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Cell Metabolism
Short Article

O-GlcNAc Signaling Entrains the Circadian Clock by Inhibiting BMAL1/CLOCK Ubiquitination

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http://dx.doi.org/10.1016/j.cmet.2012.12.015

SUMMARY

Circadian clocks are coupled to metabolic oscillations through nutrient-sensing pathways. Nutrient flux into the hexosamine biosynthesis pathway triggers covalent protein modification by *O*-linked β-D-N-acetylglucosamine (*O*-GlcNAc). Here we show that the hexosamine/*O*-GlcNAc pathway modulates peripheral clock oscillation. *O*-GlcNAc transferase (OGT) promotes expression of BMAL1/CLOCK target genes and affects circadian oscillation of clock genes in vitro and in vivo. Both BMAL1 and CLOCK are rhythmically *O*-GlcNAcylated, and this protein modification stabilizes BMAL1 and CLOCK by inhibiting their ubiquitination. In vivo analysis of genetically modified mice with perturbed hepatic OGT expression shows aberrant circadian rhythms of glucose homeostasis. These results establish the counteraction between *O*-GlcNAcylation and ubiquitination as a key mechanism that regulates the circadian clock and suggest a crucial role for *O*-GlcNAc signaling in transducing nutritional signals to the core circadian timing machinery.

INTRODUCTION

Almost all mammalian cells contain a self-sustained circadian (about 24 hr) clock that runs in tight synchrony with environmental cues including light and food (Bass and Takahashi, 2010). While the master pacemaker residing in the hypothalamic suprachiasmatic nucleus (SCN) is entrained directly by light, peripheral circadian oscillators can be entrained by diurnal feeding (Schibler and Sassone-Corsi, 2002). Among various macronutrients, glucose is a particularly potent entraining cue for peripheral clocks (Stephan and Davidson, 1998). Cellular nutrient sensors such as nuclear receptors have been proposed as mechanisms for entrainment by food (Asher and Schibler, 2011; Yang, 2010; Yang et al., 2006), but the molecular basis for glucose-mediated entrainment remains a mystery.

Circadian timekeeping occurs at the cellular level by virtue of transcriptional-translational autoregulatory feedback loops (Mohawk et al., 2012). The transcriptional activators BMAL1 and CLOCK drive expression of *Period* (*Per1* and *Per2*) and *Cryptochrome* (*Cry1* and *Cry2*) genes. PER and CRY proteins accumulate progressively and in turn inhibit BMAL1/CLOCK activity, thus generating the approximate 24 hr cycle of clock gene expression. The pace of oscillation of this autofeedback loop is controlled by various regulatory mechanisms including posttranslational modifications of clock proteins (Bass and Takahashi, 2010).

Cells possess a distinct form of posttranslational modification that is highly sensitive to nutrient availability. Glucose flux via the hexosamine biosynthesis pathway leads to intracellular glycosylation by addition of β-D-N-acetylglucosamine (GlcNAc) to many cytoplasmic and nuclear proteins at the hydroxyl groups of serine and threonine residues (Hanover et al., 2012; Hart et al., 2011). This widespread and dynamic glycosylation is mediated by *O*-linked GlcNAc transferase (OGT) and *O*-GlcNAcase (OGA), which catalyze sugar addition and removal, respectively. *O*-GlcNAc modification is increasingly recognized as a key regulator of diverse cellular processes. *O*-GlcNAcylation of a number of transcription factors mediates the effects of glucose on transcription of genes involved in key metabolic processes (Hart et al., 2011). A recent study has shown that *O*-GlcNAcylation links the cardiomyocyte circadian clock to metabolic outputs (Durgan et al., 2011). In *Drosophila*, *O*-GlcNAcylation of the PER protein has been shown to contribute to setting the clock speed (Kim et al., 2012). The present study provides direct evidence that glucose availability regulates cellular clock oscillation through the hexosamine/*O*-GlcNAc pathway. We further demonstrate that BMAL1 and CLOCK are key targets of *O*-GlcNAcylation, and this modification inhibits ubiquitination and degradation of these proteins. Accordingly, this work establishes a new mechanism for metabolic entrainment of the circadian clock by covalent modification of core clock components.

RESULTS

The Hexosamine/*O*-GlcNAc Pathway Modulates Cellular Clock Oscillation

In light of the important role of food-derived signals for peripheral clock entrainment, we examined whether the nutrient-sensing

