Inactivation of NF-κB2 (p52) restrains hepatic glucagon response via preserving PDE4B induction

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Glucagon promotes hepatic gluconeogenesis and maintains whole-body glucose levels during fasting. The regulatory factors that are involved in fasting glucagon response are not well understood. Here we report a role of p52, a key activator of the noncanonical nuclear factor-kappaB signaling, in hepatic glucagon response. We show that p52 is activated in livers of HFD-fed and glucagon-challenged mice. Knockdown of p52 lowers glucagon-stimulated hyperglycemia, while p52 overexpression augments glucagon response. Mechanistically, p52 binds to phosphodiesterase 4B promoter to inhibit its transcription and promotes cAMP accumulation, thus augmenting the glucagon response through cAMP/PKA signaling. The anti-diabetic drug metformin and ginsenoside Rb1 lower blood glucose at least in part by inhibiting p52 activation. Our findings reveal that p52 mediates glucagon-triggered hepatic gluconeogenesis and suggests that pharmacological intervention to prevent p52 processing is a potential therapeutic strategy for diabetes.
Insulin and glucagon are responsible for maintaining whole-body glucose homeostasis. Insulin reduces postprandial hyperglycemia by promoting glucose disposal to target tissues, while glucagon stimulates hepatic gluconeogenesis to maintain blood glucose levels during fasting or starvation. Accumulating evidence from human and animal studies shows that plasma glucagon concentrations are abnormally elevated in individuals with obesity and/or diabetes. Dysregulation of the hepatic glucagon response induces excessive hepatic glucose production, contributing to fasting hyperglycemia in diabetes. Mechanisms to reduce circulating glucagon levels and antagonize the hepatic glucagon response in target tissues are well-recognized means to reduce hyperglycemia in diabetes.

The hepatic glucagon response is mediated by activation of the cyclic AMP (cAMP)/protein kinase A (PKA) pathway. By binding to the G-protein-coupled receptor, glucagon activates adenyl cyclase signaling. This leads to production of cAMP, which activates PKA and induces phosphorylation of cAMP-response element-binding protein (CREB). After phosphorylation, CREB promotes gluconeogenesis through induction of glucose-6-phosphatase (G6Pase), phosphoenolpyruvate carboxykinase (PPECK), and peroxisome proliferator-activated receptor gamma coactivator-1 alpha (PGC-1α). Because CREB acts as a second messenger to initiate downstream signaling cascades in the gluconeogenic pathway, regulation of cAMP is a key step in the control of hepatic glucagon response. Inhibition of adenyl cyclase enzymes reduces hepatic glucose production by suppressing hepatic glucagon signaling. cAMP can be degraded by phosphodiesterases (PDEs) to prevent excessive accumulation. Metabolic alterations can affect PDEs activity and thus influence hepatic gluconeogenesis. AMP-activated protein kinase (AMPK) and hypoxia-inducible factor 2α increase PDEs activity and thus antagonize hepatic glucagon-stimulated cAMP signaling. However, the intracellular mechanisms that regulate glucagon-mediated responses are incompletely understood.

The nuclear factor-kappa B (NF-kB) family of transcription factors includes NF-κB1 (p50), NF-κB2 (p52), RelA (p65), RelB, and c-Rel, and these regulate diverse cellular processes. Various extracellular signals activate NF-κB intracellular signaling, which results in translocation of DNA-binding heterodimers and homodimers to the nucleus and transcriptional activation of target genes. Canonical NF-κB1 and NF-κB2 signaling is well recognized as the central regulator of inflammatory responses, but the role of NF-κB2 in human diseases is relatively less studied. Roles of p52 in lymphoid organogenesis, B-cell maturation, and osteoclast differentiation have only recently been appreciated, and aberrant overexpression of p52 has been reported in genetic and nutritionally obese mice. However, the role of p52 in hepatic gluconeogenesis remains largely unknown.

To know if NF-κB2 activation is involved in metabolic disorders, this study aims to investigate the role of p52 activation in hepatic gluconeogenesis. Our work shows that p52 binds to PDE4B promoter to inhibit its transcription and promotes cAMP accumulation, thus augmenting the glucagon response through cAMP/PKA signaling. We also show that metformin and ginsenoside Rb1 restrain hepatic glucagon response through a p52-dependent manner.

