Molecules and Cells

Regulation of Hepatic Gluconeogenesis by Nuclear Receptor Coactivator 6

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Nuclear receptor coactivator 6 (NCOA6) is a transcriptional coactivator of nuclear receptors and other transcription factors. A general *Ncoa6* knockout mouse was previously shown to be embryonic lethal, but we here generated liver-specific *Ncoa6* knockout (*Ncoa6* LKO) mice to investigate the metabolic function of NCOA6 in the liver. These *Ncoa6* LKO mice exhibited similar blood glucose and insulin levels to wild type but showed improvements in glucose tolerance, insulin sensitivity, and pyruvate tolerance. The decrease in glucose production from pyruvate in these LKO mice was consistent with the abrogation of the fasting-stimulated induction of gluconeogenic genes, phosphoenolpyruvate carboxykinase 1 (*Pck1*) and glucose-6-phosphatase (*G6pc*). The forskolin-stimulated inductions of *Pck1* and *G6pc* were also dramatically reduced in primary hepatocytes isolated from *Ncoa6* LKO mice, whereas the expression levels of other gluconeogenic gene regulators, including cAMP response element binding protein (*Creb*), forkhead box protein O1 and peroxisome proliferator-activated receptor γ coactivator 1α, were unaltered in the LKO mouse livers. CREB phosphorylation via fasting or forskolin stimulation was normal in the livers and primary hepatocytes of the LKO mice. Notably, it was observed that CREB interacts with NCOA6. The transcriptional activity of CREB was found to be enhanced by NCOA6 in the context of *Pck1* and *G6pc* promoters. NCOA6-dependent augmentation was abolished in cAMP response element (CRE) mutant promoters of the *Pck1* and *G6pc* genes. Our present results suggest that NCOA6 regulates hepatic gluconeogenesis by modulating glucagon/cAMP-dependent gluconeogenic gene transcription through an interaction with CREB.

Keywords: cAMP response element-binding protein, gluconeogenesis, glucose-6-phosphatase, nuclear receptor coactivator 6, phosphoenolpyruvate carboxykinase

INTRODUCTION

Glucose is a major metabolic fuel for energy production in most organisms. Glucose homeostasis is therefore an important process and is maintained within a narrow range by various pathways of glucose metabolism, including glycogenesis, glycogenolysis, glycolysis and gluconeogenesis (Petersen et al., 2017). In the fed state, glycogen synthesis and glycolysis are dominant processes in the liver. In contrast, glycogen breakdown and gluconeogenesis mainly occur in a fasted state. Hepatic gluconeogenesis is the primary mechanism of endogenous glucose production during prolonged fasting or starvation because glycogen storage in the liver is rapidly

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depleted in the fasted state (Rui, 2014). Importantly in this regard, increased hepatic gluconeogenesis is considered to be a major contributor to the hyperglycemia observed in patients with type 2 diabetes, whereas glycogenolysis was found not to contribute (Cline et al., 1994; Magnusson et al., 1992). In addition, gluconeogenesis is a linking factor in the causal relationship between hepatic fat accumulation and hepatic insulin resistance (Samuel et al., 2004). Hence, a better understanding the regulation of gluconeogenesis is fundamentally critical to the development of new treatments for type 2 diabetes.

The main substrates for human gluconeogenesis are lactate, glycerol, and glucogenic amino acids (particularly alanine and glutamine). Pyruvate is first generated for gluconeogenesis from lactate or an α-keto acid (e.g., α-ketoglutarate) derived from amino acid breakdown. Pyruvate is then transformed via carboxylation into oxaloacetate by pyruvate carboxylase (PC) in the mitochondria. After leaving the mitochondria via malate, oxaloacetate is converted to phosphoenolpyruvate by phosphoenolpyruvate carboxykinase 1 (PCK1). After five reverse steps of glycolysis, fructose-6-phosphate is formed from fructose-1,6-bisphosphate by fructose-1,6-bisphosphatase 1 (FBP1). Fructose-6-phosphate is then converted to glucose-6-phosphate by phosphoglucose isomerase. Glucose-6-phosphate is finally dephosphorylated by glucose -6-phosphatase (G6PC) to form free glucose. The reactions catalyzed by the PC, PCK1, FBP1, and G6PC enzymes are rate-limiting steps in gluconeogenesis. It is noteworthy that the gluconeogenic *PC*, *PCK1* and *G6PC* genes are all directly activated by the cAMP response element binding protein (CREB) transcription factor (Thiel et al., 2005; Thonpho et al., 2010; Xing and Quinn, 1993). Latent nuclear CREB is phosphorylated by the cAMP/PKA pathway and then operates the gluconeogenic program through the transcriptional activation of the *PC*, *PCK1* and *G6PC* genes (Benchoula et al., 2021).

Nuclear receptor coactivator 6 (NCOA6), also known as activating signal cointegrator-2 (ASC-2), nuclear receptor coregulator (NRC), peroxisome proliferator-activated receptor interacting protein (PRIP), nuclear receptor-activating protein 250 (RAP250), and thyroid hormone receptor-binding protein (TRBP), is a transcriptional coactivator of nuclear receptors and many other transcription factors (Caira et al., 2000; Ko et al., 2000; Lee et al., 1999; Mahajan and Samuels, 2000; Zhu et al., 2000). *Ncoa6* null mouse embryos show growth retardation, hypoplastic heart development, defective placentation, and embryonic lethality (Antonson et al., 2003; Mahajan et al., 2004; Zhu et al., 2003). We previously reported using *Ncoa6* heterozygous knockout mice that NCOA6 is involved in glucose homeostasis via the regulation of insulin secretion from pancreatic β-cells and hepatic insulin sensitivity (Kim et al., 2012; Yeom et al., 2006). In our present study, we generated liver-specific *Ncoa6* knockout (*Ncoa6* LKO) mice to enable us to study the metabolic function of hepatic NCOA6. Interestingly, we found from our analyses that pyruvate tolerance is improved in *Ncoa6* LKO mice compared to wild type. This led us to investigate the molecular function of NCOA6 during the process of hepatic gluconeogenesis. Our results demonstrate that NCOA6 plays a crucial role as a gluconeogenic factor by stimulating the transcriptional activity of CREB towards the *Pck1* and *G6pc* gene promoters.

MATERIALS AND METHODS

Mice

Ncoa6 LKO mice were generated by crossing homozygous floxed *Ncoa6* (*Ncoa6*$^{fl/fl}$) mice with hemizygous albumin-Cre mice (B6.Cg-*Speer6-ps1*$^{Tg(Alb\text{-}cre)21Mgn}$/J, Stock No 003574; The Jackson Laboratory, USA). *Ncoa6*$^{fl/+}$ mice were provided by Janardan K. Reddy from Northwestern University, Feinberg School of Medicine, Chicago, IL (Zhu et al., 2003). For genotyping, floxed *Ncoa6* and *Cre* alleles were polymerase chain reaction (PCR) amplified from tail genomic DNA using the following primers: floxed *Ncoa6* allele, 5′-GGC TCA TTT TCT AGC CCA TGA-3′ and 5′-AGG ACC AGC TCC TTG ACC ACC-3′; and *Cre* allele, 5′-CTG GTT ATG CGG CGG ATC CGA-3′ and 5′-GGC GCG AGT TGA TAG CTG GCT-3′. The genomic deletion of *Ncoa6* exon 8 was confirmed by PCR amplification of liver genomic DNA using the floxed *Ncoa6* allele primers. The mice were housed in a temperature-controlled facility with a 12-h light/12-h dark cycle and provided free access to water and regular rodent chow. All animal protocols were approved by the Institutional Animal Care and Use Committee of the Asan Institute for Life Sciences, Asan Medical Center (approval No. 2016-12-201).

Cell culture

Mouse primary hepatocytes were prepared from *Ncoa6*$^{fl/fl}$ and *Ncoa6* LKO mice by collagenase digestion and Percoll density gradient centrifugation using a previously described procedure (Oh et al., 2015). After isolation, cells were incubated in M199 (Cat. No. 31100-035; Gibco, USA) supplemented with antibiotics (Cat. No. 15140-122; Gibco). HepG2 and 293T cells were maintained in DMEM (Cat. No. 12800-017; Gibco) supplemented with 10% fetal bovine serum (FBS) (Cat. No. 16000-044; Gibco) and antibiotics.

