Regulation of hepatic gluconeogenesis by nuclear factor Y transcription factor in mice

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Hepatic gluconeogenesis is essential to maintain blood glucose levels, and its abnormal activation leads to hyperglycemia and type 2 diabetes. However, the molecular mechanisms in the regulation of hepatic gluconeogenesis remain to be fully defined. In this study, using murine hepatocytes and a liver-specific knockout mouse model, we explored the physiological role of nuclear factor Y (NF-Y) in regulating hepatic glucose metabolism and the underlying mechanism. We found that NF-Y targets the gluconeogenesis pathway in the liver. Hepatic NF-Y expression was effectively induced by cAMP, glucagon, and fasting *in vivo*. Lentivirus-mediated NF-Y overexpression in Hepa1-6 hepatocytes markedly raised the gluconeogenic gene expression and cellular glucose production compared with empty vector control cells. Conversely, CRISPR/Cas9-mediated knockdown of NF-Y subunit A (NF-YA) attenuated gluconeogenic gene expression and glucose production. We also provide evidence indicating that CRE-loxP-mediated, liver-specific NF-YA knockout compromises hepatic glucose production. Mechanistically, luciferase reporter gene assays and ChIP analysis indicated that NF-Y activates transcription of the gluconeogenic genes *Pck1* and *G6pc*, by encoding phosphoenolpyruvate carboxykinase (PEPCK) and the glucose-6-phosphatase catalytic subunit (G6Pase), respectively, via directly binding to the CCAAT regulatory sequence motif in their promoters. Of note, NF-Y enhanced gluconeogenesis by interacting with cAMP-responsive element–binding protein (CREB). Overall, our results reveal a previously unrecognized physiological function of NF-Y in controlling glucose metabolism by up-regulating the gluconeogenic genes *Pck1* and *G6pc*. Modulation of hepatic NF-Y expression may therefore offer an attractive therapeutic approach to manage type 2 diabetes.

Glucose is a primary fuel for mammals. Blood glucose levels are mainly maintained by the hormone-regulated processes of hepatic glucose output and peripheral glucose uptake (1). Under short-term fasting conditions, the continued demand for glucose is supplied by the process of glycogenolysis, which is the breakdown of glycogen into free glucose. Prolonged fasting or starvation will cause the depletion of the finite hepatic glycogen storage. To avoid hypoglycemia, new glucose is synthesized via the process of gluconeogenesis (2). Gluconeogenesis is stimulated by glucagon and glucocorticoids, and massive elevation of serum glucose concentrations is observed upon simultaneous treatment with glucocorticoid and glucagon in animals, whereas fasting significantly elevates the serum concentration of these hormone (3, 4). It is generally accepted that excessive hepatic gluconeogenesis is the major contributor to the elevated blood glucose levels observed in patients with type 2 diabetes (5).

Gluconeogenesis in the liver is largely regulated at the transcriptional level by control of PEPCK[3] and G6Pase, which are the major rate-limiting enzymes for this glucose-generating pathway (6). These enzymes catalyze the conversion of oxaloacetate and glucose-6-phosphate to phosphoenolpyruvate and glucose, respectively. Expression of these enzymes is controlled via hormonal modulation of transcription factors and coactivators (7). Abundant evidence indicates that the cAMP signaling pathway is central to the hormonal control of gluconeogenesis (8), and cAMP-responsive element (CRE)-binding protein (CREB) is a particularly important effector of the cAMP pathway in liver (9, 10). Both *Pck1* and *G6pc* genes encoding PEPCK and G6Pase, respectively, possess CRE in their promoter regions, and binding of CREB to CRE increases the transcriptional activity of these genes. In addition, CREB could induce expression of peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α), which in turn stimulates the transcriptional activity of factors such as glucocorticoid receptor and FOXO1 on the promoters of gluconeogenic genes (11). However, hepatocytes lacking PGC-1α retain some ability to respond to cAMP (12), indicating that other pathways also contribute to control of gluconeogenic gene expression by the cAMP axis.

Nuclear factor-Y (NF-Y), a ubiquitous transcription factor conserved in evolution, is composed of three subunits including

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[3] The abbreviations used are: PEPCK, phosphoenolpyruvate carboxykinase; G6Pase, glucose-6-phosphatase catalytic subunit; NF-Y, nuclear factor Y; CRE, cAMP-responsive element; CREB, cAMP-responsive element-binding protein; sgRNA, small guide RNA; PAS, periodic acid–Schiff; LKO, liver-specific *Nf-ya* knockout; qPCR, quantitative PCR.

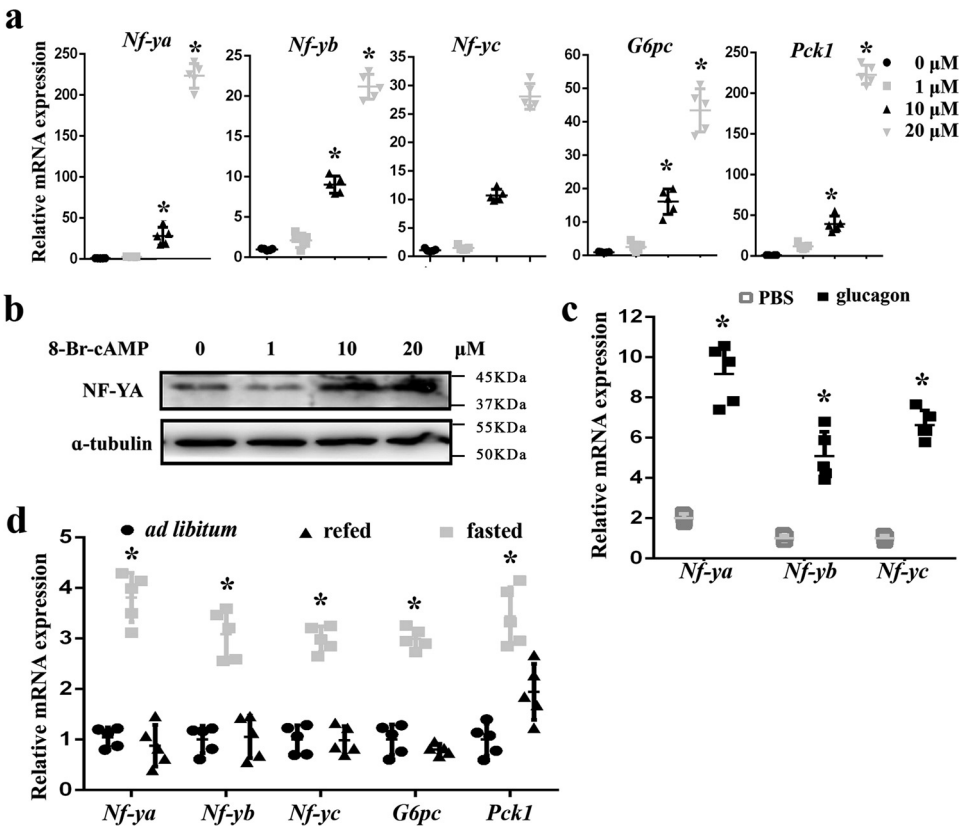

Figure 1. Induction of NF-Y expression in liver in response to the glucagon-cAMP axis and fasting. *a* and *b*, Hepa1-6 mouse hepatocytes were stimulated with different concentration (0, 1, 10, and 20 μM) of 8-Br-cAMP. *a*, mRNA expression levels of NF-Y subunits and gluconeogenic genes were measured by qRT-PCR. *b*, NF-YA protein levels were determined by Western blotting. *c*, C57BL/6 mice ($n = 5$/group) were challenged for 1 h with 300 μg/kg body weight glucagon; hepatic mRNA expressions of NF-Y subunit genes were analyzed by RT-qPCR. *d*, C57BL/6 mice ($n = 5$/group) fed *ad libitum*, fasted for 24 h, or fasted for 24 h and then refed for 24 h. Hepatic mRNA expression levels of NF-Y subunits and gluconeogenic genes were measured by RT-qPCR. The data represent means ± S.D. of three independent experiments. *, $p < 0.05$ *versus* vehicle treatment.