**Results**

**Hepatic NF-κB2 is abnormally activated in obese humans.** In search of a correlation between hepatic NF-κB2 expression and BMI, the RNAseq data of the Genotype-Tissue Expression Project (GTEx) were downloaded from the Genotype and Phenotypes (dbGaP, phs000424.v7.p2) database. As shown in Supplementary Fig. 1a, NF-κB2 expression in liver samples of 51 obese individuals correlated positively with BMI ($r = 0.36, p = 0.01$). In close agreement, when we examined livers of fasted high-fat diet (HFD)-fed mice, we observed a significant increase in p52 activation compared with those of chow-fed mice (Supplementary Fig. 1b).

**p52 mediates HFD-induced hepatic gluconeogenesis.** To investigate the role of p52 in hyperglycemia in vivo, we fed mice a HFD and silenced p52 using a siRNA transfection technology (Fig. 1a). HFD feeding resulted in fasting hyperglycemia (Fig. 1b) and impaired oral glucose tolerance (Supplementary Fig. 2a). We found that p52 knockdown lowered fasting blood glucose levels (Fig. 1b) and improved oral glucose tolerance (Supplementary Fig. 2a) in HFD-fed mice. Pyruvate provides a substrate for hepatic gluconeogenesis, and is an indicator of hepatic glucose production. HFD-fed mice had increased blood glucose levels in response to a pyruvate load compared with normal chow diet (NCD)-fed mice, but p52 knockdown reversed the glucose increase (Fig. 1c). As expected, fasting serum glucagon levels were increased in HFD-fed mice, p52 knockdown did not impact glucagon secretion in HFD-fed mice (Fig. 1d), suggesting that p52 may regulate glucagon signaling rather than glucagon secretion. Of note, p52 knockdown resulted in reduced body weight gain, less fat mass, and decreased lipid deposition in the livers of mice fed with HFD for 8 weeks (Supplementary Fig. 2b–d). Food intake was not changed by p52 knockdown (Supplementary Fig. 2b).

To observe the impact of p52 on glucagon response, we employed an acute glucagon-challenged mice model. After overnight fasting, hepatic glycogen was breakdown completely, and glucagon was not able to activate glycogenolysis (data not shown). We observed that p52 knockdown attenuated glucagon-stimulated hyperglycemia (Fig. 1e). In addition, we used AAV8-shRNA to establish liver-specific p52 knockdown mice (Supplementary Fig. 3a) for further confirmation. The results in liver-specific knockdown mice mirrored the results in siRNA transfection mice (Supplementary Fig. 3b, c). Along the same line, we used hepatotropic AAV-8 as vehicle to specifically overexpress p52 in the liver (Supplementary Fig. 3d). The results showed that p52 liver-specific overexpression increased fasting blood glucose (Fig. 1f) and augmented glucagon-stimulated hyperglycemia in mice (Fig. 1g).

**p52 knockdown blocks cAMP/PKA signaling.** Glucagon-induced hepatic gluconeogenesis relies on cAMP/PKA signaling. HFD feeding increased hepatic cAMP accumulation, whereas knockdown of p52 reduced cAMP accumulation and effectively prevented PKA activation (Fig. 2a, b). In response to PKA activation, CREB was phosphorylated to upregulate key gluconeogenesis-associated enzymes. HFD increased CREB phosphorylation (Fig. 2c) and upregulated transcription of G6Pase, PPECK, and PGC-1α (Fig. 2d). Knockdown of p52 inactivated CREB through dephosphorylation (Fig. 2c), and reversed gluconeogenesis-associated genes alterations (Fig. 2d). In close agreement, glucagon challenge increased p52 expression (Fig. 2e), stimulated cAMP accumulation (Fig. 2f), activated PKA (Fig. 2g), phosphorylated CREB (Fig. 2h), and increased G6Pase, PPECK, and PGC-1α mRNA expression in mice (Fig. 2i; Supplementary Fig. 3c). Correspondingly, p52 siRNA transfection reversed these alternations in the liver of glucagon-challenged mice. These results showed that inactivation of p52 restrained hepatic glucagon response.