Glucose, glucagon, insulin, and pyruvate tolerance tests

For glucose or pyruvate tolerance testing, 10-week-old *Ncoa6*$^{fl/fl}$ and *Ncoa6* LKO mice were intraperitoneally injected with 2 g/kg glucose or 2 g/kg pyruvate, respectively, after a 16 h overnight fast. For glucagon or insulin tolerance test, mice were intraperitoneally injected with 15 μg/kg glucagon (Cat. No. G2044; Sigma-Aldrich, USA) or 0.5 U/kg insulin (Cat. No. 12585-014; Thermo Fisher Scientific, USA), respectively, after a 6 h fast. Blood glucose concentrations were measured at 0, 30, 60, 90, and 120 min after injection using a One Touch Ultra Blood Glucose Monitoring System (LifeScan, USA).

Serum insulin and glucagon measurements

For insulin and glucagon measurements, serum was prepared by allowing whole blood to clot for 30 min followed by centrifugation at 1,000 × *g*, 4°C for 10 min. Serum insulin and glucagon levels were determined using an LBIS Mouse Insulin ELISA kit (Cat. No. 638-01489; Fujifilm Wako Shibayagi, Japan) and a Glucagon Enzyme Immunoassay kit (Cat. No. K4756; BioVision, USA), respectively, in accordance with the

manufacturer's instructions.

Glucose production assay

To assay glucose production, primary hepatocytes were plated into 6-well plates and incubated in M199 medium supplemented with antibiotics. The following day, cells were washed twice with 37°C pre-warmed phosphate-buffered saline (PBS) followed by the addition of 1 ml of glucose production buffer consisting of glucose-free DMEM (pH 7.4, without L-glutamine, phenol red, sodium pyruvate and sodium bicarbonate, Cat. No. D5030; Sigma-Aldrich) supplemented with 4 mM L-glutamine, 44 mM sodium bicarbonate, 20 mM sodium lactate, 2 mM sodium pyruvate, and 15 mM HEPES. The cells were then treated with 10 μM forskolin or DMSO and incubated at 37°C for 4 h. The glucose concentration in the glucose production buffer was measured using a Glucose (HK) Assay Kit (Cat. No. GAHK20-1KT; Sigma-Aldrich) in accordance with the manufacturer's instructions.

RNA preparation and quantitative real-time PCR (qRT-PCR)

Total RNA was isolated from mouse tissues or primary hepatocytes using TRIzol reagent (Cat. No. 15596018; Thermo Fisher Scientific) as per the manufacturer's protocol. Purified total RNA (1 μg) was reverse-transcribed using M-MLV reverse transcriptase (M1705; Promega, USA). The transcript levels of each gene were analyzed by real-time qRT-PCR using the LightCycler 480 System (Roche, Switzerland) and SYBR Green PCR Master Mix (Cat. No. 04887352001; Roche). The $2^{-\Delta\Delta Ct}$ method was used to calculate the relative transcript levels compared to internal control *Rps29*. The following primers were used for these qRT-PCR analyses: *Rps29*, 5′-CGC AAA TAC GGG CTG AAC A-3′ and 5′-GCC TAT GTC CTT CGC GTA CTG-3′; *Ncoa6*, 5′-GAA GAA ACC GCC TCG GAA GA-3′ and 5′-CCT CTA GAC CAG TTG GAC GAT TAT CT-3′; *G6pc*, 5′-CCA TGC AAA GGA CTA GGA ACA A-3′ and 5′-TAC CAG GGC CGA TGT CAA C-3′; *Pck1*, 5′-CCA CAG CTG CTG CAG AAC A-3′ and 5′-GAA GGG TCG CAT GGC AAA-3′; *Pc*, 5′-CCA CCT GGA TCC CCA ACT T-3′ and 5′-GCG TTC TCA TAG CCT ACC TGC TT-3′; *Creb1*, 5′-GGA GTG CCA AGG ATT GAA GA-3′ and 5′-CTG TCC ACT GCT AGT TTG GTA A-3′, *Foxo1*, 5′-CCA GCT CAA ATG CTA GTA CCA TCA-3′ and 5′-GTC CCC ATC TCC CAG GTC AT-3′; *Pgc1a*, 5′- AAG TGT GGA ACT CTC TGG AAC TG-3′ and 5′-GGG TTA TCT TGG TTG GCT TTA TG-3′.

Immunoblotting

For immunoblotting analysis, total cell lysates were first prepared from mouse livers and primary hepatocytes using lysis buffer containing 20 mM Tris-Cl, pH 7.5, 150 mM NaCl, 1% NP-40, and protease inhibitor cocktail (Cat. No. 4693132001; Roche). Lysate proteins (50 μg) were separated by SDS-PAGE and transferred onto a PVDF membrane. After membrane blocking with 5% skim milk, the membrane was probed with primary antibodies against NCOA6 (Cat. No. NB200-335; Novus Biologicals, USA), PCK1 (Cat. No. sc-32879; Santa Cruz Biotechnology, USA), CREB (Cat. No. 9197; Cell Signaling Technology, USA), p-CREB (Cat. No. 9198S; Cell Signaling Technology), or α-tubulin (Cat. No. T9026; Sigma-Aldrich). The blots were then incubated with HRP-conjugated secondary antibodies and visualized using ECL substrate and the Chemi-Smart system (Vilber Lourmat, France). The intensities of the protein bands were determined using ImageJ software (NIH, USA).

Immunoprecipitation (IP)

293T cells were plated onto 100-mm dishes and transfected with 3xFLAG-hNCOA6 and HA-CREB Y134F (constitutively active CREB) using Lipofectamine 2000 (Cat. No. 11668019; Thermo Fisher Scientific), in accordance with the manufacturer's instructions. After 24 h, the cells were washed with cold PBS and whole cell lysates were then prepared via the addition of lysis buffer. Aliquots of protein lysates (500 μg) were pre-cleared using protein G-agarose beads (Cat. No. sc-2002; Santa Cruz Biotechnology) and immunoprecipitated with anti-FLAG M2 affinity gel (Cat. No. A2220; Sigma-Aldrich) or anti-HA (Cat. No. MMS-101R; Covance, USA) antibody in conjunction with protein G-agarose at 4°C overnight. After IP, the beads were washed three times with IP buffer (20 mM Tris-Cl pH 7.5, 150 mM NaCl, and 0.1% NP-40) and then boiled for 5 min in SDS loading buffer to solubilize the proteins. The immunoprecipitated proteins were subsequently identified by immunoblotting analysis using anti-FLAG (Cat. No. F3165; Sigma-Aldrich) or anti-HA (Cat. No. MMS-101P; Covance) antibodies.

Construction of gluconeogenic gene promoter-luciferase reporters

The human *G6PC* −903/+58 region was amplified from human genomic DNA by PCR using PrimeSTAR HS DNA polymerase with GC buffer (Cat. No. R044A; Takara, Japan) and the following primers: 5′-CGG GGT ACC TAA GAG ACA TGA GGC CAA-3′ and 5′-CCG CTC GAG GAA GAT GTC AGC AGA G-3′. The PCR product was digested with *Kpn*I and *Xho*I and inserted upstream of the luciferase gene in the pGL3-Basic vector. A rat *Pck1* −543/+73 Luc reporter was provided by JaeHun Cheong (Pusan National University, Korea). CRE mutant promoters of the *G6PC* and *Pck1* genes were prepared via a PCR cloning strategy using the following primer pairs: *G6PC* CRE-1 mutant, 5′-TTC TAT TTT AGG TCC ATC ACC CTG AAC ATG-3′ and 5′-CTA GCT AGC ACT CTT CAT CTG AGG AGC-3′; *G6PC* CRE-2 mutant, 5′-ATG TTT CTA GAA ACC TAC TGG TGA TGC ACC-3′ and 5′-CCA GTA GGT TTC TAG AAA CAT GTT CAG GGT-3′; and *Pck1* CRE mutant, 5′-CCC TTT TTT CAG AGG CGA GCC TCC-3′ and 5′-CTC TGA AAA AAG GGG CCG GCC TTT-3′.

Luciferase reporter assay

For the luciferase assays, HepG2 cells were seeded into 24-well plates in DMEM containing 10% FBS. A luciferase reporter and β-gal construct were cotransfected the next day with or without CREB and NCOA6 expression constructs using Lipofectamine 2000, in accordance with the manufacturer's instructions. After a further 18 h, the cells were treated with 10 μM forskolin for 6 h and then lysed for the measurement of luciferase and β-gal activities. Luciferase activities were determined using a luminometer Centro LB 960 (Berthold Technologies, Germany) and normalized to β-galactosidase activities.