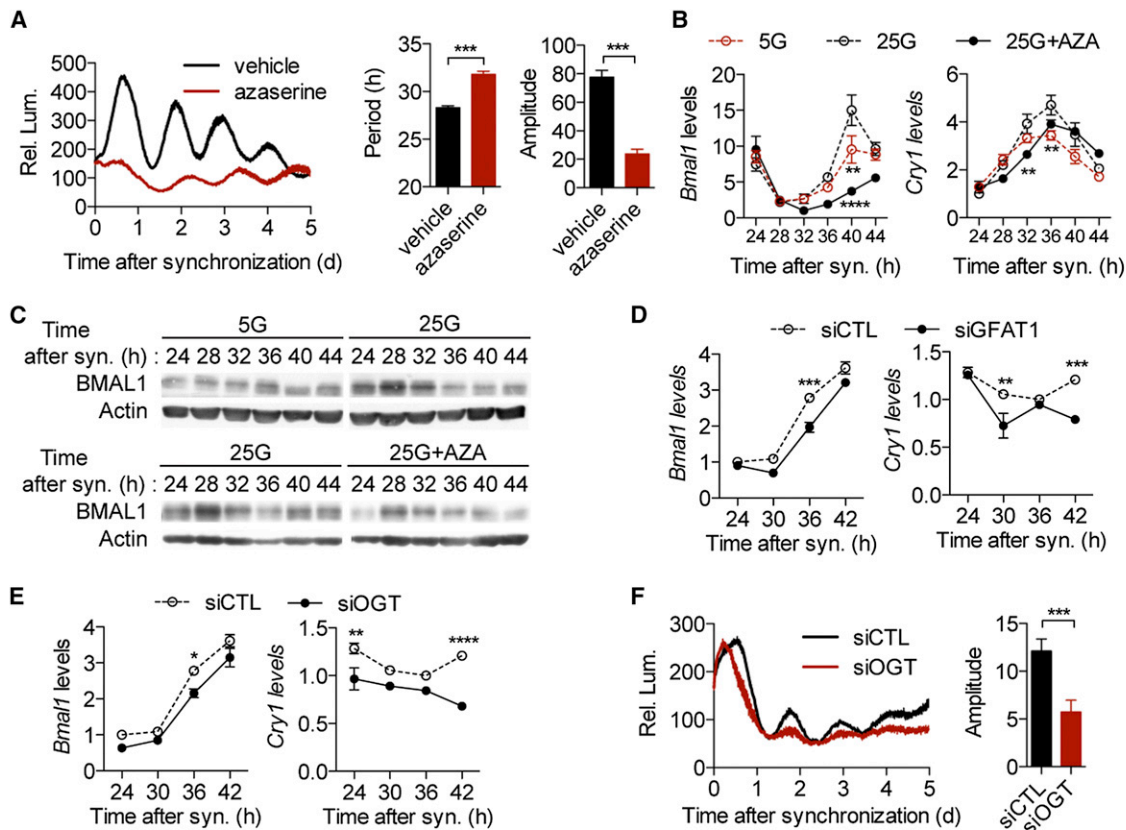

Figure 1. The Hexosamine/*O*-GlcNAc Pathway Modulates Cellular Clock Oscillation

(A) Average real-time bioluminescence from synchronized U2OS-B6 cells stably expressing a *Bmal1-luciferase* construct in the presence or absence of azaserine (n = 5). The vertical bar represents relative luminescence (Rel. Lum.). Circadian parameters were calculated by JTK_CYCLE.

(B) qRT-PCR analysis of synchronized U2OS-B6 cells (n = 3; 5G/25G, 5/25 mM glucose; AZA, azaserine).

(C) Immunoblot analysis of synchronized U2OS-B6 cells (n = 3 per lane).

(D) qRT-PCR analysis of synchronized U2OS-B6 cells transfected with GFAT1 siRNA (n = 3).

(E) qRT-PCR analysis of OGT knockdown on clock oscillation in synchronized U2OS-B6 cells (n = 3).

(F) Average real-time bioluminescence of OGT knockdown on clock oscillation in synchronized U2OS-B6 cells (n = 5). siCTL, scrambled siRNA; siGFAT1, GFAT1 siRNA; siOGT, OGT siRNA. All data are shown as mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 by ANOVA with Bonferroni's post hoc test or two-tailed Student's t test.

hexosamine pathway affects circadian oscillation. After dexamethasone synchronization, U2OS cells (U2OS-B6) stably expressing a *Bmal1-luciferase* reporter construct were grown in high (25 mM) glucose culture medium containing D-luciferin and monitored by the real-time bioluminescence recording system. Addition of azaserine, an inhibitor of hexosamine biosynthesis (Figure S1A), increases the period length and decreases the amplitude of *Bmal1* oscillation (Figure 1A). D-glucosamine is able to fuel the cellular pool of UDP-GlcNAc, the donor substrate of *O*-GlcNAcylation (Figure S1A). Addition of D-glucosamine dramatically delays the phase of *Bmal1* oscillation (Figure S1B). These data indicate a role for the hexosamine pathway in circadian regulation.

To substantiate our observations, we examined the oscillation of endogenous clock genes in synchronized U2OS-B6 cells. Compared with low (5 mM) glucose, high glucose increases the amplitude of *Bmal1* and *Cry1* messenger RNA (mRNA) oscillation, whereas azaserine suppresses them (Figure 1B). Immunoblot analysis shows that low glucose and azaserine also decrease BMAL1 protein levels as compared with high glucose (Figure 1C). Although the glucose concentrations do not affect the phase of *Bmal1* mRNA cycling (Figure 1B), low glucose delays the phase of BMAL1 protein accumulation (Figure 1C), suggesting that glucose can regulate BMAL1 levels posttranscriptionally.

