FOXO1 is an important downstream mediator of the insulin signaling pathway. In the fed state, elevated insulin phosphorylates FOXO1 via AKT, leading to its nuclear exclusion and degradation. A reduction in nuclear FOXO1 levels then leads to suppression of hepatic glucose production. However, the mechanism leading to expression of Foxo1 gene in the fasted state is less clear. We found that Foxo1 mRNA and FOXO1 protein levels of Foxo1 were increased significantly in the liver of mice after 16 h of fasting. Furthermore, dibutyryl cAMP stimulated the expression of Foxo1 at both mRNA and protein level in hepatocytes. Because cAMP-PKA regulates hepatic glucose production through cAMP-response element-binding protein co-activators, we depleted these co-activators using adeno viral shRNAs. Interestingly, only depletion of co-activator P300 resulted in the decrease of Foxo1 mRNA and FOXO1 protein levels. In addition, inhibition of histone acetyltransferase activity of P300 significantly decreased hepatic Foxo1 mRNA and FOXO1 protein levels in fasted mice, as well as fasting blood glucose levels. By characterization of Foxo1 gene promoter, P300 regulates the Foxo1 gene expression through the binding to tandem CAMP-response element sites in the proximal promoter region of Foxo1 gene.

The maintenance of blood glucose levels within a defined range (70–110 mg/dl, fasting) is critical in protecting organs against hypoglycemia during fasting and hyperglycemia during feeding given that glucose is a major energy source for mammalian cells and is the sole energy source in some mammalian tissues and cells such as neurons and erythrocytes. Hypoglycemia induces damage to tissues and cells and can even cause cell death (1, 2). However, hyperglycemia also causes serious adverse effects to mammals through nonenzymatic glycosylation of many cellular proteins; these adverse effects are evident in patients with diabetes mellitus who suffer from microvascular damage to the kidney, retina, and nerves (3, 4). In response to elevated blood glucose levels in the fed state, pancreatic β-cells secrete insulin, which suppresses glucose production in the liver and stimulates glucose uptake in muscle and adipose tissue. In the fasted state, glucagon, epinephrine, and glucocorticoids increase hepatic glucose production directly or indirectly through the cAMP-PKA signaling pathway to maintain euglycemia (5).

FOXO proteins include FOXO1, 3, 4, and 6 and are members of the Fox superfamily (6). Mounting evidence suggests that FOXO proteins play a critical role in metabolism and energy homeostasis. For example, FOXO members up-regulate gluconeogenesis through the activation of Pck1 and G6pc gene expression in the liver (7–9), activate pancreatic β-cell function (10), and promote differentiation of adipocytes (11). FOXO1 is particularly important in inhibition of hepatic gluconeogenesis by insulin; insulin inhibits FOXO1 activity through the PI3K/AKT signaling pathway (12, 13). Another layer of FOXO1 regulation is via acetylation of the cAMP-response element-binding protein (CREB) co-activators P300 and CBP (14–17).

Phosphorylation of FOXO1 by insulin leads to its nuclear exclusion and degradation in the fed state (12, 13), yet the mechanism driving Foxo1 expression in the fasted state remains unclear. Glucagon activates the cAMP-PKA signaling pathway, and phosphorylation of CREB at Ser-133 by PKA, in turn, recruits the CREB co-activators CBP, P300, and CRTC2 to CRE-containing genes and activates hepatic gluconeogenesis (18, 19). However, we have reported previously that CBP phosphorylation at Ser-436 by insulin in the fed state triggers the disassembly of the CREB-CBP-CRTC2 complex (18) and inhibits hepatic glucose production. Furthermore, phosphorylation of CRTC2 at Ser-171 by insulin leads to its nuclear exclusion and degradation (19). Considering these studies, we wanted to test the hypothesis that elevated fasting glucagon levels increase
**EXPERIMENTAL PROCEDURES**

**Plasmids and Adenoviruses**—The expression vectors for P300 and PKA used here were described previously (20). Mouse Foxo1 promoter–luciferase reporters were constructed by cloning the promoter of Foxo1 (up to −2000 to +1) into the pGL4 luciferase reporter construct. The BLOCK-iT adenoviral RNAi expression system (Invitrogen) was used to construct adenoviral shRNA for CBP, P300, CREB, and scrambled shRNA as we described previously (18).