Chromatin immunoprecipitation (ChIP) of plasmid-bound proteins

ChIP assays were performed as described previously with minor modifications (Kim et al., 2015; Wolfe and Long, 2019). Briefly, 293T cells were cotransfected with 3xFLAG-hNCOA6, HA-CREB Y134F, and *G6PC* or *Pck1* promoter constructs containing wild type or mutant CRE. The cells were incubated overnight and then fixed with 1% formaldehyde (Cat. No. F8775; Sigma-Aldrich) for 15 min at room temperature. The cells were next washed with cold PBS and lysed for 30 min at 4°C by resuspension in a buffer consisting of 50 mM Tris-Cl (pH 8.0), 1% SDS and 10 mM EDTA. Soluble chromatin was prepared by sonication (10 pulses of 10 s and 25% amplitude at 4°C using a VCX500 sonicator; Sonics & Materials, USA) and immunoprecipitated with antibodies against HA (Cat. No. MMS-101P; Covance), FLAG (Cat. No. A2220; Sigma-Aldrich) or IgG (Cat. No. ab46540; Abcam, UK). The final DNA extracts were analyzed by conventional PCR using the following primers: *G6PC* CRE forward (-196/-174), 5′-GCC GAT CAG GCT GTT TTT GTG TG-3′; *Pck1* CRE forward (-144/-124), 5′-AGG TCA GTT CCA AAC CGT GCT-3′; and a commonly used reverse primer for the pGL3 vector, 5′-CGG TTC CAT CTT CCA GCG GAT A-3′.

Statistical analysis

Data are presented as the mean ± SE. Statistical analyses were performed using a two-tailed Student's *t*-test or one-way ANOVA with SPSS software (ver. 19; IBM, USA). Differences with P values < 0.05 were considered statistically significant. Statistical results are indicated in the figures as follows: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

RESULTS

Construction of liver-specific *Ncoa6* knockout mice

Since an *Ncoa6* homozygous knockout in the mouse is embryonic lethal, we used heterozygous *Ncoa6*$^{+/-}$ and dominant-negative transgenic mice to study the physiological role of NCOA6 in the whole mouse body. Using these mouse models, we found that NCOA6 is an important transcriptional coactivator in many metabolic processes, including insulin secretion and insulin signaling (Kim et al., 2012; Yeom et al., 2006). In our present study, we generated liver-specific *Ncoa6* knockout mice by crossing *Ncoa6*$^{fl/fl}$ and albumin-Cre mice (Fig. 1A). We then used these knockout animals to investigate the metabolic role of hepatic NCOA6 under more specific and clearly defined conditions. The liver-specific deletion of *Ncoa6* exon 8 was validated by genomic conventional PCR (Fig. 1B). Consistently, the *Ncoa6* transcript and NCOA6 protein levels were dramatically decreased in the liver tissue (Figs. 1C and 1D). The successful generation of an *Ncoa6* LKO mouse was thus confirmed.

Improved pyruvate tolerance in *Ncoa6* LKO mice

In a previous study of young (10 weeks old) *Ncoa6*$^{+/-}$ mice, the level of serum insulin was decreased and the insulin sensitivity was found to be increased compared to wild type mice, whereas the fasting blood glucose level and glucose tolerance were not significantly different (Kim et al., 2012). A normal glucose tolerance level in *Ncoa6*$^{+/-}$ mice was considered to be the result of a trade-off between a decrease in insulin secretion and an increase in insulin sensitivity. In our current analyses, we first analyzed the blood glucose and insulin levels and the glucose and insulin tolerance, of *Ncoa6* LKO mice to compare their metabolic phenotypes with those of *Ncoa6*$^{+/-}$ mice. *Ncoa6* LKO mice showed no difference in their fasting glucose level but displayed an improved glucose tolerance compared with *Ncoa6*$^{fl/fl}$ mice (Figs. 2A and 2B). The glucose area under the curve (AUC) is an index of whole glucose excursion after glucose loading. The AUC from the glucose tolerance test was found to be significantly decreased in the *Ncoa6* LKO mice. As expected, the serum insulin levels of *Ncoa6* LKO mice were not significantly different from *Ncoa6*$^{fl/fl}$ mice under either fed or fasting condition, because *Ncoa6* LKO mice lack these NCOA6 expression only in liver tissue (Fig. 2C). Insulin sensitivity was found to be augmented in *Ncoa6* LKO mice, consistent with the enhancement of hepatic insulin signaling in *Ncoa6*$^{+/-}$ mice (Fig. 2D).

We speculated that the improved glucose tolerance of *Ncoa6* LKO mice was due to an increased hepatic insulin sensitivity. We next performed a pyruvate tolerance test to examine the gluconeogenic ability of *Ncoa6* LKO mice. Gluconeogenesis occurs primarily in the liver, accounting for up to 80% of the total glucose production in healthy individuals during a prolonged fasting (Ekberg et al., 1999). To evaluate gluconeogenesis as the major hepatic glucose output, our mice were fasted for 16 h to deplete hepatic glycogen storage. Surprisingly, the pyruvate tolerance was significantly increased in *Ncoa6* LKO mice (Fig. 2E), suggesting that glucose production from pyruvate is impaired in these animals.

Alteration of the gluconeogenic program in *Ncoa6* LKO mice

Gluconeogenesis is achieved via a series of enzymatic reactions to produce glucose from non-carbohydrate metabolites. The PC, PCK1, FBP1, and G6PC enzymes are unique to gluconeogenesis and considered to catalyze rate-limiting steps in this process. Among them, FBP1 is regulated allosterically by fructose 2,6-bisphosphate. In contrast, PC, PCK1, and G6PC are transcriptionally regulated by CREB. Since NCOA6 is known to be a transcriptional coactivator, we investigated the transcript levels of key gluconeogenic genes that are regulated by transcriptional mechanisms in our mouse model. The hepatic transcript levels of the *Pc*, *Pck1*, and *G6pc* genes were all increased by 24 h fasting in *Ncoa6*$^{fl/fl}$ mice, whereas the *Pck1* and *G6pc* transcripts did not show a significant increase in fasted *Ncoa6* LKO mice (Fig. 3A). The defective induction of the *Pck1* gene was evident at the protein level following fasting in *Ncoa6* LKO mice (Fig. 3B). It is well known that glucagon is markedly upregulated by fasting and is responsible for subsequent gluconeogenic gene induction. We thus performed glucagon tolerance tests to compare glucagon-stimulated glucose production between *Ncoa6* LKO and *Ncoa6*$^{fl/fl}$ mice. As expected, the glucagon-stimulated increase in the blood glucose level was significantly diminished in *Ncoa6* LKO mice (Fig. 3C).

We next examined the effects of NCOA6 on the autonomous glucose production of hepatocytes induced by forsko-

