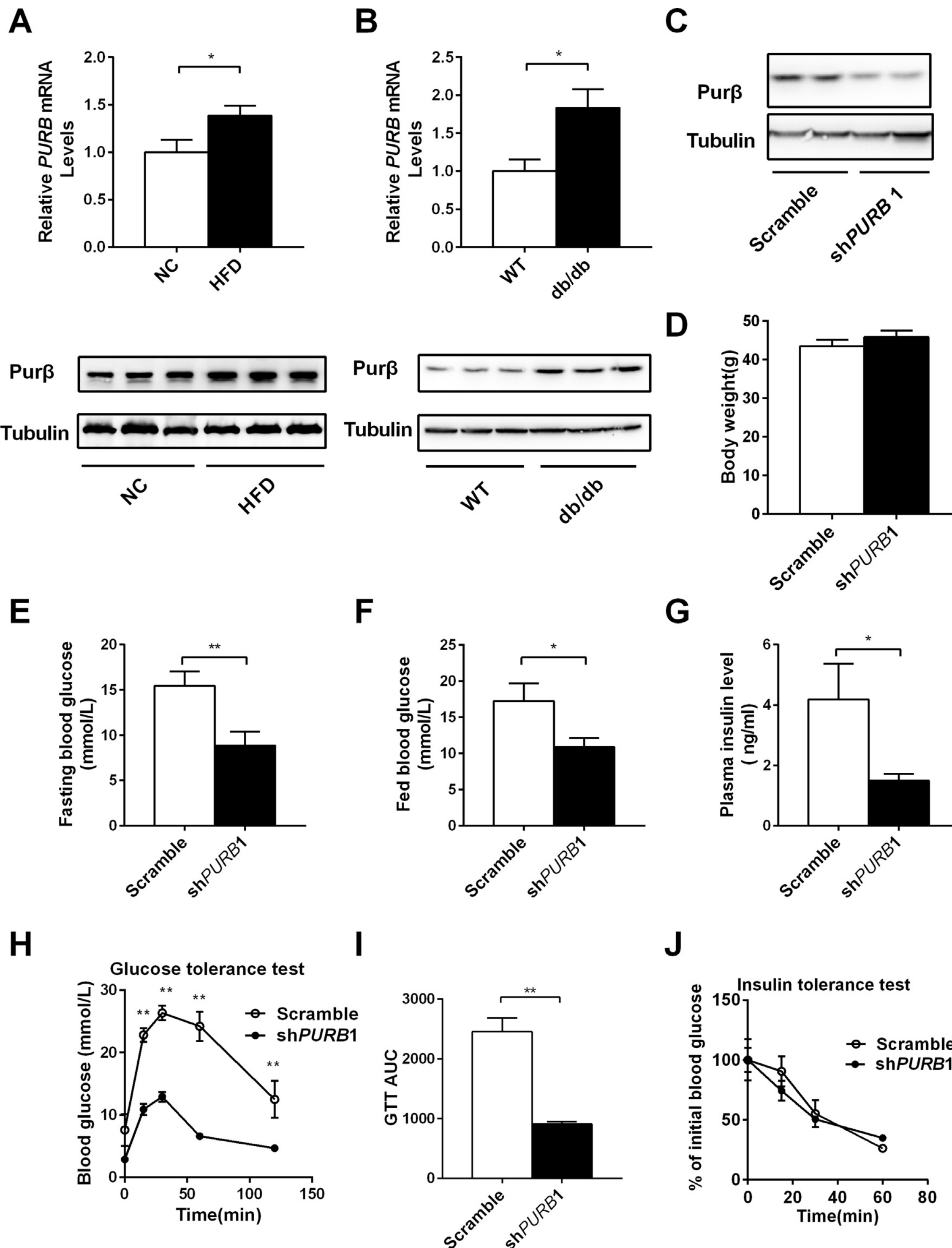

Figure 2: Liver-specific knockdown of Purβ protects against hyperglycemia and glucose intolerance in obesity. (A—B) C57BL/6 male mice (8 weeks) were fed a regular chow diet or HFD for 12 weeks; wild-type and db/db mice (11 weeks) were fed a regular chow diet. Purβ mRNA and protein levels were measured by qPCR and immunoblotting analysis. (C) Liver-specific Purβ knockdown db/db mice were generated via tail-vain injection of a purified adenovirus expressing sh*PURB*1. At the same time, db/db mice were injected with the same amount of purified Scramble adenovirus as their control. Liver extracts were immunoblotted with antibodies against Purβ or Tubulin. (D) Body weight of Purβ-KD and control db/db mice. (E) Fasting blood glucose levels. (F) Fed blood glucose levels. (G) Plasma insulin levels. (H—I) Glucose tolerance tests and AUC. (J) Insulin tolerance tests. n = 7—8. $*p < 0.05$. $**p < 0.01$.