GFAT1 is the first and rate-limiting enzyme in hexosamine biosynthesis and the target of azaserine. The small interfering RNA (siRNA)-mediated knockdown of GFAT1 decreases expression of *Bmal1* and *Cry1* (Figures 1D and S1C), which is also seen in the cells transfected with OGT siRNA (Figures 1E and S1D). Consistently, OGT knockdown reduces BMAL1 protein abundance (Figure S1E). Furthermore, OGT knockdown dramatically decreases the amplitude of the *Bmal1-luciferase* rhythm (Figure 1F). Taken together, these data demonstrate that the hexosamine/*O*-GlcNAc pathway regulates cellular clock oscillation.

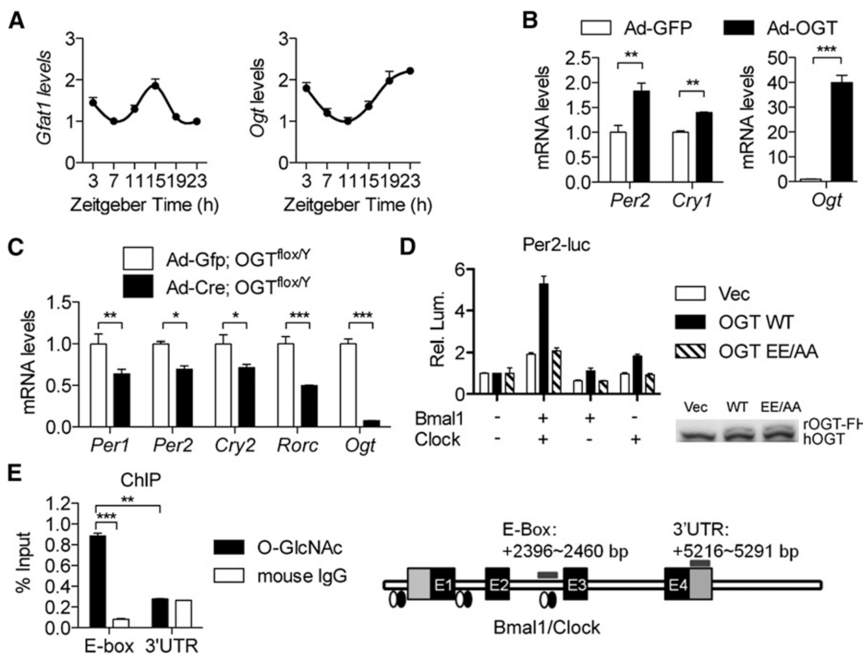

Figure 2. OGT Promotes Expression of BMAL1/CLOCK Target Genes

(A) Diurnal levels of *Gfat1* and *Ogt* mRNA in mouse livers (n = 4). Data were normalized to that of *u36b4*.

(B) qRT-PCR analysis of U2OS cells infected with the adenovirus expressing OGT (n = 3).

(C) qRT-PCR analysis of $OGT^{flox/Y}$ mouse primary hepatocytes transduced with the adenoviral vector expressing Cre recombinase (n = 3).

(D) *Per2-luciferase* assays of HeLa cells transiently expressing OGT, Myc-BMAL1, and Myc-CLOCK constructs (n = 3). GFP was used to equalize the total plasmid amount. Luminescence signals were normalized to that of GFP control groups. Immunoblot analysis of cell lysates is shown to confirm overexpression of OGT.

(E) Chromatin immunoprecipitation (ChIP)-qPCR analysis of mouse primary hepatocytes using an *O*-GlcNAc antibody (n = 3). ChIP with mouse IgG was used as the negative control. The diagram of assayed DNA regions is shown on the right. qPCR signals were normalized to those from genomic DNA inputs. All data are shown as mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001; two-tailed Student's t test.

OGT Promotes Expression of BMAL1/CLOCK Target Genes

To further dissect the circadian function of hexosamine signaling, we examined the rhythmicity of expression of key genes in this pathway. In mouse livers, diurnal levels of *Gfat1* transcripts are ultradian with a 12 hr period, whereas *Ogt* transcripts oscillate in a circadian manner (Figure 2A). *Oga* expression exhibits a weak diurnal rhythm (Figure S2A).

To investigate whether rhythmic hexosamine signaling affects expression of clock genes, we analyzed endogenous gene expression in U2OS cells transiently expressing GFP or OGT from recombinant adenovirus vectors. The results show that OGT significantly increases expression of *Per2* and *Cry1* (Figure 2B). In contrast, Cre-induced homologous recombination in $OGT^{flox/Y}$ mouse primary hepatocytes that eliminates OGT expression decreases expression of BMAL1/CLOCK target genes, including *Per1*, *Per2*, *Cry1*, and *Rorc* genes (Figure 2C). Luciferase reporter assays using a *Per2-luciferase* construct reveal that OGT promotes BMAL1/CLOCK-mediated activation of *Per2* transcription, whereas the catalytically dead $OGT_{E899A/E900A}$ ($OGT_{EE/AA}$) has no effect (Figures 2D, S2B, and S2C). These results indicate that OGT increases BMAL1/CLOCK transcriptional activity by an enzymatic mechanism and thereby promotes expression of BMAL1/CLOCK target genes.