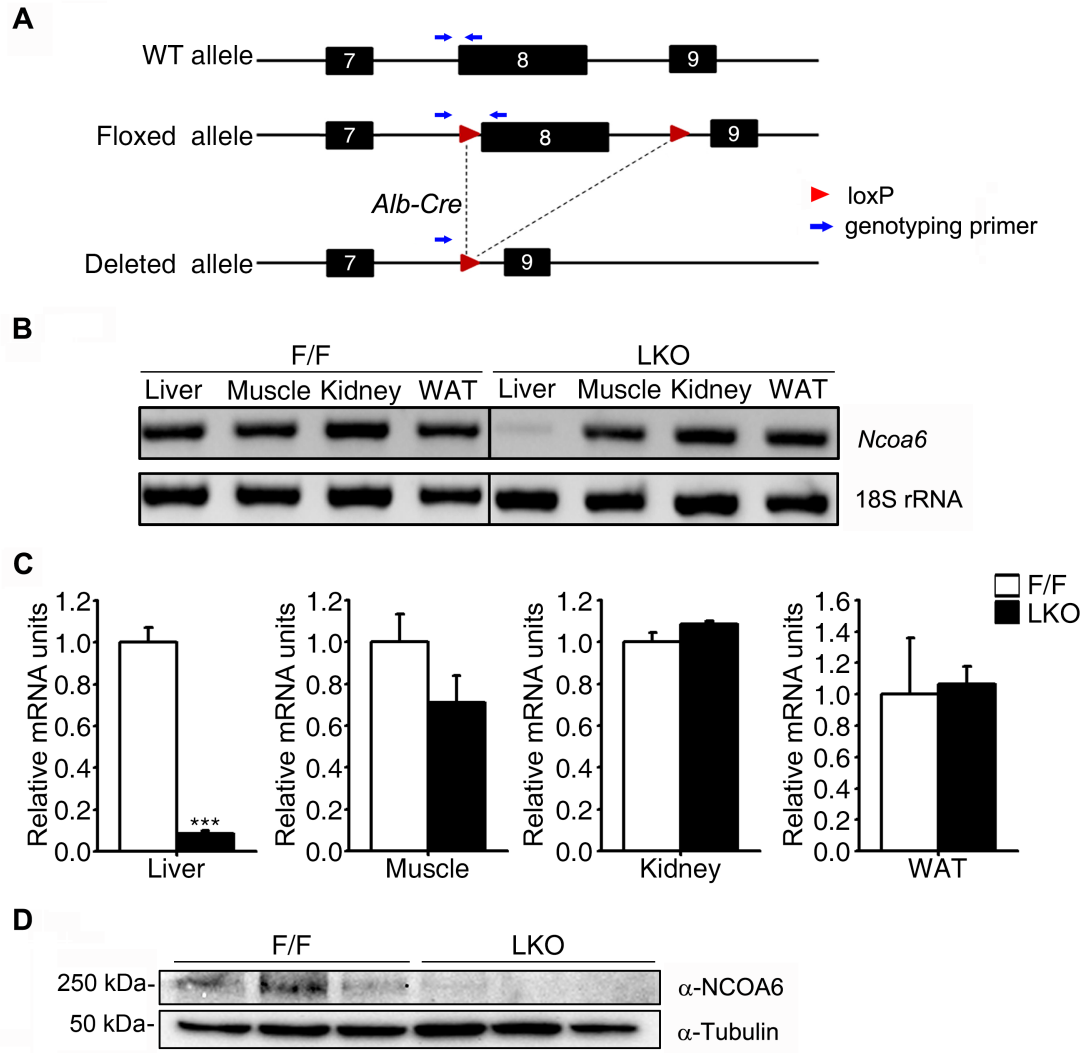

Fig. 1. Establishment of liver-specific *Ncoa6* knockout mice. (A) Schematic representation of the liver-specific knockout strategy used for the *Ncoa6* gene, based on the Cre-LoxP system. Blue arrows denote the specific primer binding sites for genotyping. (B-D) The liver specific knockout of *Ncoa6* was confirmed at the level of genomic DNA (gDNA), mRNA and protein. gDNA and mRNA were isolated from the liver, muscle, kidney and white adipose tissue (WAT) of $Ncoa6^{fl/fl}/Cre^{+/-}$ (LKO) and $Ncoa6^{fl/fl}/Cre^{-/-}$ (F/F) mice. The *Ncoa6* gDNA levels were determined by conventional PCR (B). The *Ncoa6* mRNA and NCOA6 protein levels were determined by qRT-PCR (C; n = 4) and western blotting (D; n = 3), respectively. mRNA data are presented as the mean ± SEM; $***P < 0.001$ by *t*-test comparisons of the genotypes.

lin, a potent activator of adenylate cyclase. Glucagon-receptor complex activates adenylate cyclase to generate second messenger cAMP, which in turn stimulates the PKA-CREB pathway. Thus, forskolin treatments mimic fasting signals to induce gluconeogenesis. In contrast to the normal glucose output of $Ncoa6^{fl/fl}$ hepatocytes, forskolin-stimulated glucose production was abolished in *Ncoa6* LKO hepatocytes (Fig. 3D). In addition, and consistent with the observations in the whole mouse liver, the forskolin-stimulated induction of *Pck1* and *G6pc* mRNA was dramatically decreased in primary hepatocytes isolated from *Ncoa6* LKO mice (Fig. 3E). Taken together, these results suggest that hepatic NCOA6 is required for the transcriptional induction of gluconeogenic enzyme genes, including *Pck1* and *G6pc*, by fasting signals in a cell-autonomous manner.

Gluconeogenesis regulators and CREB phosphorylation in *Ncoa6* LKO mice

In addition to the enzymes that catalyze each step of the gluconeogenesis pathway from pyruvate to glucose, other gluconeogenic regulatory factors were examined for quantitative changes caused by fasting in *Ncoa6* LKO mice in comparison with $Ncoa6^{fl/fl}$ mice. These analyses aimed to identify factors responsible for the gluconeogenic defects of *Ncoa6* LKO mice. We first examined glucagon, which is a peptide hormone produced by pancreatic α-cells and stimulates gluconeogenesis largely through transcriptional regulation. The serum glucagon levels were increased by fasting in both

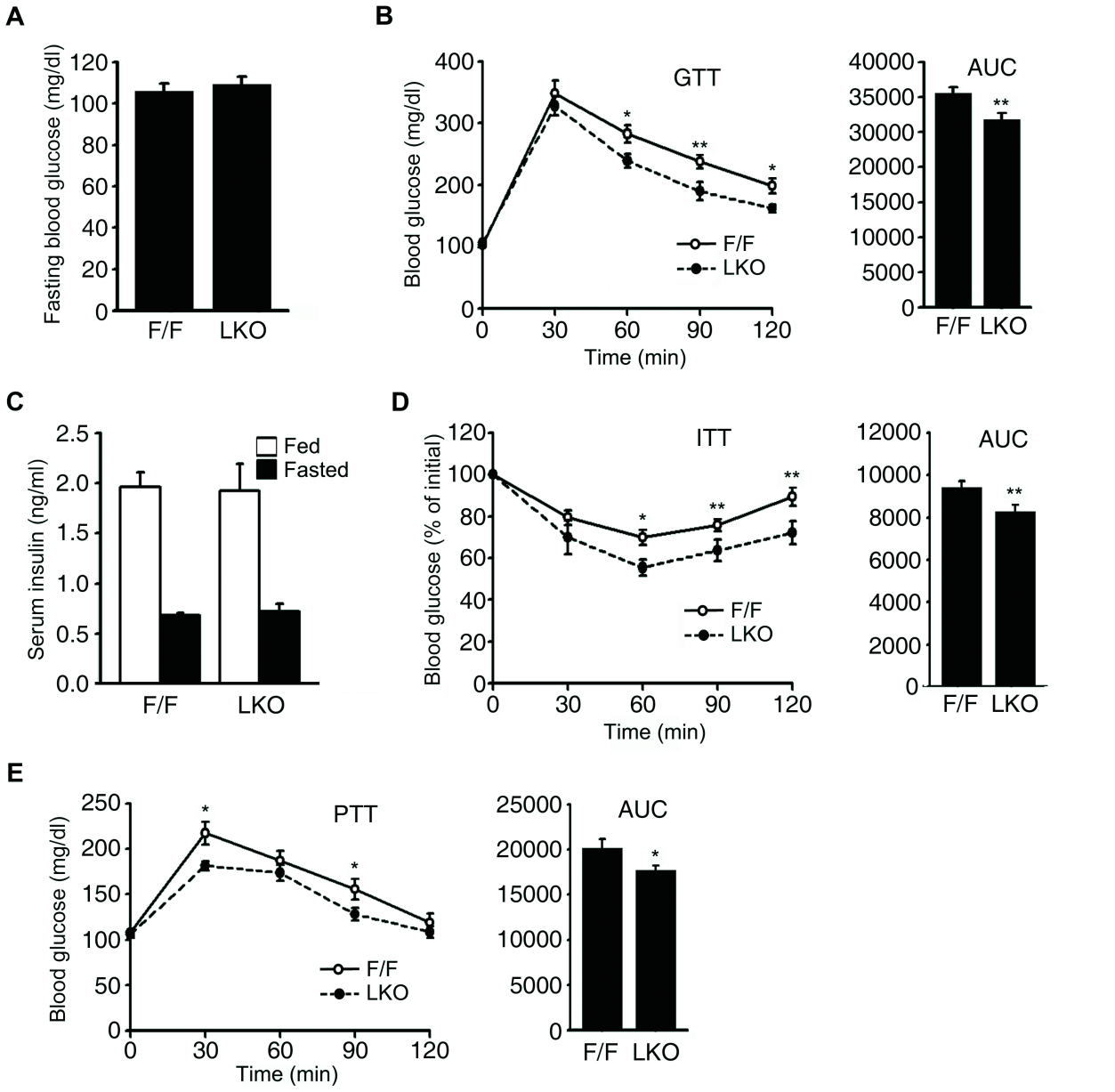

Fig. 2. Glucose metabolism in *Ncoa6* LKO mice. (A) The blood glucose levels were measured in *Ncoa6* LKO (n = 8) and WT (F/F, n = 9) mice after 16 h fasting. (B) Improved glucose tolerance in the *Ncoa6* LKO mice. Intraperitoneal glucose tolerance tests were performed in 10-week-old mice (n = 9 for F/F, n = 8 for LKO) after a 16 h fast. (C) The serum insulin levels were measured in *Ncoa6* LKO (n = 6 for fed, n = 7 for fasted) and WT mice (n = 7 for both fed and fasted) before and after a 16 h fast. (D) Enhanced insulin sensitivity in *Ncoa6* LKO mice. Intraperitoneal insulin tolerance tests were performed in 10-week-old *Ncoa6* LKO (n = 13) and WT mice (n = 10) after a 6 h fast. (E) Pyruvate tolerance was determined in 10-week-old *Ncoa6* LKO (n = 11) and WT (n = 10) mice via an intraperitoneal injection with pyruvate (2 g/kg body weight) after overnight fasting for 16 h. AUC values for GTT, ITT, and PTT were calculated and are presented on the right of each graph. Data are presented as the mean ± SEM; $*P < 0.05$, $**P < 0.01$ by *t*-test comparisons of the genotypes. WT, wild type; GTT, glucose tolerance test; ITT, insulin tolerance test; PTT, pyruvate tolerance test.