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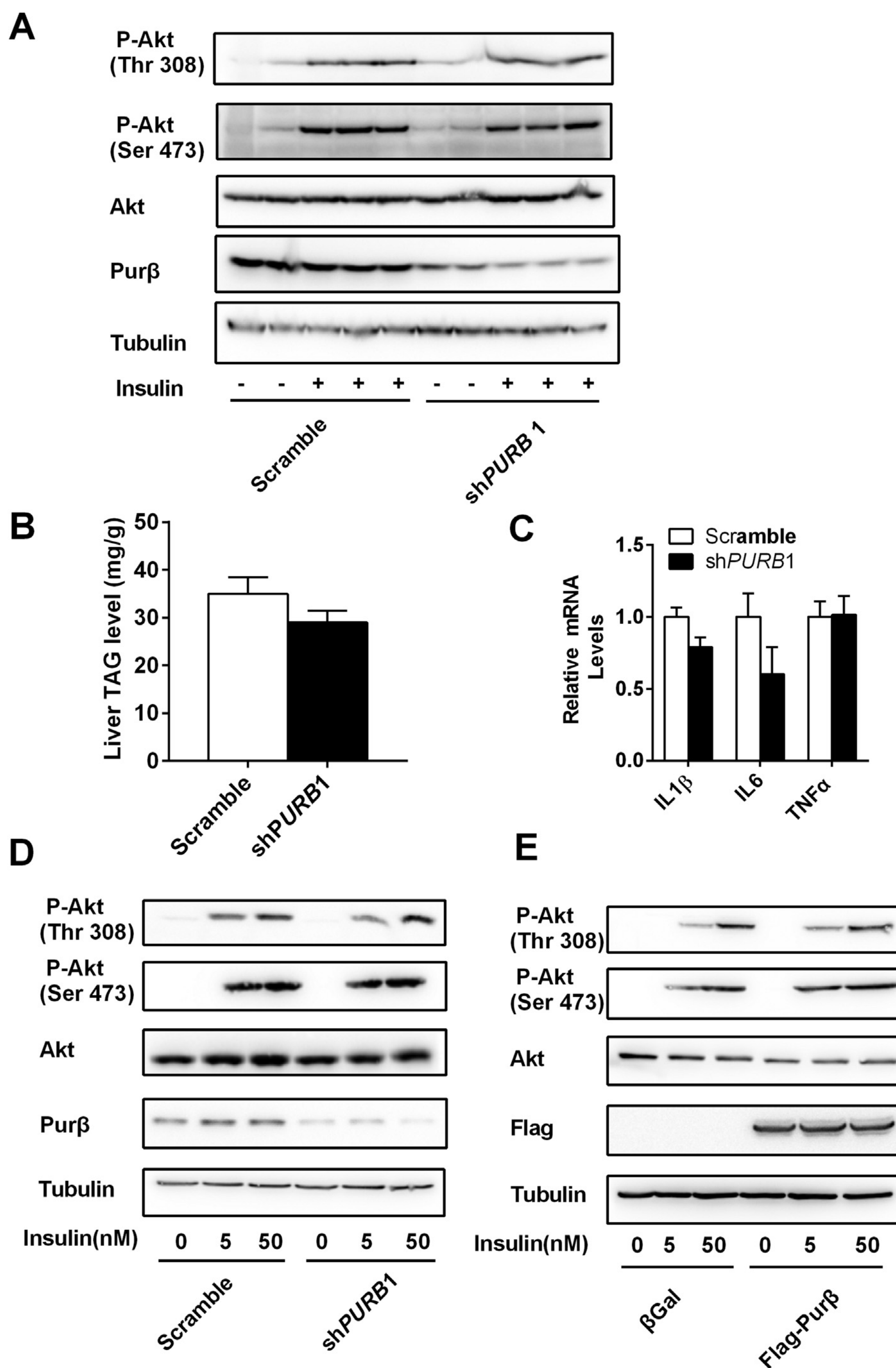

Figure 3: Purβ does not regulate hepatic insulin signaling, steatosis, or inflammation. (A) Purβ-KD and control db/db mice were fasted for 20–24 h and administered insulin (4 units/kg body weight) via the inferior vena for 5 min. The phosphorylation of Akt (at Ser 473 and Thr 308) was measured by immunoblotting. (B) Liver TAG levels. (C) *IL1β*, *IL6*, and *TNFα* mRNA levels. (D) Primary hepatocytes were infected with Scramble or shPURB1 adenoviruses for 30 h. Insulin-induced phosphorylation (Ser 473 and Thr 308) of Akt was measured by immunoblotting. (E) Primary hepatocytes were infected with βGal or Purβ adenoviruses overnight. Insulin-induced phosphorylation (Ser 473 and Thr 308) of Akt was measured by immunoblotting.

regulate hepatic glucose metabolism independent of insulin signaling, hepatic steatosis, and inflammation.

3.4. Liver-specific knockdown of Purβ decreases glucagon sensitivity and gluconeogenesis in obesity

In both patients and rodents with obesity and type 2 diabetes, plasma glucagon levels, glucagon sensitivity, and glucagon/CREB signaling are abnormally increased, contributing to higher HGP and hyperglycemia. To determine whether Purβ regulates glucagon-induced gluconeogenesis, glucagon tolerance tests and lactate tolerance tests were measured in Purβ-KD and control db/db mice. Exogenous glucagon markedly increased blood glucose levels in the control db/db mice; however, its ability to increase blood glucose was severely impaired in Purβ-KD db/db mice (Figure 4A), with the AUC decreased by 46% in