Rhythmic *O*-GlcNAcylation Stabilizes BMAL1 and CLOCK by Inhibiting Their Ubiquitination

The circadian *Dbp* gene contains the conserved E-box motifs recognized by BMAL1/CLOCK (Ripperger and Schibler, 2006). Chromatin immunoprecipitation using an anti-*O*-GlcNAc antibody reveals that *O*-GlcNAcylated proteins associate with the *Dbp* E-box region (Figure 2E). This suggests that the BMAL1/CLOCK complex itself could be *O*-GlcNAcylated. To test this possibility, we immunoprecipitated epitope-tagged BMAL1 and CLOCK proteins expressed in synchronized U2OS cells and assayed the *O*-GlcNAc levels of BMAL1 and CLOCK over a complete circadian cycle. Immunoblot analysis reveals that both BMAL1 and CLOCK are *O*-GlcNAcylated rhythmically (Figure 3A). *O*-GlcNAcylation of endogenous CLOCK proteins is decreased by azaserine treatment (Figure S3A), supporting the importance of the hexosamine pathway in circadian regulation.

It has been known that OGT can modulate protein stability (Dey et al., 2012; Ruan et al., 2012). Whether OGT regulates CLOCK stability was tested by treating HEK293T cells expressing Myc-tagged CLOCK in the presence or absence of exogenous OGT with cycloheximide (Figure 3B). OGT overexpression increases the estimated half-life of CLOCK proteins. In line with this, we found that OGT overexpression decreases the steady-state ubiquitination of CLOCK (Figure 3C).

As shown in Figure 3A, BMAL1 is also *O*-GlcNAcylated. The Database of *O*-GlcNAcylated Proteins and Sites (dbOGAP) predicts S418 in mouse BMAL1 as a putative modification site (Wang et al., 2011) (Figure S3B). Mutation of S418 to alanine (S418A) decreases, but does not abolish, *O*-GlcNAcylation of BMAL1 (Figure 3D). To investigate whether *O*-GlcNAc modification on BMAL1 regulates its stability, Myc-tagged wild-type (WT) BMAL1 or S418A mutant was transiently expressed in HEK293T cells in the presence or absence of exogenous OGT and treated with cycloheximide (Figure 3E). The BMAL1 S418A mutant degrades faster than the WT protein, and OGT overexpression increases the half-life of BMAL1, as it does on CLOCK (Figures 3B and 3E). To test whether *O*-GlcNAcylation regulates BMAL1 stability by inhibiting ubiquitination, HEK293T cells transiently expressing Myc-tagged BMAL1 WT or S418A were treated with the proteasome inhibitor MG132 in the presence or absence of the OGA inhibitor PUGNAc. Elevation of global *O*-GlcNAc levels by PUGNAc leads to decreased ubiquitination, and BMAL1 S418A has more attached ubiquitins than WT (Figure 3F). *Per2-luciferase* reporter assays show that BMAL1 S418A