NF-YA, NF-YB, and NF-YC. NF-Y induces gene expression by binding to the CCAAT box in the proximal promoter region, so it is also named as the CCAAT-binding factor (13). It has been known that many genes are regulated by NF-Y, so NF-Y plays multiple roles in various contexts, *e.g.* cell cycle (14), embryonic development (15), endoplasmic reticulum stress (16), fatty acid metabolism, muscle cell differentiation (17), neurodevelopment (18), and so on. In addition, several extracellular signals, such as hormones, inflammatory signals, and growth factors, could promote NF-Y expression in a cell type–specific manner (19). Given the diverse pathways inducing NF-Y expression, it is speculated that the NF-Y function also depends highly on cell type and context. However, the NF-Y function in the regulation of hepatic gene expression has not previously been explored.

We hypothesized that gluconeogenesis could a NF-Y target in the liver for the following reasons. 1) It is known that the NF-Y expression is responsive to the cAMP signaling pathways in pancreatic β cell line (20); 2) NF-Y is a ubiquitously evolutionally conserved CCAAT box motif-binding transcription factor; 3) the CCAAT box located at the promoter region has been shown to mediate the cAMP stimulatory effects (21–23); and 4) cAMP as the secondary messenger plays a central role in the hepatic gluconeogenesis controlled by hormones. This raised the possibility that NF-Y could be one of the cAMP downstream mediators regulating glucose metabolism. Our results showed that NF-Y expression is effectively induced by cAMP, glucagon, and fasting *in vivo*. Furthermore, we provided evidence indicating that NF-Y regulates gluconeogenic gene expression and hepatic glucose production. Our study further supports evidence that NF-Y enhances gluconeogenesis by physically interacting with CREB and activating transcription of *Pck1* or *G6pc* via directly binding to its promoter region.

Results

cAMP axis induces hepatic NF-YA expression

To determine the NF-Y function in hepatic glucose metabolism, we stimulated Hepa1-6 mouse hepatocytes with different concentrations (0, 1, 10, and 20 μmol/liter) of 8-Br-cAMP, which is a stable cAMP analog and is known to activate gluconeogenesis. As shown in Fig. 1*a*, 8-Br-cAMP treatment significantly up-regulated mRNA expressions of the gluconeogenesis genes *G6pc* and *Pck1* in a dose-dependent manner. Treatment of 8-Br-cAMP also induced mRNA expressions of all three subunits of NF-Y including NF-A, NF-YB, and NF-YC in Hepa1-6 hepatocytes (Fig. 1*a*). Simultaneously, the NF-YA protein was also significantly stimulated by 8-Br-cAMP treatment (Fig. 1*b*).

To further confirm that NF-Y is involved into the cAMP axis *in vivo*, we treated mice with 300 μg/kg glucagon, a hormone produced by α cells activates the hepatic cAMP signaling pathway (24). In line with the effects observed in cultured Hepa1-6 hepatocytes, glucagon treatment significantly stimulated mRNA

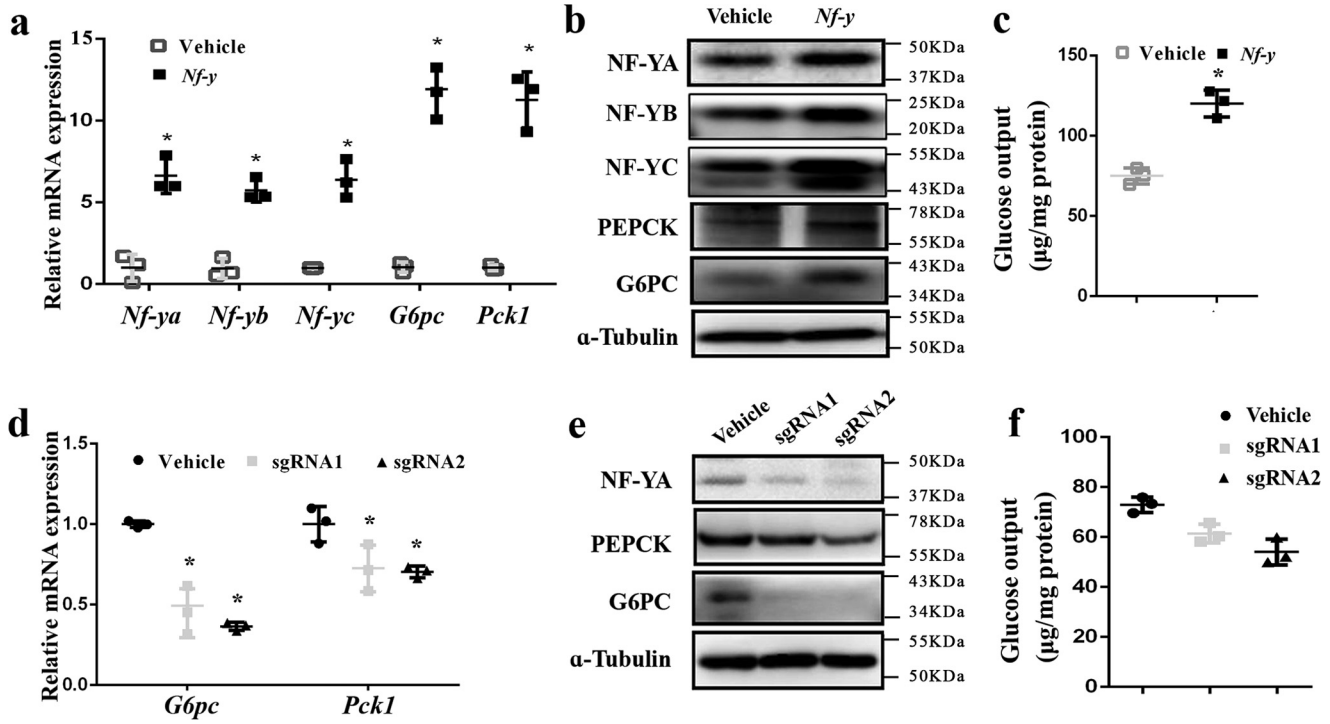

Figure 2. NF-Y regulates gluconeogenesis gene expression and glucose metabolism. *a–c*, Hepa1-6 cells were transfected with lentivirus vectors to express all three subunits of NF-Y simultaneously or empty vector control (pLVX) and then selected stable cells with 1.5 μg/ml puromycin. Expression of gluconeogenic gene mRNAs (*a*) and proteins (*b*) were induced, and glucose production (*c*) was stimulated in NF-Y over expression stable cells. *d–f*, NF-YA knockout by the CRISPR-Cas9 system in Hepa1-6 cells. Stable cells were selected with 1.5 μg/ml puromycin. Expression of gluconeogenic genes mRNAs (*d*) and proteins (*e*) were down-regulated, and glucose production (*f*) was reduced in NF-YA knockout stable cells. The protein and mRNA levels of the indicated genes were assayed by Western blotting and real-time PCR, respectively. Glucose production from pyruvate and lactate in Hepa1-6 cells were assayed as described under "Materials and Methods." The values are means ± S.D. of three independent experiments. *, $p < 0.05$ *versus* control (unpaired Student's *t* test).

expressions of NF-YA, NF-YB, and NF-YC in mouse liver (Fig. 1*c*). Furthermore, mRNA expressions of all three subunits were induced in livers of 24-h fasted mice and were reduced upon refeeding (Fig. 1*d*), a typical regulatory manner characterized by the gluconeogenic gene. These findings provided evidence of NF-Y being induced in response to cAMP.