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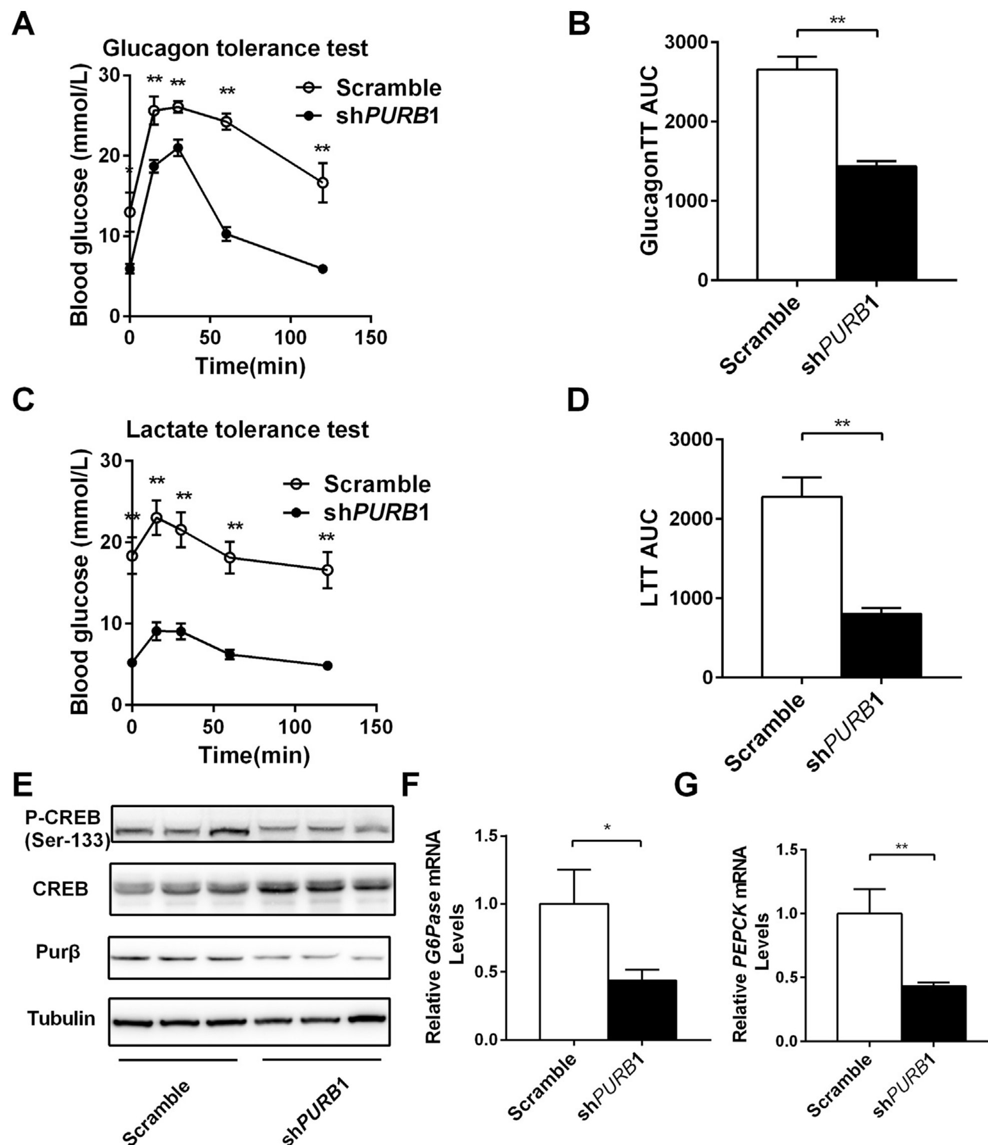

Figure 4: Knockdown of Purβ decreases glucagon sensitivity and gluconeogenesis in obesity. (A–B) Purβ-KD and control db/db mice were fasted for 6 h and intraperitoneally injected with glucagon (6 μg/kg body weight). Blood glucose was monitored after injection, and AUC was calculated. (C–D) Hepatic gluconeogenesis was measured by injection of sodium lactate (0.5 g/kg body weight) in Purβ-KD and control db/db mice fasted for 6 h, and AUC was calculated. (E) p-CREB, CREB, Purβ, and Tubulin protein levels were measured by western blot. (F–G) *G6Pase* and *PEPCK* mRNA levels were measured by qPCR. n = 7–8. $^{*}p < 0.05$. $^{**}p < 0.01$.

Purβ-KD db/db mice (Figure 4B). Hepatic gluconeogenesis, as estimated by lactate tolerance tests, was also significantly lower in Purβ-KD db/db mice, with the AUC reduced by 64.7% (Figure 4C,D).

To determine whether hepatic Purβ modulates glucagon-stimulated CREB phosphorylation, Purβ-KD and control db/db mice were fasted for ∼20 h, and liver tissue extracts were immunoblotted using a phospho-CREB (pSer133) antibody. This analysis indicated that CREB phosphorylation was decreased by 62% in Purβ-KD db/db mice (Figure 4E). In addition, total CREB levels were slightly increased in Purβ-KD db/db mice compared with the control db/db mice (Figure 4E), possibly compensating for the decreased p-CREB levels.

To determine whether Purβ regulates the hepatic gluconeogenic program, liver mRNA was extracted and used to measure the mRNA abundance of key genes via qPCR. The expression of *G6Pase* and *PEPCK* was dramatically decreased by 56.7% and 56.2% in Purβ-KD db/db mice, respectively (Figure 4F,G). These data suggest that hepatic knockdown of Purβ can ameliorate hyperglycemia and glucose intolerance, and this most likely occurs via a decrease in HGP in obese mice.