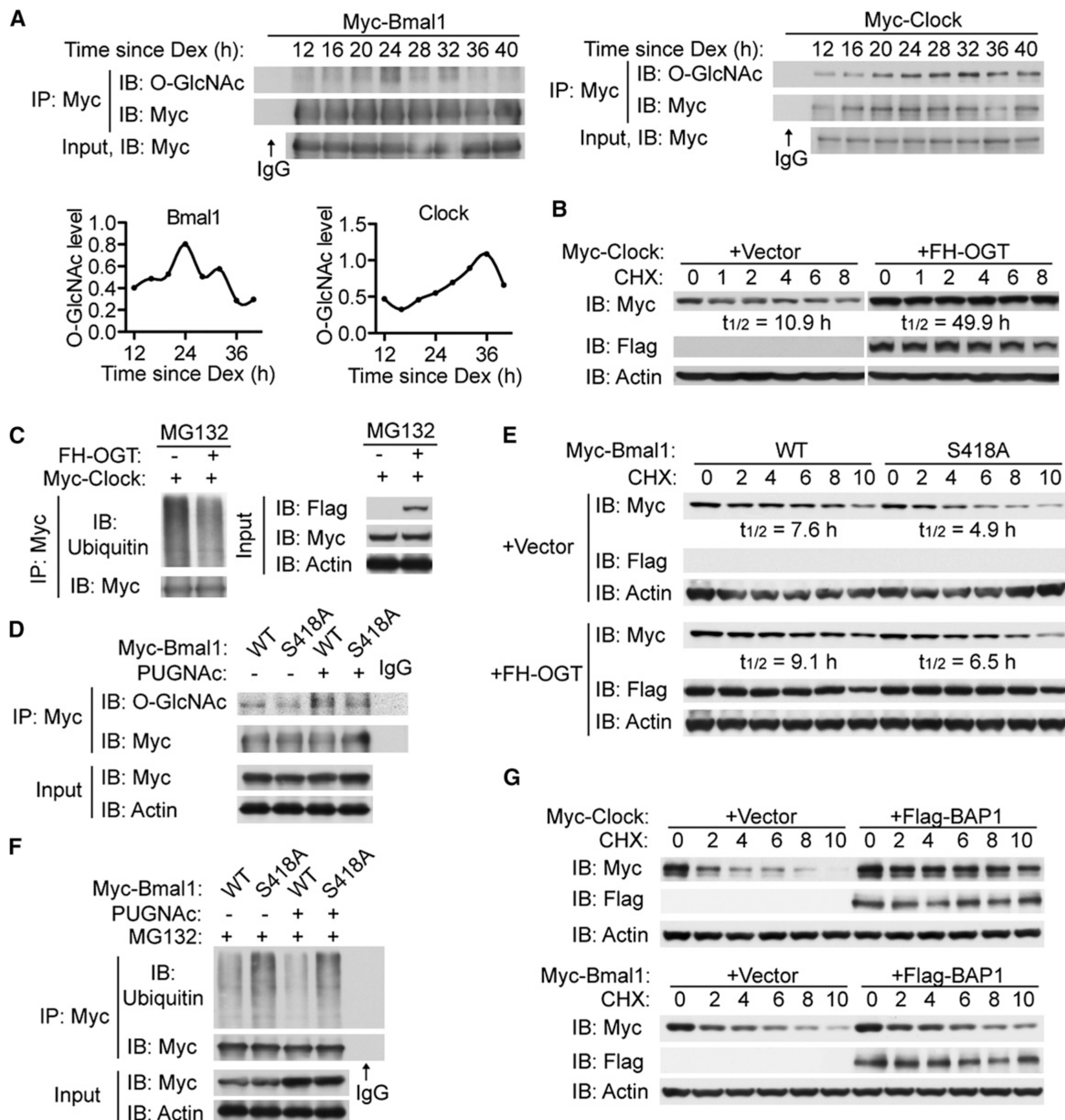

Figure 3. *O*-GlcNAcylation Stabilizes CLOCK and BMAL1 by Inhibiting Ubiquitination

(A) Circadian *O*-GlcNAc levels of BMAL1/CLOCK in synchronized U2OS cells transiently expressing Myc-tagged BMAL1 or CLOCK. *O*-GlcNAc levels normalized to levels of BMAL1 and CLOCK proteins are shown below the BMAL1 blot.

(B–C) HEK293T cells were transfected with Myc-CLOCK in the absence or presence of Flag/HA (FH)-tagged OGT. (B) Immunoblot analysis of CLOCK upon cycloheximide (CHX) treatment. Half-lives of Clock are shown. (C) Immunoblot analysis of ubiquitination of CLOCK. Cells were pretreated with MG132 and subjected to immunoprecipitation.

(D) HEK293T cells were transfected with Myc-tagged WT or S418A mutant BMAL1. *O*-GlcNAcylation of BMAL1 was determined when pretreated with or without PUGNAc.

(E) Stability of WT and S418A BMAL1 in the absence or presence of FH-OGT was determined by CHX treatment of transfected HEK293T cells.

(F) Immunoblot analysis of ubiquitination of BMAL1 WT and S418A in HEK293T cells. Cells were pretreated with MG132 and subjected to immunoprecipitation.

(G) Immunoblot analysis of Myc-tagged BMAL1/CLOCK upon CHX treatment in the presence or absence of Flag-BAP1.

exhibits impaired transcriptional activity compared to WT when coexpressed with CLOCK (Figure S3C).

The nuclear deubiquitinase BRCA1-associated protein 1 (BAP1) has recently been characterized as an OGT-binding protein that removes the ubiquitin markers on other associated proteins (Dey et al., 2012; Ruan et al., 2012). It follows that OGT-targeted proteins are likely to be regulated by BAP1. Coexpression of BAP1 in HEK293T cells transiently expressing Myc-tagged BMAL1 or CLOCK reveals that BAP1 stabilizes both proteins (Figure 3G). Based on these results, we conclude that OGT stabilizes BMAL1 and CLOCK through direct *O*-GlcNAcylation, which prevents ubiquitination and subsequent degradation.

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Hepatic Manipulation of OGT Perturbs the Diurnal Rhythm of Glucose Homeostasis

In line with the results from U2OS cells (Figure 1C), immunoprecipitation analysis of *O*-GlcNAcylated proteins from mouse livers shows diurnal variations in *O*-GlcNAcylation of BMAL1 and CLOCK that peak in the fed/dark phase (Figure 4A). Consistently, *O*-GlcNAcylation of hepatic BMAL1/CLOCK is increased by refeeding, confirming that *O*-GlcNAcylation of clock proteins is responsive to food availability (Figure S4A). To determine whether *O*-GlcNAcylation regulates circadian clocks in vivo, we generated liver-specific OGT overexpression mice by tail-vein injection of recombinant adenovirus (Figure 4B). Analysis of circadian transcripts in livers of these mice shows that OGT overexpression advances the phase of *Bmal1* and *Clock* and increases expression levels of *Per2, Cry1*, *Ror*γ, and *Dbp* during the peak phase (Figures 4B and S4B and Table S1), supporting the notion that *O*-GlcNAcylation increases BMAL1/CLOCK-mediated E-box-dependent transcription.

To study the effects of *O*-GlcNAc deficiency on clock oscillation, we generated liver-specific OGT knockout mice by tail-vein injection of the recombinant adenovirus expressing Cre recombinase into OGT-floxed mice. Immunoblot analysis shows that BMAL1 and CLOCK exhibit decreased *O*-GlcNAc levels in mouse livers (Figure S4C). The oscillation of *Bmal1* transcripts exhibits decreased amplitude due to reduced peak levels (Figure 4C). However, *Per2* and *Cry1* oscillation is unchanged (Figure S4D), suggesting the existence of compensatory mechanisms. Together, these results indicate that *O*-GlcNAcylation regulates circadian rhythms of clock gene expression in vivo.

