O-GlcNAc Transferase/Host Cell Factor C1 Complex Regulates Gluconeogenesis by Modulating PGC-1α Stability

Hai-Bin Ruan,1,2 Xuemei Han,6 Min-Dian Li,1,2,3 Jay Prakash Singh,1,2 Kevin Qian,1,2,3 Sascha Azarhoush,1,2,7 Lin Zhao,1,2,8 Anton M. Bennett,1,2,4 Varman T. Samuel,5 Jing Wu,1,2,8 John R. Yates III,6 and Xiaoyong Yang1,2,3,*

1Program in Integrative Cell Signaling and Neurobiology of Metabolism
2Section of Comparative Medicine
3Department of Cellular and Molecular Physiology
4Department of Pharmacology
5Department of Internal Medicine

Yale University School of Medicine, 333 Cedar Street, New Haven, CT 06519, USA
6Department of Chemical Physiology, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, CA 92037, USA
7School of Medicine, University of Cologne, Cologne 50931, Germany
8School of Life Science and Technology, Xi’an Jiaotong University, Xi’an, Shaanxi 710049, China

*Correspondence: xiaoyong.yang@yale.edu

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SUMMARY

A major cause of hyperglycemia in diabetic patients is inappropriate hepatic gluconeogenesis. PGC-1α is a master regulator of gluconeogenesis, and its activity is controlled by various posttranslational modifications. A small portion of glucose metabolizes through the hexosamine biosynthetic pathway, which leads to O-linked β-N-acetylglucosamine (O-GlcNAc) modification of cytoplasmic and nuclear proteins. Using a proteomic approach, we identified a broad variety of proteins associated with O-GlcNAc transferase (OGT), among which host cell factor C1 (HCF-1) is highly abundant. HCF-1 recruits OGT to O-GlcNAcylate PGC-1α, and O-GlcNAcylation facilitates the binding of the deubiquitinase BAP1, thus protecting PGC-1α from degradation and promoting gluconeogenesis. Glucose availability modulates gluconeogenesis through the regulation of PGC-1α O-GlcNAcylation and stability by the OGT/HCF-1 complex. Hepatic knockdown of OGT and HCF-1 improves glucose homeostasis in diabetic mice. These findings define the OGT/HCF-1 complex as a glucose sensor and key regulator of gluconeogenesis, shedding light on new strategies for treating diabetes.

INTRODUCTION

Glucose flux through the hexosamine biosynthetic pathway (HBP) leads to the posttranslational modification of cytoplasmic, nuclear, and mitochondrial proteins by O-linked β-N-acetylglucosamine (O-GlcNAc), termed O-GlcNAcylation (Hart et al., 2007; Torres and Hart, 1984). O-GlcNAcylation is emerging as a key regulator of diverse cellular processes, such as signal transduction, transcriptional regulation, and proteasomal degradation (Yang et al., 2002; Zachara and Hart, 2004, 2006). Aberrant O-GlcNAcylation has been linked to a plethora of human diseases, including diabetes, cancer, and neuronal diseases (Lazarus et al., 2009; Ngoh et al., 2010; Slawson et al., 2010). UDP-GlcNAc, the donor substrate, and O-GlcNAcylation levels within the cell are modulated by the availability of glucose, fatty acids, amino acids, and nucleotides. Therefore, O-GlcNAcylation is proposed as a nutrient sensor and metabolic regulator (Butkinaree et al., 2010; Hanover et al., 2012). Overexpression of the rate-limiting enzyme of the HBP, glutamine fructose-6-phosphate transaminase (GFAT), leads to peripheral insulin resistance (Hebert et al., 1996; Veerababu et al., 2000). Transgenic mice overexpressing O-GlcNAc transferase (OGT) in skeletal muscle and fat exhibit elevated circulating insulin levels and insulin resistance (McClain et al., 2002). Key components of insulin signaling can be O-GlcNAcylated (Whelan et al., 2010), and O-GlcNAcylation has been shown to be a negative regulator of insulin signaling (Yang et al., 2008). Hyperglycemia is also associated with O-GlcNAcylation of transcription factors and cofactors. O-GlcNAcylation of FOXO1, CRTC2, and PGC-1α modulate expression of gluconeogenic genes (Dentin et al., 2008; Housley et al., 2008, 2009; Kuo et al., 2008). Chronic increases in the levels of PDX1 and NeuroD1 O-GlcNAcylation may contribute to hyperinsulinemia in type 2 diabetes (Andrali et al., 2007; Gao et al., 2003). Thus, O-GlcNAc signaling is believed to serve as a nexus between nutrient flux, insulin resistance, and diabetes.

