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Hepatic ZBTB22 promotes hyperglycemia and insulin resistance via PEPCK1-driven gluconeogenesis

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Abstract

Excessive gluconeogenesis can lead to hyperglycemia and diabetes through as yet incompletely understood mechanisms. Herein, we show that hepatic *ZBTB22* expression is increased in both diabetic clinical samples and mice, being affected by nutritional status and hormones. Hepatic *ZBTB22* overexpression increases the expression of gluconeogenic and lipogenic genes, heightening glucose output and lipids accumulation in mouse primary hepatocytes (MPHs), while *ZBTB22* knockdown elicits opposite effects. Hepatic *ZBTB22* overexpression induces glucose intolerance and insulin resistance, accompanied by moderate hepatosteatosis, while *ZBTB22*-deficient mice display improved energy expenditure, glucose tolerance, and insulin sensitivity, and reduced hepatic steatosis. Moreover, hepatic *ZBTB22* knockout beneficially regulates gluconeogenic and lipogenic genes, thereby alleviating glucose intolerance, insulin resistance, and liver steatosis in db/db mice. ZBTB22 directly binds to the promoter region of *PCK1* to enhance its expression and increase gluconeogenesis. *PCK1* silencing markedly abolishes the effects of *ZBTB22* overexpression on glucose and lipid metabolism in both MPHs and mice, along with the corresponding changes in gene expression. In conclusion, targeting hepatic ZBTB22/PEPCK1 provides a potential therapeutic approach for diabetes.

Keywords gluconeogenesis; hepatosteatosis; hyperglycemia; PEPCK1; ZBTB22
Subject Categories Chromatin, Transcription & Genomics; Metabolism; Molecular Biology of Disease
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Introduction

The prevalence of Type 2 Diabetes (T2D), an ailment characterized by abnormally elevated blood glucose levels, and sustained by an excessive hepatic gluconeogenesis, is increasing worldwide in epidemic proportions. Hence, T2D has become a serious global public health issue (Santoleri & Titchenell, 2019). In Mammals, hepatic glucose synthesis and release together with the concurring glucose uptake by peripheral tissues maintain blood glucose homeostasis, preventing the onset of a dysregulated hyperglycemia. Conversely, under fasting/food deprivation conditions, hepatic gluconeogenesis quickly responds to circulating glucagon, catecholamines, and cortisol, increasing liver glucose production and output, to maintain the glycemic balance and satisfy the glucose needs of peripheral tissues (Pickett-Blakely *et al*, 2018). However, an excessive hepatic gluconeogenesis under conditions of sufficient nutrition promotes a hyperglycemia, which eventually may develop into a T2D. Thus, identifying and unraveling the function and mechanism of new molecules regulating hepatic gluconeogenesis may single out potential targets and indicated novel strategies to prevent and treat T2D.

Hepatic gluconeogenesis plays an important role in hepatic glucose metabolism and is regulated by a number of transcription factors. The transcription of key genes encoding for two gluconeogenic rate-limiting enzymes such as glucose-6-

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phosphatase (G6Pase) and phosphoenolpyruvate carboxykinase 1 (PEPCK1) crucially drives hepatic gluconeogenesis (Radziuk & Pye, 2001; Korenfeld *et al*, 2021). Several transcription factors including the hepatic nuclear factor-4 (HNF4), glucocorticoid receptor (GR), forkhead factor O1 (FOXO1), and cyclic AMP (cAMP) responsive element binding protein (CREB) regulate the expression of hepatic *G6PC* and *PCK1* genes, respectively, encoding for G6Pase and PEPCK1, tightly maintaining the blood glucose homeostasis through a precise regulation of liver gluconeogenesis (Hirota *et al*, 2008; Cui *et al*, 2016, 2019; Lee *et al*, 2018). During prolonged fasting or food deprivation, the increases in circulating gluconeogenic hormones stimulate cAMP synthesis and the cAMP response element-binding protein (CREB)/transducer of regulated CREB activity (TORC) pathways to induce the expression of the transcriptional factor peroxisome proliferator-activated receptor γ coactivator-1α (*PGC1α*). The latter is the master regulator that co-activating multiple transcription factors regulates the expression of two key gluconeogenic genes, that is, *PCK1* and *G6PC*, whose product can promote hepatic gluconeogenesis to meet the ongoing body's demands for blood glucose (Herzig *et al*, 2001; Lin *et al*, 2004; Koo *et al*, 2005; Li *et al*, 2007; Schmidt & Mandrup, 2011; Waldman *et al*, 2018).

Being a critical enzyme of the gluconeogenic program, PEPCK1 crucially regulates mitochondrial function, glucose and fatty acids metabolism, and adaptive heat production (Moura *et al*, 2005; Millward *et al*, 2010; Vincent *et al*, 2015; Yu *et al*, 2021). In the liver, PEPCK1 expression is regulated by the body's response to hypoglycaemia (Beale *et al*, 2007; Yang *et al*, 2009). Under normal feeding conditions, PEPCK1 expression is low. Conversely, fasting increases PEPCK1 expression and promotes liver glucose production by stimulating gluconeogenesis and decreasing fatty acid oxidation (Hanson & Reshef, 2003; Banerjee *et al*, 2020). Various lines of evidences indicated that mice with genetically or pharmacologically reduced PEPCK1 expression exhibited a lower fasting blood glucose level, and a decreased glucose output from *PCK1*-deficient hepatocytes, and their response to gluconeogenic hormones, was also impaired, resulting in clear therapeutic effect in diabetic animals (Liu *et al*, 2005, 2017; Gómez-Valadés *et al*, 2008; Tsuneki *et al*, 2015). Despite the fact that multiple factors, at both transcriptional and posttranscriptional levels, reportedly regulate the expression of PEPCK1, the exact regulatory mechanism regarding how to regulate *PCK1* expression and its activity, the underlying mechanism(s) allowing the latter to maintain hepatic glucose homeostasis remains unclear (Lee *et al*, 2002; Le Lay *et al*, 2009).

Zinc Finger And BTB Domain Containing 22 gene (*ZBTB22*) belongs to the POK (POZ and Krüppel)/BTB family, which participate in a variety of cellular functions, including proliferation, apoptosis, DNA damage response, and tumor onset and development (Albagli *et al*, 1995; Costoya, 2007). Previous observations suggested that ZBTB22 was closely related to maternal sensitivity and offspring DNA methylation, and interacted with and transcriptionally regulated genes involved in RNA Polymerase (Pol III) transcription, (Zhang *et al*, 2012). A mutation of *ZBTB22* was implicated in the colorectal cancer biopathways (Liu *et al*, 2014). In this study, we investigated the molecular mechanisms by which ZBTB22 regulates hepatic glucose and lipid metabolism.

Results

Hepatic ZBTB22 expression is increased in diabetic mice and regulated by nutritional status

We performed RNA sequencing on health donor and high blood glucose diabetic patients to confirm this result, and our results strengthened the previous finding and showed that hepatic ZBTB22 expression was increased in diabetic patients (Appendix Fig S1A and B). We performed real-time PCR and Western immunoblotting to verify this finding in diabetic mice models, including db/db, ob/ob, and DIO mice. Consistently, the liver *ZBTB22* mRNA and its protein levels significantly increased in these diverse strains of diabetic mice, as compared to their respective control (Fig 1A–C). Besides, there was a positively expressing correlation between ZBTB22 and PEPCK1 in these disease models (Fig 1A–C). Moreover, we found that the hepatic *ZBTB22* expression could rapidly respond to the ongoing nutritional status, as shown by its upregulation in prolongedly fasting wild-type mice followed by recovery 2 h after refeeding (Fig 1D). To identify the potential triggers of *ZBTB22* expression under fasting conditions and other pathophysiological conditions, MPHs were treated with forskolin (FSK), which could activate the cAMP signaling pathway, thereby mimicking the effect of both glucagon and catecholamines on hepatic glucose production (Nixon *et al*, 2016). Thus, FSK significantly increased MPHs' *ZBTB22* mRNA and its protein levels. Conversely, adding insulin attenuated MPHs' ZBTB22 mRNA and protein expression levels (Fig 1E). More importantly, compared with healthy subjects, we found a prominent upregulation of *ZBTB22* in liver tissue samples of diabetic patients. This upregulation was closely and significantly related to the increased levels of fasting blood glucose in the diabetic patients (Fig 1F; Appendix Fig S1A and B). These findings suggested that *ZBTB22* might be involved in hepatic glucose metabolism and T2D pathogenesis.

ZBTB22 drives gluconeogenic and lipogenic reprogramming in primary hepatocytes

To explore the *ZBTB22* physiological modulation of the liver glucose and lipid programming response in relation to the ongoing nutritional state, we performed a gain-and-loss-of-function assay using cultured MPHs. The adenovirus vector-mediated *ZBTB22* overexpression significantly increased the expression of the gluconeogenic genes *PGC1α*, *G6PC*, *PCK1*, and of the lipogenic genes sterol regulatory element-binding protein 1c (*SREBP-1C*), *FAS* and Acetyl-CoA Carboxylase (*ACC*), while suppressing genes involved in fatty acid oxidation, such as *PPARα*, Carnitine Palmitoyl Transferase 2 (*CPT2*), and Carnitine Palmitoyl Transferase 1B (*CPT1B*) (Fig 2A and B). Correspondingly, the forced expression of *ZBTB22* increased the glucose production and lipid accumulation in the MPHs (Fig 2C–E). Besides, we have detected the *PCK1* expression in ad-*ZBTB22*-infected MPHs after insulin treatment and found that *ZBTB22* forced overexpression significantly suppressed the insulin-induced suppression of *PCK1* (Appendix Fig S2A and B), suggesting an important role of *ZBTB22* in mediating insulin-induced protective effects on gluconeogenesis. By contrast, *ZBTB22* knockdown significantly decreased the expression of the just-mentioned gluconeogenic and lipogenic genes, while