**p52 inhibits PDE4B expression to promote cAMP accumulation.** To investigate the underlying mechanism by which p52
mediates glucagon response, we focused on phosphodiesterase family. PDE4B is the predominant isof orm of phosphodiesterase responsible for cAMP degradation in the liver. HFD and glucagon challenge inhibited PDE4B expression in the livers of mice, and this suppression was prevented by p52 knockdown (Fig. 3a, b). We then investigated the effect of p52 activation on the regulation of PDE4B and cAMP in HepG2 cells. In response to glucagon stimulation, PDE4B expression at the mRNA (Fig. 3c) and protein levels (Fig. 3d) decreased with cAMP accumulation (Fig. 3e), whereas silencing of p52 with siRNA preserved PDE4B expression and effectively ameliorated glucagon-stimulated cAMP production (Fig. 3c–e). In contrast, overexpression of p52 enhanced glucagon-stimulated reduction in PDE4B mRNA (Fig. 3f) and protein levels (Fig. 3g), and promoted cAMP production (Fig. 3h).

To verify whether or not p52 silencing antagonizes glucagon signaling was dependent on PDE4B, p52 siRNA and PDE4B siRNA were co-transfected in primary hepatocytes (Supplementary Fig. 4a, b). We observed that the inhibitory effects of p52 silencing were blocked by p52 siRNA and PDE4B siRNA co-transfection (Fig. 3i), indicating that p52 silencing inhibited gluconeogenesis in a PDE4B-dependent manner.

PDE3B is also expressed in the liver and is responsible for cAMP degradation. However, when stimulated by glucagon, PDE3B mRNA expression levels did not change significantly in vitro or in vivo (Fig. 3j). These results showed that p52 activation selectively suppressed PDE4B induction to increase cAMP accumulation in response to glucagon stimulation.

**Fig. 1** Treatment with p52 siRNA reduces fasting hyperglycemia in HFD-fed mice. a p52 protein level in liver tissue of NCD-fed, HFD-fed, and HFD-fed mice treated with p52 siRNA or NC siRNA. Liver tissues were collected from the mice after 8 weeks feeding (n = 3). b Fasting blood glucose in mice in panel a after 7 weeks feeding (n = 6). c Pyruvate tolerance test (2 g/kg body weight) in the mice in panel a after 7 weeks of feeding. AUC is indicated on the right (n = 6). d Fasting serum glucagon levels in mice in panel a fed with NCD or HFD for 8 weeks (n = 6). e Blood glucose levels in normal mice subjected to glucagon challenge (2 mg/kg body weight) and treatment with p52 siRNA or NC siRNA. AUC is indicated on the right (n = 6). f Fasting blood glucose of liver-specific p52 overexpression mouse (n = 8). g Blood glucose curve and AUC for mice that are either treated with AAV8-p52 or AAV8-NC after glucagon injection (n = 6). AAV adeno-associated virus, NCD normal chow diet, HFD high-fat diet, AUC area under the curve, NS normal saline. Bars represent mean ± SEM values. Statistical difference in panel g was determined by a two-tailed Student’s t test, and all others were used one-way ANOVA. *p < 0.05 vs. the control group, **p < 0.01 vs. the control group. Source data are provided as a Source Data file.