3.5. Purβ promotes the hepatic gluconeogenic program in primary hepatocytes

Knockdown of Purβ decreased HGP, whereas overexpression of Purβ increased HGP in primary hepatocytes (Figure 1E-H), indicating that Purβ could cell-autonomously regulate HGP. To further confirm that Purβ cell-autonomously regulates the hepatic gluconeogenic program, the expression of *G6Pase* and *PEPCK* and its upstream regulator p-CREB levels were measured in Purβ knockdown or overexpressing hepatocytes. The expression of *G6Pase* and *PEPCK* was significantly reduced in Purβ knockdown hepatocytes (Figure 5A,B), which was most likely due to decreased p-CREB levels (Figure 5C). Conversely, the expression of *G6Pase* and *PEPCK* was significantly increased in

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hepatocytes overexpressing Purβ (Figure 5D,E), likely because of increased p-CREB levels (Figure 5F). However, CREB protein levels did not show any change in Purβ-overexpressing and Purβ KD hepatocytes (Figure 5C, F, and Supplemental Fig. 1), suggesting that Purβ does not regulate CREB expression.

3.6. Purβ promotes hepatic cAMP production and PKA activity

Both cAMP and PKA function upstream of the CREB signaling pathway. To determine whether Purβ can regulate hepatic cAMP production and PKA activity, intracellular cAMP levels and PKA activity were measured in Purβ-overexpressing hepatocytes, PurβKD hepatocytes, and livers of PurβKD db/db mice. Overexpression of Purβ in primary hepatocytes increased cAMP levels and PKA activity (Figure 6A,B), whereas knockdown of Purβ decreased cAMP levels and PKA activity both *in vitro* (Figure 6C,D) and *in vivo* (Figure 6E,F). These data suggest that Purβ could promote the CREB signaling pathway and hepatic gluconeogenic program by increasing hepatic cAMP production and PKA activity.