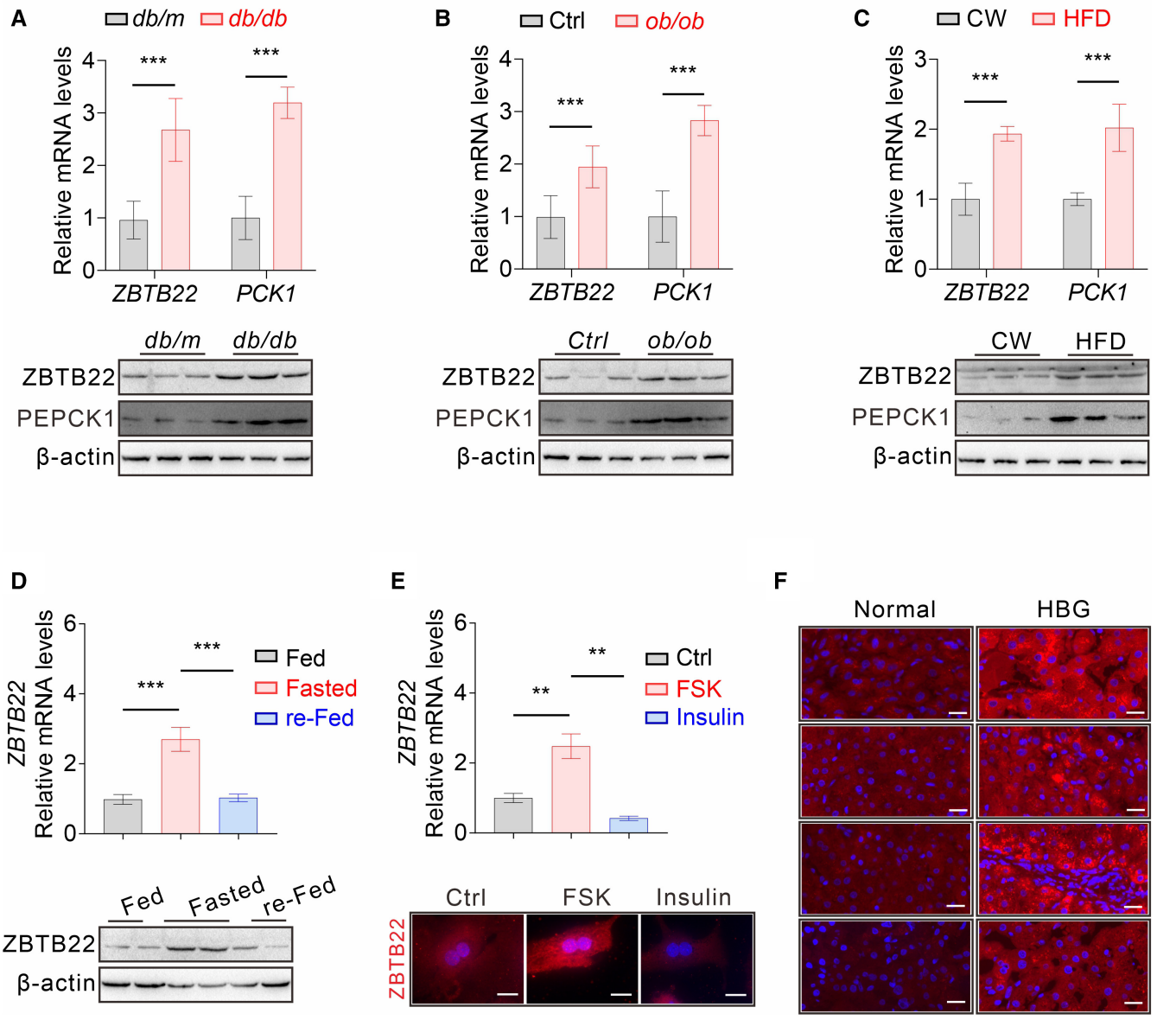

Figure 1. Hepatic ZBTB22 expression is induced in diabetic mice and regulated by nutritional status.

A–C qPCR and Western blot analysis indicated an upregulation of hepatic ZBTB22 in diabetic db/db mice, obese ob/ob mice and high-fat diet for 12 weeks mice (n = 6 mice).

D qPCR and Western blot analysis showed an increase in hepatic ZBTB22 in normal C57 mice after overnight fasting, and a recovery of ZBTB22 expression after refeeding (n = 6 mice).

E qPCR and immunofluorescence analysis displayed an enhanced expression of *ZBTB22* in MPHs after FSK treatment for 24 h and a reduction in ZBTB22 in MPHs after insulin treatment for 15 min (n = 4 biological replicates), scale bars represent 10 μm.

F Immunofluorescence experiments indicated an increase in ZBTB22 expression in clinical NAFLD samples with higher blood glucose levels (HBG), scale bars represent 20 μm.

Data information: Data are means ± SEM. $^{**}P < 0.01$, $^{***}P < 0.001$ by Student's t-test.
Source data are available online for this figure.

upregulating the fatty acid oxidation-involved genes in MPHs (Fig 2F and G). And these same MPHs produced less glucose and accumulated less lipids (Fig 2H–J). Moreover, *ZBTB22* knockdown effectively decreased the (FSK+Dex)-induced expression of gluconeogenic genes (Fig 2K). Such findings suggested that *ZBTB22* crucially mediated the glucagon-induced gluconeogenic process, which mimics the fasting-stimulated hepatic gluconeogenesis and lipogenic reprogramming observed in MPHs.

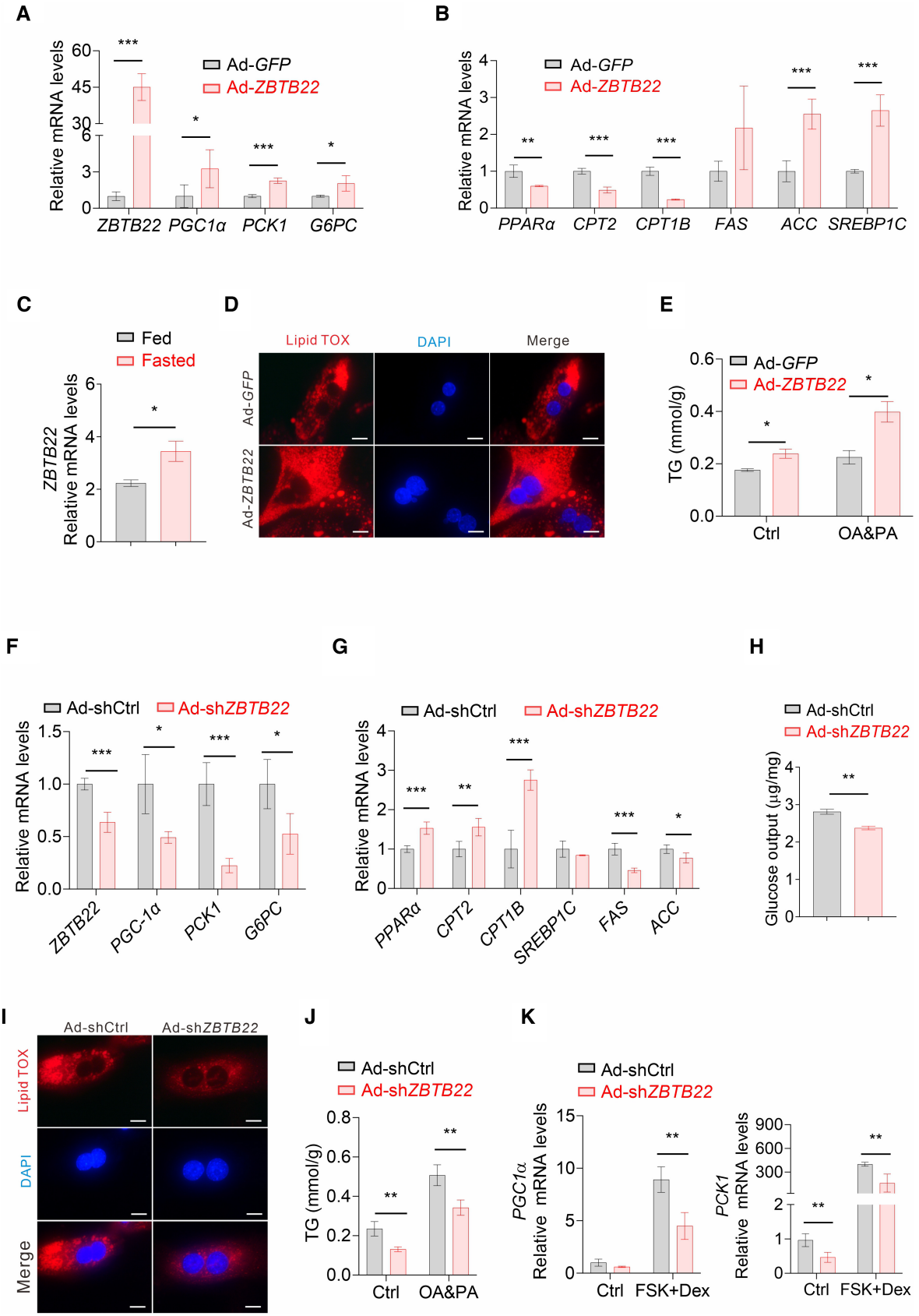

Figure 2.

Figure 2. ZBTB22 regulates gluconeogenesis and lipid deposition in primary hepatocytes.

A, B qPCR analysis the change of genes involved in gluconeogenesis, lipogenesis, and fatty acid oxidation in primary hepatocytes after *ZBTB22* overexpression (n = 4 biological replicates).

C *ZBTB22* overexpression increase the glucose generation in primary hepatocytes (n = 4 biological replicates).

D, E *ZBTB22* overexpression increase lipid deposition in primary hepatocytes, as shown by the enhanced lipid TOX probes and TG content (n = 4 biological replicates), scale bars represent 10 μm.

F, G qPCR analysis the change of genes involved in gluconeogenesis, lipogenesis, and fatty acid oxidation in primary hepatocytes after *ZBTB22* knockdown (n = 4 biological replicates).

H *ZBTB22* knockdown decrease glucose output in primary hepatocytes (n = 4 biological replicates).

I, J *ZBTB22* knockdown suppress lipid deposition in primary hepatocytes, as shown by the weakened lipid TOX probes and TG content (n = 4 biological replicates), scale bars represent 10 μm.

K qPCR analysis of mRNA levels of Pgc1α, *PCK1* in primary hepatocytes infected with Ad-shCtrl or Ad-sh*ZBTB22* with or without forskolin treatment (n = 4 biological replicates).

Data information: Data are means ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001 by Student's t-test.
Source data are available online for this figure.

ZBTB22 deficiency protects mice against HFD-induced obesity and hyperglycemia

To further verify the *ZBTB22*'s requirements to regulate endogenous gluconeogenesis and lipogenesis, we generated *ZBTB22*$^{-/-}$ knockout mice and verified their *ZBTB22* mRNA expression via qPCR and Western blot (Appendix Fig S3A).

Interestingly, *ZBTB22*$^{-/-}$ mice fed a HFD displayed a lower body weight, decreased fat mass, and reduced hypertrophy of the white adipose tissue, as shown by the adipocytes' shrunk lipid droplets. Conversely, no change in body composition occurred in the mice fed a standard chow diet (Fig 3A–C; Appendix Fig S3B). Moreover, *ZBTB22*$^{-/-}$ mice on a HFD showed an enhanced expression of thermogenic genes in the brown adipose tissue, which leads to a higher whole-body energy expenditure, as shown by increasing heat production, CO2 generation, and O2 consumption. By contrast, no difference observed among occurred in basal metabolism between *ZBTB22*$^{-/-}$ mice and littermate controls when both were fed a standard chow diet. These observations implied that a *ZBTB22* deficit would protect against a HFD-induced obesity (Fig 3D and E; Appendix Fig S3C). However, *ZBTB22* did not regulate appetite since *ZBTB22*'s deletion barely decreased food intake in the HFD mice (Fig 3F).