NF-Y regulates gluconeogenesis gene expression and glucose metabolism

PEPCK and G6Pase are the primary enzymes in the gluconeogenic pathway in the liver. It is well known that the cAMP signaling pathway plays a central role in controlling the expression of *G6pc* and *Pck1* (25, 26). The response of NF-Y to cAMP challenge in the mouse liver suggested that this transcription factor might mediate gluoconeogenic gene expression. To prove this hypothesis, we used lentivirus vectors to express all three subunits of NF-Y simultaneously or empty vector control (pLVX) in Hepa1-6 hepatocytes. Transduction of recombinated lentivirus encoding NF-Y significantly induced both mRNA expression (Fig. 2*a*) and protein levels (Fig. 2*b*) of G6Pase and PEPCK. The functional consequence of forced expression of NF-Y is confirmed by the stimulation of glucose production from pyruvate and lactate in Hepa1-6 hepatocytes (Fig. 2*c*).

To further confirm the effect of NF-Y expression on the gluconeogenic pathway, we employed the CRISPR-Cas9 system to knock out NF-YA in Hepa1-6 hepatocytes. The small guide RNA (sgRNA) effectively abolished NF-YA expression. Simultaneously, NF-YA knockout attenuated both mRNA levels (Fig. 2*d*) and protein expressions (Fig. 2*e*) of G6Pase and PEPCK. Correspondingly, the diminished expression of NF-YA significantly blunted glucose production (Fig. 2*f*). Taken together, these findings suggest that NF-Y might be involved in the transcriptional process of hepatic gluconeogenesis.

Basal phenotyping of liver-specific Nf-ya knockout mice

Subsequently, we examined the effects of NF-Y on hepatic glucose metabolism *in vivo*. A total body knockout of *Nf-ya* is lethal (15). Thus, to study the function of NF-Y in liver in animals, we generated a liver-specific NF-Y knockout by crossing an albumin-CRE (*Alb-Cre*) mouse to an *Nf-ya* flox/flox mouse, in which exons 3–8 of 9 total coding exons are flanked by loxP sequences (Fig. 3*a*). Through two rounds of crossing, we obtained liver-specific *Nf-ya* knockout mice *Nf-ya* flox/flox/Alb-Cre, which was referred as *Nf-ya* LKO and was confirmed by genotyping as well as by performing Western blotting to assay *Nf-ya* protein expression. The littermates *Nf-ya* flox/flox were used as controls.

Nf-ya LKO mice showed significant reductions in body weight (Fig. 3*b*) and the total body fat percentage (Fig. 3*c*), although liver weight/body weight ratio remained similar to that in control mice (Fig. 3*d*). Under fasting conditions, the *Nf-ya* LKO mice showed significantly reduced levels of blood glucose (Fig. 3*e*) and serum glucagon (Fig. 3*f*) with similar serum insulin levels (Fig. 3*g*) compared with littermate controls. Periodic acid–Schiff (PAS) staining indicated that the

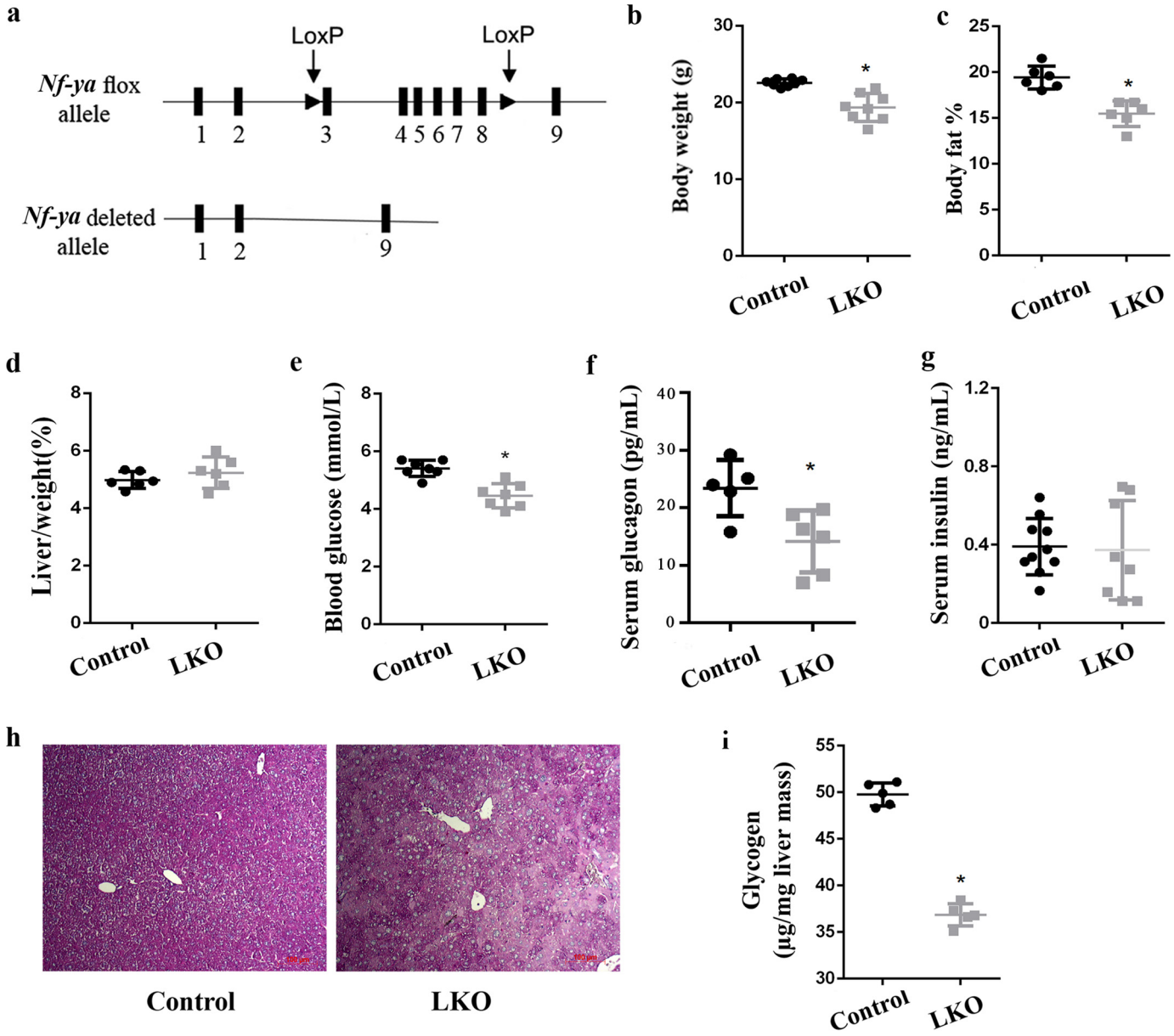

Figure 3. Metabolic characteristics of *Nf-ya* LKO mice. LKO mice were generated by crossing *Nf-ya* flox/flox mice with albumin-Cre transgenic mice, CRE-negative *Nf-ya* flox/flox animals mice were used as control. *a*, schematic of the *Nf-ya* gene showing exons 3–8 flanked by two loxP sites indicated as *triangles* and the subsequent excision of exons 3–8 by Cre-mediated gene recombination. *Vertical thick bars* show relative locations of exons. *b–d*, body weight (*b*), the total body fat percentage (*c*), and the ratio of liver weight/body-weight (*d*) of 10-week-old male mice fed with normal chow diet. *e–g*, blood glucose levels (*e*), serum glucagon levels (*f*), and serum insulin levels (*g*) of these mice under overnight fasting conditions ($n = 5–10$ for each group). *h*, PAS staining of hepatic glycogens in the livers of *Nf-ya* LKO and WT control male mice (magnification, 100×). *i*, quantitative enzymatic analysis of hepatic glycogen in the livers of *Nf-ya* LKO and WT control male mice ($n = 4–6$ mice/group). The values are expressed as means ± S.D. *, $p < 0.05$ *versus* control (unpaired Student's *t* test).

production of hepatic glycogens in the *Nf-ya* LKO mice was significantly lower than that of littermate control mice (Fig. 3*h*), which was further confirmed by quantitative enzymatic assay of hepatic glycogen content (Fig. 3*i*).