**Cell Cultures**—Equal amounts of plasmids were transfected using Lipofectamine 2000 (Invitrogen) or adenoviral shRNAs into mouse hepatoma Hepa1–6 cells. After 48 h of incubation, cells were exposed to 0.2 mM dibutyryl cAMP for 5 h, 20 μM P300-specific histone acetyltransferase (HAT) inhibitor C646 or its inactive C37 analog (21). The C37 analog differs from P300-specific histone acetyltransferase (HAT) inhibitor C646 by only one double bond but is completely silent as a P300 inhibitor, serving to control for off-target effects of C646 (22).

**Glucose Production Assays**—Mouse primary hepatocytes were cultured in 6-well plates with William’s medium E supplemented with ITS (BD Biosciences) and 27.5 nm dexamethasone. 18 h after the planting, primary hepatocytes were treated with 20 μM C37 or C646 for 3 h during serum starvation. Then, medium was replaced with 1 ml of glucose production buffer consisting of glucose-free DMEM supplemented with 20 mM sodium lactate and 2 mM sodium pyruvate or with 0.2 mM 8-bromo-cAMP and 20 μM C37 or C646 chemicals, and incubated for another 3 h.

**Animal Experiments**—All animal protocols were approved by the Institutional Animal Care and Use Committee of the Johns Hopkins University. C57BL/6 mice were purchased from The Jackson Laboratory, and 10-week-old mice were used. Mice were given C37 or C646 (30 nmol/g) through intraperitoneal injection and then subjected to fasting. Mice were sacrificed after an 8-h fast. In adenoviral shRNA knockdown experiments, 48 h after mice were injected with the adenovirus through tail vein, mice were subjected to an 16-h fast before being sacrificed (23).

**RESULTS**

**Co-activator P300 Regulates Foxo1 Gene Expression**

**Immunoblotting, Real-time qPCR, and Chromatin Immunoprecipitation**—Immunoblotting was conducted as described previously (18, 20). Cellular lysates were sonicated for 15 s three times sequentially and immunoblotted to examine the target proteins with antibodies against CBP, P300 (Santa Cruz Biotechnology), CREB, pCREB, and FOXO1 (Cell Signaling) at concentrations recommended by the manufacturers. Secondary antibodies were used at the concentrations of approximately 1:5000. The primers used for the measurement of mouse Foxo1 mRNA in real-time qPCR were as follows: 5′-primer (5′-ACATTTCTGCTCTCGAACCAGCTCA-3′) and 3′-primer (5′-ATTTCAGACAGACTGGGCAGCGTA-3′). The chromatin immunoprecipitation assay was performed as we described previously (20); eluted DNA was amplified by real-time PCR with a 5′-primer (5′-TACCCCAACCCGCCCCACCAA-3′) and a 3′-primer (5′-GACTGACGGCTGCGGCGCCA-3′) specific for the CRE-containing regions in the mouse Foxo1 promoter. To examine the specific binding in ChIP assay, eluted DNA was also amplified with primers targeting Gapdh promoter, 5′-primer (5′-ACCTCAACTATGTTGTTACATGTT-3′) and 3′-primer (5′-CAAAAATGGGGGATCATCGGCGGAGAA-3′).

**Statistical Analyses**—Statistical significance was calculated with Student’s t test and analysis of variance. Significance was accepted at the level of p < 0.05.

**Fasting Resulted in a Marked Increase of Hepatic Foxo1 mRNA and FOXO1 Protein Levels**—Because phosphorylation of FOXO1 by activated AKT in the insulin signaling pathway results in FOXO1 nuclear exclusion and degradation in the fed state (12, 13), it is conceivable that fasting might increase FOXO1 levels due to its important role in stimulating gluconeogenesis and maintaining euglycemia (7–9). Indeed, hepatic Foxo1 mRNA levels increase approximately 3-fold in the liver of mice sacrificed at fed or fasted (16 h) states (n = 5). Real-time qPCR was used to measure gene expression (normalized to 36B4 expression levels). Asterisk (*) signifies that groups with same treatment are significantly different (p < 0.05). Error bars, S.E.