Moreover, *ZBTB22* deficiency effectively restrained the expression of the gluconeogenic genes *PGC1α*, *PCK1*, and of the lipogenic genes *SREBP-1C*, and *ACC* expression in the liver, thereby decreasing fasting blood glucose and improving glucose tolerance, while not affecting pyruvate tolerance and insulin sensitivity (Appendix Fig S4A–D). Also, the liver weight/body weight ratio value was decreased in the *ZBTB22*$^{-/-}$ mice (Appendix Fig S4E). While hepatic and serum triglyceride levels did no change (Appendix Fig S4F). Of note, *ZBTB22* deletion more effectively attenuated the disordered glucose and lipid metabolism in the MPHs from HFD-fed mice, as shown by the remarkably decreased fasting blood glucose, and hepatic and serum triglyceride levels (Fig 4A and B). Correspondingly, *ZBTB22* deletion significantly alleviated the HFD-induced hepatic steatosis, as shown by the decreasing liver weight/body weight ratio value, hepatocytes' lipid droplets size, and ballooning degree ratio, along with corresponding changes in the expression of genes involved in hepatic glucose and lipid metabolism (Fig 4C and D; Appendix Fig S5A and B). Moreover, *ZBTB22* knockout significantly increased the phosphorylation level of AKT and GSK-3β in the liver, thereby improving the effect of insulin signaling (Fig 4E). Additionally, tolerance tests revealed that *ZBTB22* deletion remarkably ameliorated glucose tolerance, pyruvate tolerance, and insulin sensitivity, even when the animals were fed on a HFD (Fig 4F). To further verify the effects of hepatic *ZBTB22* on glucose and lipid metabolism, we injected an AAV-*ZBTB22* into *ZBTB22*$^{-/-}$ mice to specifically rescue its expression in the liver. Thus, we found that *ZBTB22* forced overexpression caused a clearly disordered glucose and lipid metabolism in these mice, as shown by the increasing fasting blood glucose and lipid mobilization, and by decreasing glucose tolerance and insulin sensitivity, coupled with the changes in the expression of the corresponding genes (Fig 4G–K). To wit, these data suggested that hepatic *ZBTB22* deficiency should improve glucose and lipid metabolism disorders.

Hepatic ZBTB22 silencing improves hyperglycemia and liver steatosis in db/db diabetic mice

To further investigate how *ZBTB22* regulates glucose and lipid metabolism in the liver, we generated a strain of Adenovirus-mediated hepatic *ZBTB22* silenced db/db mice via tail vein injection of an AAV-sh*ZBTB22*, which did not affect *ZBTB22* expression in other tissues. Consistently, the hepatic *ZBTB22* knockdown resulted in the suppressed expression of the gluconeogenic genes *PGC1α*, *PECPK1*, and of the lipogenic genes *SREBP-1C*, *FAS*, while the expression of genes involved in fatty acid oxidation increased (Fig 5A and B). Moreover, hepatic *ZBTB22* suppression significantly reduced the fasting blood glucose and serum insulin levels in the db/db mice (Fig 5C). Later, tolerance tests also showed a remarkable improvement of glucose tolerance, pyruvate tolerance, and insulin sensitivity (Fig 5D). Meanwhile, in db/db mice, hepatic *ZBTB22* knockdown was also coupled with the insulin-induced phosphorylation of AKT and GSK3β in the liver, which implied a rescued response to insulin (Fig 5E). Additionally, a neat attenuation of hepatic steatosis occurred in the AAV-sh*ZBTB22* injected db/db mice displayed an obvious attenuation of hepatic steatosis, as shown by the decreased in liver weight/body weight ratio values and in hepatic triglyceride levels (Fig 5F and G; Appendix Fig S6A and B). Moreover, hepatic *ZBTB22* silencing clearly reversed the otherwise massive accumulations of large lipid droplets in and the ballooning degeneration of the hepatocytes (Fig 5H). Moreover, the AAV-sh*ZBTB22*-induced *ZBTB22* knockdown decreased serum ALT and AST levels, which implied an improved hepatic

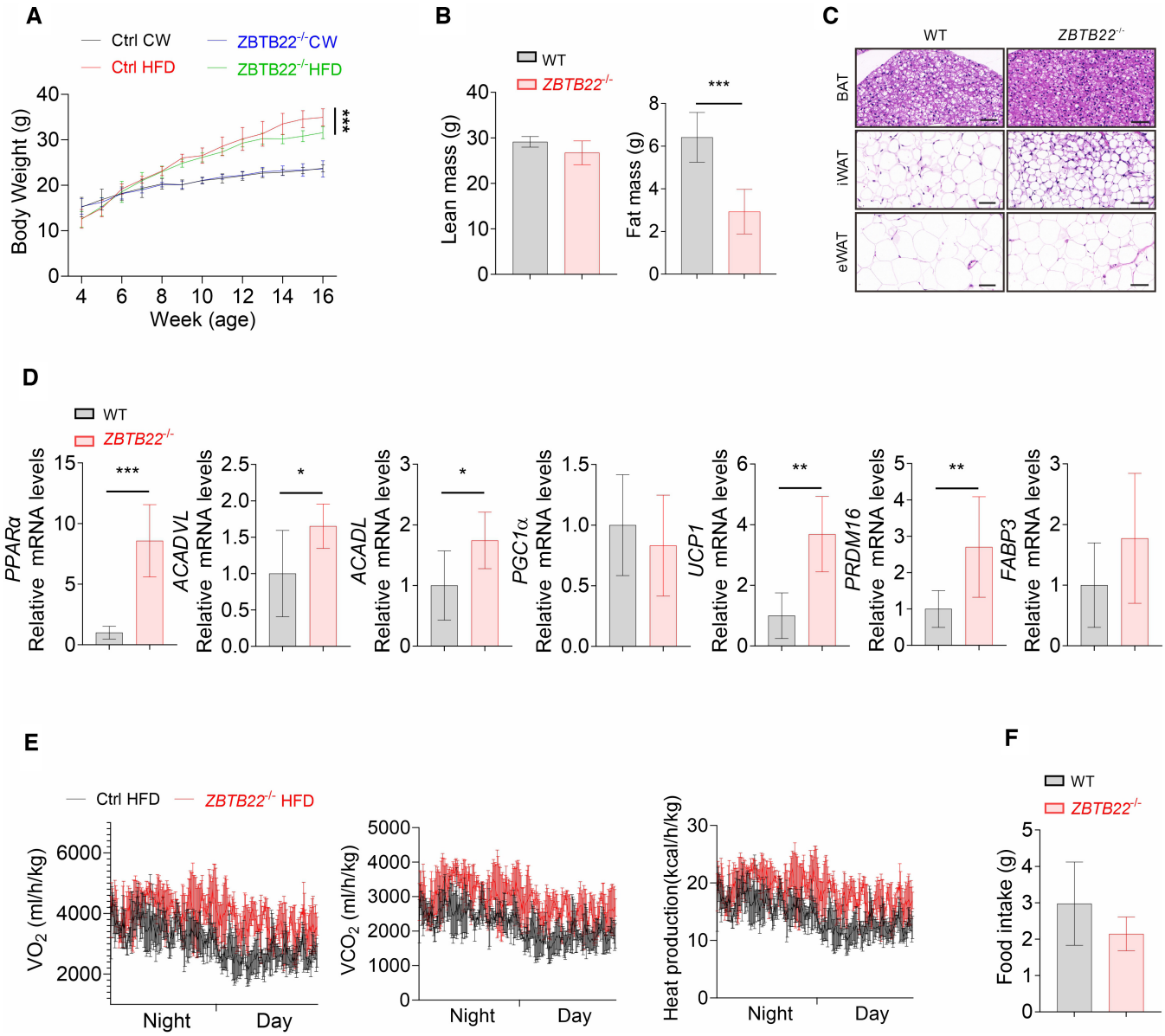

Figure 3. *ZBTB22* deficiency protects mice against HFD-induced obesity.

A, B *ZBTB22* deletion effectively decrease the body weight and fat mass of mice fed on HFD (n = 6 mice).

C *ZBTB22* deficiency significantly reduced adipocyte hypertrophy of adipose tissue from mice fed on HFD, scale bars represent 20 μm.

D *ZBTB22* deletion enhanced the expression of genes involved in thermogenesis and fatty acid oxidation in brown adipose tissue from mice fed on HFD (n = 6 mice).

E *ZBTB22* deletion increased the heat production, CO2 generation, and O2 consumption in mice fed on HFD (n = 3 mice).

F *ZBTB22* deletion barely changed in HFD intake in mice (n = 6 mice).

Data information: Data are means ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001 by Student's t-test.
Source data are available online for this figure.

function in db/db mice (Fig 5I). These same mice also displayed a significant fall of both proinflammatory cytokines serum levels and of mRNA levels of the hepatic inflammatory genes, thereby alleviating the disordered metabolism of glucose and lipids in db/db mice (Fig 5J and K). However, the body weight of these mice did not change. Collectively, these data suggested that *ZBTB22* exerted a critical regulatory effect on the glucose and lipid metabolism in the liver of db/db mice.