Nutrient-dependent peripheral clock entrainment allows metabolic tissues to optimize the timing of their metabolic processes. Accordingly, we examined whether glucose-responsive *O*-GlcNAc signaling in the liver affects diurnal rhythms of glucose metabolism. The results show that overexpression of OGT boosts the diurnal rhythm of blood glucose, whereas control mice maintain a weak diurnal variation of circulating glucose (Figure 4D). Knockout of OGT advances the circulating glucose rhythm by 6–8 hr and induces hyperglycemia in the daytime (Figure 4E). To assess the circadian metabolic effects of reduced *O*-GlcNAc signaling, we assayed the circadian responses of OGT deficient animals to intraperitoneal injection of a bolus of glucose. While control mice exhibit diurnal changes in glucose tolerance, depletion of OGT exacerbates the already poor glucose tolerance at ZT1 (1 hr into the light phase), which is not seen at ZT13 (Figure 4F). Gluconeogenesis is known to be circadian. qRT-PCR analysis of liver transcripts shows that rhythmic expression of gluconeogenic genes is perturbed by OGT overexpression or depletion (Figures S4B and S4D). This indicates that *O*-GlcNAc signaling is important for diurnal regulation of glucose metabolism in vivo and supports the conclusion that OGT acts as a nutrient-sensing mediator that resets peripheral circadian clocks.

DISCUSSION

Here we have shown that the hexosamine/*O*-GlcNAc pathway regulates the circadian clock in peripheral tissues. It has been known for a decade that diurnal variation in nutrient availability can override the light/dark cycle to entrain circadian rhythms in peripheral tissues (Damiola et al., 2000; Stokkan et al., 2001). How metabolic signals entrain the circadian clock remains a central question in circadian biology (Bass and Takahashi, 2010). Among macronutrients, glucose has a prominent role in metabolic entrainment (Hirota et al., 2002; Stephan and Davidson, 1998). Extracellular glucose levels modulate intracellular UDP-GlcNAc and subsequent *O*-GlcNAc levels through the hexosamine biosynthesis pathway (Figure S1A). OGT overexpression increases the amplitude of clock oscillation in vivo (Figure 4B), whereas OGT knockout decreases *O*-GlcNAcylation and protein abundance of BMAL1 and CLOCK as well as *Bmal1* oscillation (Figures 4C and S4B). Notably, depletion of OGT in liver fails to perturb oscillation of the core oscillator genes *Per* and *Cry* (Figure S4D). This conundrum may be explained by the dominant effect of cryptic oscillating systemic cues (Hughes et al., 2012; Kornmann et al., 2007). For instance, Hughes et al. (2012) found that recovery of the SCN clock in clock mutant mice is sufficient to reestablish the circadian rhythm of the liver clock. Whether *O*-GlcNAc signaling is integral to food entrainment in peripheral clocks is an important subject for further investigation.

We further demonstrate that *O*-GlcNAcylation on BMAL1 and CLOCK prevents their protein degradation by inhibiting ubiquitination. The control of BMAL1/CLOCK protein stability is emerging as a critical layer of regulation on the amplitude and phase of clock oscillation (Cardone et al., 2005; Lee et al., 2008; Sahar et al., 2010; Stratmann et al., 2012). We have demonstrated that *O*-GlcNAcylation stabilizes BMAL1/CLOCK and thereby increases BMAL1/CLOCK-mediated transcription of genes in the negative limb of the clock such as *Per* and *Cry* (Figures 3 and 4). *O*-GlcNAcylation of PER and other components could further stabilize the negative limb (Kim et al., 2012). Together, our study helps establish a framework for understanding the crosstalk between different protein modifications on the positive limb of the circadian clock and provide a potential mechanism for food entrainment.

In the physiological context, perturbation of *O*-GlcNAc signaling in liver affects the diurnal rhythm of glucose homeostasis (Figures 4D–4F). OGT has been established as a suppressor of insulin signaling and a mediator of glucocorticoid transrepression and gluconeogenesis (Dentin et al., 2008; Housley et al., 2009; Li et al., 2012; Ruan et al., 2012; Yang et al., 2008). Thus, changes in plasma glucose rhythm are likely due to the combined effects of OGT on the circadian clock and nutrient/hormone signaling.

In summary, the present study establishes the crosstalk between *O*-GlcNAcylation and ubiquitination as a key molecular mechanism underlying metabolic entrainment of the circadian clock, supporting the concept that various post-translational modifications on the clock proteins integrate environmental and physiological cues to control circadian rhythms. Diurnal rhythms of *O*-GlcNAc signaling have broad implications for the circadian regulation of physiological processes in peripheral tissues, and the *O*-GlcNAc cycling enzymes OGT and OGA are thus potential drug targets for treating disorders at the interface of nutrient metabolism and circadian rhythms.