Unlike the presence of hundreds of protein kinases and phosphatases in the human genome, O-GlcNAc cycling is modified only by one O-GlcNAc transferase (OGT) and one O-GlcNAcase (OGA). It is largely unknown how the substrate specificity of OGT and OGA is achieved. It has been proposed that OGT recognizes substrates primarily though the tandem tetratricopeptide repeats (TPRs). Indeed, different OGT isoforms with various lengths in TPRs show different substrate specificities. Another possibility is that OGT forms dynamic holoenzymes with various protein partners that facilitate substrate recognition.
(Butkinaree et al., 2010; Chikanishi et al., 2010). For instance, interaction of OGT and p38MAPK activates O-GlcNAcylation of neurofilament H (Cheung and Hart, 2008). We hypothesize that OGT recognizes its substrates by association with a hierarchy of highly conserved adaptor proteins, analogous to the ubiquitin system in which dual E1 enzymes interact with dozens of E2 and hundreds of E3 ligases for substrate recognition.

In this study, we show that OGT and its interacting protein host cell factor C1 (HCF-1) cooperatively upregulate gluconeogenesis by stabilizing PGC-1α. O-GlcNAcylation of PGC-1α decreases its ubiquitination by recruiting the deubiquitinase BAP1. Glucose homeostasis in diabetic animals can be improved by knocking down OGT and HCF-1 in liver. Hence, OGT and HCF-1 may serve as potential targets for treating diabetes.

RESULTS

Proteome-wide Analysis Identifies HCF-1 and PGC-1α as OGT-Interacting Proteins

To identify candidate adaptor proteins that mediate substrate recognition of OGT on a proteome-wide level, we performed tandem affinity purification of OGT-binding proteins in HEK293T cells (Figure S1A). Purified proteins were then identified by Multidimensional Protein Identification Technology (MudPIT) (Washburn et al., 2001) and subjected to pathway analysis using MetaCore software (Figure 1A). A total of 853 putative OGT-interacting proteins involved in a wide range of biological processes were identified (Table S1). Strikingly, a large majority of these proteins participate in signal transduction and metabolism, supporting the notion that O-GlcNAcylation is a sensor and regulator of metabolic homeostasis.

One of the highly abundant proteins copurified with OGT is HCF-1 (Figure 1B). HCF-1 is an essential transcriptional cofactor that has been shown to be required for herpes virus gene expression, cell-cycle regulation, and stem cell growth (Dejosez et al., 2010; Julien and Herr, 2003; Peng et al., 2010). Consistent with previous reports (Capotosti et al., 2011; Daou et al., 2011), coimmunoprecipitation analysis of endogenous proteins confirms the interaction of OGT and HCF-1 and shows that HCF-1 is highly O-GlcNAcylated (Figures 1C and S1B). HCF-1 exists as a complex of the N- and C-terminal fragments that result from proteolytic processing of the precursor protein (Wilson et al., 1993). We found that both the N- and C-terminal fragments of HCF-1 interact with OGT and are O-GlcNAcylated (Figures S1C and S1D).

PGC-1α is a key transcriptional coactivator that regulates mitochondrial biogenesis and hepatic gluconeogenesis (Fernandez-Marcos and Auwerx, 2011). PGC-1α harbors a HCF-1 binding motif (HBM) (Lin et al., 2002). A proteomic approach to identify PGC-1α-interacting proteins shows that HCF-1 and OGT are among them (Figures 1D and S1E). Coimmunoprecipitation analysis confirms the interaction between PGC-1α and HCF-1 overexpressed in HEK293T cells and demonstrates O-GlcNAcylation of PGC-1α (Figure 1E). It is known that PGC-1α is strongly induced in liver during fasting (Puigserver et al., 2003). To test the endogenous interactions between these proteins, HCF-1 was immunoprecipitated from the liver of mice after 24 hr fasting. The results show that both OGT and PGC-1α are enriched in the HCF-1 immunoprecipitate (Figure 1F). After 3 hr refeeding, the PGC-1α level is largely reduced, which is coincident with a decreased interaction between HCF-1 and OGT (Figure 1F). O-GlcNAcylation of PGC-1α is also evident in fasted mouse liver (Figure 1F). Taken together, these observations suggest that OGT/HCF-1 complex formation and O-GlcNAcylation of PGC-1α are sensitive to food availability.

OGT and HCF-1 Cooperatively Regulate Gluconeogenesis

Next, we examined the functional consequence of the OGT/HCF-1 complex on PGC-1α. The presence of HCF-1 increases the transcriptional activity of PGC-1α, which is further augmented by OGT (Figure 2A). It has been known that PGC-1α coactivates FOXO1 via the insulin response sequence (IRS) to induce the expression of gluconeogenic genes such as glucose-6-phosphatase (G6pc) and phosphoenolpyruvate carboxykinase (Pck1) (Puigserver et al., 2003). We show that OGT and HCF-1 synergistically upregulate FOXO1 activity on both the G6pc promoter (Figure 2B) and the synthetic IRS DNA binding element (Figures S2A and S2B). Such synergistic effect on FOXO1 activity is abolished by PGC-1α knockdown (Figure 2C). Overexpression of either the N- or C-terminal fragment of HCF-1 has an effect similar to the precursor HCF-1 (Figures S2C and S2D). We further show that the synergistic induction of G6pc promoter activity by OGT and HCF-1 is more dependent on FOXO1 than other factors such as CREB/CRTC2, despite the fact that OGT can promote CRTC2 activity (Dentin et al., 2008) (Figures S2E–S2G). PGC-1α also coactivates HNF4α to regulate gluconeogenic genes (Yoon et al., 2001). We found that OGT and HCF-1 coexpression can promote the activity of the HNF4α responsive reporter as well (Figures S2H and S2I), suggesting a general effect of OGT/HCF-1 on PGC-1α regulation.