Deletion of NF-Y suppresses the hepatic gluconeogenesis in vivo

In line with the difference in fasting glucose levels, mRNA expression of the gluconeogenic genes *G6pc* and *Pck1* was inhibited in *Nf-ya* LKO mice after 24 h of fasting compared with littermate controls (Fig. 4*a*). Consistent with mRNA expression, the protein levels of G6PC and PEPCK were significantly lowered in *Nf-ya* LKO mice (Fig. 4*b*). To further investigate whether knockout of *Nf-ya* in the liver give rise to a decreased glucose production, we conducted a pyruvate-tolerance test, which consisted of analyzing the rise of glycemia in response to a pyruvate injection in a fasted subject. An obviously elevated glucose level was observed in WT control mice, and the maximal increase of 64% is observed after 30–60 min. In contrast, the blood glucose level in overnight fasted *Nf-ya* LKO mice was already reduced by 30% compared with control animals and then stayed significantly lower after the pyruvate injection (Fig. 4*c*). The corresponding glycemia area under the curve value in *Nf-ya* LKO mice was 60% of that observed in control

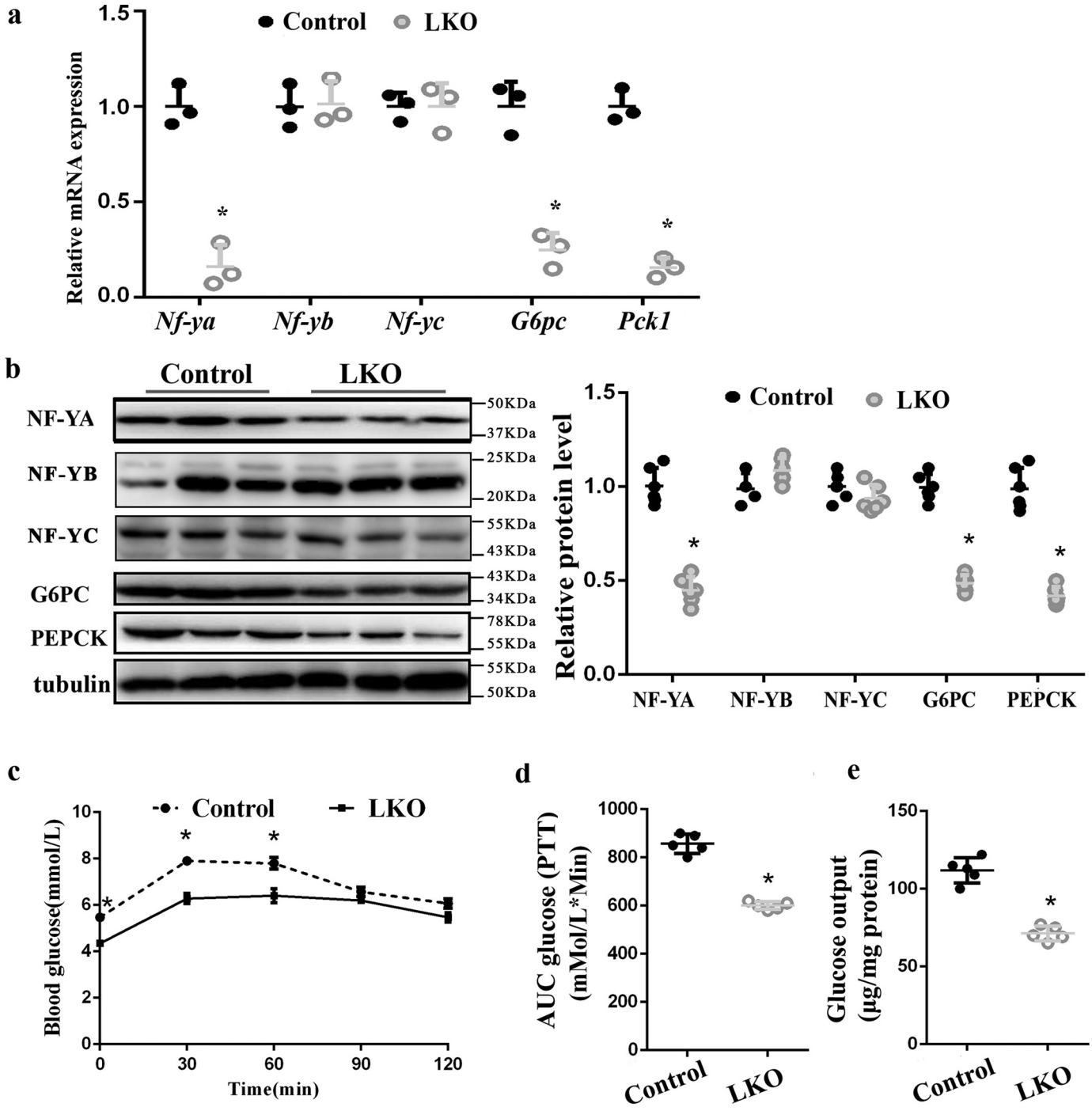

Figure 4. Liver-specific deletion of *Nf-ya* suppresses the hepatic gluconeogenic program. 10-week-old male mice fed with normal chow diet ($n = 4–6$ mice/group). *a*, real-time PCR analysis showing hepatic mRNA expressions of *Nf-ya*, *Nf-yb*, *Nf-yc*, and gluconeogenic gene. *b*, Western blotting analysis showing hepatic NF-YA, NF-YB, NF-YC, and gluconeogenic protein levels. *c*, pyruvate-tolerance test showing glucose production in response to pyruvate challenge. The mice were fasted for 16 h and intraperitoneally injected with sodium pyruvate (2 g/kg body weight). After injection, blood glucose was monitored at the designated time points. *d*, the areas under the curves (*AUC*) were calculated. *e*, glucose production (glucose output) was measured in the culture medium of primary hepatocytes isolated from *Nf-ya* LKO and littermate control mice. The values are expressed as means ± S.D. *, $p < 0.05$ *versus* control (unpaired Student's *t* test).

mice (Fig. 4*d*). These results clearly indicated an attenuated gluconeogenesis ability in the liver of *Nf-ya* LKO mice. We further directly measured glucose production in isolated hepatocytes from *Nf-ya* LKO or control mice. Indeed, glucose production was dramatically reduced in primary hepatocytes from *Nf-ya* LKO mice compared with controls (Fig. 4*e*). Together, these observations clearly indicate that NF-Y drives glucose production in mice livers.

Additionally, we also explored the effect of hepatic *Nf-ya* knockout on lipogenesis. We found that *Nf-ya* LKO mice had increased expression of lipolytic gene *Pnpla2* (also known as *Atgl*) and decreased expression of lipogenic gene *Acaca* and *Fasn* (also known as *Acc1* and *Fas*, respectively) (Fig. 5*a*). Correspondingly, *Nf-ya* LKO mice displayed reduced serum levels of triglyceride (Fig. 5*b*) and cholesterol (Fig. 5*c*), implying that NF-Y has a marked regulatory impact on lipid metabolism.