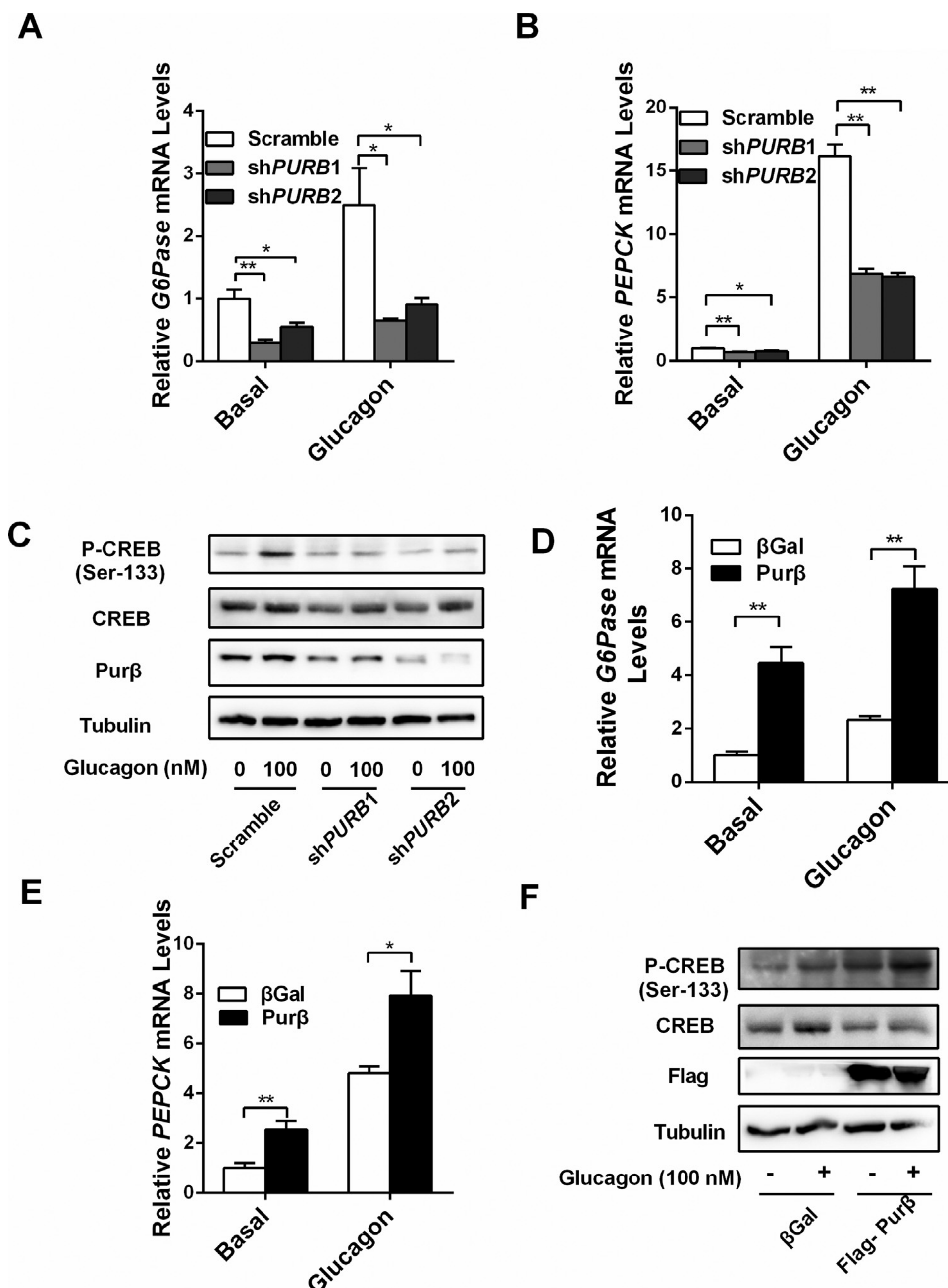

Figure 5: Purβ promotes the hepatic gluconeogenic program in primary hepatocytes. (A–B) Primary hepatocytes were prepared from C57BL/6 males (8–10 weeks) and infected with Scramble, sh*PURB*1, or sh*PURB*2 adenoviruses for 30 h and then treated with or without glucagon for 2 h. The abundance of *G6Pase* and *PEPCK* mRNA levels was measured by qPCR (n = 3–4). (C) Primary hepatocytes were infected with Scramble, sh*PURB*1, or sh*PURB*2 adenoviruses for 30 h and then treated with or without glucagon for 30 min. p-CREB, CREB, Purβ, and Tubulin protein levels were measured by immunoblotting. (D–E) Primary hepatocytes were infected with βGal or Purβ adenovirus overnight and then treated with or without glucagon for 2 h. The abundance of *G6Pase* and *PEPCK* mRNA levels was measured by qPCR (n = 4). (F) Primary hepatocytes were infected with βGal or Purβ adenovirus overnight and then treated with or without glucagon for 30 min. p-CREB, CREB, Flag, and Tubulin protein levels were measured by immunoblotting. *$p < 0.05$. **$p < 0.01$.

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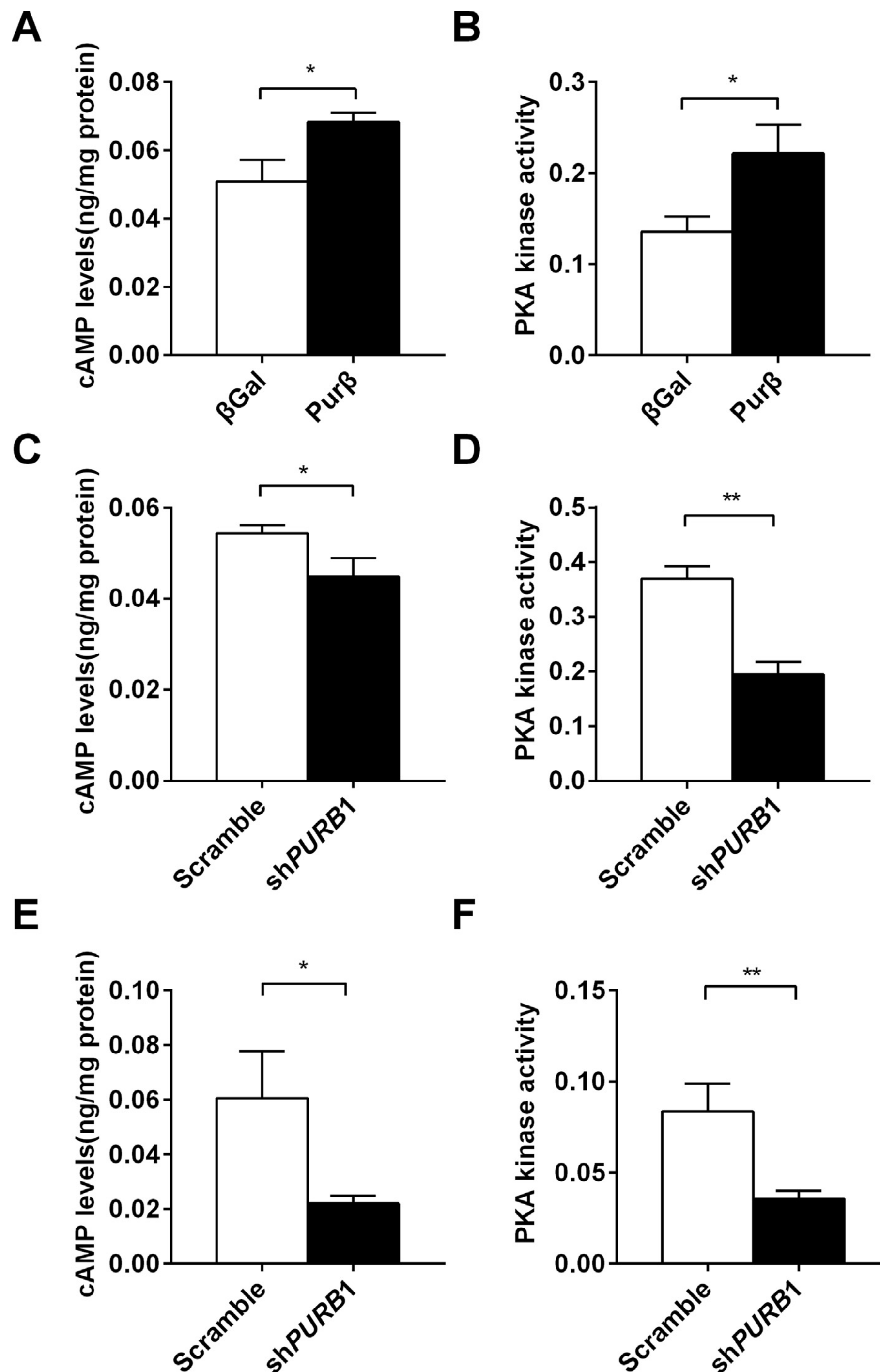

Figure 6: Purβ promotes hepatic cAMP production and PKA activity. (A—B) Primary hepatocytes were infected with βGal or Purβ adenovirus overnight and then treated with glucagon (100 nM) for 10 min. cAMP levels and PKA activity were measured using commercial kits ($n = 5–6$). (C—D) Primary hepatocytes were prepared from C57BL/6 males (8—10 weeks) and infected with Scramble or sh*PURB*1 adenoviruses for 30 h and then treated with glucagon (100 nM) for 10 min. cAMP levels and PKA activity were measured using commercial kits ($n = 5–6$). (E—F) cAMP levels and PKA activity in Purβ-KD and control db/db mice were measured using commercial kits ($n = 7–8$). *$p < 0.05$. **$p < 0.01$.