Chromatin immunoprecipitation was performed to detect O-GlcNAc and HCF-1 at the promoter of the G6pc gene. Both O-GlcNAc and HCF-1 are highly enriched in the FOXO1/HNF4α binding region of the G6pc promoter (Figures 2D and 2E). To test the effects of OGT and HCF-1 on expression of endogenous gluconeogenic genes, adenoviruses encoding OGT and HCF-1 were used to infect hepatoma FAO cells (Figure 2F). Overexpression of either OGT or HCF-1 stimulates endogenous G6pc and Pck1 expression, and coexpression of the two proteins has a cooperative effect (Figures 2G and 2H). By measuring glucose secretion into the culture medium, we also observed a cooperative effect of OGT and HCF-1 on glucose output (Figure 2I). On the other hand, knockdown of OGT and HCF-1 in FAO cells by adenoviruses encoding the corresponding shRNAs (Figure 2J) decreases gluconeogenic gene expression (Figures 2K and 2L) and glucose production (Figure 2M). Treatment of FAO cells with PUGNAc, a potent OGA inhibitor that increases global O-GlcNAcylation levels, also significantly increases gluconeogenic gene expression and glucose output (Figures S2J and S2K). Collectively, these results indicate that the OGT/HCF-1 complex promotes glucose production in hepatocytes.

O-GlcNAcylation and HCF-1 Interaction Stabilize PGC-1α

An O-GlcNAcylation site of PGC-1α (Ser333) was previously identified; however, the functional role of this site was not fully determined (Housley et al., 2009). Here we show that PUGNAc
treatment increases PGC-1α O-GlcNAcylation, and mutation of Ser333 to Ala (S333A) substantially decreases PGC-1α O-GlcNAcylation (Figure 3A). Although the S333A mutation does not affect the transcriptional activity, phosphorylation, or subcellular localization of PGC-1α (Figures S3 A–S3C) (Roth et al., 2009), elevating the O-GlcNAc level by PUGNAc increases PGC-1α stability. In contrast, the S333A mutant shows slightly decreased stability and is refractory to PUGNAc treatment (Figures 3B and S3D). PGC-1α can be targeted for ubiquitin-mediated proteolysis (Olson et al., 2008; Trausch-Azar et al., 2009).

Figure 1. Identification of OGT/HCF-1/PGC-1α Protein Complex
(A) Pie chart of functional distribution of identified putative OGT-binding proteins.
(B) The top of the list of putative OGT-binding proteins. Spectrum counts in GFP and OGT samples are shown.
(C) The interaction between OGT and HCF-1 was confirmed by coimmunoprecipitation of endogenous proteins from hepatoma FAO cells. A total of 0.1 M free GlcNAc was added to control for the specificity of O-GlcNAc antibody.
(D) List of top proteins identified in PGC-1α complex purification.
(E) HEK293T cells were cotransfected with HA-tagged PGC-1α and V5-tagged HCF-1, and their interaction and PGC-1α O-GlcNAcylation were determined.
(F) Interactions of HCF-1 with OGT and PGC-1α (middle) and O-GlcNAcylation of PGC-1α (top) in the livers from 24 hr-fasted and 3 hr-refed mice are shown.
We found that OGT overexpression, which increases PGC-1α O-GlcNAcylation (Figure S3E), decreases ubiquitination of PGC-1α. While the S333A mutant has a dramatic increase in ubiquitination compared with the wild-type (Figure 3C), OGT overexpression still slightly decreases ubiquitination of the S333A mutant, suggesting the existence of other potential O-GlcNAcylation sites (also see Figure 3A). Moreover, the S333A mutant has less ability to induce G6pc and Pck1 transcription compared with the wild-type (Figure S3F). Taken together, these data indicate that O-GlcNAcylation protects PGC-1α from proteasomal degradation.

Because HCF-1 and OGT have a cooperative effect on PGC-1α activity and gluconeogenic gene expression (Figure 2), we hypothesized that HCF-1 is an adaptor protein for targeting OGT to PGC-1α. To test this, a Y385A mutation in the HCF-1 binding motif (HBM) of PGC-1α (PGC-1αHBMmt) was generated. The PGC-1αHBMmt protein shows no change in transcriptional activity, phosphorylation, or localization (Figures 3A–S3C). Real-time PCR was performed with primers flanking FOXO1/HNF4α binding region or 3' UTR (a negative control) of the G6pc gene.