Ncoa6$^{fl/fl}$ and *Ncoa6* LKO mice at a comparable level (Fig. 4A). We next determined the mRNA levels of transcriptional regulators involved in glucose production, which included two transcription factors, CREB and forkhead box protein O1 (FOXO1), and a transcriptional coactivator, peroxisome proliferator-activated receptor γ coactivator 1α (PGC-1α). Notably however, *Ncoa6* LKO mice did not show significant differences from *Ncoa6*$^{fl/fl}$ mice in the hepatic transcript levels of *Creb1*, *Foxo1*, or *Pgc1a* under either fed or fasted conditions (Fig. 4B). Additionally we compared the effects of an NCOA6 deficiency on the hepatic transcript levels of CREB- and FOXO1-specific target genes because the *Pck1* and *G6pc*

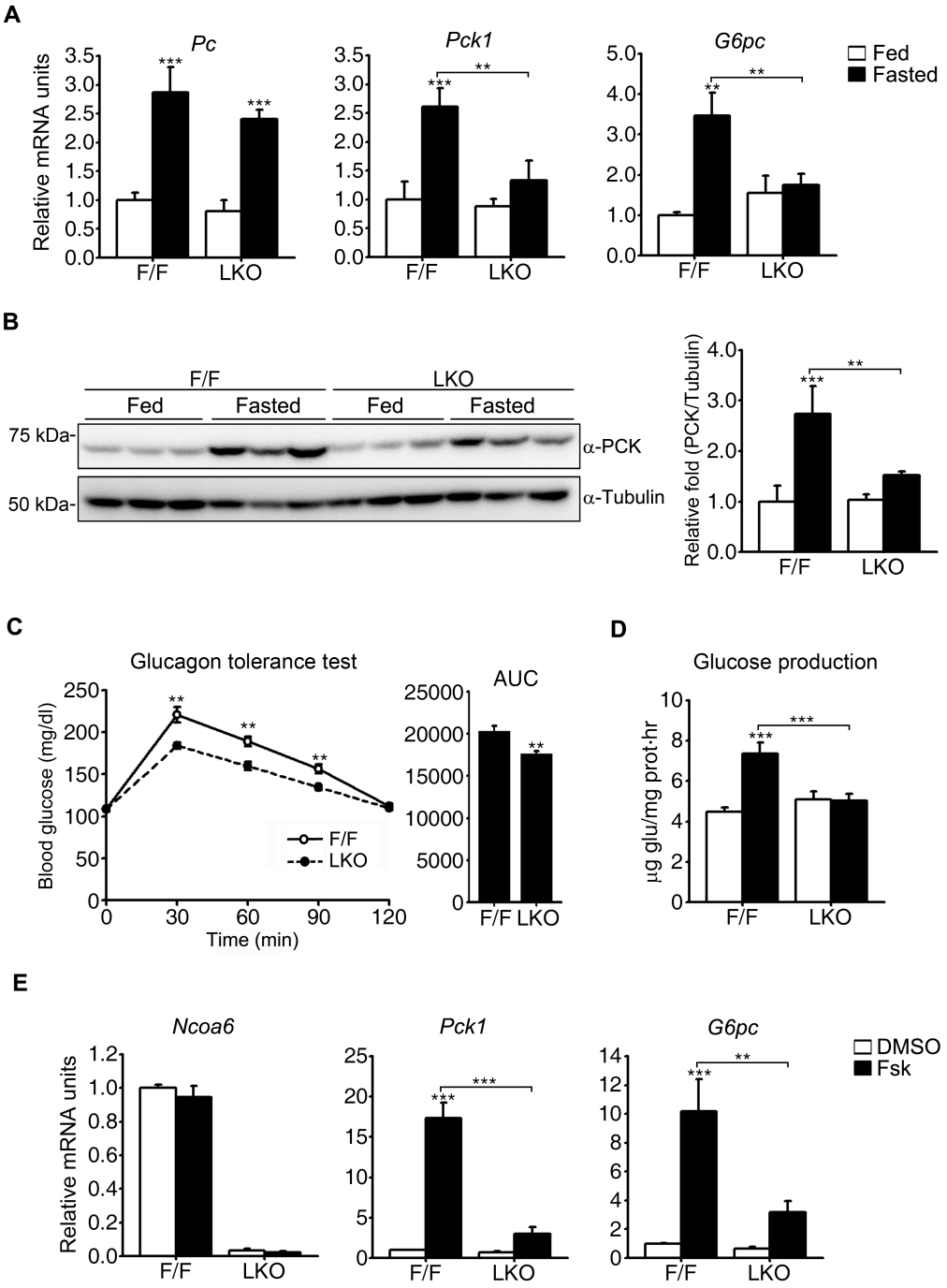

Fig. 3. Alterations in the gluconeogenic enzyme expression levels in *Ncoa6* LKO mice. (A) Decreased induction of gluconeogenic gene transcripts in the *Ncoa6* LKO liver following fasting. Liver RNAs were isolated from *Ncoa6* LKO and WT mice after a 24 h fast (n = 6 for each group). The mRNA levels of *Pc*, *Pck1*, and *G6pc* were determined by real-time qRT-PCR. (B) Attenuated induction of PCK protein in the *Ncoa6* LKO liver after fasting. Protein expression was analyzed in the liver of ad libitum fed mice and 16 h fasted mice (n = 7 for each group) by western blot analysis. Intensities of the protein bands were measured using the ImageJ program. (C) Glucagon tolerance of *Ncoa6* LKO mice (n = 10 for F/F, n = 14 for LKO). Ten-week-old mice were injected intraperitoneally with glucagon (15 μg/kg) after 6 h of fasting. Blood samples were prepared at 0, 30, 60, 90, and 120 min after the glucagon injection. AUC values are shown on the right. (D) Effects of NCOA6 on the glucose production level in primary hepatocytes. Glucose production was compared between *Ncoa6* LKO and WT mouse primary hepatocytes after a 4 h forskolin treatment using glucose free media supplemented with lactate and sodium pyruvate (n = 4 independent experiments). (E) Alteration of *Pck1* and *G6pc* transcript induction in *Ncoa6* LKO primary hepatocytes following a 4 h treatment of these cells with forskolin (n = 5 independent experiments). Transcript levels were analyzed by real-time qRT-PCR. Data are presented as the mean ± SEM; $^{**}P < 0.01$, $^{***}P < 0.001$, determined using one-way ANOVA. Fsk, forskolin.

genes are targeted by both of these transcription factors. By contrast, *Nr4a1* (nuclear receptor subfamily 4 group A member 1) and *Igfbp1* (insulin-like growth factor binding protein 1) are specific target genes of CREB and FOXO1, respectively. However, *Ncoa6* LKO hepatocytes did not exhibit any significant alteration in the transcript levels either *Nr4a1* or *Igfbp1* irrespective of forskolin treatment (Fig. 4C).

CREB phosphorylation is important for the recruitment of coactivators to promoter regions and for the subsequent activation of gluconeogenic gene transcription. We therefore compared the levels of CREB phosphorylation induced by fasting in the *Ncoa6*$^{fl/fl}$ and *Ncoa6* LKO mice. *Ncoa6* LKO mice exhibited fasting-induced phosphorylation of CREB to a similar level as *Ncoa6*$^{fl/fl}$ mice (Fig. 4D). We then examined forskolin-stimulated CREB phosphorylation in primary hepatocytes and found that CREB was phosphorylated by forskolin treatment to a similar degree in both *Ncoa6*$^{fl/fl}$ and *Ncoa6* LKO hepatocytes (Fig. 4E). These results indicated that the alterations of gluconeogenic activity by an NCOA6 deficiency are not due to changes in the levels of serum glucagon or of intracellular gluconeogenic transcriptional factors such as CREB, FOXO1, and PGC-1α.