**FIGURE 1. Fasting induces Foxo1 gene expression.** a, Foxo1 mRNA levels compared in the liver of mice sacrificed at fed or fasted (16 h) states (n = 5). Real-time qPCR was used to measure gene expression (normalized to 36B4 expression levels). Asterisk (*) signifies that groups with same treatment are significantly different (p < 0.05). Error bars, S.E. b, phosphorylation status of CREB, AKT and total AKT, CREB, and FOXO1 protein levels in the liver from fed and 16 h fasted mice are shown (n = 5). c, fasting led to the early induction of Foxo1 expression. The Foxo1 protein levels in the liver of mice sacrificed are shown at the indicated fasting time points. Each lane represents sample pooled from two mice.
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Dibutyrl cAMP (Bt-cAMP) Stimulated the Foxo1 Gene Expression in Hepatocytes—To test whether the activation of cAMP-PKA pathway is able to increase the expression of the Foxo1 gene, we treated hepatoma Hepa1–6 cells with Bt-cAMP, a nonhydrolyzable cAMP analog that activates the cAMP-PKA signaling pathway. As shown in Fig. 2, Bt-cAMP treatment led to increased FOXO1 protein (Fig. 2a) as well as Foxo1 mRNA levels (Fig. 2b). Furthermore, the inhibition of Foxo1 transcription by actinomycin D blocked induction of FOXO1 protein levels by cAMP (Fig. 2c). These data suggest that the cAMP-PKA pathway activates Foxo1 gene expression and that the activation of the gluconeogenic enzyme profile by glucagon stimulation during fasting is mediating at least in part by an increase in Foxo1 gene expression.

CREB Co-activator P300 Regulates Foxo1 Gene Expression—Having seen that Bt-cAMP treatment increased Foxo1 gene expression (Fig. 2), we investigated the potential involvement of the CREB co-activators CBP, P300, and CRTC2 in regulating Foxo1 gene expression. We employed adenoviral shRNAs to deplete these co-activators individually in Hepa1–6 cells. Depletion of CBP and CRTC2 did not change the FOXO1 protein levels compared with cells treated with scrambled shRNA adenovirus (Fig. 3, a and b). In contrast, depletion of P300 decreased FOXO1 protein levels.

We examined further the role of CBP and P300 in the regulation of FOXO1 expression in primary hepatocytes. As shown in Fig. 3c, cAMP treatment increased FOXO1 protein levels in hepatocytes treated with scrambled shRNA adenovirus. Depletion of P300 by adenoviral shRNA blocked the induction of FOXO1 by Bt-cAMP. In comparison, depletion of CBP did not affect the overall protein levels of FOXO1. Moreover, depletion of P300 and CREB also blocked the induction of Foxo1 mRNA by Bt-cAMP. Again, depletion of CBP did not significantly affect Foxo1 mRNA levels in Hepa1–6 cells (Fig. 3d). The above data suggest that cAMP-induced Foxo1 gene expression is mediated by CREB and P300.

Given the importance of CBP and P300 in regulation of gluconeogenic gene expression in the liver, we next assessed the role of CBP and P300 in regulation of Foxo1 gene expression in vivo. We used adenoviral shRNAs to deplete CBP or P300 in the liver of mice through tail vein injection. Depletion of P300 significantly decreased Foxo1 mRNA levels in the liver of fasted mice (Fig. 4a), whereas the depletion of CBP had a minimal effect on Foxo1 mRNA levels (Fig. 4b).

The Foxo1 Promoter Contains CREs—To characterize the Foxo1 promoter, a series of mouse Foxo1 promoter luciferase constructs were made and tested in Hepa1–6 cells. Fig. 5a is an illustration of the construct used and the 5′-end point. Reporter activity is relative to the −2000-bp construct, which was assigned a value of 1 in the absence of co-transfected PKA (white bars). PKA co-transfection increased promoter activity of all constructs, suggesting that a CRE may be located in the proximal 125 bp of the promoter. After analysis of the −125 bp region of the Foxo1 proximal promoter using the TransFac Database, three putative CREs were identified as shown and labeled CRE1, 2, and 3 (Fig. 5b). To define the role of these CREs on the Foxo1 gene in cAMP-PKA induction, these CREs were individually deleted in the context of a −125-bp promoter construct and tested again in Hepa1–6 cells. Deletion of either CRE1 or CRE2 significantly reduced PKA-stimulated reporter activity (Fig. 5c), whereas deletion of CRE3 had no effect on the promoter activity. By using these constructs, we assessed further their effect on reporter activity after co-transfection with P300 and PKA expression plasmids in Hepa1–6 cells. In the wild type construct, P300 co-transfection markedly increased promoter activity, whereas deletion of either CRE1 or CRE2 in the reporter construct negated the P300 effect (Fig. 5d). Deletion of CRE3 had no effect. Because P300 is known to interact with CREB, these data suggest that P300 may interact with CREB and bind to CRE1 and CRE2 of the Foxo1 promoter.