(F–M) FAO cells were infected with overexpression (F–I) or knockdown (J–M) adenoviruses as indicated, then protein expression (F and J), G6pc (G and K), and Pck1 (H and L) gene expression and glucose output (I and M) were determined (n = 3). All values represent mean ± SEM of data from three independent experiments. *p < 0.05; **p < 0.01; ***p < 0.001 by ANOVA with a Bonferroni's post hoc test compared with the vector control (A, B, G–I, and K–M). *p < 0.05 by Student's t test (C–E).

Figure 2. OGT and HCF-1 Cooperatively Upregulate Gluconeogenesis

(A and B) Luciferase assays were performed in HepG2 cells; luciferase activity was normalized to cotransfected β-Gal activity. (A) Gal4-PGC-1α transactivation assay (n = 3). (B) G6pc-luciferase assay in the absence or presence of FOXO1 (n = 3). (C) G6pc-luciferase assay following infection of FOXO1-transfected FAO cells with adenovirus encoding PGC-1α shRNA or scrambled control shRNA (n = 3).

(D and E) Chromatin immunoprecipitation assays of primary hepatocytes using O-GlcANc (D) and HCF-1 (E) antibodies (n = 3). IgG served as a negative control.

(F–M) FAO cells were infected with overexpression (F–I) or knockdown (J–M) adenoviruses as indicated, then protein expression (F and J), G6pc (G and K), and Pck1 (H and L) gene expression and glucose output (I and M) were determined (n = 3). All values represent mean ± SEM of data from three independent experiments. *p < 0.05; **p < 0.01; ***p < 0.001 by ANOVA with a Bonferroni's post hoc test compared with the vector control (A, B, G–I, and K–M). *p < 0.05 by Student's t test (C–E).
OGT/HCF-1 Complex Mediates Regulation of Gluconeogenesis by Glucose

Hormones such as insulin, glucagon, and glucocorticoids tightly control glucose homeostasis. Although insulin, forskolin (a cAMP agonist mimicking the action of glucagon), and dexamethasone (a synthetic glucocorticoid) are potent regulators of gluconeogenic gene expression (Figures S4A and S4B), our results show that they do not modulate the association between OGT and HCF-1 (Figure S4C). It has been known that glucose itself regulates gluconeogenesis (Rigoulet et al., 1987; Sacca et al., 1978; Seglen, 1974). However, the underlying mechanism is still obscure. Consistent with previous in vivo studies (Rigoulet et al., 1987), we show that glucose treatment can directly modulate gluconeogenesis in primary hepatocytes, with a maximal effect observed at 5 mM glucose (Figure 4A). Consistent with this observation, the expression of G6pc and Pck1 is also modulated by glucose availability, with the peak level at 5 mM glucose (Figures 4B and 4C). Since O-GlcNAc is a glucose sensor, we tested whether OGT/HCF-1 complex formation responds to glucose availability. Interestingly, OGT/HCF-1 interaction peaks under euglycemic conditions, although their expression levels are relatively constant at different glucose concentrations (Figure 4D). The levels of PGC-1α proteins also peak at 5 mM glucose (Figure 4D). Following treatment with MG132, which blocks protein degradation and normalizes PGC-1α levels, we observed that PGC-1α O-GlcNAcylation reaches its maximum level at 5 mM glucose (Figure 4E). Chromatin immunoprecipitation assays also show that O-GlcNAc and HCF-1 are most abundant at the G6pc promoter upon 5 mM glucose treatment (Figures 4F, 4G, S4D, and S4E). These findings suggest that the OGT/HCF-1 complex senses glucose availability, thereby regulating PGC-1α stability and glucose production.

Knockdown of OGT and HCF-1 Decreases Gluconeogenesis

To determine whether OGT/HCF-1 regulates gluconeogenesis in a PGC-1α-dependent manner, primary hepatocytes from fed mice were infected with adenoviruses encoding shRNAs targeting OGT and/or HCF-1. The efficacy of OGT and HCF-1 knockdown was validated by examining their mRNA and protein levels (Figures 5A and S5A). Along with the observation that PGC-1α expression is not detectable in fed mice (Figure 5A), OGT and/or HCF-1 knockdown has no effect on expression of gluconeogenic genes G6pc and Pck1 (Figure 5B). However, OGT and/or HCF-1 knockdown can significantly suppress the expression of gluconeogenic genes induced by PGC-1α overexpression (Figure 5B). As PGC-1α expression is induced in primary hepatocytes from overnight-fasted mice, OGT and/or HCF-1 knockdown significantly suppresses G6pc expression, which is abrogated by PGC-1α knockdown (Figure S5B). Compared with fed mice, we observed the increased occupancies of O-GlcNAc and HCF-1 at the FOXO1/HNF4α binding region of the hepatic G6pc promoter in fasted mice (Figures 5C and 5D). Taken together, these data show that the modulation of gluconeogenic gene expression by OGT and HCF-1 is dependent on PGC-1α.