Hepatic ZBTB22 forced overexpression leads to hyperglycemia and lipid disorder in wild-type C57BL/6J mice

To further prove that hepatic *ZBTB22* physiologically regulates glucose and lipid metabolism, we generated and injected an AAV-*ZBTB22* into wild-type C57BL/6J mice via the tail vein, which led to a hepatic-specific forced overexpression of *ZBTB22*. Contrary to the

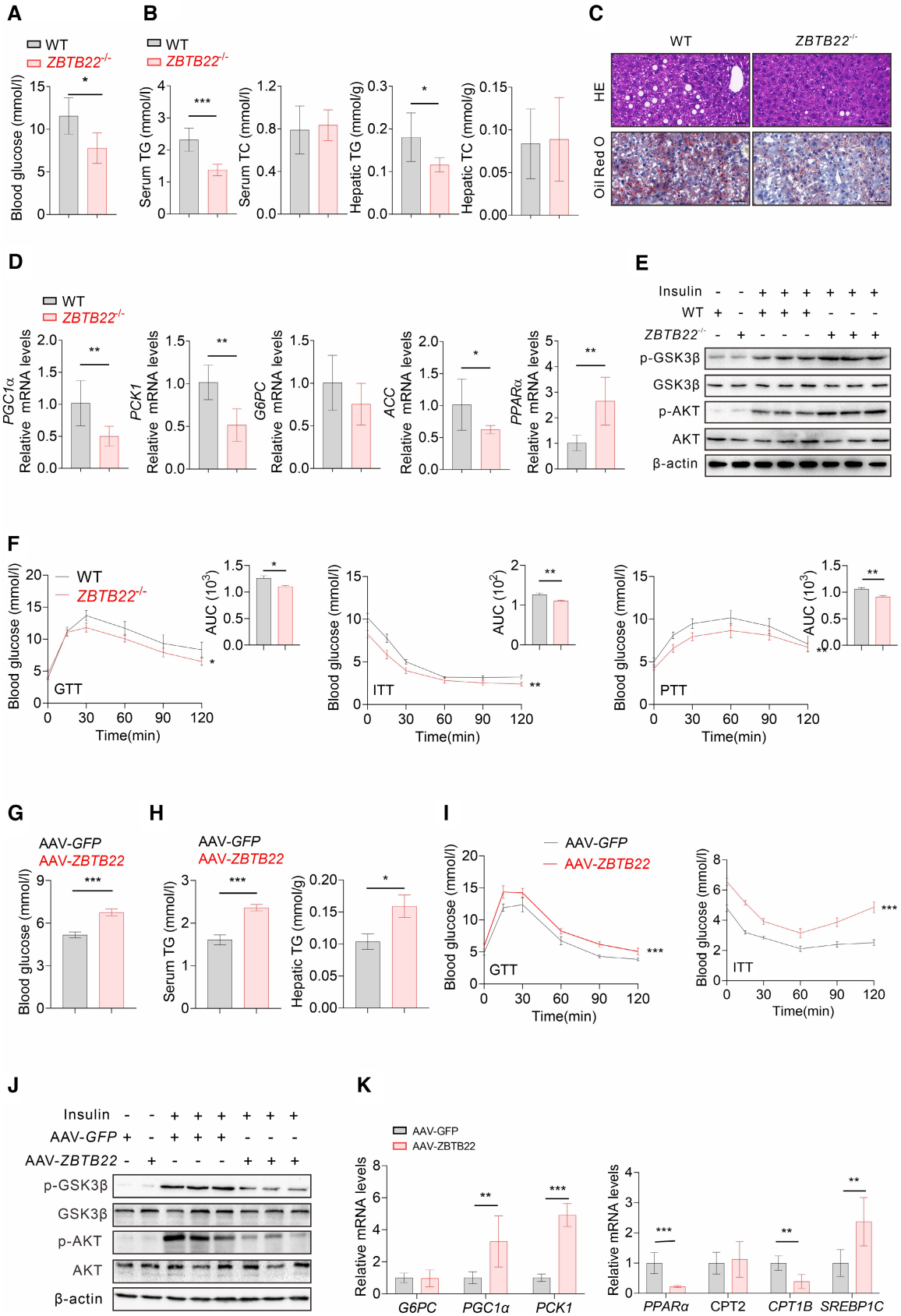
A
B
C
WT
ZBTB22-/-
Blood glucose (mmol/l)
Serum TG (mmol/l)
Serum TC (mmol/l)
Hepatic TG (mmol/g)
Hepatic TC (mmol/g)
HE
Oil Red O
D
E
PGC1α Relative mRNA levels
PCK1 Relative mRNA levels
G6PC Relative mRNA levels
ACC Relative mRNA levels
PPARα Relative mRNA levels
Insulin
p-GSK3β
GSK3β
p-AKT
AKT
β-actin
F
GTT
ITT
PTT
AUC (10^3)
AUC (10^2)
Time(min)
G
H
I
AAV-GFP
AAV-ZBTB22
J
K
G6PC
PGC1α
PCK1
PPARα
CPT2
CPT1B
SREBP1C
Relative mRNA levels

Figure 4.

Figure 4. Hepatic *ZBTB22* induces glucose and lipid disorder.

A, B *ZBTB22* deletion effectively decrease the fasting blood glucose and TG levels in the liver and serum of mice fed on HFD (n = 6 mice).
C HE and oil red O staining showed a reduction in hepatic lipid deposition in mice fed on HFD, scale bars represent 20 μm.
D *ZBTB22* deletion significantly changed hepatic expression of genes involved in gluconeogenesis, lipogenesis, and fatty acid oxidation in mice fed on HFD (n = 6 mice).
E *ZBTB22* deletion significantly improved the phosphorylation of AKT and GSK3β in the liver of mice fed on HFD.
F *ZBTB22* deficiency obviously improved the glucose tolerance, insulin sensitivity and pyruvate tolerance in mice fed on HFD (n = 6 mice).
G, H Hepatic *ZBTB22* overexpression increase the fasting blood glucose and TG levels in serum and liver of *ZBTB22* deficiency mice fed on HFD (n = 6 mice).
I Hepatic *ZBTB22* overexpression induces glucose intolerance and insulin resistance in *ZBTB22* deficiency mice fed on HFD (n = 6 mice).
J Hepatic *ZBTB22* overexpression decreased the insulin-induced phosphorylation of AKT and GSK3β in the liver of *ZBTB22* deficiency mice fed on HFD.
K qPCR analysis of hepatic genes involved in gluconeogenesis, lipogenesis, and fatty acid oxidation in *ZBTB22* deficiency mice fed on HFD (n = 6 mice).

Data information: Data are means ± SEM. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ by Student's t-test.
Source data are available online for this figure.

above results, hepatic *ZBTB22* overexpression increased the expression of gluconeogenic genes, *PGC1α* and *PCK1*, but not *G6PC*, thereby raising fasting blood glucose levels (Fig 6A and B). Additionally, *ZBTB22* forced overexpression dramatically curbed the glucose tolerance of wild-type C57BL/6J mice, even when fed a standard chow diet, while their insulin sensitivity and pyruvate tolerance were slightly yet significantly impaired (Fig 6C; Appendix Fig S7A). Also, the insulin-induced phosphorylation of AKT and GSK3βwas slightly decreased, suggesting a dysfunctional insulin response in the liver (Fig 6D). We also found that the liver *ZBTB22* forced overexpression also induced the expression of the lipogenic genes, *SREBP-1C*, *FAS*, and *ACC*. By contrast, suppressed the expression of the genes involved in fatty acid oxidation, *PGC1* and *CP1B*, resulting in an increasing levels of both hepatic and serum triglyceride (Fig 6E and F; Appendix Fig S7B–D). HE and Oil Red O staining also showed a slight but visible lipid accumulation in the liver (Fig 6G). However, hepatic *ZBTB22* forced overexpression did not change ALT and AST serum levels, implying an unaltered hepatic function in mice kept on standard chow diet mice (Fig 6H). However, the liver *ZBTB22* overexpression heightened the serum levels of proinflammatory cytokines and the expression of hepatic inflammatory genes (Fig 6I and J; Appendix Fig S7E). These changes led to a dysfunctional metabolism of glucose and lipid. Taken together, these results showed that by accelerating gluconeogenesis and lipogenesis hepatic *ZBTB22* plays essentially roles to promote the development of diabetes and hepatic steatosis.

ZBTB22 actives PCK1 transcription by directly binding to its promoter region, thereby accelerating hepatocellular gluconeogenesis and lipogenesis

To probe the *ZBTB22*-specific actions associated with the downstream mechanisms controlling hepatic glucose and lipid metabolism, we performed RNA-seq analysis on MPHs infected with an Ad-*ZBTB22* or Ad-*GFP*. As expected, our results revealed an *ZBTB22*-induced expression of gluconeogenic genes, especially of *PCK1* and *G6PC*. Conversely, the expression of *PGC1α* did not change, which was inconsistent with the above results and suggested a direct regulatory function of *ZBTB22* on *PCK1* expression compensatively affecting other gluconeogenic and lipogenic genes (Fig 7A). Moreover, we further verified that *ZBTB22* overexpression induced PEPCK1 level in Ad-*ZBTB22* infected MPHs (Fig 7B and C). To further explore the underlying mechanism, we conducted a luciferase reporter gene assay by generating a series of luciferase reporter plasmids holding shorter fragments of the *PCK1* gene promoter (−1284Luc, −783Luc, and −254Luc). We found that the *ZBTB22* forced overexpression enhanced the transcription of −1284Luc and −783Luc, while no effect obtained on the truncated −254 bp (−254Luc). This suggested that the *ZBTB22* transcription factor's putative binding and working site was located from 783 to 254 bp (Fig 7D left). To validate this hypothesis, we generated a luciferase reporter construct containing a mutation of the –670/–654 nt site and found that this mutation greatly diminished the ZBTB22 protein-dependent activation of the *PCK1* promoter (Fig 7D right). This result was also confirmed by ChIP and ChIP-qPCR results, as shown by the amplified binding regions of the precipitates obtained from the anti-ZBTB22 antibody group, but not from the normal rabbit IgG group (negative control) in the C57BL/6J mice liver tissue lysates (Fig 7E and F).

To corroborate *PCK1* expression's import in mediating ZBTB22-induced glucose and lipid reprogramming, we generated a small interfering (si)RNA against *PCK1* mRNA and transfected it into MPHs. Next, we also infected the cells with an Ad-*ZBTB22* or Ad-*GFP*. Interestingly, *PCK1* silencing effectively abrogated the stimulatory effects of the *ZBTB22* transcription factor on lipogenesis (Fig 7G–I). Moreover, *ZBTB22* forced overexpression did not change *SREBP-1C* expression and glucose accumulation in MPHs (Fig 7J and K). Additionally, we injected AAV-*GFP* or AAV-*ZBTB22* via the tail vein into AAV-sh*PCK1*-pretreated mice to generate *PCK1* silenced mice, which did or did not overexpress *ZBTB22*. Of note, we found that *PCK1*silencing abrogated the effects of ZBTB22 on glucose and lipid metabolism, as shown by the lack of effects on fasting blood glucose levels and hepatic lipid deposition, and by the unchanged glucose tolerance and insulin sensitivity (Fig 8A–G).

Overall, these results suggested that *ZBTB22* transcription factor could separately increase the gluconeogenesis and lipogenesis by directly binding to the promoter region of *PCK1* and transcriptionally activate the gluconeogenic pathway while in a compensatory fashion affecting cellular lipogenesis.