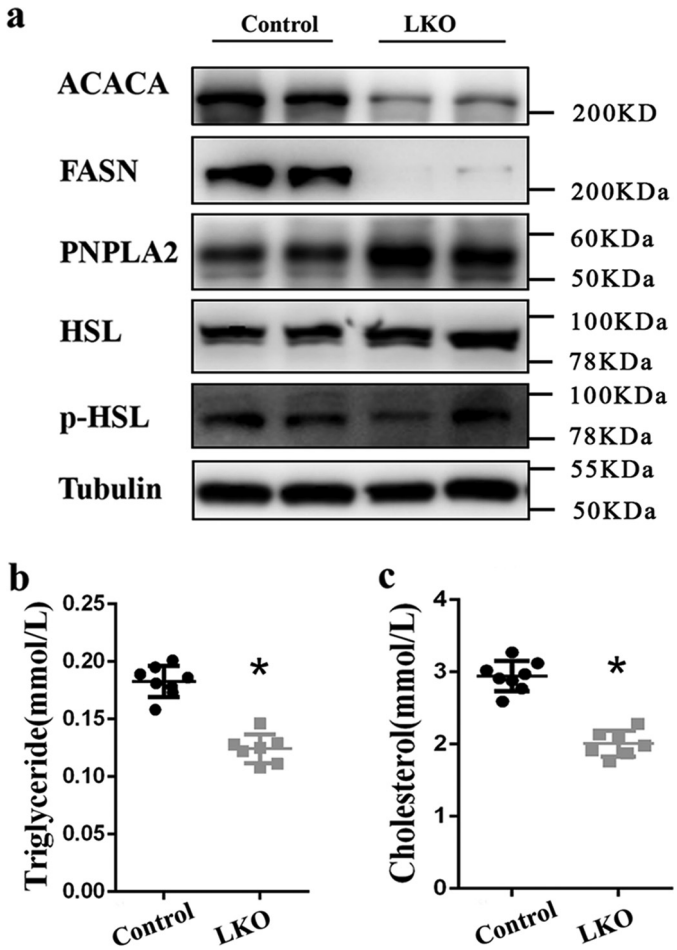

Figure 5. Effects of hepatic *Nf-ya* knockout on lipogenesis. 10-week-old male mice fed with normal chow diet ($n = 4–6$ mice/group). *a*, Western blotting analysis showing hepatic expression of lipogenic genes including ACACA and FASN (also known as ACC1 and FAS, respectively) and lipolytic genes including PNPLA2 (also known as ATGL), HSL, and its phosphorylation status (*p-HSL*). *b* and *c*, overnight fasting levels of triglyceride (*b*) and cholesterol (*c*). The data are means ± S.D. *, $p < 0.05$ *versus* control (unpaired Student's *t* test).

NF-Y is not necessary for glucagon-mediated gluconeogenesis in vivo

In the fasted state, glucagons secreted from pancreatic α cells to increase hepatic glucose production. We explored whether NF-Y is necessary for glucagon induction of gluconeogenic genes. *Nf-ya* in Hepa1-6 hepatocytes was knocked out via the CRISPR-Cas9 system, and then the cells were treated with cAMP. Nf-ya knockout was found to significantly reduce the gluconeogenic gene expression and the cAMP stimulatory effects on gluconeogenic genes *Pck*1 and *G6pc* (Fig. 6*a*).

To further explore the physiological effects of NF-Y deficiency on hepatic glucose metabolism in response to the glucagon stimulation, we performed a glucagon challenge assay to determine hepatic glucagon sensitivity in *Nf-ya* LKO mice. Prior to the glucagon administration, mice were injected with somatostatin intraperitoneally to inhibit endogenous glucagon production. *G6pc* and *Pck1* were significantly down-regulated at the transcript levels at both baseline and in response to glucagon stimulation in *Nf-ya* LKO mice compared with control mice (Fig. 6*b*). However, the *Nf-ya* LKO mice respond robustly to glucagon administration with respect to the expression of *G6pc* and *Pck1*. Moreover, the relative fold increase in *G6pc* and *Pck1* expression with glucagon treatment appears similar in *Nf-ya* LKO mice and littermate controls. (Fig. 6*b*). Consistent with the stimulatory effects on the gluconeogenic gene expression, glucagon produces a robust increase in the glucose excursion in *Nf-ya* LKO mice, although this appears modestly enhanced in littermate controls (Fig. 6*c*). These data indicated that NF-Y is not required for glucagon-mediated induction of the expressions of gluconeogenic genes *in vivo*.

The induction of gluconeogenesis by glucagon-dependent fasting depends on CREB and PGC-1α transcriptional activation, CREB is one of the PGC-1α transcription factors (11, 27). We examined the phosphorylation status of CREB. Although the fasting-induced hepatic CREB phosphorylation was markedly lower in *Nf-ya* LKO mice compared with control mice, the protein levels of PGC-1α were similar in *Nf-ya* LKO and control livers (Fig. 6*d*). Taken together, these observations suggested that NF-Y largely regulates gluconeogenic gene expression, which may be cAMP/CREB axis-independent.

NF-Y is a direct regulator of gluconeogenesis genes

We next wanted to specify the mechanism for NF-Y–mediated gluconeogenic gene expression. Because NF-Y is a transcription factor, we determined whether NF-Y can modulate gluconeogenic gene promoter transcriptional activity. Hepa1-6 hepatocytes were transiently cotransfected with a luciferase reporter vector driven by the mouse *G6pc* (−1441 to +38 bp) or *Pck1* (−1265 to +308 bp) promoter fragment and all three lentiviral NF-Y subunits. The results in Fig. 7*a* showed that inclusion of NF-Y resulted in markedly higher activities of both *G6pc* and *Pck1* promoters in transient transfection assays.

We further examine whether NF-Y exerts its effect on gene expression through direct promoter interactions. NF-Y often binds to CCAAT motif in regulated genes promoters. Sequence analysis showed the presence of a potential CCAAT motif within 1.5 kb upstream from mRNA start site of *G6pc* and *Pck1* promoters, respectively (Fig. 7*b*, *left panel*). The occupancy of NF-Y over each putative CCAAT motif on *G6pc* and *Pck1* promoters was confirmed by ChIP assay (Fig. 7*b*, *right panel*).

To further determine whether NF-Y activates *G6pc* and *Pck1* promoters through binding to the CCAAT motif, constructs with site-directed mutagenesis (deletions) of CCAAT motif in the *G6pc* and *Pck1* promoters were generated. When the CCAAT motif in the *G6pc* or *Pck1* promoter was deleted, the stimulating effect was ablated (Fig. 7*c*).

CREB is one of important effectors of the hepatic cAMP signal pathways, the cAMP–CREB axis is generally accepted as a critical signal for the gluconeogenesis stimulation, prompting us to determine whether CREB would interact with NF-Y. Indeed, CREB interacted with NF-YA in a coimmunoprecipitation study (Fig. 7*d*), suggesting that NF-Y could function as a coactivator for CREB. Taken together, these data demonstrated that gluconeogenic genes *G6pc* and *Pck1* were direct targets of NF-Y in hepatocyte cell lines.

Discussion

Gluconeogenesis is essential for the glucose production during fasting to maintain circulating levels of glucose. Glucagon