**Glucagon induced p52 activation by the cAMP/PKA pathway.** To explore the underlying mechanisms by which glucagon induced p52 activation, we stimulated primary hepatocytes with glucagon. We observed that the increase in p52 protein levels was prevented by the glucagon receptor inhibitor adomeglivant (Supplementary Fig. 5a). Forskolin activates adenylyl cyclase to generate cAMP from ATP. Bt₂-cAMP is typically used to mimic cellular cAMP. Similar to stimulation by glucagon, forskolin and Bt₂-cAMP increased p52 protein levels in hepatocytes (Supplementary Fig. 5b, c). H89, a PKA inhibitor, inhibited p52 expression stimulated by glucagon, forskolin, and Bt₂-cAMP (Supplementary Fig. 5b–d). H89 also abrogated glucagon-induced p100 phosphorylation increase and p100 protein levels decrease (Supplementary Fig. 5e). Moreover, when hepatocytes stimulated with glucagon for different times, p100 decreased in a time-dependent manner while p52 rise accordingly (Supplementary Fig. 5f). But when pretreated with MG132, a cell-permeable proteasome inhibitor, p100 remained unchanged (Supplementary Fig. 5g). These results indicated that glucagon induced proteasome-mediated cleavage of p100 to p52. Interestingly, the transcriptional levels of NF-κB2 by glucagon stimulation was increased (Supplementary Fig. 5h), but diminished when pretreated with MG132 (Supplementary Fig. 5i).
Fig. 2 p52 knockdown blocks cAMP/PKA signaling. a Hepatic cAMP accumulation in the liver tissue of NCD-fed, HFD-fed, and HFD-fed mice with p52 silencing. Liver tissues were collected from the mice after 8 weeks feeding (n = 6). b, c Western blot analysis of phospho-PKA substrates (b) and p-CREB (c) expression in the liver tissue from the mice in panel a (n = 3). d qRT-PCR determination of mRNA levels of G6pase, PEPCK, and PGC-1α in the livers from the mice in panel a (n = 6). e Western blot analysis of p52 expression using lysates of the liver tissue from normal mice treated with glucagon (2 mg/kg body weight) and p52 siRNA or NC siRNA (n = 4). f Hepatic cAMP accumulation in the liver tissue of the mice (n = 6). g, h Hepatic phospho-PKA substrates and p-CREB protein levels in the mice (n = 3). i Hepatic mRNA levels of G6pase, PEPCK, and PGC-1α in the mice (n = 6). NCD normal chow diet, HFD high-fat diet, PKA protein kinase A, CREB cAMP-response element-binding protein, qRT-PCR quantitative real-time polymerase chain reaction, NS normal saline, G6pase glucose-6-phosphatase, PEPCK phosphoenolpyruvate carboxykinase, PGC-1α peroxisome proliferator-activated receptor gamma coactivator-1 alpha. Each bar represents mean ± SEM values. Statistical differences were determined by one-way ANOVA. *p < 0.05 vs. the control group, **p < 0.01 vs. the control group. Source data are provided as a Source Data file.
Fig. 3 p52 inhibits PDE4B expression to promote cAMP accumulation. a, b Liver PDE4B protein expression in HFD-fed (a) and glucagon-stimulated mice (b). Bar graphs represent data normalized to β-actin levels (n = 3). c The mRNA levels of PDE4B in HepG2 cells transfected with p52 or NC siRNA (n = 5). Bar graphs represent the levels of genes normalized to β-actin. d The protein expression of PDE4B in p52 knocked down HepG2 cells, β-actin levels served as loading control (n = 5). e cAMP level in HepG2 cells transfected with p52 siRNA (n = 6). f The mRNA levels of PDE4B in HepG2 cells transfected with p52 overexpression plasmid (n = 5). g Western blotting of PDE4B in p52 overexpression cells (n = 4). h cAMP level in HepG2 cells transfected with p52 overexpression plasmid (n = 6). Bars represent mean ± SEM values. i Intracellular cAMP levels and glucose output in primary hepatocytes transfected with p52 siRNA with or without PDE4B siRNA (n = 6). j Relative mRNA abundance of PDE3B in glucagon stimulated HepG2 cells (100 nM glucagon for 1 h, in vitro) or mice liver tissue (2 mg/kg glucagon for 1 h, in vivo), β-actin levels used as a reference (n = 6). PDE phosphodiesterase, HFD high-fat diet, NS normal saline, ns not statistically significant, PBS phosphate buffer solution. Values represent mean ± SEM. Statistical differences were determined by one-way ANOVA. *p < 0.05 vs. the control group, **p < 0.01 vs. the control group. ###p < 0.001 vs. AAV8-p52 or p52 plasmid group. Source data are provided as a Source Data file.
Metformin suppresses hepatic p52 activation in HFD-fed mice. To determine if pharmacological intervention could inhibit NF-κB2 activation, we examined the effect of metformin on hepatic p52 expression. Metformin attenuated p52 expression in the livers of HFD-fed mice (Fig. 5a), preserved PDE4B induction (Fig. 5b), and prevented cAMP accumulation (Fig. 5c). Metformin had similar effects in glucagon-treated mice (Fig. 5d–f). In primary hepatocytes, metformin inhibited p52 nuclear translocation in response to glucagon (Fig. 5g). These results indicate that metformin suppresses NF-κB2 activation, contributing to reduced cAMP accumulation. Consistent with this, metformin inhibited PKA activation (Supplementary Fig. 8a), inactivated CREB by dephosphorylation (Supplementary Fig. 8b), and consequently suppressed expression of G6Pase, PEPCK, and PGC1-α (Supplementary Fig. 8c) in the livers of HFD-fed mice. As a result, metformin improved pyruvate tolerance in HFD-fed mice and attenuated the hyperglycemic response in glucagon-treated mice (Supplementary Fig. 8d, e).