3.7. Purβ promotes *Adcy6* expression

Hepatic cAMP homeostasis is controlled by adenylate cyclases (ADCYs) and phosphodiesterases (PDEs), and Purβ promotes hepatic cAMP production either by increasing expression of ADCYs or by decreasing expression of PDEs. To address this possibility, RNA sequencing (RNA-seq) analysis was performed in Purβ-overexpressing and PurβKD hepatocytes. As shown in Figure 7A, the expression of liver-enriched PDEs, such as *Pde4b*, *Pde3b*, *Pde8a*, *Pde7a*, *Pde6d*, and *Pde5a*, did not show any correlated change in Purβ-overexpressing and Purβ KD hepatocytes. However, the expression of liver-enriched *Adcy6* was upregulated by overexpression of Purβ and down-regulated by knockdown of Purβ (Figure 7B). RT-qPCR and

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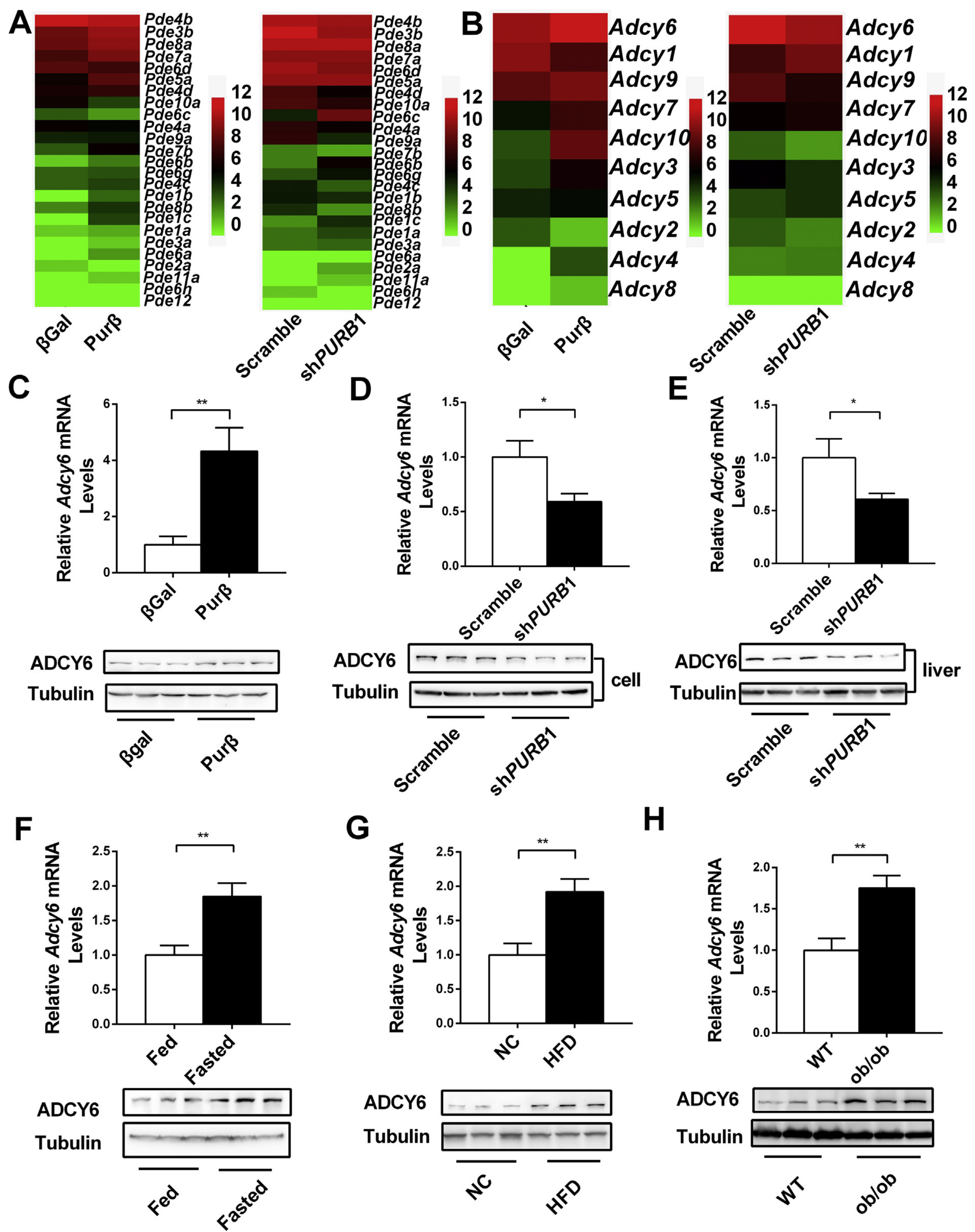

Figure 7: Purβ promotes *Adcy6* expression. Primary hepatocytes were infected with Scramble or sh*PURB*1 adenoviruses for 30 h or infected with βGal or Purβ adenovirus overnight. Total RNA was isolated, and RNA-seq was performed. (A) Heat map of PDEs in Purβ-overexpressing and PurβKD hepatocytes. (B) Heat map of ADCYs in Purβ-overexpressing and PurβKD hepatocytes. (C) ADCY6 mRNA and protein levels in Purβ-overexpressing hepatocytes (n = 4). (D) ADCY6 mRNA and protein levels in PurβKD hepatocytes (n = 4). (E) ADCY6 mRNA and protein levels in PurβKD and control db/db mice (n = 7–8). (F) ADCY6 mRNA and protein levels in fasted or fed mice (n = 6). (G–H) ADCY6 mRNA and protein levels in the livers of HFD or ob/ob mice (n = 6–8). *$p < 0.05$. **$p < 0.01$.