To examine the physiological function of OGT/HCF-1, adenoviruses encoding shRNAs were delivered to the liver of C57BL/6 mice by systemic injection to knock down OGT and/or HCF-1 (Figure 5E). No significant difference in the expression of inflammatory markers Tnfa and Ifng in the liver was observed (Figure S5C). Pyruvate tolerance tests were performed to assess gluconeogenesis (Figure 5F). Compared with the scrambled shRNA control, OGT shRNA mice show an initial decrease in blood glucose, but maintain high levels after 60 min (Figure 5F), which may be due to their glucose intolerance (Figure 5G). OGT/HCF-1 double knockdown decreases glucose production from pyruvate to the extent similar to HCF-1 knockdown, suggesting that the effect of OGT on gluconeogenesis is dependent on
HCF-1 (Figure 5F). Although OGT shRNA mice are glucose intolerant, and HCF-1 shRNA mice behave similarly to the control, mice with OGT/HCF-1 double knockdown show improved glucose tolerance (Figure 5G). No significant difference in serum insulin was observed between the different groups (Figure S5D). Basal glucose levels are lower in the double knockdown mice, further indicating a gluconeogenic defect (Figure S5E). However, there is no statistically significant difference in insulin sensitivity between those mice (Figure S5F). In further support of our hypothesis, OGT and HCF-1 knockdown decreases PGC-1α protein levels (Figure 5E) and expression of gluconeogenic genes in the liver (Figure 5H). Consistent with the role of PGC-1α as a master regulator of genes involved in fatty acid oxidation and mitochondrial biogenesis, we observed decreased expression of genes in fatty acid oxidation and mitochondrial respiration in the liver following OGT/HCF-1 double knockdown (Figure 5G). Taken together, these results strongly argue that OGT/HCF-1 knockdown in C57BL/6 mice can suppress gluconeogenesis through the downregulation of PGC-1α.

O-GlcNAcylation Recruits BAP1 to Deubiquitinate PGC-1α
BAP1, a deubiquitinating enzyme, has been shown to interact with HCF-1 (Figure 6A), yet its targets are largely unknown (Machida et al., 2009; Misaghi et al., 2009). We tested the hypothesis that BAP1 is responsible for PGC-1α deubiquitination. Overexpression of BAP1 does not increase global ubiquitination, but substantially increases the level of PGC-1α and decreases PGC-1α ubiquitination as normalized to the protein level (Figure 6B). This suggests that the regulation of PGC-1α by BAP1 is target specific. PUGNAc treatment promotes the interaction between PGC-1α and BAP1, while it has no effect on the S333A mutant (Figure 6C), suggesting that O-GlcNAcylation of PGC-1α facilitates BAP1 binding. Overexpression of OGT, HCF-1, and BAP1 individually or in combination enhances PGC-1α stability (Figure 6D). OGT, HCF-1, and BAP1 stabilize PGC-1α by blocking its ubiquitination (Figure 6E). Conversely, knockdown of BAP1 in cells (Figure 6A) decreases the stability of PGC-1α (Figure 6F). In agreement with others’ observations (Daou et al., 2011), we noticed that HCF-1 can stabilize OGT (Figures 6E and S6B). BAP1 also increases OGT protein levels, suggesting that BAP1 is a potential deubiquitinase for OGT as well (Figure S6C). These data indicate that HCF-1 recruits OGT to O-GlcNAcylate PGC-1α, which facilitates the recruitment of BAP1 to deubiquitinate PGC-1α and OGT, thereby sustaining levels of PGC-1α in the cell.

Interestingly, Bap1 gene expression is significantly increased in high fat diet (HFD) and db/db mice (Figure 6G). To further determine the physiological role of BAP1 in gluconeogenesis, Flag-tagged BAP1 was overexpressed in mouse liver by adenoviral infection. In line with our observation that BAP1 is a deubiquitinase for PGC-1α in vitro, hepatic BAP1 overexpression increases the protein level of PGC-1α (Figure 6H). Furthermore, BAP1-overexpressing mice show increased expression of Pck1 transcripts (Figure 6I) and increased gluconeogenesis as seen in the pyruvate tolerance test (Figure 6J). These data demonstrate that BAP1 deubiquitinase stabilizes PGC-1α and promotes gluconeogenesis in vivo.