NCOA6 as a transcriptional coactivator of CREB

Our current results had demonstrated that the transcriptional activation of *Pck1* and *G6pc* by gluconeogenic signaling is impaired in *Ncoa6* LKO mice and in the primary hepatocytes derived from these animals (Fig. 3). Both the *Pck1* and

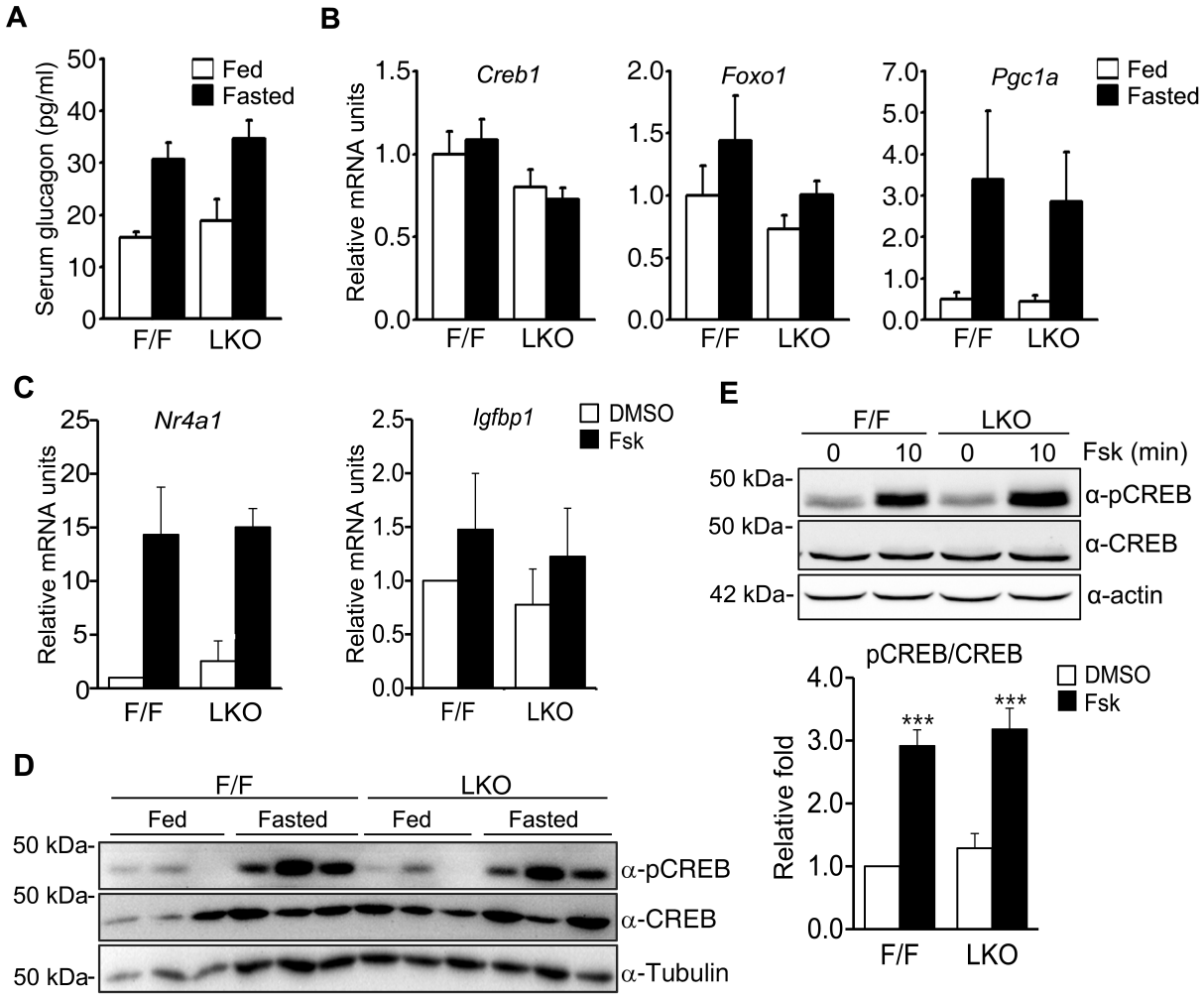

Fig. 4. Gluconeogenic factors and CREB phosphorylation in the *Ncoa6* LKO mouse. (A) Serum glucagon levels in the *Ncoa6* LKO (n = 6 for fed, n = 7 for fasted) and WT mice (n = 7 for both fed and fasted) before and after 16 h of fasting. (B) mRNA levels of gluconeogenic transcriptional factors in the liver of *Ncoa6* LKO and WT mice in a fed or 24 h fasted state (n = 6 for each group). Hepatic mRNAs were analyzed by real-time qRT-PCR. (C) Transcript levels of the CREB target gene *Nr4a1* and FOXO1 target gene *Igfbp1* in the primary hepatocytes of *Ncoa6* LKO (n = 4) and WT mice (n = 3). (D) Induced phosphorylation of CREB by 16 h of fasting in the livers of *Ncoa6* LKO and WT mice (n = 3 for each group). (E) Forskolin-induced phosphorylation of CREB in primary hepatocytes of *Ncoa6* LKO and WT mice. Protein levels were determined by western blot analyses using anti-CREB, anti-pCREB (Ser133) or anti-tubulin antibodies (n = 5 independent experiments). The intensities of the protein bands were measured using the ImageJ program. Data are presented as the mean ± SEM; ***$P < 0.001$, determined using one-way ANOVA. Fsk, forskolin.

G6pc genes are activated by the binding of CREB to CRE in response to glucagon or forskolin. However, there has been no evidence of any interaction between CREB and NCOA6 to date. We here examined the interaction between HA-CREB and FLAG-NCOA6 following the cotransfection of these constructs into 293T cells. CREB and NCOA6 were successfully co-immunoprecipitated in both directions, i.e., using either anti-HA or anti-FLAG antibodies (Fig. 5A). NCOA6 is a bona