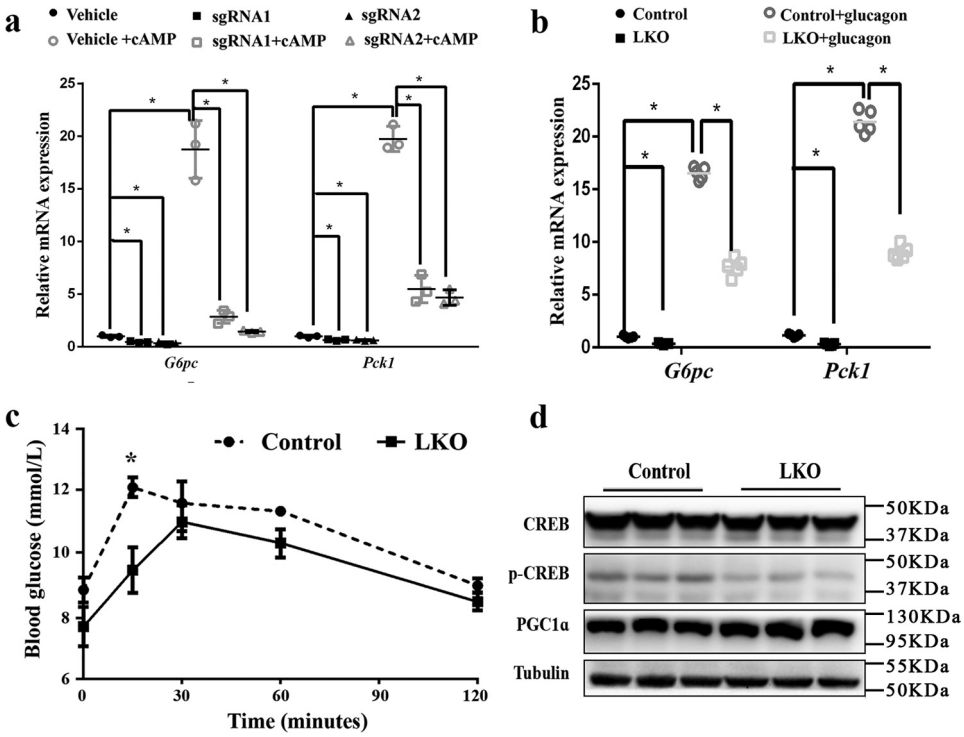

Figure 6. Glucagon sensitivity was attenuated in the absence of NF-Y. *a*, real-time PCR analysis of mRNA levels of *G6pc* and *Pck1* in Hepa1-6 mouse hepatocytes infected with recombinant lentivirus containing *Nf-ya* sgRNA1 or sgRNA2 or in the presence or absence of 8-Br-cAMP (10 μM). *b* and *c*, overnight fasted 10-week-old male mice were injected intraperitoneally with glucagon (300 μg/kg) 15 min after intraperitoneal injection of somatostatin (10 mg/kg) (n = 4–6 mice/group). *b*, real-time PCR analysis of hepatic mRNA levels of *G6pc* and *Pck1* in WT control and *Nf-ya* LKO mice. *c*, a glucagon challenge was performed to assess liver glucagon sensitivity. Glucagon increased glucose levels over time to a much lower degree in *Nf-ya* LKO mice relative to control mice. *d*, Western blotting analysis measured the hepatic protein levels of CREB, CREB phosphorylation (*p-CREB*), and PGC-1 in 10-week-old male mice fed a normal chow diet (n = 4–6 mice/group). The data are means ± S.D. *, $p < 0.05$ in unpaired Student's t test.

and its downstream cAMP/CREB–signaling pathway play a crucial role in the activation of glucose production especially in the early phase of fasting (8). CREB promotes the transcription of gluconeogenic genes by binding to the promoter. In addition, cAMP–CREB axis also stimulates the expression of PGC-1α, which is a transcriptional coactivator facilitating the biological activities of other transcription factors, such as the glucocorticoid receptor, FOXO1, and HNF4α (11).

In this study, we identified NF-Y as a previously unrecognized modulator mediating hepatic glucose production. Hepatic NF-Y expression is potently induced in response to treatments of cAMP, glucagon, and fasting. Gluconeogenesis involves multiple stepwise chemical reactions in which G6Pase and PEPCK are the rate-limiting enzymes, NF-Y activates the transcription of *Pck1* or *G6pc* via a unique regulatory sequence in their promoters. Our results also indicate that NF-Y stimulates CREB expression both *in vitro* and *in vivo*, and it works together with CREB to promote transcription of gluconeogenic genes. The exact underlying mechanism is not known, but it appears that NF-Y has a role in the assembly of transcription complexes at the proximal promoter region. NF-Y interacts with other transcriptional protein, such as TATA box-binding protein, to form promoter complexes and to facilitate the initiation of the gene transcription (13).

NF-Y is an evolutionarily conserved CCAAT motif-binding transcription factor, and the NF-Y–binding CCAAT box is usually located between −60 and −100 bp upstream of the transcription starting site (28). In the present study, an inverted NF-Y–binding CCAAT motif was identified in the promoters of *G6pc* and *Pck1*, which is speculated to be responsible for the cAMP-stimulated gene expression. Indeed, NF-Y–binding CCAAT box has been involved into the cAMP stimulation of some genes (21–23, 29). In human pinealocytes, an inverted NF-Y–binding CCAAT box in the promoter of the *Tph* (tryptophan hydroxlase) gene is necessary for mediating cAMP stimulatory effects (30). Additionally, a NF-Y–binding CCAAT motif in the rat hexokinase II (Hk2) gene promoter can confer cAMP inducibility sufficiently in L6 myotubes (31). Our study clearly demonstrates that NF-Y confers cAMP responsiveness to gluconeogenic genes in mice hepatocytes.

Glucagon and its downstream cAMP/CREB–signaling pathway play a fundamental role in the hepatic glucose production in the fasted state. In the current study, *Nf-ya* LKO mice showed reduced glycemia and gluconeogenic gene expression compared with littermate controls. Surprisingly, the serum glucagon level is also significantly lower in the *Nf-ya* LKO mice than that of controls. There is no expected counter-regulation to compensate the reduction in gluconeogenic enzymes in the *Nf-ya* LKO mice. This may be attributed to the glucagon role in regulating hepatic glucose metabolism. Glucagon produced by α cells of the pancreas generally elevates the blood glucose concentration by promoting glycogenolysis in the liver; it has hardly any effect on gluconeogenesis (32). However, the production of hepatic glycogens in the *Nf-ya* LKO mice is significantly lower than that of control mice. Thus, *Nf-ya* LKO mice might not require much more glucagon to promote glycogenolysis than control mice.