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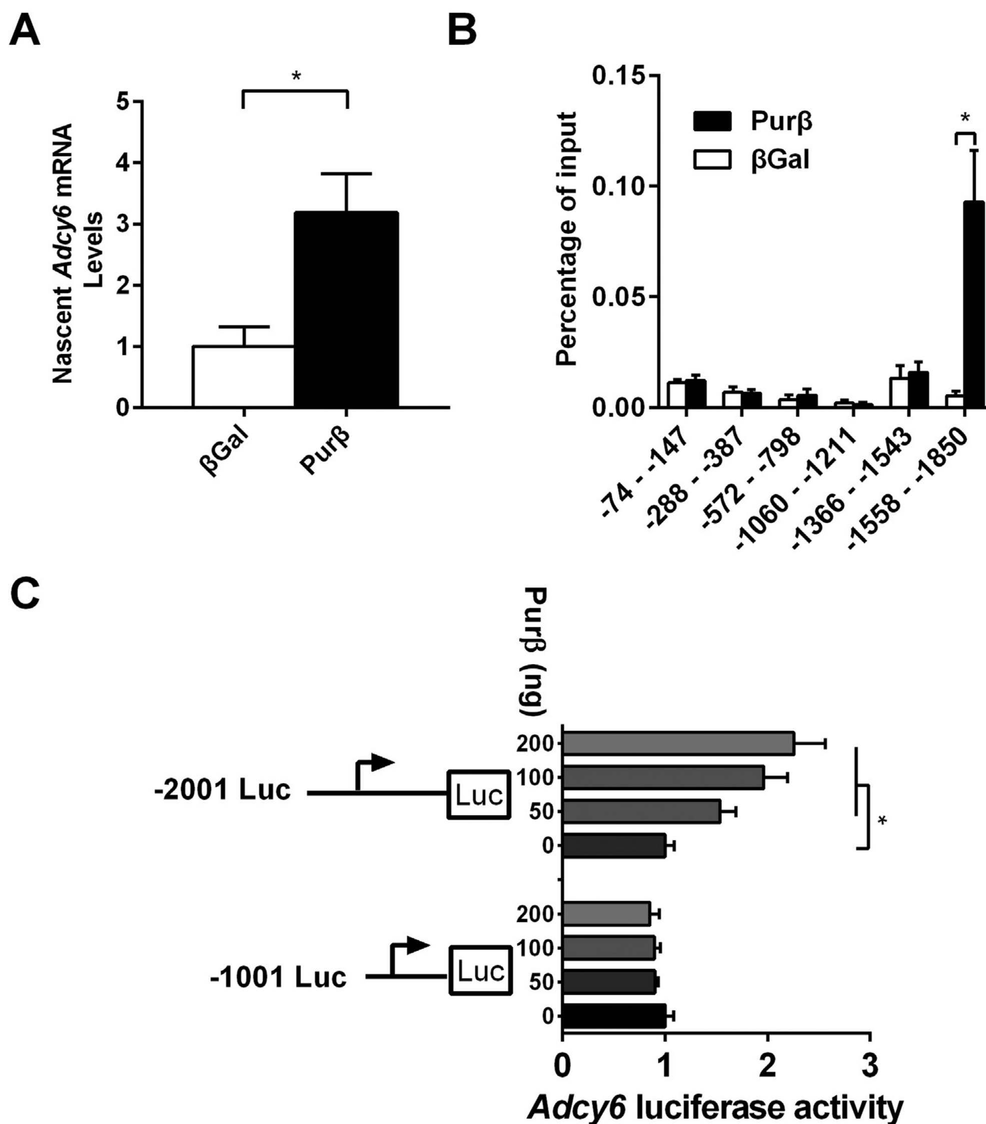

Figure 8: Purβ directly binds to the *Adcy6* promoter and increases its transcription. (A) Nascent *Adcy6* mRNA levels were measured by nuclear run-on RT-qPCR in Purβ-overexpressing hepatocytes (n = 3). (B) ChIP assays showing Purβ occupancy at the promoter of *Adcy6* (n = 3). (C) Mouse *Adcy6* promoter (−2001 to −1 or −1001 to −1) luciferase reporter plasmids and β-galactosidase reporter plasmids were transiently cotransfected with Purβ expression plasmids into HEK293T cells. Luciferase activity was measured 24 h after transfection and normalized to βGal levels (n = 6). $^{*}p < 0.05$. $^{**}p < 0.01$.

immunoblotting analysis showed that overexpression of Purβ increased both the ADCY6 mRNA and protein levels (Figure 7C), whereas knockdown of Purβ decreased ADCY6 expression (Figure 7D). Furthermore, liver-specific knockdown of Purβ in db/db mice also resulted in a significant decrease in both the ADCY6 mRNA and protein levels (Figure 7E). Interestingly, similar to Purβ, hepatic ADCY6 expression could also be induced by fasting (Figure 7F) and glucagon (Supplemental Fig. 2). Hepatic ADCY6 expression was also abnormally elevated in the two obese mouse models (Figure 7G,H). These data demonstrate that Purβ could promote HGP and lead to hyperglycemia by increasing *Adcy6* expression.