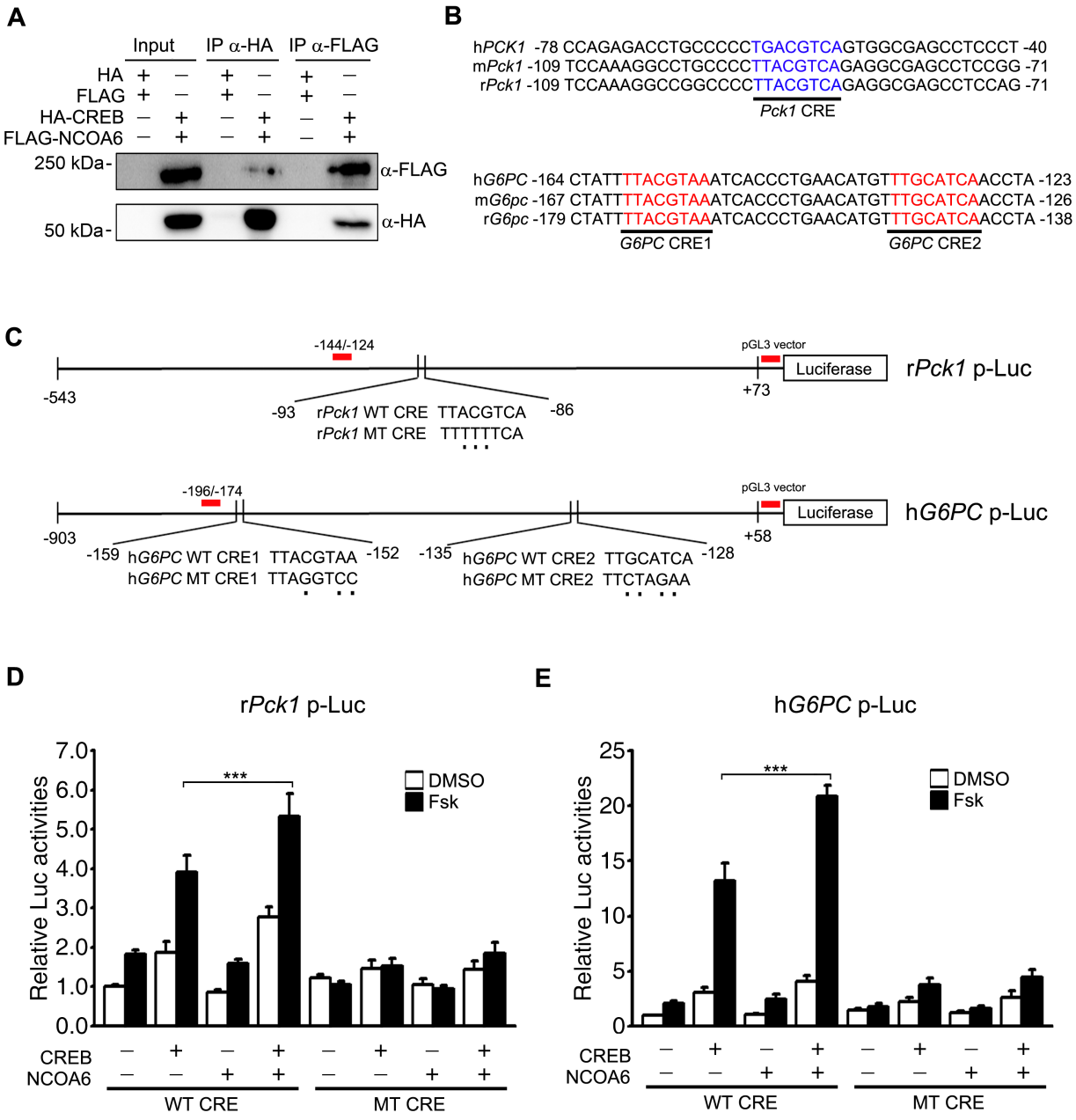

Fig. 5. Transcriptional activation of the *Pck1* and *G6PC* genes by NCOA6. (A) Interactions between CREB and NCOA6. 293T cells were transfected with HA-CREB and FLAG-NCOA6 and lysates were immunoprecipitated from these cells using anti-HA or anti-FLAG antibody. The coimmunoprecipitates were then analyzed by western blotting with anti-HA or anti-FLAG antibody. (B) Sequence alignment of the CRE-containing promoter regions of the *Pck1* or *G6pc* gene among human, mouse, and rat. (C) Schematic diagram of the r*Pck1* or h*G6PC* promoter-Luc reporter containing WT or MT CRE. The red bars indicate the loci of the ChIP primers used in Figs. 6A and 6B. (D and E) Effects of NCOA6 on the CREB-mediated transcriptional activation of the r*Pck1* (D) and h*G6PC* (E) promoter (n = 5 independent experiments). WT or MT CRE-containing *Pck1* or *G6PC* promoter-Luc reporters were cotransfected into HepG2 cells with or without HA-CREB, FLAG-NCOA6 and actin-β-galactosidase. Luciferase activities were then measured with a luminometer and normalized using β-galactosidase activities. Data are presented as the mean ± SEM; ***$P < 0.001$, determined using one-way ANOVA. IP, immunoprecipitation; WT, wild type; MT, mutant; Fsk, forskolin.

fide coactivator of many transcription factors, in addition to nuclear hormone receptors. We thus investigated the coactivator function of NCOA6 in the context of CRE-containing promoters of gluconeogenic biosynthetic genes (Figs. 5B and 5C). The transcriptional activities of CREB were further enhanced at the promoters of both *Pck1* and *G6pc* by NCOA6 (Figs. 5D and 5E). Notably, these additional activation events were abolished by using CRE mutant promoters of *Pck1* and *G6pc* genes (Figs. 5D and 5E). The CRE-dependent transactivation of NCOA6 was also confirmed in the context of a synthetic CRE-Luc reporter (Supplementary Fig. S1). NCOA6 alone had no effect on reporter activity, and a further activation only occurred when it was cotransfected with CREB. We next determined the extent of the recruitment of CREB and NCOA6 to the CRE regions of *Pck1* and *G6pc* genes using a modified ChIP assay and promoter-Luc reporters. Both CREB and NCOA6 were found to be recruited to the CREs of the *Pck1* and *G6pc* gene promoters (Figs. 6A and 6B). Moreover, these recruitments were markedly reduced in the CRE mutants of *Pck1* and *G6pc* promoter-Luc reporters (Figs. 6A

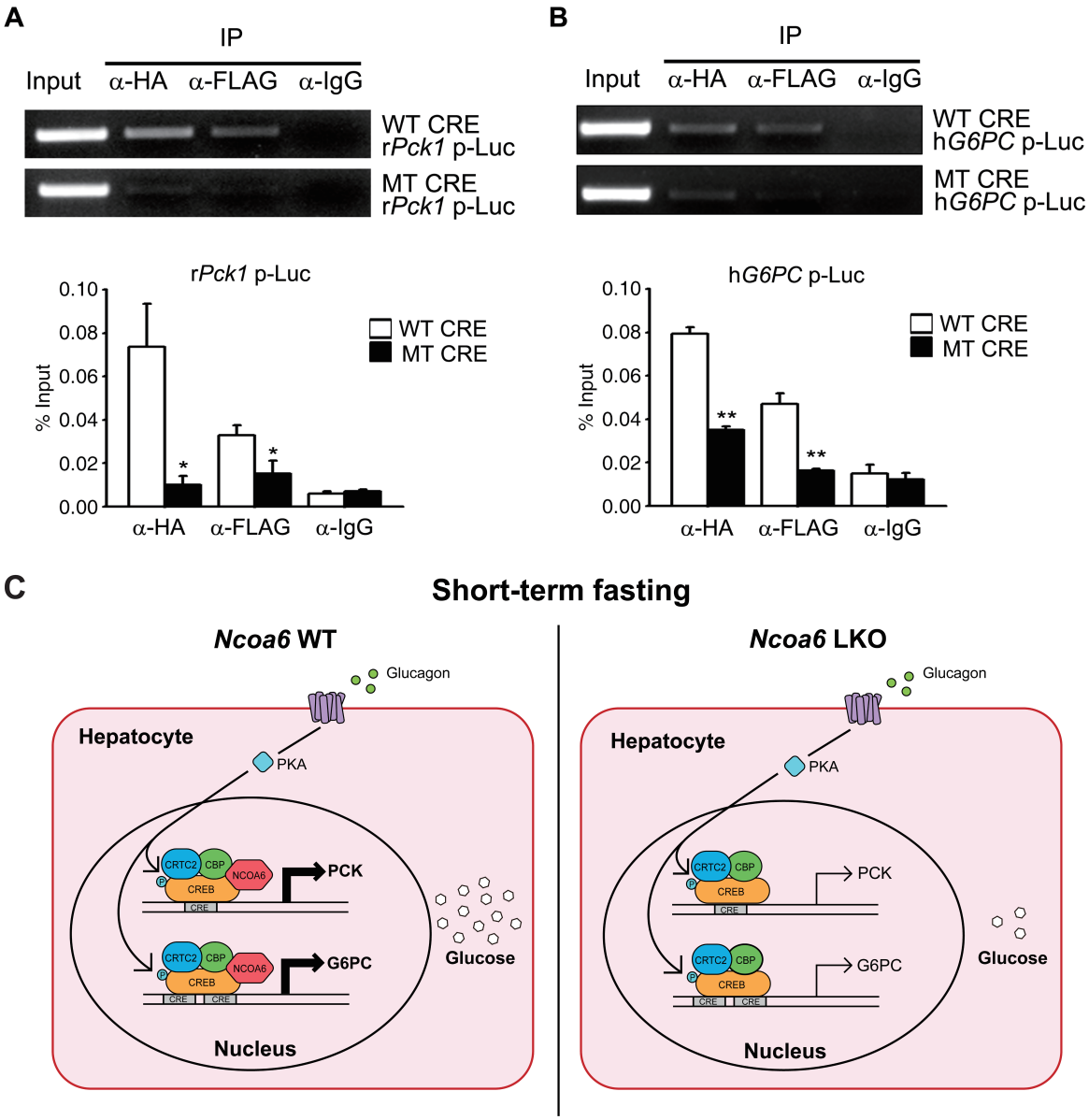

Fig. 6. Recruitment of CREB and NCOA6 to the CRE regions of the *Pck1* and *G6PC* promoter. (A and B) 293T cells were cotransfected with a *Pck1* (A) or *G6PC* (B) promoter-Luc reporter, HA-CREB and FLAG-NCOA6. Modified ChIP assays were then performed using anti-HA, anti-FLAG or IgG antibodies, followed by analysis using conventional PCR (n = 4 independent experiments). The intensities of the PCR bands were measured using the ImageJ program. Data are presented as the mean ± SEM; $*P < 0.05$, $**P < 0.01$, determined by *t*-test. WT, wild type; MT, mutant; IP, immunoprecipitation. (C) Hypothetical model for the gluconeogenic transcription of the *Pck1* and *G6pc* genes mediated via NCOA6.

and 6B). Taken together, these results suggest that NCOA6 regulates gluconeogenesis by enhancing the transcriptional activity of CREB at the *Pck1* and *G6pc* genes (Fig. 6C).