Knockdown of OGT and HCF-1 Improves Glucose Homeostasis in Diabetic Mice
Insulin resistance and diabetes are associated with increased O-GlcNAc modification (Cooksey and McClain, 2002; Hart et al., 2004). In the present study, we observe that BAP1 overexpression decreases O-GlcNAcylation of PGC-1α, which facilitates the recruitment of BAP1 to deubiquitinate PGC-1α and OGT, thereby sustaining levels of PGC-1α in the cell.
et al., 2011). We observed an increase in OGT expression and global O-GlcNAcylation in the liver of HFD mice (Figures 7A, S7A, and S7C). Although hepatic OGT expression is slightly decreased in genetically diabetic db/db mice, global O-GlcNAcylation is increased, suggesting a possible increase in OGT activity (Figures 7A, S7B, and S7D). Interestingly, HCF-1 protein levels are also significantly elevated in both HFD and db/db mice (Figures 7B and S7E). To evaluate whether OGT/HCF-1 knockdown ameliorates diabetes, shRNA adenoviruses were administered to db/db mice. No significant differences in expression of inflammatory genes were observed between the different groups (Figure S7F). Individual knockdown of OGT or HCF-1 improves glucose homeostasis, and double knockdown further normalizes blood glucose, as shown by the glucose tolerance test (Figure 7C). Insulin sensitivity is relatively unchanged (Figure S7G). The salutary effects of OGT/HCF-1 knockdown in db/db mice can be attributed, at least in part, to the suppression of gluconeogenesis through the downregulation of G6pc expression (Figure 7D). These results suggest that the OGT/HCF-1 complex may contribute to the pathogenesis of diabetes by upregulating gluconeogenesis in vivo.

**DISCUSSION**

PGC-1α is a critical metabolic node subjected to both transcriptional and posttranslational regulation (Fernandez-Marcos and Auwerx, 2011). The expression of PGC-1α can be stimulated by exercise, cold, and fasting through Ca2+, p38MAPK, AMPK, and PKA pathways (Fernandez-Marcos and Auwerx, 2011). The activity of PGC-1α is fine-tuned by phosphorylation, acetylation, and methylation. PGC-1α has also been shown to be O-GlcNAcylated (Housley et al., 2009), and PGC-1α acts to target OGT to FOXO1 and FOXO3, resulting in their increased O-GlcNAcylation and transcriptional activity. Nevertheless, the role of O-GlcNAcylation on PGC-1α remains elusive. In this study, we show that O-GlcNAcylation of PGC-1α can stabilize PGC-1α by inhibiting its ubiquitination. PGC-1α can be regulated by ubiquitin-dependent proteasomal degradation (Olson et al., 2008; Trausch-Azar et al., 2010). Skp1/Cullin/F-box-cell division control 4 (SCF Cdc4) was identified as the E3 ubiquitin ligase for PGC-1α. Here, we identify BAP1, a member of ubiquitin C-terminal hydrolases, as the PGC-1α deubiquitinase. This suggests that a dynamic balance of ubiquitination and deubiquitination is a key mechanism controlling cellular PGC-1α levels. Along with the findings by Housley et al., it is conceivable that O-GlcNAcylation of PGC-1α and FOXO transcription factors may have sequential and concerted action in promoting gluconeogenesis.

Interplay between O-GlcNAcylation and phosphorylation has been well documented (Zeidan and Hart, 2010). These two modifications can either competitively occupy single or proximal sites or coexist at different sites to cooperatively regulate protein functions. The copurification of protein kinase(s) and phosphatase(s) with OGT and OGA also suggests an intrinsic relationship between O-GlcNAcylation and phosphorylation (Slawson et al., 2008; Wells et al., 2004). However, a central question yet to be answered is whether and how O-GlcNAcylation interplays with other posttranslational modifications. An important study by the Lefebvre group reveals that O-GlcNAcylation can positively...
regulate global protein ubiquitination (Guinez et al., 2008). Here we report that O-GlcNAcylation of PGC-1α does not affect phosphorylation, but antagonizes ubiquitination of PGC-1α by recruiting the deubiquitinase BAP1 (Figure 6). This is the first evidence for a direct crosstalk between O-GlcNAcylation and ubiquitination on a single protein. Previous studies show that O-GlcNAcylation can modulate proteasome function (Guinez et al., 2008; Zhang et al., 2003). However, this is unlikely to account for the stabilization of PGC-1α by OGT because (1) the S333A mutation, which bypasses the effects of O-GlcNAcylation on the proteasome, increases PGC-1α ubiquitination and decreases its stability, and (2) HCF-1 and BAP1 have no effect on proteasome function or global ubiquitination (Figure 6B and data not shown), but can decrease PGC-1α ubiquitination and increase its stability.