Discussion

An abnormal hepatic glucose metabolism is the main pathological driver of various metabolic syndromes such as T2D, obesity, and NAFLD. Their most apparent pathophysiological characteristics are a disordered gluconeogenesis, whose role is preeminent, and an

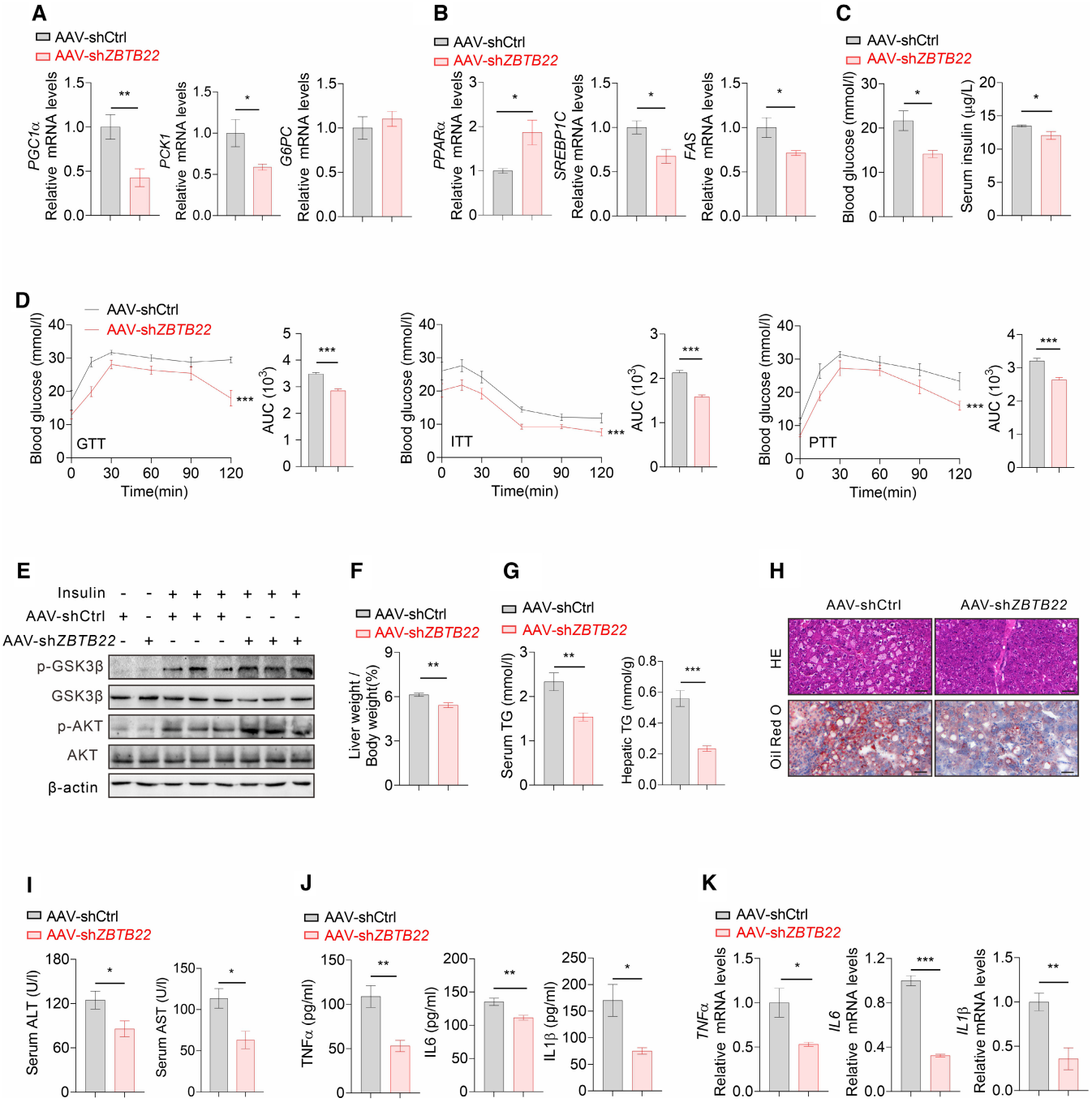

Figure 5. Hepatic *ZBTB22* silencing improves hyperglycemia and liver steatosis in db/db diabetic mice.

A, B Hepatic *ZBTB22* silencing decreases the expression of gene involved in gluconeogenesis, lipogenesis, and fatty acid oxidation in db/db mice (n = 6 mice).

C Hepatic *ZBTB22* silencing reduces the fasting blood glucose and insulin levels in db/db mice (n = 6 mice).

D Hepatic *ZBTB22* silencing improved the glucose tolerance, insulin sensitivity, and pyruvate tolerance in db/db mice (n = 6 mice).

E Hepatic *ZBTB22* silencing improves the insulin-induced phosphorylation of AKT and GSK3β in the liver of db/db mice.

F, G Hepatic *ZBTB22* silencing decrease the ratio of liver weight relative to body weight, TG contents in the serum and liver of db/db mice (n = 6 mice).

H HE and oil red O staining showed a reduction in hepatic lipid deposition in db/db mice after *ZBTB22* silencing, scale bars represent 20 μm.

I Hepatic *ZBTB22* silencing decreases the serum ALT and AST levels in db/db mice (n = 6 mice).

J, K Hepatic *ZBTB22* silencing decreases serum inflammatory cytokines levels and hepatic inflammatory genes expression (n = 6 mice).

Data information: Data are means ± SEM. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ by Student's t-test.
Source data are available online for this figure.

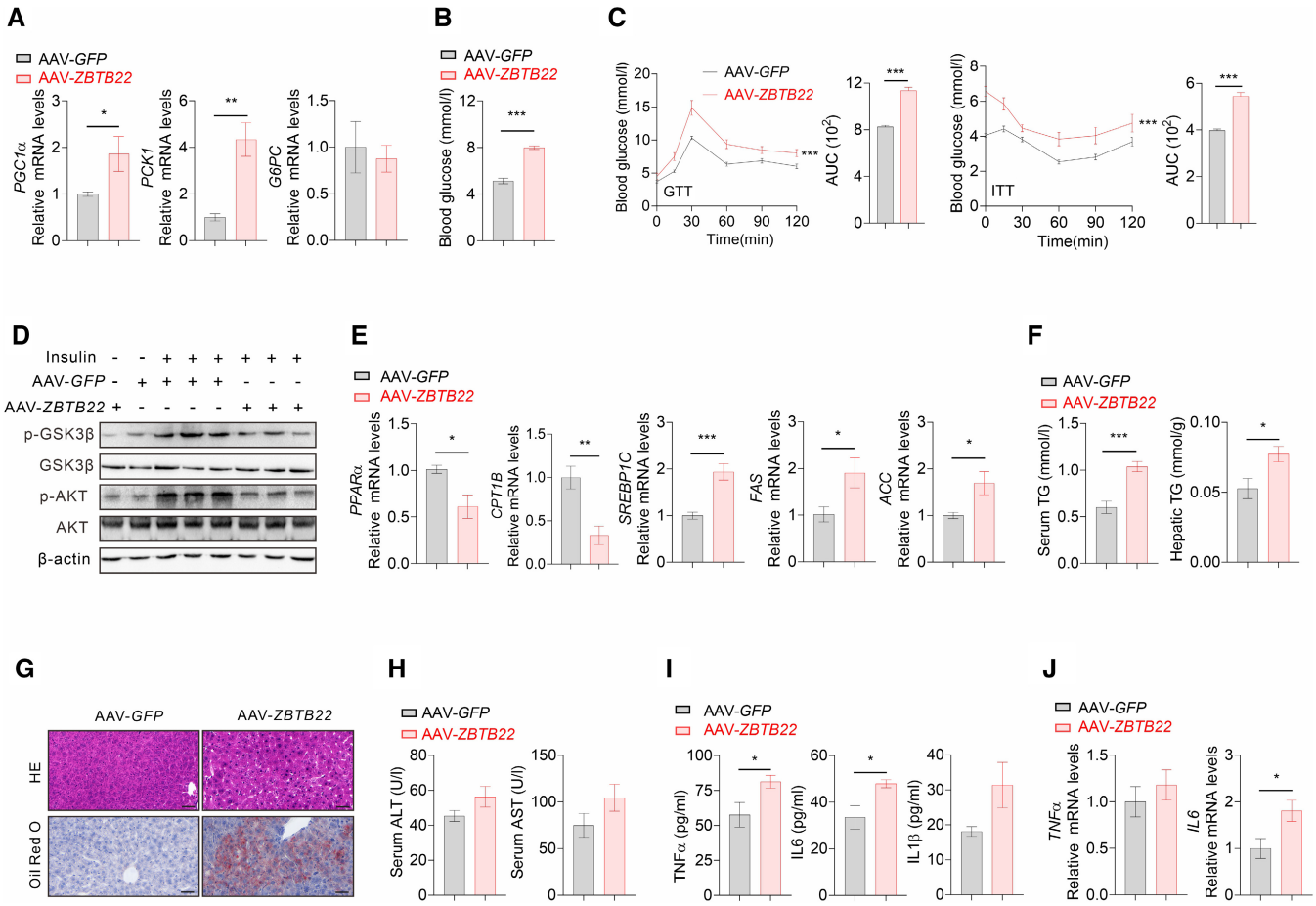

Figure 6. Hepatic *ZBTB22* overexpression leads to hyperglycemia and lipid disorder in wild-type C57BL/6J mice.

A, B Hepatic *ZBTB22* overexpression increases the expression of genes involved in gluconeogenesis and fasting blood glucose levels in normal C57 mice (n = 6 mice).

C Hepatic *ZBTB22* overexpression significantly induced glucose intolerance and insulin resistance in normal C57 mice (n = 6 mice).

D Hepatic *ZBTB22* overexpression significantly decreased insulin-induced phosphorylation of hepatic AKT and GSK3β in normal C57 mice.

E Hepatic *ZBTB22* overexpression significantly affects the expression of genes related to lipogenesis and fatty acid oxidation in normal C57 mice (n = 6 mice).

F Hepatic *ZBTB22* overexpression increased hepatic and serum TG levels in normal C57 mice (n = 6 mice).

G HE and oil red O staining showed a slight increase of hepatic lipid deposition in Ad-*ZBTB22* infected mice, scale bars represent 20 μm.

H Hepatic *ZBTB22* overexpression increases serum ALT and AST levels in normal C57 mice (n = 6 mice).

I, J Hepatic *ZBTB22* overexpression increase serum inflammatory cytokines levels and hepatic expression of inflammatory genes (n = 6 mice).

Data information: Data are means ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001 by Student's t-test.
Source data are available online for this figure.

Figure 7. *ZBTB22* transcriptional actives *PCK1* to accelerate cellular gluconeogenesis and lipogenesis via directly binding to its promoter region.

A RNA sequencing analysis indicated that *ZBTB22* overexpression obviously increase the gluconeogenic genes, especially *PCK1*.

B, C Immunofluorescence and Western blot analysis showed that ZBTB22-mediated induced the upregulated expression of PEPCK1 in Ad-ZBTB22-infected mice primary hepatocytes (B) and livers (C), scale bars represent 10 μm.

D Luciferase reporter gene assay showed potential binding sites of *ZBTB22* on the promoter region of *PCK1* (−670 to −654 bp) (n = 3 biological replicates).

E, F Chip and Chip-qPCR displayed increase occupancy of *ZBTB22* on the promoter region of *PCK1* (n = 3 biological replicates).