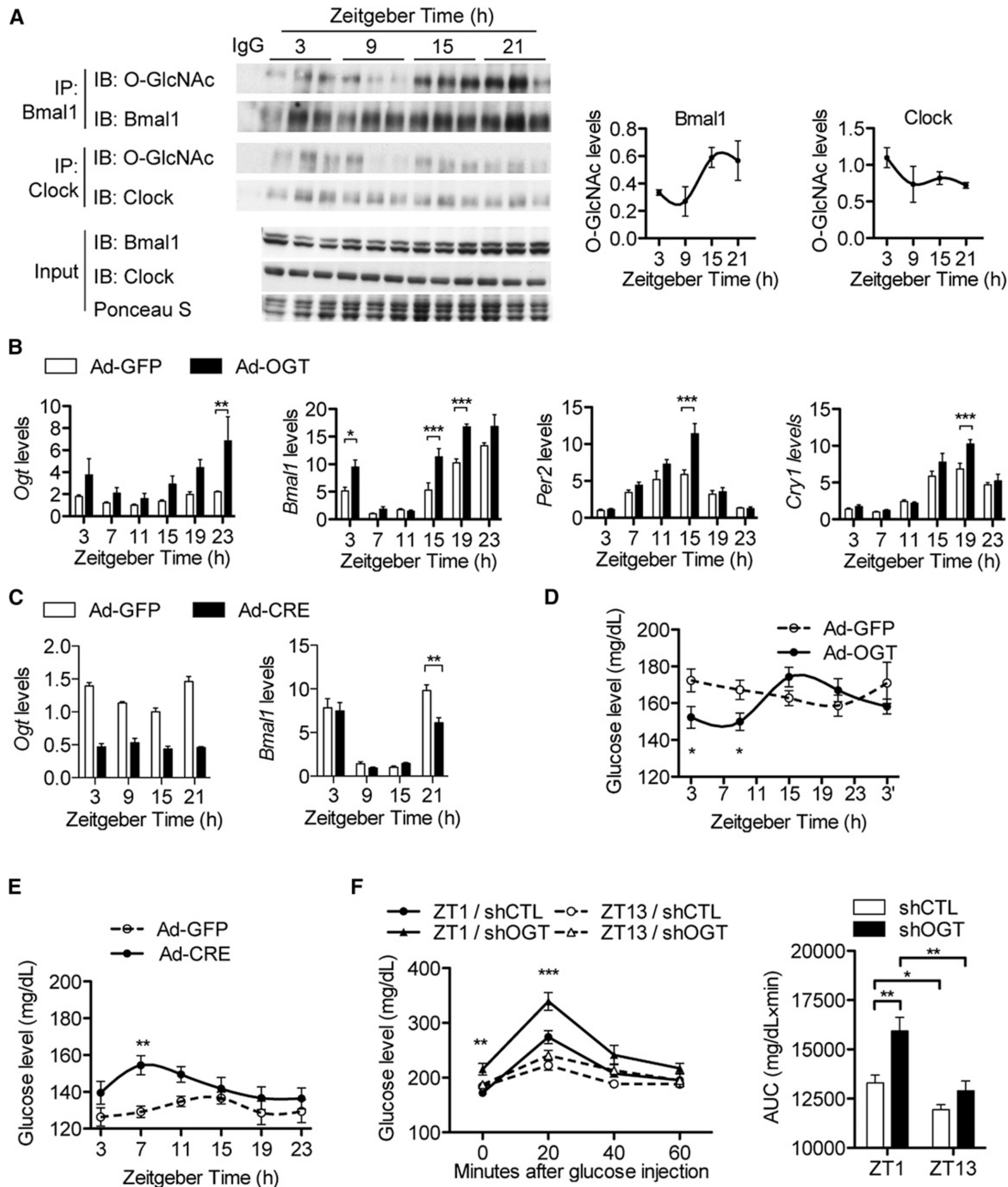

Figure 4. OGT Regulates Expression of Clock Genes and Glucose Homeostasis in Mouse Livers

(A) Diurnal *O*-GlcNAc profiles of BMAL1/CLOCK in mouse livers (n = 3 per time point). Results of densitometry analysis are shown on the right.

(B) Diurnal gene expression profiles of male mouse livers transduced with Ad-OGT (n = 4 per time point). Data were normalized to *u36b4*.

(C) Diurnal gene expression profiles of *Ogt*-floxed female mouse livers transduced with Ad-Cre (n = 3 per time point). Data were normalized to *Gapdh*.

(D) Diurnal plasma glucose levels in male mice overexpressing OGT in livers (n = 7).

(E) Diurnal plasma glucose levels in female $OGT^{flox/flox}$ mice overexpressing Cre in livers (n = 12).

(F) Diurnal plasma glucose responses to intraperitoneal glucose tolerance tests (GTT) at week 12 in male mice expressing scrambled shRNA (shCTL) or OGT shRNA (shOGT) in the liver (n = 7). Average areas under curve (AUC) of GTT curves are shown on the right. All data are shown as mean ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; ANOVA with Bonferroni's multiple comparison test.