HCF-1 is required for its proteolytic maturation, and OGT itself is suggested as the protease that catalyzes HCF-1 cleavage (Capotosti et al., 2011; Daou et al., 2011). Approximately 50% of nuclear OGT is associated with HCF-1, yet the targets and functions of this abundant nuclear complex are poorly understood (Daou et al., 2011). HCF-1 is known as a scaffold protein for multiple histone-modifying enzymes (Kristie et al., 2010). We also identified that a variety of enzymes that catalyze diverse posttranslational modifications are associated with OGT and/or HCF-1 (Table S1 and data not shown). Drosophila OGT is one of the polycomb group genes involved in epigenetic silencing (Gambetta et al., 2009) and, remarkably, histones are modified by O-GlcNAc (Sakabe et al., 2010). Therefore, we propose that HCF-1, as an adaptor protein, facilitates the recognition of OGT substrates involved in epigenetic regulation.

Metabolism is tightly controlled by hormonal and nutritional signals. Glucose effectiveness refers to the ability of high glucose per se to suppress endogenous glucose production, and it has an important role in glucose homeostasis (Tonelli et al., 2005). Here, we describe a mechanism whereby glucose determines its own production, which is through regulation of OGT/HCF-1 complex formation and subsequent O-GlcNAcylation and stabilization of PGC-1α. Compared to no glucose, low levels of glucose stimulate gluconeogenesis; on the other hand, hyperglycemia inhibits gluconeogenesis (Rigoulet et al., 1987; Seglen, 1974). Consistent with this phenomenon,
for preventing hyperglycemia and treating type 2 diabetes. Logical inhibition of this pathway represents a potential strategy. The pathway diminishes glucose effectiveness. Hence, pharmacological inhibition of the OGT/HCF-1/PGC-1 pathway in maintaining normal glucose levels and mediating glucose effectiveness. It has been shown that loss of glucose effectiveness contributes to type 2 diabetes (Mevorach et al., 1998). Our finding that expression of HCF-1 and BAP1 is upregulated in diabetic mice strongly suggests that the hyperactivation of the OGT/HCF-1/PGC-1 pathway diminishes glucose effectiveness. Hence, pharmacological inhibition of this pathway represents a potential strategy for preventing hyperglycemia and treating type 2 diabetes.

**EXPERIMENTAL PROCEEDURES**

**Cell Culture**
HEK293T cells were cultured in DMEM with 10% fetal bovine serum (FBS), HepG2 and FAO hepatoma cells were cultured in RPMI with 10% FBS, 2 mM sodium pyruvate, 1% insulin and 1% dexamethasone, 0.1 μM insulin on Collagen I-coated plates. HEK293T and HepG2 cells were transfected with FuGENE HD (Roche). FAO and primary hepatocytes were infected with adenovirus in medium containing 0.5% BSA. Treatment with shRNA adenovirus constructs was established using AdEasy system (Luo et al., 2007). Adenoviruses were amplified in HEK293 cells and purified using the kit from Virapar.

**Tandem Purification and MudPIT**
FH-OGT and FH-PGC-1α protein complexes were isolated from cell extracts by immunoprecipitation with α-Flag antibody followed by α-HA antibody according to established procedures with certain modifications (Nakatani and Ogryzko, 2003). For the MudPIT analysis, the precipitated protein was resuspended and subjected to digestion with trypsin. The resulted protein digest was pressure-loaded onto a Kasil-fritted fused silica capillary column (250 μm i.d.) packed with 3 cm 5 μm Partisphere strong cation exchange resins (Whatman, Clifton, NJ) and 3 cm 5 μm Aqua C18 resins (Phenomenex, Venture, CA). After desalting, this sample-loaded back-end column was then connected to a 100 μm i.d. capillary column with a 5 μm pulled tip packed with 12 cm 3 μm Aqua C18 material through a zero-dead-volume union (Upchurch Scientific, Oak Harbor, WA), and the entire three phase column was placed in line with an Agilent 1200 quaternary HPLC (Agilent, Palo Alto, CA) and analyzed using a modified 11-step separation described previously. As peptides were eluted from the microcapillary column, they were electrospayed directly into a hybrid LTQ-Orbitrap mass spectrometer (Thermo Fisher Scientific, San Jose, CA) with the application of a distal 2.5 kV spray voltage. A cycle of one FT full-scan mass spectrum (400–1400 m/z, 60,000 resolution) followed by eight data-dependent LTQ MS/MS spectra at a 35% normalized collision energy was repeated continuously throughout each step of the multidimensional separation. Application of mass spectrometer scan functions and HPLC solvent gradients was controlled by the Xcalibur data system (Thermo Fisher Scientific, San Jose, CA). MS/MS spectra were processed and searched with the ProLuCID algorithm (Xu et al., 2008) against the EBI human IPI database (ftp://ftp.ebi.ac.uk/pub/databases/IPI/, version 3.30), that was concatenated to a decay database in which the sequence for each entry in the original database was reversed. ProLuCID results were assembled and filtered using the DTASelect (version 2.0) program (Cociorva et al., 2007; Tabb et al., 2002). The MudPIT data from FH-OGT, FH-PGC-1α, and EGFP control was compared. Proteins were designated as putative interacting proteins if they were identified only in FH-OGT or FH-PGC-1α samples but not in the EGFP control, or the spectral count of each protein copurified with FH-OGT or FH-PGC-1α was at least four times larger than that in the EGFP control.