To provide evidence that metformin acts through inhibiting p52 to increase PDE4B expression, we overexpressed p52 in mice liver by AAV8-p52, and then detected the hypoglycemic effects of ginseng, also inhibited glucagon-induced p52 expression and ginsenoside Rb1, the most abundant active component in ginseng, also inhibited glucagon-induced p52 expression and hyperglycemia at least in part by inhibiting p52 activation. Interestingly, ginsenoside Rb1, the most abundant active component in ginseng, also inhibited glucagon-induced p52 expression and hyperglycemia.
nuclear translocation, thereby antagonizing hepatic glucose production (Supplementary Fig. 9a–c). In order to verify that Rb1 exerts its hypoglycemic effects dependent on p52, we overexpressed p52 in mice liver with AAV8-p52 injection. The hypoglycemic effects of Rb1 were diminished in p52 overexpressed mice (Supplementary Fig. 9d). In addition, we transfected p52 plasmid in primary hepatocytes and detected the hepatic glucose production. The inhibitory effects of Rb1 on hepatic glucose production were reversed (Supplementary Fig. 9e).

Fig. 5 Metformin alleviates hyperglycemia by inhibiting p52 activation. a, b Western blotting analysis of p52 (a) and PDE4B (b) using liver lysates of mice fed with the indicated diet for 8 weeks. Each lane represents a liver lysate from a different animal. Bar graphs represent the data normalized to β-actin (n = 3). Metformin (200 mg/kg/d) was administrated by gavage for 8 weeks. c The cAMP levels in the liver of NCD-fed or HFD-fed mice (n = 6). d, e Western blotting analysis of hepatic p52 (d) and PDE4B (e) in mice injected with 2 mg/kg glucagon (n = 3). In total, 200 mg/kg metformin was pre-administrated by gavage 1 h before glucagon injection. f Hepatic cAMP levels in glucagon-injected mice (n = 6). g Representative confocal images of primary hepatocytes exposed to glucagon (100 nM, 1 h) pretreated with metformin (1 mM, for 4 h), or PBS. Scale bar represents 5 μm. h Blood glucose curve and AUC for mice that are either treated with AAV8-p52 or AAV8-NC after glucagon injection (n = 6). Metformin (200 mg/kg) or normal saline was administrated 1 h before glucagon injection by gavage. i Hepatic glucose production in p52 overexpression primary hepatocytes treated with or without 1 mM metformin (n = 8). HFD high-fat diet, Met metformin, PDE phosphodiesterase, NCD normal chow diet, NS normal saline, PBS phosphate buffer saline, AUC area under the curve, DAPI 4′,6-diamidino-2-phenylindole, AAV adeno-associated virus. Bars represent mean ± SEM values. Statistical differences were determined by one-way ANOVA. *p < 0.05 vs. the control group, **p < 0.01 vs. the control group. ##p < 0.01 vs. AAV8-p52 or p52 plasmid group. Source data are provided as a Source Data file.
Discussion

Dysregulation of hepatic gluconeogenesis is a major contributing factor to the pathogenesis of type 2 diabetes. Until now, the signaling pathways that regulate the key transcription factors of gluconeogenesis remain largely unknown. Better understanding of the precise mechanisms underlying the regulation of gluconeogenesis is crucial for the management of diabetes. Here, we identified a critical role of p52 in glucagon-induced hepatic gluconeogenesis during fasting. The major findings of this work include: (1) p52 was activated by glucagon in livers of HFD-fed mice, and the activated p52 in turn augmented glucagon response as a positive feedback loop; (2) liver-specific p52 knockdown lowered glucagon-stimulated hyperglycemia, while p52 overexpression augmented glucagon response; (3) p52 inhibited PDE4B gene promoter activity to promote cAMP accumulation, augmenting glucagon response via cAMP/PKA signaling; (4) metformin and ginsenoside lowered blood glucose at least in part by inhibiting p52 activation. The proposed mechanism is illustrated in Fig. 6. Our data point to a novel therapeutic strategy of targeting p52 activation for the treatment of type 2 diabetes.