Recruitment of P300 to the Foxo1 Promoter after PKA Stimulation—To investigate whether recruitment of CREB and P300 to CREs was induced by PKA stimulation, we conducted a ChIP assay. Hepa1–6 cells were transfected with control or PKA-containing plasmids to assess the occupancy of CREB and P300 on Foxo1 and a negative control Gapdh promoter. Fig. 6a shows the quantitative PCR for Foxo1 or the negative control Gapdh promoter of either CREB- or P300-immunoprecipitated chromatin. PKA transfection markedly increased the occupancy of both CREB and P300 on the Foxo1 promoter but not on the negative control Gapdh promoter. These data correlate with the functional data obtained in the same mouse hepatocyte cultures as shown in Fig. 5. To probe further whether PKA stimulated the occupancy of P300 on the proximal Foxo1 promoter, we overexpressed HA-tagged P300 together with PKA and used a HA tag-specific antibody to immunoprecipitate the cross-linked Foxo1 promoter in a ChIP assay. The binding of HA-tagged P300 increased >6-fold in the presence of PKA stimulation (Fig. 6b).
HAT Activity of P300 Is Important for Regulating Foxo1 Gene Expression—P300 contains intrinsic HAT activity, and histone acetylation by this co-activator leads to the chromatin remodeling and an increase in gene transcription (24). To test whether P300 HAT activity has a role in regulating Foxo1 gene expression, adenoviral shRNA was employed to deplete P300 in Hepa1–6 cells. After washing with PBS, 0.2 mM Bt-cAMP was added in glucose production medium. In Hepa1–6 cells, 48 h after the addition of adenoviral shRNAs of scrambled, CREB, CBP, and P300, growth medium was changed to FBS-free DMEM and incubated for 3 h, then 1 mM Bt-cAMP was added and incubated for 5 h before harvesting. Real-time qPCR was used to measure gene expression ($n$ = 3), and data were normalized to 36B4 expression levels. Each lane represents sample pooled from three treatments. Asterisk (*) signifies that groups with the same treatment are significantly different ($p < 0.05$). Error bars, S.D.
Identification of the functional regions in Foxo1 gene promoter. a, schematic shows reporter constructs tested. Nucleotides are numbered relative to the transcription start site (bp +1). 20 ng of reporter constructs was transfected into Hepa1–6 cells together with 400 ng of control RSV-cat or PKA expression plasmids. Reporter activities were measured 48 h after the transfection. b, three CRE site were identified in the proximal promoter region of the Foxo1 gene. c, three CREs were individually deleted in the context of the proximal promoter construct (−125 bp) and tested again. Hepa1–6 cells were transfected with 20 ng of each construct together with 200 ng of control RSV-cat or PKA expression plasmids. d, 20 ng of the proximal promoter construct and its mutants and 200 ng of PKA expression plasmid were co-transfected into Hepa1–6 cells together with 400 ng of control pcDNA or P300 expression vector. Reporter activities were measured 48 h after the transfection. Asterisk (*) signifies that groups with same treatment are significantly different (p < 0.05). Error bars, S.D.

P300 binds to the proximal promoter region of the Foxo1 gene. a, PKA stimulated the binding of CREB and p300 to CREs in the proximal promoter of the Foxo1 gene. 48 h after the transfection of PKA expression plasmid, Hepa1–6 cells were fixed with formaldehyde followed by immunoprecipitation with CREB and P300 antibodies. The DNA in the immunoprecipitates was amplified using primers encompassing 150 bp including the CREs in the promoter of the mouse Foxo1 gene (n = 3). b, Hepa1–6 cells were transfected with PKA or together with HA-tagged P300 expression plasmid, followed by the fixation and immunoprecipitation using anti-HA tag-specific antibody. The binding of HA-tagged P300 was determined by real-time PCR (n = 3). Asterisk (*) signifies that groups with same treatment are significantly different (p < 0.05). Error bars, S.D.
Co-activator P300 Regulates Foxo1 Gene Expression

**Hepa1–6 cells.** Then, cells were treated with Bt-cAMP and/or the HAT inhibitor C646, which is a P300 HAT-specific inhibitor (21, 22). C646 treatment decreased FOXO1 protein levels, demonstrating that the HAT activity of P300 is indeed important in Foxo1 gene expression (Fig. 7a).

Moreover, P300 is also important in regulating basal expression of the Foxo1 gene as shown in the shRNA knockdown of P300. We next sought to determine the mechanism of HAT inhibition on Foxo1 gene expression. Using Foxo1 luciferase reporters, we show that the HAT inhibitor C646 significantly decreased PKA-stimulated reporter activity, whereas a CRE mutant construct was not affected (Fig. 7b). A control compound C37 without HAT inhibitory properties was without effect. Moreover, treatment with C646 markedly decreased both basal and Bt-cAMP-stimulated glucose production in primary hepatocyte, whereas the Bt-cAMP-stimulated glucose production was intact after treatment with the control compound C37 (Fig. 7c). Finally, mice administrated with C646 through intraperitoneal injection exhibited significantly lower fasting blood glucose levels (Fig. 7d) accompanied by decreased levels of Foxo1 mRNA and FOXO1 protein levels (Fig. 7, e and f).