3.8. Purβ directly binds to the *Adcy6* promoter and increases its transcription

To further test whether Purβ promotes *Adcy6* expression at the transcriptional level, nascent *Adcy6* mRNA levels were measured by performing nuclear run-on RT-qPCR. As shown in Figure 8A, the nascent *Adcy6* mRNA level was significantly increased in Purβ-overexpressing hepatocytes, indicating that Purβ promoted *Adcy6* transcription. Furthermore, ChIP assays showed that Purβ directly binds to the *Adcy6* promoter at −1558 to −1850 (Figure 8B), which is a purine-enriched region. In addition, Purβ increased *Adcy6* promoter-controlled luciferase activity in a dose-dependent manner, and deletion of the purine-enriched region abolished this enhancement (Figure 8C). These results demonstrated that Purβ directly binds to the *Adcy6* promoter and increases its transcription.

4. DISCUSSION

HGP is controlled by glucagon and insulin, and enhanced glucagon signaling in the liver leads to increased HGP, contributing to hyperglycemia in both patients and rodents with obesity and type 2 diabetes

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[3,4]. The detailed molecular mechanisms underlying this enhanced HGP are not yet fully understood. In this study, Purβ was identified as a positive regulator of HGP and was shown to promote HGP by increasing *Adcy6* expression and subsequent activation of the cAMP/PKA/CREB signaling pathway, which contributes to the occurrence of hyperglycemia in obesity.

This study provides several lines of evidence indicating that Purβ acts as a positive regulator of HGP. First, hepatic Purβ expression was increased by fasting and glucagon. Glucagon could activate CREB via the cAMP/PKA signaling pathway. We noted that there are four binding sites of CREB at the promoter (−22 to −29; −98 to −105; −1337 to −1344; and −1349 to −1356) of *PURB* gene by searching the JASPAR database [24], indicating that glucagon induces Purβ expression through the activation of CREB. Knockdown of Purβ decreased the ability of glucagon to stimulate CREB phosphorylation, the expression of *G6Pase* and *PEPCK*, and glucose production in primary hepatocytes, whereas Purβ overexpression had the opposite effects. Purβ did not regulate CREB expression, indicating that Purβ enhances glucagon-induced CREB phosphorylation mainly through the activation of the upstreams of CREB. Mechanistically, Purβ likely increases *Adcy6* transcription by directly binding to its promoter, which then leads to increased cAMP production, enhanced PKA activity, and increased HGP. Both Purβ and ADCY6 were induced at a later stage by fasting and then promoted HGP for survival during prolonged fasting.

Second, hepatic Purβ and ADCY6 are abnormally elevated in obesity and type 2 diabetes mouse models. Hepatocyte-specific knockdown of Purβ in db/db mice severely impaired the ability of glucagon to increase blood glucose levels and inhibited CREB phosphorylation and PKA activity (due to decreased cAMP production) by suppressing *Adcy6* expression in the liver. These observations indicate that hepatic Purβ/ADCY6 could promote the glucagon signaling pathway, contributing to the occurrence of hyperglycemia in type 2 diabetes.

Surprisingly, Purβ does not regulate insulin signaling, although Purβ-KD db/db mice displayed reduced plasma insulin levels, probably secondary to a decrease in blood glucose in Purβ-KD db/db mice given that low blood glucose stimulates less insulin secretion from β cells. Hepatic deletion of *TRAF2* also shows similar phenotypes [20]. Liver-specific knockdown of Purβ in db/db mice did not alter hepatic insulin signaling, steatosis, or inflammation. Exogenous insulin reduced blood glucose to a similar degree in both Purβ-KD and control db/db mice, suggesting that hepatic Purβ does not alter hepatic or systemic insulin sensitivity. Consistently, in isolated primary hepatocytes, neither knockdown nor overexpression of Purβ altered the insulin-induced phosphorylation of Akt, further supporting the conclusion that hepatic Purβ does not regulate insulin sensitivity.

In summary, Purβ was shown to positively regulate HGP by directly binding to the promoter of the *Adcy6* gene, increasing its expression and thereby enhancing the glucagon/cAMP/PKA/CREB signaling pathway. Purβ/ADCY6 is abnormally elevated in obese mice and is also increased by fasting or via the glucagon signaling pathway. Hepatic knockdown of Purβ in db/db mice significantly ameliorated hyperglycemia and glucose intolerance by suppressing the ADCY6/cAMP/PKA/CREB signaling pathway. These data suggest that Purβ/ADCY6 might serve as an important drug target for the treatment of hyperglycemia in patients with type 2 diabetes.

DISCLOSURE STATEMENT

All authors have nothing to declare.

AUTHOR CONTRIBUTIONS

L.J. performed most of the experiments. Y.J. and X.L. researched data. Z.C. designed the project and wrote the manuscript.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

APPENDIX A. SUPPLEMENTARY DATA

Supplementary data to this article can be found online at https://doi.org/10.1016/j.molmet.2019.11.008.

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