EXPERIMENTAL PROCEDURES

Cell Culture

U2OS, HeLa, and HEK293T cells were maintained in high glucose Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS). U2OS-B6 cells were maintained in high glucose DMEM with 2 μg/ml of Puromycin (Sigma-Aldrich) and 10% FBS. Primary hepatocytes were isolated by Yale Liver Center Core Facility and plated in DMEM with 10% FBS, 2 mM sodium pyruvate, 1 μM dexamethasome, and 0.1 μM insulin on Collagen I coated plates. U2OS-B6 cells were transfected by Lipofectamine 2000 (Invitrogen). U2OS, HeLa, and HEK293T cells were transfected with FuGENE HD (Promega). For time course studies of BMAL1/CLOCK *O*-GlcNAc modification, U2OS cells were transfected upon confluence, cultured for 2 days, then shocked by 100 nM dexamethasone for 90 min and switched to fresh high glucose DMEM with 10% FBS. For expression assays, primary hepatocytes and U2OS cells were infected with adenoviruses in serum-free DMEM containing 0.5% BSA. Azaserine (20 μM), D-glucosamine (5 mM), PUGNAc (10 μM, 16 hr), MG132 (20 μM, 4 hr), and Cycloheximide (100 μg/ml) were added to the cultures as indicated.

Real-Time Recordings of Bioluminescence

At 48 hr after transfection, cells were shocked for 90 min at 37°C in a final concentration of 100 nM dexamethasone. Following dexamethasone shock, the medium was replaced with high glucose phenol red-free DMEM (GIBCO; supplemented with 10% FBS, 10 mM HEPES [pH 7.3], nonessential amino acids, sodium pyruvate, and 100 μM D-Luciferin). The plate was sealed with a plastic cover and read in a temperature-controlled TECAN M200 Luminometer with iTecan Software (Tecan Group, Ltd) (Vollmers et al., 2008). Luminescence for each well was integrated over 5 s and read at 30 min intervals for 5 days at a temperature setting of 37°C. LumiCycle data were statistically assessed for rhythmicity using JTK_Cycle (Hughes et al., 2010) using a period length window of 18–40 hr. Analyses used 3 days of data spanning 24–96 hr after changing to the assay culture media. JTK_Cycle was implemented in R (×64 v2.12.1) (Hughes et al., 2010; Miyazaki et al., 2011). All scripts are available on demand.

RNA Extraction, cDNA Synthesis, and Real-Time Quantitative PCR

Procedures were described previously (Ruan et al., 2012). qPCR data were normalized to either *u36b4* or *Gapdh* as indicated. Primer sequences are listed in Table S2.

Antibodies, Immunoprecipitation, and Immunoblotting

Anti-Flag (F3165) and anti-β-Actin (A5441) antibodies were from Sigma-Aldrich. Anti-Bmal1 (A302-616A) and anti-Clock (A302-617A) were from Bethyl Laboratories. Anti-*O*-GlcNAc (RL2, ab2739) and anti-OGT (ab50270) were from Abcam. Anti-Ub (P4D1, sc-8017) and anti-Myc (9E10, sc-40) antibodies were from Santa Cruz Biotechnology. Anti-HA antibody (12CA5) was from Roche. Procedures for immunoprecipitation and immunoblotting assays were described previously (Ruan et al., 2012).

Chromatin Immunoprecipitation

Procedures were described previously (Ruan et al., 2012). The 3′ UTR of *Dbp* was used as the negative control. A small aliquot of untreated sonicated chromatin was reverse crosslinked and used as the total input DNA control.

Animal Studies

All procedures have been approved by the Institutional Animal Care and Use Committee of Yale University. Male C57Bl/6 mice (10 weeks old) were purchased from NCI/NIH. Female $OGT^{flox/flox}$ (5 months old) mice were generated previously (Shafi et al., 2000; Watson et al., 2010). Mice were maintained under 12 hr light/12 hr dark cycle with free access to food and water. Recombinant adenoviruses (2×10^9 plaque-forming units [pfu] for males, 5×10^8 pfu for females) were delivered by systemic tail-vein injection to mice. At 3–6 days after viral infection, mice were subjected to glucose tolerance tests. Ad libitum fed male mice were injected intraperitoneally with glucose (1.5 g/kg body weight) at ZT1 or ZT13. Blood glucose was measured from tail-vein blood collected at the designated times using Nova Max Glucometer. Tissues were collected for RNA and protein isolation.

Statistical Analysis

Data are presented as means ± SEM. Statistical analysis was performed with GraphPad Prism by ANOVA using Bonferroni's post hoc test or t test where appropriate. Statistical analysis was accepted as significant if p value was <0.05.

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures, two tables, and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.cmet.2012.12.015.

ACKNOWLEDGMENTS

We thank P. Sassone-Corsi for Myc-BMAL1 and Myc-CLOCK constructs, K. Lamia for pGL3-*Per2*, and S. Panda for U2OS-B6 cell line. We are grateful to Clifford Slayman and Yao Wu for inspiring discussions. This work was supported by NIH R01 DK089098, P01 DK057751, American Diabetes Association, and Ellison Medical Foundation to X.Y.; NIH R21 NS058330, R01 NS055035, R01 NS056443, and R01 GM098931 to M.N.N.; NIH R01 HL083320, R01 HL094419, P01 HL078825, and P20 RR024489 to S.P.J.; CSC-Yale World Scholars Program scholarship to M.-D.L.; Brown-Coxe fellowship to H.-B.R.; and NIH F32GM096577 to M.E.H.

Received: May 29, 2012
Revised: October 25, 2012
Accepted: December 21, 2012
Published: February 5, 2013

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