G Western blot analysis showed the expression of PEPCK1 after transfected small interfering (si)RNA against *PCK1* into MPHs, following by Ad-*ZBTB22* or Ad-*GFP* infection (n = 4 biological replicates).

H–K TG level (H) (n = 4 biological replicates) and Immunofluorescence (I) showed *PCK1* silencing an invalid effect on lipid accumulation in MPHs, as well as the stimulatory effects of *ZBTB22* on *SREBP1C* expression (J) (n = 4 biological replicates) and cellular glucose output (K) (n = 4 biological replicates), scale bars represent 10 μm.

Data information: Data are means ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001 by Student's t-test.
Source data are available online for this figure.

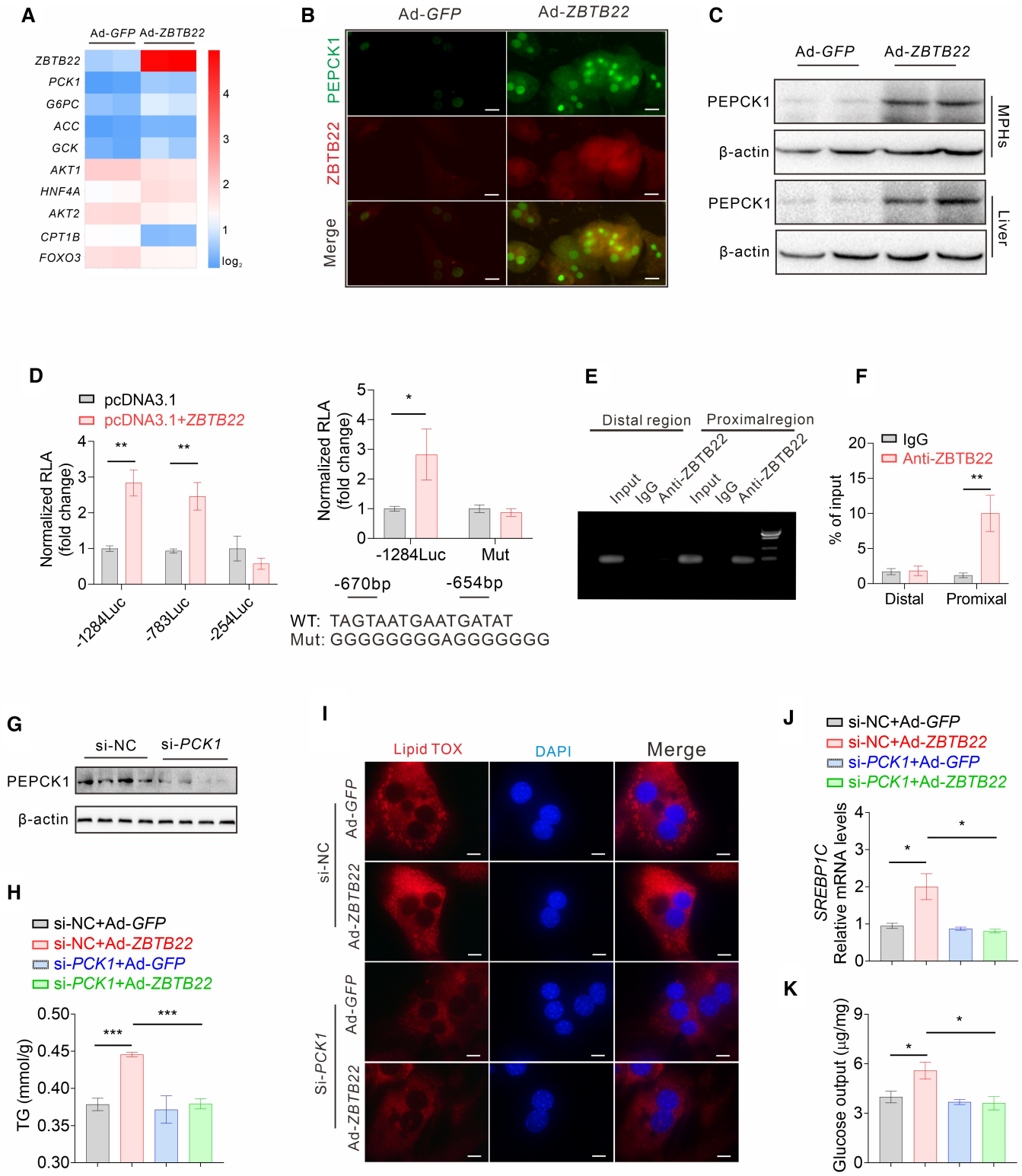

Figure 7.

altered glycogenolysis, both of which concur to increase the hepatic glucose output (Moller, 2001). Therefore, effectively inhibiting liver's excessive gluconeogenesis and glycogenolysis will be an important approach to therapeutically target T2D and the glucometabolic syndromes. Our work has revealed the role and underlying mechanism of action of the novel ZBTB22 transcription factor in the enhanced gluconeogenesis proper of T2D. Therefore, ZBTB22 is an important therapeutic target for T2D therapy.

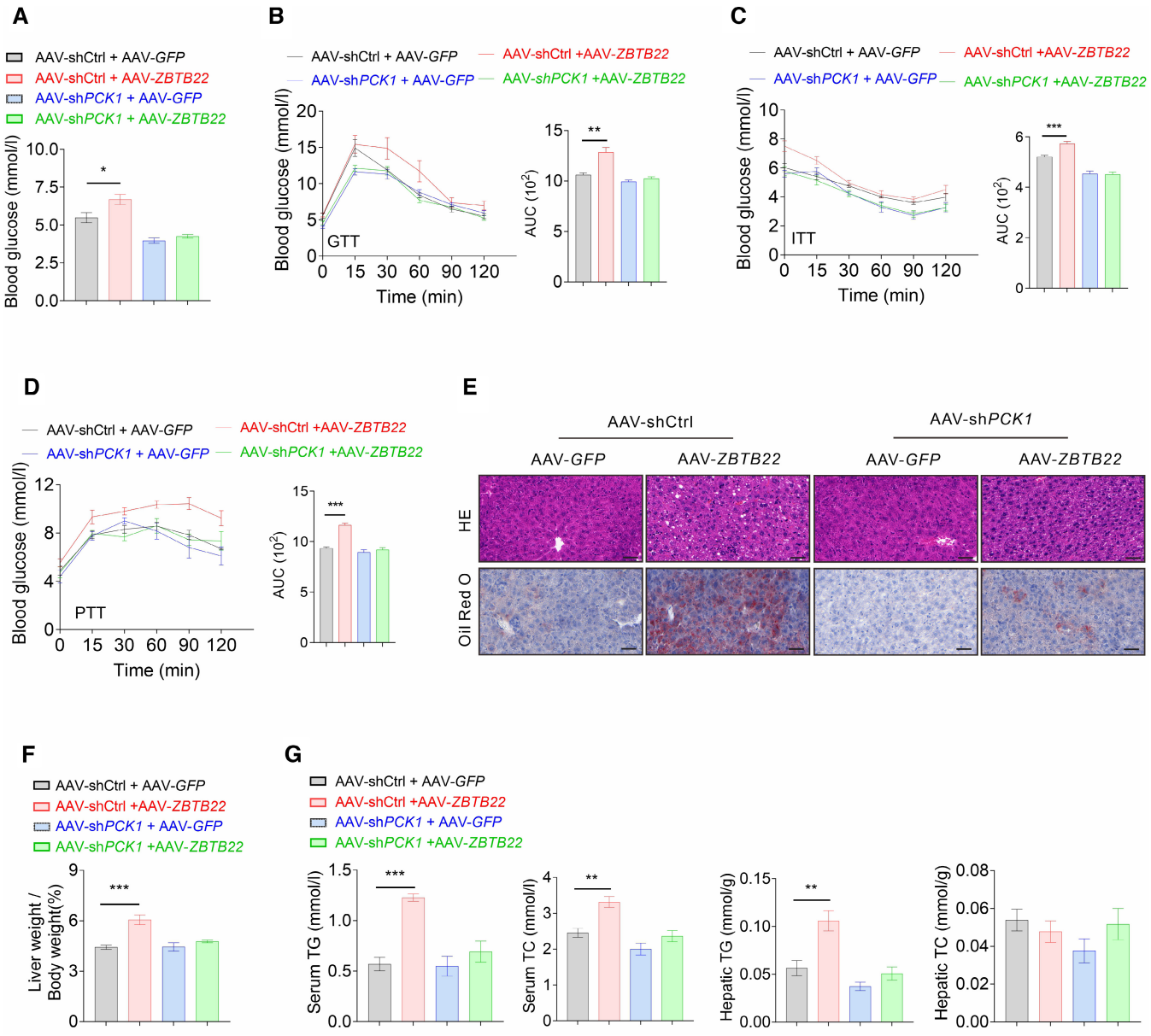

Figure 8. Silencing of hepatic *PCK1* expression abrogated *ZBTB22* overexpression-induced glucose and lipid disorder in normal C57 mice.

A Hepatic *ZBTB22* overexpression failed to change the fasting blood glucose levels in *PCK1*-silencing C57 mice (n = 6 mice).

B–D Hepatic *ZBTB22* overexpression failed to change glucose tolerance, insulin sensitivity, and pyruvate tolerance in *PCK1*-silencing C57 mice (n = 6 mice).

E HE and oil red O staining of liver sections from mice with indicated adenovirus treatment, scale bars represent 20 μm.

F Hepatic *ZBTB22* overexpression failed to change the ratio of liver weight relative to body weight in *PCK1*-silencing C57 mice (n = 6 mice).

G Hepatic *ZBTB22* overexpression failed to change serum and hepatic TG and TC levels in *PCK1*-silencing C57 mice (n = 6 mice).

Data information: Data are means ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001 by Student's t-test.
Source data are available online for this figure.