DISCUSSION

The blood glucose levels are maintained within a very narrow range by a complex network of metabolic organs. In simple terms however, this control represents a balance between glucose uptake by peripheral tissues and glucose release by the liver. Hence, hepatic gluconeogenesis is a very important process for regulating glucose homeostasis *in vivo*, especially during prolonged fasting. In addition, gluconeogenesis is aberrantly stimulated in type 2 diabetes and its elevation is associated with high fasting glucose and excessive postprandial hyperglycemia in this disease (Basu et al., 2005; Chevalier et al., 2006; Wajngot et al., 2001). Further elucidating the regulatory mechanisms of gluconeogenesis will therefore be critical not only for a fuller understanding of physiology of glucose homeostasis, but also for developing novel therapeutic agents for type 2 diabetes. In our present study, we demonstrate that the coactivator NCOA6 affects glucose production in the mouse by regulating the transcription of the *Pck1* and *G6pc* genes, both of which express rate-limiting enzymes in the gluconeogenic process. However, as shown in Fig. 2A, the 16 h fasting glucose level in *Ncoa6* LKO mice did not differ from that in their wild type counterparts. The hepatic fasting response maintains glucose homeostasis through a feedforward mechanism that operates in two stages, depending on the duration of fasting (Altarejos and Montminy, 2011; Zhang et al., 2014). During short-term fasting, CREB acts as a major transcription factor for the expression of gluconeogenic enzymes and transcription factors, and then under conditions of prolonged fasting, PGC-1α, NR4A1, and FOXO1 participate in the maintenance of glucose homeostasis. Further to this, our present findings indicated that NCOA6 does not affect the transcription of the gluconeogenic FOXO1 target gene *Igfbp1*, but selectively contributes to the transcription of gluconeogenic CREB target enzyme genes including *Pck1* and *G6pc*. Hence, in a long-term fasting state, it would be expected that the relative contribution of NCOA6 to the entire gluconeogenesis process decreases, and that the differences caused by an NCOA6 deficiency during short-term fasting would be offset by the homeostasis mechanism.

Glucose homeostasis is essentially controlled by two opposing hormones, insulin and glucagon. In our previous study, we demonstrated that NCOA6 inhibits hepatic insulin signaling through the induction of the insulin signaling inhibitors SOCS1 and SOCS3, and hence that hepatic insulin sensitivity is enhanced by a decreased expression of NCOA6 (Kim et al., 2012). Here, we have demonstrated that NCOA6 is required to deliver glucagon signals to induce the transcription of gluconeogenic genes by stimulating CREB activity, and therefore that gluconeogenesis is impaired by an *Ncoa6* knockout in fasting conditions. We speculate from this that the modulation of hepatic NCOA6 activity could potentially repress the increase in the blood glucose level after feeding and block the hyperglycemia that arises due to increased gluconeogenesis under diabetic conditions. NCOA6 could therefore be further investigated as a promising therapeutic target for the treatment of type 2 diabetes.

NCOA6 is known to function as a coactivator not only for nuclear hormone receptors, but also for other transcription factors such as AP-1, NFκB, and SRF. In our present analyses, CREB was found for the first time to be a transcription factor that interacts and cooperates with NCOA6 to control gluconeogenesis. As the expression of both the *G6pc* and *Pck1* genes is regulated by many transcription factors including FOXO1, HNF4α, and glucocorticoid receptor, in addition to CREB, it will be worth investigating the possible association of these other transcription factors with NCOA6 in terms of gluconeogenesis in a future study. FOXO1, HNF4α, and glucocorticoid receptor are all involved in the activation of gluconeogenesis during fasting. Therefore, the activity of other gluconeogenic transcription factors may be impaired in the *Ncoa6* LKO mouse. In this regard, it is noteworthy that the transcriptional activity of HNF4α was found previously to be enhanced by NCOA6 cotransfection in a synthetic promoter reporter assay using HeLa cells (Yeom et al., 2006).

CREB is well known to be modified posttranslationally in the course of the gluconeogenic program. First, CREB is phosphorylated at Ser133 through the cAMP-PKA-CREB pathway by a fasting signal. CREB binding protein (CBP), one of the known coactivators of CREB, is then recruited to the phosphorylated CREB and acetylates Lys91, Lys94, and Lys136 within the CREB activation domain. The resulting doubly modified phospho (Ser133)-acetyl (Lys136) CREB protein then further potentiates CBP recruitment to it (Paz et al., 2014). In this context, it will be very interesting in a future study to determine the point at which NCOA6 participates in the CREB activation process and how these known modifications (phosphorylation and acetylation) of CREB affect NCOA6 recruitment to it. It would then be possible to obtain information about the crosstalk among CREB, CBP, and NCOA6 by investigating the binding of specific domains and particular modification sites of CREB with NCOA6. NCOA6 recruitment to *Pck1* and *G6pc* promoters would be enhanced under the CREB modification conditions in which its binding to NCOA6 is stronger. Conversely, CREB-NCOA6 binding may affect the recruitment of other coactivators including CBP.

Interestingly, the *Pc* gene, which encodes the enzyme that catalyzes the first committed step of the gluconeogenesis pathway, showed fasting-induced increases in its mRNA level in the *Ncoa6* LKO liver, in contrast to the *Pck1* and *G6pc* genes. PC is a mitochondrial enzyme that performs anaplerotic carboxylation from pyruvate to oxaloacetate. On the other hand, both PCK1 and G6PC are cytosolic enzymes that determine the overall rate of the gluconeogenic process. There are two isoforms of the PCK enzyme, cytosolic PCK1 (PEPCK-C) and mitochondrial PCK2 (PEPCK-M), which are encoded by two different nuclear genes (Nordlie and Lardy, 1963). While PCK1 expression can be strongly stimulated by glucagon and inhibited by insulin, PCK2 is constitutively expressed without hormonal control (Hanson and Patel, 1994; Modaressi et al., 1998). Thus, PCK2 expression would not be altered during the transient increase of glucose production under fasting conditions. In conclusion therefore, NCOA6 appears to take part selectively in the transcription of cytosolic gluconeogenic

genes, but not in the expression of mitochondrial enzymes under fasting conditions. Further studies are required to elucidate the differential regulation of these cytosolic and mitochondrial enzymes, even though they are all involved in the gluconeogenesis pathway.

The regulatory regions of the *Pc*, *Pck1*, and *G6pc* genes all have CREs. Jitrapakdee et al. (1997; 2001) demonstrated that the rat *Pc* gene has two promoter regions, i.e., a proximal and distal promoter. The proximal promoter (P1) of rat *Pc* is active in the liver and adipose tissue, while the distal promoter (P2) is active in the pancreatic islets. The CRE sequence is present in the proximal promoter region of the rat *Pc* gene. In addition, CREB reportedly binds to the -1639/-1631 CRE of the mouse *Pc* gene and transactivates the CRE-containing 1.95 kb 5' flanking sequence of mouse *Pc* (Thonpho et al., 2010). As mentioned above, the *Pc*, *Pck1*, and *G6pc* genes are all activated by CREB. However, NCOA6 is involved in the fasting-induced transcription of *Pck1* and *G6pc* selectively, but not in the transcription of the *Pc* gene, even though NCOA6 and CREB were shown in our present study to interact with each other using IP experiments with transfected 293T cells. This suggests that the interaction between CREB and NCOA6 is not the only condition necessary for the expression of the *Pck1* and *G6pc* genes by NCOA6. The characteristics of the CRE-adjacent sequences of the *Pc* gene could affect the recruitment of relevant factors to the promoter region. The interactions between CREB and other transcription factors may also alter the binding and action of NCOA6. Therefore, it is reasonable to assume that NCOA6 does not always act as a major coactivator for CREB, that is, NCOA6 is not always involved in the transcription of all CREB target genes. Nevertheless, our present findings have revealed the significance of the interaction between NCOA6 and CREB in gluconeogenesis. To further our understanding of the transcriptional effects of NCOA6 on overall gluconeogenic regulation, it would be helpful in the future to analyze the gene profile when NCOA6 is recruited after a fasting signal, and also to analyze changes in the hepatic transcript levels under conditions of an *Ncoa6* KO or overexpression.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

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AUTHOR CONTRIBUTIONS

S.W.K. conceived the experiments, wrote the manuscript, and secured funding. G.S.O. and S.R.K. wrote the initial draft of the manuscript and analyzed the data. G.S.O., S.R.K., E.S.L., J.Y., M.K.S., H.K.R., and D.S.K. performed the experiments.

CONFLICT OF INTEREST

The authors have no potential conflicts of interest to disclose.

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