Although the role of the canonical NF-κB pathway in type 2 diabetes is well documented, the role of the alternative pathway in gluconeogenesis remains largely unknown. Better understanding of the precise mechanisms underlying the regulation of gluconeogenesis is crucial for the management of diabetes. Here, we identified a critical role of p52 in glucagon-induced hepatic gluconeogenesis during fasting. The major findings of this work include: (1) p52 was activated by glucagon in livers of HFD-fed mice, and the activated p52 in turn augmented glucagon response as a positive feedback loop; (2) liver-specific p52 knockdown lowered glucagon-stimulated hyperglycemia, while p52 overexpression augmented glucagon response; (3) p52 inhibited PDE4B gene promoter activity to promote cAMP accumulation, augmenting glucagon response via cAMP/PKA signaling; (4) metformin and ginsenoside lowered blood glucose at least in part by inhibiting p52 activation. The proposed mechanism is illustrated in Fig. 6. Our data point to a novel therapeutic strategy of targeting p52 activation for the treatment of type 2 diabetes.

Fig. 6 NF-κB2 (p52) activation restrains the hepatic glucagon response by preserving PDE4B induction. Glucagon induces p52 activation. Activated p52 binds to PDE4B promoter to inhibit its transcription, leading to cAMP accumulation. Accumulated cAMP augmented glucagon response by cAMP/PKA signaling. Metformin and ginsenoside Rb1 inhibited p52 activation to restrain hepatic glucagon response by preserving PDE4B induction.
might be a candidate target to reduce excessive hepatic glucose output by restraining the hepatic glucagon response.

Methods

Animals and treatments. Six to eight-week-old male C57BL/6j mice were purchased from the Comparative Medical Center of Yangzhou University (Yangzhou, China). After 1 week of acclimation, the mice were fed with a HFD (60% kcal from fat; TP2330; Trophic, China) or a NCD (10% kcal from fat; Xietong Organism, China) for 8 weeks. The mice were raised in a temperature-controlled facility on a 12 h light–dark cycle with free access to food and water. The mice were given metformin (Sigma, America; 200 mg/kg body weight/day) by gavage during the 8 weeks of HFD feeding. Body weights and food intake were recorded every day. Blood was collected after an overnight fast.

The procedures for experiments and animal care were approved by the Institutional Animal Care and Use Committee of China Pharmaceutical University (Nanjing, China). Animal testing and research conforms to all relevant ethical regulations.

p52 siRNA transfection in vivo. p52 knockdown mice were created using in vivo transfection technology. In brief, 8-week-old C57BL/6j mice were injected with Entranster-in vivo reagent (Engreen Biosystem, China) carrying p52 small interfering RNA (siRNA) through the caudal vein weekly during HFD feeding. Negative control siRNA oligos were used as controls in chronic and acute experiments. The siRNA oligos are as follows:

- Negative control siRNA: UUCGCGACGUGUCACTGGTT
- p52 siRNA: UCCAGAGGGAGUCCCAAATT

Liver-specific p52 overexpression. Male C57BL/6j mice at 6–8-week old were purchased from Sino-British SIPPB/KB Lab. Animal Ltd (Shanghai, China). Liver-specific overexpression of p52 in mice was created based on AAV8-p52 plasmid transfection. In brief, after 1 week of acclimation, mice were injected with 7 μl of AAV8-plasmid virus suspension (virus titer > 1012) blend with 193 μl normal saline. Control mice were injected with AAV8-normal vector (AAV8-NC). Three weeks later, mice were randomly divided into different groups for testing fasting blood glucose and glucagon challenge experiments.

Pyruvate and glucagon tolerance tests. For pyruvate tolerance tests, fasted mice were injected intraperitoneally with pyruvate (2 g/kg body weight). For glucagon tolerance tests, fasted mice were injected with p52 siRNA oligo via tail vein 48 h before the test. The mice were fasted overnight and then injected with glucagon (2 mg/kg body weight). During glucagon tolerance test, metformin (200 mg/kg body weight) or ginsenoside Rb1 (50 mg/kg body weight) was administrated by gavage 1 h before glucagon injection. Blood glucose levels were measured using ONE-TOUCH glucose meters (Johnson, America) from tail tips at indicated times.