Previous lines of evidence indicated that hepatic gluconeogenesis is affected and regulated by a variety of physiological or environmental factors. Especially, the family of endogenous zinc finger transcription factors plays an important role by regulating the expression of gluconeogenic gene to keep the whole body's glucose balance. We and others have confirmed that Krüppel-like factors (KLFs) bearing zinc finger domains could directly bind to the promoter region of key genes involved in glucose and lipid metabolism, such as *PGC1α*, and *PPARα*, to regulate hepatic metabolism processes. The ZBTB22 protein (aka ZNF297 or BING1) is a transcription factor abundantly expressed in the kidneys, liver, gall bladder, and other tissues. Its N-terminus contains a typical POK/BTB domain while its C-terminus owns the C2H2-zinc-finger domain that recognizes promoter or enhancer DNA sequences to play a

transcriptional regulatory role (Herberg *et al*, 1998). The transcription factors of the POK/BTB family have been associated with the regulation of several key cellular processes, such as transcriptional regulation, proliferation, differentiation, development, oncogenesis, and tumor suppression (Kim *et al*, 2013). Available evidence has suggested that multiple POK/BTB family transcription factors regulate hepatic glucose, insulin, and lipid metabolism. For instance, ZBTB7A promotes lipid accumulation through the activation of *SREBP1* and the NF-κB pathway and plays a significant role in NAFLD development (Zhou *et al*, 2020). The Th-POK (ZBTB7b) transcription factor directly modulates the expression of the insulin receptor substrate-1 (IRS-1) and of the insulin-induced AKT–mTOR-SREBP signaling (Zhang *et al*, 2018). Even though PLZF (Zbtb16) has been reported to regulate hepatic gluconeogenesis potentially through regulation of Pck1, the detailed mechanism and binding site is far from clear (Chen *et al*, 2014). ZBTB20-deficient mice displayed a hypoglycemic phenotype, which proved the essential role of ZBTB20 in the maintaining of glucose homeostasis (Sutherland *et al*, 2009; Jeon *et al*, 2012). The Kr-POK (ZBTB7c) transcription factor enhanced FASN expression by regulating the DNA binding of *SREBP-1C* and *SP1* at the proximal promoter, a finding indicating that Kr-POK might importantly control fatty acid synthesis (Choi *et al*, 2019). These results indicated the members of ZBTBs manipulate glucose and lipid metabolism in different manner. Besides, these previous studies mainly focused on the effects of ZBTB7, ZBTB16, and ZBTB20 on manipulating glucose and lipid metabolism in different manner under the pathological condition. In the present study, we uncover an alternative POK/BTB family transcription factor, ZBTB22, to ZBTB22 upregulates SREBP1C to contribute to fatty liver formation and insulin resistance under pathological context. It also was induced by fasting stimulation, and suggesting the important role of Zbtb22 in the physiological condition.

Previous studies had evidenced that the focus of the functional role of the ZBTB22 transcription factor lies mainly on cancer development (Douglas *et al*, 2020), thereby identifying ZBTB22 as a prognostic marker of oncogenesis. Hitherto, the role of ZBTB22 in the pathological development of T2D remains unknown. To uncover that, ZBTB22 is linked to glucose metabolism, we first proved that hepatic ZBTB22 expression significantly increased in db/db mice, ob/ob mice, and HFD-induced obesity, respectively. We also showed that hepatic ZBTB22 expression was modulated by nutritional conditions, fasting included, and by pathophysiological contexts (e.g., insulin resistance and diabetes). Actually, fasting induces ZBTB22 expression, whereas refeeding after fasting restores *ZBTB22* mRNA and its protein to normal levels. Under fasting conditions, the output of glucagon and glucocorticoids surges while insulin levels are decreased, increasing hepatic glucose output (Yoon *et al*, 2001; Puigserver *et al*, 2003; Cui *et al*, 2019). Our data demonstrated that glucagon, insulin, and glucocorticoids could regulate the expression of ZBTB22, indicating that ZBTB22 may be involved in the control of hepatic glucose homeostatic response to circulating hormones, especially in health and disease contexts. Theoretically, an increased hepatic ZBTB22 expression in diabetic mice might contribute to the diabetic phenotype. Therefore, we developed two mice models, including global ZBTB22$^{-/-}$ mice and liver-specific *ZBTB22* knockdown db/db mice. Next, we proved that the expression of gluconeogenic genes was decreased in both these two mice models—a condition reducing hepatic glucose output and preventing hyperglycemia from occurring (Yoon *et al*, 2001; Puigserver *et al*, 2003; Cui *et al*, 2019). Conversely, an Adenovirus-mediated liver-specific overexpression of *ZBTB22* led to the induction of hepatic gluconeogenic genes and to a diabetic phenotype in normal C57BL/6J mice. Collectively, our results support the view that ZBTB22 plays critical roles in regulation of hepatic glucose homeostasis in diabetes by decreasing hepatic gluconeogenesis, and by improving hepatic impaired glucose tolerance and insulin sensitivity. Altogether, our findings indicating that ZBTB22 may be a therapeutic target for T2D treatment.

In mammals, blood glucose levels are tightly kept within a very narrow range through the precise control of the balance between glucose output from the liver and glucose uptake by peripheral tissues. Fasting increased glucagon's circulating levels and induced *PGC1α* gene expression via the CREB/TORC pathway (Herzig *et al*, 2001; Yoon *et al*, 2001; Puigserver *et al*, 2003; Koo *et al*, 2005), thereafter, by directly interacting and co-activating with various transcription factors, including HNF-4α, FoxO1, and GR, the PGC-1α transcription factor induces the expression of *PCK1* and *G6PC*, which activate hepatic gluconeogenesis (Yoon *et al*, 2001; Puigserver *et al*, 2003). Conversely, a meal increases blood glucose availability, heightens serum insulin levels, which promotes glucose uptake by peripheral tissues while blocking hepatic gluconeogenesis though the repression of PEPCK1 and G6Pase activities via various mechanisms (Nakae *et al*, 2002; Li *et al*, 2007). Therefore, suppressing the abnormal PEPCK1 and G6Pase activation might be an attractive therapeutic strategy for T2D treatment. In the present study, our transcriptomic data indicated that *ZBTB22* mRNA and its protein overexpression could effectively increase *PCK1* expression. Besides, the change of *ZBTB22* expression slightly but not significantly altered other gluconeogenic genes, including *G6PC* and *PGC1α*, suggesting that *PCK1* maybe the specific target gene of the ZBTB22-induced hepatic gluconeogenesis. This hypothesis is also strengthened by the results of our *in vivo* tests showing that *ZBTB22* knockout/knockdown decreased *PCK1* expression, while *ZBTB22* forced overexpression elevated *PCK1* expression. Thus, these results indicated that the ZBTB22, as a transcriptional factor, might regulate *PCK1* transcription by directly binding to its promoter region. Mechanistically, being a member of the POK family of transcription factors, to exert its transcriptional effects the ZBTB22 protein binds GC-rich DNA sequences through its zinc finger domain (Maeda *et al*, 2005; Molloy *et al*, 2018). Consistently, via a luciferase reporter analysis driven by the *PCK1* promoter section of 1,284 base pairs placed upstream from the promoter start site and through a ChIP assay, we proved that ZBTB22 controls *PCK1* transcriptional activity by directly binding to the latter's promoter region (from 670 to 654 bp), thereby modulating *PCK1* expression at the transcriptional level to accelerate hepatic gluconeogenesis. Otherwise, ZBTB22 overexpression could not only induce the expression of *PCK1* and of its compensatory target gluconeogenic genes, such as *PGC1α* and *G6PC*, but also enhance the FSK-mediated increase in gluconeogenic genes expression both *in vivo* and *in vitro*, consequently resulting in an upsurge of glucose output. In contrast, *ZBTB22* knockdown or knockout attenuated the induction of *PCK1* expression. And interfering with *PCK1* expression completely abolished the ZBTB22-induced hepatic gluconeogenesis in MPHs or in mouse models. Collectively, our results strongly support the notion that the ZBTB22 transcription

factor regulates *PCK1* expression at the transcriptional level by directly binding to its promoter and upregulating its expression and that of its compensatory target genes *PGC-1α* and *G6P*, thereby inducing an abnormally elevated hepatic gluconeogenesis. Therefore, *PCK1* is the typical target gene of ZBTB22 transcription factor.

Of note, we also found corresponding differences in the metabolism of hepatic lipids elicited by changes in *ZBTB22* expression. ZBTB22$^{-/-}$ mice displayed a higher energy expenditure and a lower fat mass, which would suggest a potential *ZBTB22* role in the regulation of adipogenesis and thermogenesis at the adipose tissue levels. However, when we focused the study on the liver, we found that *ZBTB22*'s effective role was the regulation of lipid homeostasis, which may depend on the transcriptional activation of *PCK1*-mediated gluconeogenesis. This observation is consistent with the fact that diabetic patients often have lipid metabolism disorders. In db/db mice, hepatic *ZBTB22* knockdown not only improved the glucose disorder but also mediated the expression of genes involved in lipid metabolism, and induced the remission of liver steatosis. Moreover, *ZBTB22* knockout significantly attenuated the HFD-induced fatty liver phenotype. On the contrary, in hepatic *ZBTB22* overexpressing C57BL/6J mice, a fatty liver phenotype occurred, which may be the upshot of a compensatory effect counteracting the increased gluconeogenesis. Interestingly, interfering with liver *PCK1* expression almost abrogate the ZBTB22-induced lipogenic genes expression and hepatosteatosis in MPHs, suggesting that ZBTB22 transcription factor activated *PCK1*'s expression plays a pivotal role in the hepatic metabolism of glucose and lipid metabolism. Otherwise, we conducted the GTT, ITT, and PTT experiments on *ZBTB22* KO female mice or AAV-induced *ZBTB22* overexpression female mice, and these results further showed similar effects of ZBTB22 on regulating blood glucose homeostasis, suggesting that the effects of ZBTB22 is independent on gender (Appendix Fig S8A and B).

In conclusion, our study has revealed that *ZBTB22* plays crucial physiological role in the regulation of hepatic glucose and lipids metabolism. A raised *ZBTB22* expression might be the underlying factor increasing hepatic gluconeogenesis, thereby promoting the T2D development: directly binding of the ZBTB22 transcription factor to the specific promoter region of *PCK1*, which would accelerate gluconeogenesis and lipogenesis. Conversely, *ZBTB22* deletion decreases *PCK1*'s expression in mouse liver, thereby suppressing hepatic gluconeogenesis and decreasing blood glucose levels. Therefore, our results show that ZBTB22 is a novel target for the prevention and treatment of T2D, an achievement of hopefully important theoretical and practical significance.

Materials and Methods

Ethics statement

Each patient had written informed consent and the study protocol conforms to the ethical guidelines of the 2008 Declaration of Helsinki as reflected in *a priori* approval by the ethics committees of the First Affiliated Hospital of Guangzhou University of Chinese Medicine (K-2022-079). All animal care and experimental studies were approved by and in accordance with the guidelines of the Animal Ethics Committee of Guangzhou University of Chinese Medicine (20220228002).

Clinical samples

Human specimens were taken from patients with cholecystitis who underwent liver biopsy at the First Affiliated Hospital of Guangzhou University of Chinese Medicine.

Animals and experimental design

Male db/db, ob/ob, and their control mice aged 6–8 weeks were purchased from GemPharmatech Co., Ltd (Guangdong, China). C57BL/6J mice aged 6–8 weeks were purchased from the Animal Experimental Centre of Guangzhou University of Chinese Medicine (Guangdong, China) and *ZBTB22* knockout mice were generated by Cyagen Bioscience using CRISPR/Cas-mediated genome engineering (Jiangsu, China). Exon 2 will be selected as target site. The gRNA to mouse *ZBTB22* gene, and Cas9 mRNA were co-injected into fertilized mouse eggs to generate targeted knockout offspring. F0 founder animals were identified by PCR followed by sequence analysis, which were bred to wild-type mice to test germline transmission and F1 animal generation. *ZBTB22* knockout was confirmed by qPCR targeting Exon 2 with following primers: F: ACTTCCACGACC AGGTCCTACT; R: GTGAGGAAGTTGACAATGTCCGC.