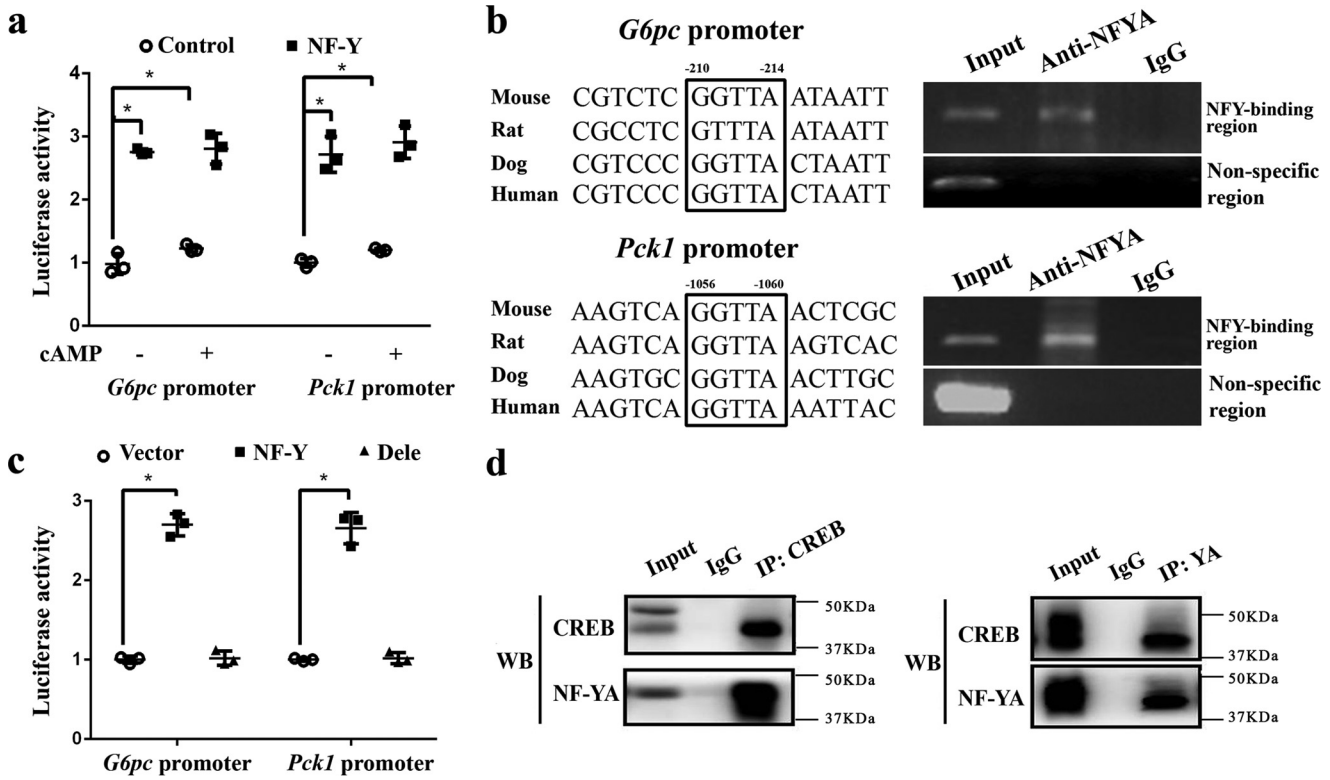

Figure 7. NF-Y forms complex with CREB and interacts with gluconeogenic promoter directly. *a*, luciferase assay using HEK293 cells transiently transfected with *G6pc* (*left symbols*) or *Pck1* (*right symbols*) luciferase construct together with expression vectors for *Nf-ya*, *Nf-yb*, and *Nf-yc* or in the presence or absence of 8-Br-cAMP (10 μM). The values are means ± S.D. of three independent experiments. *, $p < 0.05$ *versus* control (unpaired Student's *t* test). *b*, occupancy of *Nf-ya* on distinctive its binding site gluconeogenic promoters. *Left panel*, location of NF-YA–binding site (GGTTA) on *G6pc* or *Pck1* promoter. *Right panel*, ChIP assay showing a binding of NF-Y on *G6pc* (*top panels*) or *Pck1* promoter (*bottom panels*). *c*, luciferase assay using HEK293 cells transiently transfected with WT or GGTTAT-motif deleted *G6pc/Pck1* luciferase reporter constructs together with expression vectors for *Nf-ya*, *Nf-yb*, and *Nf-yc*. *Vector*, empty vector + WT *G6pc/Pck1*-luciferase; *NF-Y*, NF-Y + WT *G6pc/Pck1*-luciferase; *Dele*, NF-Y + deleted *G6pc/Pck1*-luciferase (GGTTA motif deleted). The values are means ± S.D. of three independent experiments. *, $p < 0.05$ in unpaired Student's *t* test. *d*, coimmunoprecipitation (*IP*) assay showing endogenous interaction between CREB and NF-Y in Hepa1-6 hepatocyte. Representative Western blotting (*WB*) analysis is shown. *Left panel*, NF-Y was precipitated by using CREB antibody. *Right panel*, CREB was precipitated by using NF-Y antibody.

In the present study, we also explored whether NF-Y is necessary for glucagon-mediated gluconeogenesis in mice livers. We found that the stimulatory effects of glucagon on the expression of the gluconeogenic genes are similar between control and *Nf-ya* LKO mice. Furthermore, glucagon also induce a robust increase in glycemia in *Nf-ya* LKO mice. Our data suggest that NF-Y is not necessary for glucagon-mediated induction of the expression of gluconeogenic genes *in vivo*. Thus, we speculated that NF-Y largely regulates gluconeogenic gene expression independently of the cAMP/CREB axis.

The cAMP pathway is also used by other hormones to signal the glucose metabolism (33). Thus, it is very likely that NF-Y also takes part in mediating their responsiveness. Postprandially, blood insulin levels elevate. Insulin pathway has long been recognized to be a dominant over gluconeogenic stimuli and suppress hepatic glucose production (6). Therefore, it is required to explore where insulin fits in the NF-YA pathway in the future study. In addition, the biology of the three subunits of NF-Y is complex. NF-YA and NF-YC have multiple splicing isoforms with different function and expression levels in different tissues, and the subunits are subject to post-translational modifications, such as phosphorylations, acetylations, and ubiquitinations (34). It is possible that insulin-signaling pathways modify NF-Y and alter the protein degradation of NF-Y. Additionally, because insulin signaling is essential to the activities of some gluconeogenic regulator, such as PGC-1α and FOXO1, NF-YA may be involve in the insulin pathway by altering the activities of such factors.

As a whole, this work reveals a previously unrecognized physiological function of NF-Y in controlling glucose metabolism. We identified that NF-Y binding to the promoter of *Pck1* or *G6pc* to transcriptionally modulate hepatic gluconeogenesis. Elevated hepatic gluconeogenesis is primarily responsible for the increase in fasting hepatic glucose production in patients with diabetes. Effective management of dysregulated gluconeogenesis is emerging as a promising therapeutic strategy to alleviate hyperglycemia in diabetes. Several antidiabetic drugs, such as metformin, regulate blood glucose by transcriptional inhibition of the gluconeogenic program (35). Modulation of hepatic NF-Y expression may therefore offer an attractive therapeutic approach to manage type 2 diabetes.

Materials and methods

Reagents

DMSO, DMEM, streptomycin, penicillin, 8-Br-cAMP, puromycin, glutamine, glucagon, glucose, insulin, Triton X-100, sodium pyruvate, type II collagenase, Schiff's reagent, and sec-

ondary antibodies were obtained from Sigma–Aldrich. Fetal bovine serum was from Hyclone (Logan, UT). Sperm DNA and BSA were from New England BioLabs. ELISA kits for glucagon and insulin were from Crystal Chem (Elk Grove Village, IL). Glycogen assay kit was from BioAssay Systems (Hayward, CA). The ChIP assay kit and PCR purification kit were from Beyotime Biotechnology (Shanghai, China). pGL3-basic reporter vector and Dual-Glo™ luciferase assay kit were from Promega (Shanghai, China). Site-directed mutagenesis kit was from Stratagene (La Jolla, CA). Lipofectamine™ 2000 and TRIzol reagent were from Invitrogen. Antibodies against PEPCK and α-tubulin were from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against G6PC, PEPCK, NF-YA, NF-YB, and NF-YC were from Cell Signal Technology (Danvers, MA). Antibody against CREB and pCREB were from HuaAn Biotechnology (Hangzhou, China). SYBR Green Master Mix was from Applied Biosystems (Foster City, CA). All of the chemicals were dissolved in the appropriate media solution or DMSO and then used at the indicated concentrations.

Mice

The mice were housed under a 12-h light/12-h dark cycle and followed free access to normal chow diet. All animal experiments were carried according to the National Institute of Health ethical guidelines. Animal care and experimental procedures were approved by the ethics committee of animal experimentation of Sichuan University. *Nf-ya* flox mice were a gift from N. Nobuyuki's laboratory at Juntendo University Graduate School of Medicine (Tokyo, Japan). *Nf-ya* flox mice were maintained on a C57BL6J background and genotyped according to the protocol described previously (15). Alb-Cre mice harboring a transgene containing Cre recombinase driven by the albumin promoter (36) were purchased from The Jackson Laboratory (Bar Harbor, ME). The *Nf-ya* flox/flox mice bred with the Alb-Cre mice (homozygous for WT *Nf-ya*) generated *Nf-ya* flox/wt;Cre mice. These mice were then mated via brother and sister generate *Nf-ya* flox/flox;Cre mice, which were used as liver-specific NF-YA knockout (LKO) mice. The littermates *Nf-ya* wt/flox;Cre and *Nf-ya* flox/flox were used as controls for each subsequent experiment. 10-week-old male mice were overnight fasted and then killed. Their livers and plasma were collected for further analysis.

Metabolic analysis

Serum concentrations of insulin and glucagon were quantified by ELISA according to the manufacturer's instructions. Serum concentrations of triglyceride and total cholesterol were measured by an automated Monarch device (Instrumentation Laboratory, Lexington, MA). Body fat mass was measured using peripheral dual-energy X-ray absorptiometry in a Lunar PIXImus II densitometer (PIXImus; GE-Lunar, Madison, WI) as described previously (37), and anesthetized mice were placed on a positioning tray ventral side down with the legs extended away from the body. A pyruvate-tolerance test was performed after 16 h of fasting (5:00 p.m. to 9:00 a.m.). 10-week-old male mice were injected intraperitoneally with sodium pyruvate (2 g/kg). The glucagon challenge was performed by injecting glucagon (300 μg/kg) subcutaneously 15 min after the intraperitoneal injection of somatostatin (10 mg/kg). Blood glucose levels were determined from the tail vein at the indicated time points using an automatic glucose monitor (OneTouch; LifeScan).