**DISCUSSION**

Blood glucose levels are finely regulated by the opposing actions of the insulin and glucagon signaling pathways. In the fed and postprandial states, the uptake of glucose from the gastrointestinal tract causes elevation in blood glucose levels, which in turn, triggers the secretion of insulin from pancreatic β-cells. Insulin stimulates glucose utilization in the muscle and adipose tissue and suppresses endogenous glucose production in the liver, so that glucose eventually returns to baseline levels. The molecular mechanism for the suppression of hepatic glucose production by insulin is complicated and likely involves a number of transcriptional factors and co-activators, and several mechanisms have been reported. One mechanism involves phosphorylation of FOXO1 by insulin through PI3K-AKT, which excludes FOXO1 from nucleus and promotes cytoplasmic ubiquitinylation and degradation (25, 26). Another mechanism suggests that CRTC2 may undergo a similar exclusion from the nucleus after phosphorylation of Ser-171 by the insulin (19). Our laboratory has proposed a third mechanism to ensure a sufficient control of hepatic gluconeogenesis, where insulin regulates the composition of CREB co-activator co-

![Figure 7. P300 binds to the proximal promoter region of the Foxo1 gene.](image-url)
plex. Phosphorylation of CBP at Ser-436 by insulin leads to the disassembly of the CREB-CBP-CRTC2 complex (18).

In the fasting state, glucagon activates phosphorylation of CREB at Ser-133 through a cAMP-PKA pathway, which leads to recruitment of the co-activators CBP, P300, and CRTC2 to CRE-containing genes such as Pck1 and G6pc. The increased expression of gluconeogenic genes is thought to be a major way in which glucagon increases hepatic gluconeogenesis (18, 19); and accordingly, FOXO1 induces Pck1 and G6pc gene expression (8, 9). In light of this information, we reasoned that glucagon might increase Foxo1 gene expression in the fasted state through CREB co-activators. In this way, FOXO1 would further enhance gluconeogenesis. We found that depletion of CREB and P300 abolished the induction of Foxo1 mRNA by cAMP (Fig. 3d); however, depletion of CBP and CRTC2 had no effect on Foxo1 mRNA and FOXO1 protein induction by cAMP (Fig. 3, a–d). These data suggest that CREB-P300 mediates the Foxo1 induction, revealing a unique role for P300 in regulating Foxo1 gene expression (Fig. 8). This notion was further substantiated by data obtained after depletion of hepatic CBP and P300 in the fasted mice, in which only the depletion of p300 resulted in a significant decrease in Foxo1 mRNA (Fig. 4). P300 has HAT activity, and in accordance with a previous report, the inhibition of HAT activity of CBP and/or P300 significantly decreased fasting blood glucose levels (Fig. 7c) (8). These effects might be mediated, in part, through the suppression of Foxo1 gene expression, because P300 is known to regulate Foxo1 gene expression (14–16). Indeed, administration of a P300 HAT-specific inhibitor C646 decreased reporter activity (Fig. 7b) and lowered the fasting blood glucose levels together with decreasing Foxo1 mRNA and FOXO1 protein levels (Fig. 7, e and f). Although we report here that the HAT activity of P300 is important for the transcription of Foxo1 gene, the specific acetylation target may involve lysines on histones or other proteins. For example, the HAT activity of P300 might acetylate FOXO1 protein and affect its protein stability because deacetylation of FOXO by sirtuin 1 reduces levels of mammalian FOXO transcriptional factors (27–29). Of note, some investigators suggest that the acetylation of FOXO1 facilitates FOXO1 phosphorylation by insulin, which should reduce FOXO1 levels by promoting its nuclear exclusion (17, 30).

In summary, we demonstrate that the P300 specifically activates Foxo1 gene expression at the transcriptional level. Previous reports have shown that insulin signaling terminates nuclear FOXO1 action by phosphorylating the protein, which promotes its nuclear exclusion and degradation. This is the first report to our knowledge, however, to show that co-activator P300 mediates a large increase in FOXO1 protein levels during fasting state and suggests that FOXO1 might have a much greater role in regulating gluconeogenesis in the fasting state.

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