All mice were housed and maintained in 12-h light/dark photoperiod with unrestricted water and food. In order to establish a diet-induced obese (DIO) model, wild-type C57BL/6J mice were fed a 60% high-fat diet (D12492, Research Diets NewBrunswick, NJ, USA) with free access to water.

Adenovirus expressing green fluorescent protein (Ad-GFP), adenovirus expressing *ZBTB22* (Ad-*ZBTB22*), control adenovirus expressing short hairpin (sh)RNA against negative control sequence (Ad-shCtrl), adenovirus expressing shRNA against *ZBTB22* (Ad-sh*ZBTB22*), Adeno-associated virus expressing green fluorescent protein (AAV-*GFP*), Adeno-associated virus expressing *ZBTB22* (AAV-*ZBTB22*), control Adeno-associated virus expressing short hairpin (sh) RNA against negative control sequence (AAV-shCtrl), Adeno-associated virus expressing shRNA against *ZBTB22* (AAV-sh*ZBTB22*) or Adeno-associated virus expressing shRNA against *PCK1* (AAV-sh*PCK1*) and all plasmids were constructed and purchased from Shanghai ObioTechonology Company (Shanghai, China) and mice were intravenously injected through the tail vein with the indicated Adeno-associated virus ($1.0–1.5 \times 10^{11}$ active viral particles in 200 μl saline). At 4 weeks after infection, anesthetized mice were fasted for 6 h, and their livers and plasma were collected for further analyses.

In vivo glucose, insulin, and pyruvate tolerance tests

ZBTB22 knockout mice or mice infected with the indicated Adeno-associated virus were subjected to the following tests. For GTT and PTT, mice were fasted overnight and injected with D-glucose (1–2 g/kg) or pyruvate sodium (0.5–1.5 g/kg) via i.p. injection. For ITT, mice were fasted 6 h and injected with insulin (0.5–0.75 U/kg) via i.p. injection. Blood glucose levels were measured at 0, 15, 30, 45, 60, 90, and 120 min from the tail vein using a glucose monitor (OnCallEZIV, China).

RNA interference

The sequence of small interfering (si)RNA against luciferase Negative Control siRNA (NC siRNA) and *PCK1* siRNA sequence was purchased from Suzhou GenePharma Co., Ltd. The sequence of NC siRNA was 5′-UUGUCCGAACGUGUCACGUTT-3′; and the sequence of siRNA against *PCK1*-Mus-1102 (si*PCK1*) was 5′-GCAACUUAAGGGCUAUCAA TT-3′. *PCK1* siRNA or NC siRNA was performed according to the manufacturers Lipofectamine (CA, USA).

Cell culture

Mouse primary hepatocytes (MPHs) were isolated from livers of male C57BL/6J mice (6–8 weeks of age) and cultured as previously reported (Cabral *et al*, 2018). Mouse primary hepatocytes were infected with Ad-*ZBTB22*, Ad-*GFP*, Ad-shCtrl, or Ad-sh*ZBTB22* for 24–36 h, and cells were treated with Forskolin (FSK, 10 μmol/l, 24 h), oleic acid and palmitic acid (OA&PA, OA: 200 μmol/l; PA: 200 μmol/l, 24 h) and insulin (10 mg/l, 30 min), and then cells were harvested for further analyses. For coinfection experiments, 24 h after transfection with *PCK1* siRNA or NC siRNA, cells were coinfected with Ad-*ZBTB22* or Ad-*GFP* for another 24 h and treated with above items subsequently. Cells were then harvested for further analyses.

Metabolic cages and MRI

For energy expenditure monitoring, mice were housed and adapted for 24 h individually in metabolic cages (Promethion monitoring system (SABLE)). And then the heat production, respiratory exchange ratio (RER), carbon dioxide production (VCO2), oxygen consumption (VO2), and X-axis ambulatory (XAMB) for next 36 h were recorded and data from 24 h were chose to show as previously reported. Body composition including fat mass and lean mass was detected by MRI (Echomri.Combo-700).

RNA-seq

RNA-seq of MPHs infected with Ad-*GFP* or Ad-*ZBTB22* was conducted and analyzed by Berry Genomics Corporation, Beijing, China. Briefly, RNA sample preparations with 1 μg RNA from above samples and sequencing were performed using NEBNext® Ultra™ RNA Library Prep Kit for Illumina® (NEB, USA) according to the manufacturer's recommendations. To attribute sequences to above samples, Index codes were added and cBot Cluster Generation System using TruSeq PE Cluster Kit v3-cBot-HS (Illumia) was used for the clustering of the index-coded samples following the manufacturer's instructions. After that, the library preparations were sequenced on an Illumina NovaSeq platform and 150 bp paired-end reads were generated. Raw data (raw reads) of fastq format were first processed and counted with HTSeq v0.6.1. And then FPKM of each gene was calculated based on the length of the gene and reads count mapped to this gene.

Real-time quantitative PCR (qPCR)

Total RNA was extracted from cells or liver tissues of indicated mice with TRIzol (Invitrogen), NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) was used to determine the quality and quantity. And then reverse transcription was conducted using the SuperScript™ VILO™ cDNA Synthesis Kit, and qPCR was carried out as previously described. The expression of specific genes was normalized by β-actin mRNA levels. Primers used in these experiments are listed in Appendix Table S1.

Western blot analysis

Proteins were extracted from fresh livers or cultured hepatocytes using a RIPA lysis buffer containing a cocktail of protease inhibitors (KeyGEN BioTECH, China), about 80 μg of protein was loaded and resolved using 10% SDS–PAGE (Bio-Rad Laboratories, Hercules, CA, USA) and separated proteins were eletrotransferred to immuno-Blot PVDF membranes. Western blot assays were conducted with primary antibodies: anti-ZBTB22 (1:1,000, NBP1-92603, Novus, USA), anti-PEPCK1 (TD6770), anti-AKT (T55551), anti-p-AKT (T40067), anti-GSK (T52434), anti-p-GSK (TA2016) (1:1,000, Abarmat, China), and anti-β-actin (1:2,000, AC038, Abclonal, China). The blots were then incubated with corresponding HRP-conjugated secondary antibodies and detected using enhanced chemiluminescence using Molecular Imager ChemiDOC™ XBS imaging systems (Bio-Rad Laboratories).

Immunofluorescence

The MPHs were treated as we mentioned before, then immersed in 1% PFA for 1 h. For Lipid TOX, cells were stained by HCS LipidTOX™ Deep Red Neutral Lipid Stain (1:1,000, Thermo Fisher Scientific, USA) for 30 min. For immunofluorescence staining, cells were sealed using 1% BSA at room temperature, then incubated with anti-ZBTB22 antibody (1:500, Bioss, China), PEPCK1 antibody (1:500, abarmat, China) at 4°C overnight, and further incubated with Alexa Fluor 488-conjugated anti-rabbit IgG (1:1,000). Images were obtained on an inverted Leica TCS SP8 confocal microscope (Leica, Germany).

Hematoxylin–Eosin staining (H&E staining) and oil red O staining

For H&E staining, livers were fixed with 4% paraformaldehyde, embedded in paraffin wax, cut into 5 μm sections, and stained by using hematoxylin and eosin. For oil red O staining, livers were cryosectioned into 7-μm sections, stained by oil red O staining solution. Images were obtained by using a microscope (Nikon, Japan).

Luciferase reporter gene assay

Luciferase reporter gene assays were performed as previously, hepG2 cells were grown in 24-well plates using Dulbecco's Modified Eagle Medium containing 10% (vol./vol.) FBS (Invitrogen). Luciferase reporter plasmids containing shorter fragments of the *PCK1* gene promoter and *ZBTB22* overexpressed plasmids were cotransfected into cells, together with aramlila luciferase expression vector as an internal control. Forty-eight hours later, cells were harvested and lysis, and the luminescence was detected using a luciferase assay with the Dual-Glo luciferase reporter assay system and Enspire Multimode Plate Reader (PerkinElmer, Waltham, MA, USA). Renilla luciferase activity of pCMV-RL-TK was used to correct the Relative luciferase activity and normalized to the activity of the control.

Chromatin immunoprecipitation (ChIP) assay

Chromatin immunoprecipitation assay was conducted as we previously reported. Briefly, liver tissues from indicated mice were lysed and sonicated. The protein–DNA complexes were immunoprecipitated with mouse IgG antibody (control) or anti-ZBTB22 antibody. And primers targeting the specific promoter region of *PCK1* were used to amplify with by PCR or qPCR. The primer as followed: F: 5′-CCAGCCGGCTGGGACCTGTTTA-3′; R: 5′-ACCCCTGCTCTAAA TGTTTTCT-3′.

Statistical analysis

Data were presented as the mean ± standard error of mean (SEM). After data were tested for normality, the differences among the intervention groups were determined. using the unpaired two-tailed Student's *t*-test for a single variable and one-way analysis of variance (ANOVA) followed by the *post hoc*, Tukey's test for multiple comparisons. Values with $P < 0.05$ were considered statistically significant (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

Data availability

The datasets produced in this study are available in the following databases: RNA sequencing data for ZBTB22 overexpression in mouse primary hepatocytes: NCBI Sequence Read Archive PRJNA930420 (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA930420). RNA sequencing data from high blood glucose patients: NCBI Sequence Read Archive PRJNA930561 (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA930561).

Expanded View for this article is available online.

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Author contributions
Naihua Liu: Formal analysis; funding acquisition; validation; visualization; methodology; writing – original draft. **Xiaoying Yang:** Supervision; funding acquisition; writing – review and editing. **Jingyi Guo:** Validation; methodology. **Lei Zhang:** Validation; methodology. **Shangyi Huang:** Methodology. **Jiabing Chen:** Methodology. **Jiawen Huang:** Formal analysis; methodology; writing – original draft. **Yingjian Chen:** Formal analysis; methodology; writing – original draft. **Tianqi Cui:** Methodology. **Yi Zheng:** Methodology. **Tianyao Li:** Methodology. **Kaijia Tang:** Methodology. **Yadi Zhong:** Methodology. **Siwei Duan:** Methodology. **Lili Yu:** Supervision; writing – review and editing. **Ying Tang:** Formal analysis; writing – original draft. **Dayong Zheng:** Supervision; funding acquisition; writing – review and editing. **Huafeng Pan:** Supervision; funding acquisition; writing – review and editing. **Yong Gao:** Conceptualization; supervision; funding acquisition; investigation; project administration; writing – review and editing.

Disclosure and competing interests statement
The authors declare that they have no conflict of interest.

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