Hepatic glycogen staining and measurement

Hepatic glycogen was stained by PAS following the standard protocol. Briefly, formalin-fixed, paraffin-embedded mice liver tissue was sectioned, deparaffinized, rehydrated, and oxidized in 0.5% periodic acid solution. Then the oxidized tissue sections were incubated in Schiff's reagent. To quantify hepatic glycogens, mice liver tissue were homogenized in ice-cold citrate buffer (0.1 M, pH 4.2). Homogenates were immediately subjected to glycogen measurement using the commercial glycogen assay kit following the manufacturer's instructions. Hepatic glycogen content was presented after normalization to liver mass.

Plasmid and virus

The promoters of *G6pc* (−1441 to +38 bp) and *Pck1* (−1265 to +308 bp) were amplified from C57BL6J mouse genomic DNA and cloned into pGL3-basic reporter vector. The putative NF-Y binding site in the *G6pc* or *Pck1* promoter was mutated or deleted using a QuikChange lightning site-directed mutagenesis kit, and the mutations were further confirmed by DNA sequencing. The cDNAs encoding full-length mouse *Nf-ya*, *Nf-yb*, and *Nf-yc* were amplified by RT-PCR, respectively, which were then subcloned into the pLVX-puro vector via EcoRI and XbaI restriction sites to produce the recombinant lentivirus plasmid. In CRISPR-Cas9 system, two pairs of sgRNAs (pair 1, 5′-CACCGATTGTGCCATCTGCATTAAC-3′ and 5′-AAACGTTAATGCAGATGGCACAATC-3′; and pair 2, 5′-CACCGCAGATACAGTTGGTGCCTCC-3′ and 5′-AAACGGAGGCACCAACTGTATCTGC-3′) targeting the coding region in exon 4 or 5 of *Nf-ya* were synthesized according to CRISPR Design (Massachusetts Institute of Technology) and cloned into the pSpCas9(BB)-2A vector (Addgene plasmid entry 48138) to produce the recombinant lentivirus plasmid. Each recombinant lentivirus plasmid was then transfected into HEK293 cells with viral packaging vectors (pMD2G and psPAX2) by Lipofectamine™ 2000 to allow packaging and amplification of the recombinant lentivirus.

Cell culture

Hepa1-6 cells and 293T cells were purchased from American Type Culture Collection (Manassas, VA). Mouse primary hepatocytes were isolated from *Nf-ya* flox/flox;Cre mice and their littermate control mice by liver perfusion with type II collagenase and grown on collagen-coated plates, and cell number and viability were assessed using trypan blue. The cells were cultured in DMEM containing 4.5 g/liter glucose and 2 mM L-glutamine at 37 °C with a humidified 5% CO_2 atmosphere. The medium was supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 50 units/ml penicillin, and 50 mg/ml streptomycin.

NF-Y overexpression and knockdown

Hepa1-6 cells plated on 6-well plates were coinfected with recombinant *Nf-ya*, *Nf-yb*, and *Nf-yc* lentiviruses in overexpression assay, and infected with recombinant *Nf-ya* Crispr-Cas9

lentivirus in knockdown assay. 1.5 μg/ml puromycin was used for stable selection.

Dual-luciferase activity measurement

Luciferase activity assay was carried out as described previously (38). Briefly, HEK293 cells plated on 96-well plates were transfected transiently with 20 ng/well of *G6pc* or *Pck1* promoter reporter plasmid or control vector using Lipofectamine™ 2000. After 12 h of treatment, the cells were infected with vehicle or *Nf-ya*, *Nf-yb*, and *Nf-yc* lentiviruses for an additional 24 h. The luciferase activity was assayed by using the Dual-Glo™ luciferase system according to the manufacturer's protocols. The firefly luciferase activity was normalized to the *Renilla* luciferase activity (firefly luciferase/*Renilla* luciferase) and presented as relative luciferase activity.

Glucose output assay

Stable Hepa1-6 cells with *Nf-y* overexpression or *Nf-ya* knockdown and primary mouse hepatocytes seeded on 6-well plates were subjected to glucose production. The cells were washed three times with PBS and then incubated in glucose production medium (phenol-red–free, glucose-free DMEM containing 10 μM 8-Br-cAMP, 20 mM sodium lactate, and 2 mM pyruvate) for 6 h. Glucose concentrations in the media were quantified in triplicate using a colorimetric glucose oxidase assay (Applygen Technologies, Beijing, China) and normalized to the total protein content of cells.

ChIP assays

ChIP followed by PCR was conducted using the inputs according to the ChIP assay kit protocol. Briefly, the cells were fixed, lysed, and sonicated. The protein–DNA complexes were immunoprecipitated with the indicated antibodies. Purified DNA was subsequently subjected to PCR using the following primers. The primers amplifying *G6pc* promoter region are 5′-GCAGCCTCTAGCACTGTCAAGCA-3′ and 5′-GCCCTGGATTCAGTCTGTAGGTCAA-3′; the primers amplifying *Pck1* promoter region are 5′-AGTTGCCTGTCACCATCCTCC-3′ and 5′-GTAAAGACTTCTCTGCGAGCCAC-3′. Meanwhile, the region flanking the putative binding site in the promoter was amplified by following primer pairs to be as negative controls: for the *G6pc* promoter, 5′-TGTAGTTCAATCCTCAATACC-3′ and 5′-GAGGTCAGCGATCACAGACAC-3′; and for the *Pck1* promoter, 5′-TTAGAGCAGGGGTCAGTA TG-3′ and 5′-GAGACGCCTCTTGGACTTCA-3′.

Coimmunoprecipitation

Proteins were extracted from NF-Y stable cells by using radioimmune precipitation assay lysis buffer, and protein extracts were quantified using BCA assay. 2 μg of antibody against NF-Y or CREB and 20 μl of protein A/G resin were suspended in 500 μl of cold PBS binding for 9 h and were washed with cold PBS before being blocked overnight with 1% BSA in PBS. The antibody–resin complex was then incubated with 2 mg of total protein in 1 ml of lysis buffer overnight at 4 °C on a rotating wheel. The immunoprecipitated complexes were collected by centrifugation at 4 °C and then eluted with the lysis buffer. The eluted protein samples were separated on SDS-PAGE and followed by immunoblotting using the anti-NF-Y or CREB body.

Real-time quantitative PCR analysis

Total RNA was extracted from Hepa1-6 cells, primary hepatocytes, and mouse liver using TRIzol reagent and quantified on a NanoDrop Spectrophotometer (NanoDrop Technologies, Wilmington, DE). cDNA synthesis and real-time quantitative PCR (qPCR) analysis were performed as described (39). In brief, qPCRs were performed using ABI PRISM 7500 sequence detection system (Applied Biosystems) using the mixture composed of cDNA, SYBR Green PCR Master Mix, and gene-specific primers (Table S1). All measurements were normalized to *Gapdh* expression, and the relative gene expression between groups was calculated using a comparative *Ct* (ΔΔ*Ct*) method. qPCRs were conducted in triplicate.

Protein extraction and Western blotting

As previously described (40), liver tissue or Hepa1-6 were lysed in radioimmune precipitation assay buffer. The proteins were quantified using a BCA protein assay kit. For Western blotting, proteins (20 μg) from each sample was separated by SDS-PAGE (10%) and then transferred onto a polyvinylidene difluoride membrane. The membranes were blocked with 5% nonfat milk repaired in Tween-TBS buffer and immunoblotted with the antibodies against target proteins overnight. The blots were subsequently washed and then incubated with the horseradish peroxidase– conjugated secondary antibodies. The immunoreactive bands were visualized by using an enhanced chemiluminescence kit and quantified by volume densitometry using the UN-SCAN-IT Gel 5.1 software (Silk Scientific Inc., Orem, UT).

Statistical analyses

The data are presented as means ± S.D. of at least three independent experiments, unless otherwise indicated in the figure legends. Statistical analyses were performed with GraphPad Prism. Differences between two groups were analyzed by using unpaired two-tailed Student's *t* test. A p value of <0.05 was considered statistically significant.

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