**Antibodies, Immunoprecipitation, and Western Blotting**
Anti-OGT, anti-Flag, anti-HA antibodies were from Sigma. Anti-O-GlcNAC (RL2), anti-V5 antibodies were from Abcam. Anti-HCF-1 antibody was from Betyl Laboratories. Anti-ubiquitin antibody was from Santa Cruz Biotechnology. Anti-PGC-1α (4C13.1) antibody was from EMD chemicals. Tissue and cell proteins were lysed in 1% Nonidet P-40, 50 mM Tris-HCl, 0.1 mM EDTA, 150 mM NaCl, protease inhibitors, and protein phosphatase inhibitors. For immunoprecipitation, whole-cell lysates were mixed with various antibodies as specified in the text and precipitated with Protein A/G agarose beads (Santa Cruz). Equal amounts of whole lysates or immunoprecipitates were electrophoresed on 8% SDS-PAGE gels and transferred to PVDF membrane. Primary antibodies were incubated at 4°C overnight. Western blotting was visualized by peroxidase conjugated secondary antibodies and ECL chemiluminescent substrate.

**Transcriptional Activity Assay**
HepG2 and FAO cells were transfected with expression plasmids, luciferase reporters, and β-galactosidase. Cells were lysed and luciferase and β-galactosidase enzyme activities were measured using kits from Promega. Relative luciferase activity was determined by normalizing to β-galactosidase activity.

**Chromatin Immunoprecipitation**
Primary hepatocytes were fixed with formaldehyde, lysed in buffer containing 50 mM Tris-HCl (pH 8.0), 10 mM EDTA, and 1% SDS, and then sonicated. Soluble chromatin was diluted and then immunoprecipitated with anti-O-GlcNac (plus 1 M free GlcNac as a negative control), anti-HCF-1 (H12) antiserum, or IgG. DNA was extracted using Chelex-100 and subjected to real-time PCR using primers flanking the FOXO1/HNF4α binding region of...
G6pc promoter. The 3' UTR of G6pc was used as the negative control (Nelson et al., 2006).

Real-Time PCR
Total RNA was extracted from cells or mouse liver using TRIzol reagent (Invitrogen). Complementary DNA was synthesized from total RNA with Superscript III enzyme and random hexamer primers (Invitrogen). cDNAs were amplified with SYBR Green I Master and the LightCycler 480 Real-Time PCR system (Roche). All data were normalized to the expression of 18s or 36b4. Primer sequences are available from the authors on request.

Glucose Output Assay
Medium of primary hepatocytes or FAO cells was replaced with 20 mM sodium lactate and 2 mM sodium pyruvate and various glucose levels when indicated. After a 6 h incubation, medium was collected and the glucose concentration was measured using glucose oxidation kit (Sigma). The readings were then normalized to the total protein content. To measure gluconeogenesis, primary hepatocytes were treated with gluconeogenic medium containing 13C pyruvate, lactate, and different levels of glucose. Newly synthesized glucose was calculated based on the distribution of glucose M+1 isotopomers.

Animal Studies
C57BL/6 mice were purchased from NCI/NIH, and db/db mice (Stock No. 000697) were purchased from Jackson Laboratory. Recombinant adenovirus (6 x 10⁹ plaque forming units [pfu] to C57BL/6 mice, and 2 x 10⁹ pfu to db/db mice) was delivered by systemic tail-vein injection to mice. Three to seven days after viral infection, mice were subjected to pyruvate, glucose, and insulin tolerance tests. Sixteen hour fasted mice were injected intraperitoneally with sodium pyruvate (2 g/kg body weight) or glucose (1.5 g/kg body weight); 6 h fasted mice were injected with insulin (0.75 U/kg body weight to C57BL/6 mice, 2.5 U/kg body weight to db/db mice). Blood glucose was measured from tail-vein blood collected at the designated times using Nova Max Glucometer.

Statistical Analyses
Results are shown as mean ± SEM. The comparisons between two groups were carried out using two-tailed unpaired Student’s t test. For multiple-group comparisons, one-way ANOVAs were used to test for no differences among the group means. Post hoc comparisons were adjusted using Bonferroni corrections. Mixed-model ANOVAs were used to analyze data from glucose comparisons, one-way ANOVAs were used to test for no differences among datasets using the Corrected Akaike Information Criterion and the Schwarz Bayesian Information Criterion. Bonferroni corrections were employed for data set using the Corrected Akaike Information Criterion and the Schwarz Bayesian Information Criterion. Covariance structure was optimized for each corrections. Mixed-model ANOVAs were used to analyze data from glucose deprivation.

Supplemental Information
Supplemental Information includes seven figures and one table and can be found with this article online at http://dx.doi.org/10.1016/j.cmet.2012.07.006.

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