Primary hepatocyte isolation and cell culture. Primary hepatocytes were isolated from 6–8-week-old male C57BL/6j mice using an in situ liver perfusion approach26. Isolated primary hepatocytes were cultured in the William E Medium supplemented with 10% (v/v) fetal bovine serum (FBS). HepG2 cell line was purchased from Sino-British SIPPR/BK Lab. Animal Ltd (Shanghai, China). Liver-specific p52 plasmid transfection in vivo was performed on the Roche LightCycler 96 System using the Fast SYBR Green Master Mix (Roche, America). The mRNA expression levels of target genes were normalized to β-actin expression levels. All the primer pairs used are listed in Supplementary Table 1.

Western blotting. Liver tissue and cell protein were extracted with a radioimmunoprecipitation assay lysis buffer. Nuclear extracts were prepared using an NE-PER Nuclear Cytoplastic Extraction Reagent kit (Pierce, America). Protein extracts were separated on 8–10% SDS-PAGE gels and transferred onto nitrocellulose membranes. Protein expression was visualized by incubating primary antibodies (Supplementary Table 2) overnight at 4 °C followed by the corresponding secondary antibodies. Unprocessed scans of the most important blots in the Supplementary Fig. 14.

Hepatic glucose production. HepG2 cells or primary hepatocytes were maintained in the DMEM with 10% FBS. At 50% confluence, the cells were transfected with p52 siRNA or plasmid. PDE4B siRNA or plasmid as mentioned previously. After 24 h, the medium was replaced with Krebs-Ringer HEPES (KRH) buffer to fast the cells for 2 h. After washing with PBS three times, the cells were incubated in KRH buffer supplemented with 10 mM pyruvate, 100 nM glucagon (Novo Nordisk, Denmark), or with 1 mM metformin and 10 μM ginsenoside Rb1 for 6 h. The cell supernatant was then collected for glucose analysis using the Glucose Assay Kit, and normalized to total cellular protein content.

Luciferase reporter assay. Transfection of the pGL3-basic PDE4B promoter and p52 plasmid was conducted in 293T cells using Lipofectamine™ 2000. After 48 h, cell lysates were used for luciferase assays using a 96-well luminometer with a dual-luciferase substrate system (Promega, America). A Renilla Luciferase plasmid was used at 1:0.1 pg/well as an internal control.

ChIP assays. Chromatin immunoprecipitation (ChIP) assays were performed using a kit (Magna ChIP HSens) purchased from Millipore (Temecula, America) according to the manufacturer’s instructions. Briefly, chromatin in HepG2 cells pretreated with 100 nM glucagon or vehicle was cross-linked in 1% formaldehyde and subsequently lysed using 1% SDS lysis buffer. Chromatin was fragmented by SFX250 Ultrasonic Cell Disruption System (Branson, America). Soluble chromatin was immunoprecipitated with an NF-κB antibody (Santa Cruz Biotechnology, Europe). The de-cross-linked samples were incubated with RNAse A and proteinase K. DNA was purified using a Phenol/Chloroform extraction method. The following primers were used during qRT-PCR detection:

- Site A: Forward primer GCCATGTCCTGCTGTGTTGA
- Site B: Forward primer CCTTGCGAGCTCTCTCTTT

Primers for quantitative reverse transcription PCR were designed using the primer-3 program (IDT). The primers used are listed in Supplementary Table 1.

Quantitative real-time reverse transcription PCR (qRT-PCR). The total mRNA was isolated using TRizol™ Reagent (Invitrogen, America) from the livers of mice, primary hepatocytes or HepG2 cells and cDNAs were synthesized. qRT-PCR was performed on the Roche LightCycler 96 System using the Fast SYBR Green Master Mix (Roche, America). The mRNA expression levels of target genes were normalized to β-actin expression levels. All the primer pairs used are listed in Supplementary Table 1.

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Author contributions Q.L., L.-W.Q., and P.L. conceived and designed the experiments. W.-S.Z., Q.L., X.Z., and A.Y. performed the experiments. G.-X.M. and W.-S.Z. analyzed the data. Q.L., B.-L., L., and W.-S.Z. wrote the paper. L.-W.Q. and P.L. improved the paper. All authors contributed to the discussion of the results and paper corrections.

Competing interests
The authors declare no competing interests.

Additional information
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