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Glucose Sensor *O*-GlcNAcylation Coordinates with Phosphorylation to Regulate Circadian Clock

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SUMMARY

Post-translational modifications play central roles in myriad biological pathways including circadian regulation. We employed a circadian proteomic approach to demonstrate that circadian timing of phosphorylation is a critical factor in regulating complex GSK3β dependent pathways and identified *O*-GlcNAc transferase (OGT) as a substrate of GSK3β. Interestingly, OGT activity is regulated by GSK3β, hence OGT and GSK3β exhibit reciprocal regulation. Modulating *O*GlcNAcylation levels alter circadian period length in both mice and *Drosophila*, and conversely protein *O*-GlcNAcylation is circadianly regulated. Central clock proteins, Clock and Period, are reversibly modified by *O*-GlcNAcylation to regulate their transcriptional activities. In addition, *O*-GlcNAcylation of a region in PER2 known to regulate human sleep phase (S662–S674) competes with phosphorylation of this region, and this interplay is at least partly mediated by glucose levels. Together, these results indicate that *O*-GlcNAcylation serves as a metabolic sensor for clock regulation and works coordinately with phosphorylation to fine tune circadian clock.

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SUPPLEMENTAL INFORMATION Supplemental information includes Extended Experimental Procedures, seven figures, four tables, and can be found with this article online.

INTRODUCTION

Circadian rhythms in physiology and behavior are present in organisms from plants and bacteria to humans. These rhythms are controlled by endogenous molecular clocks even in the absence of external cues (e.g. light). The fact that circadian clocks are evolutionarily conserved supports the view that precise rhythms are essential for organisms to survive. Perturbations of circadian rhythms and sleep have been associated with many human ailments such as metabolic syndrome, cardiovascular disease, depression, epilepsy, and cancer (Bass and Takahashi, 2010; Climent et al., 2010; Duez and Staels, 2010; Wulff et al., 2010).

Glycogen Synthase Kinase 3β (GSK3β) is an important signaling mediator that has central functions in diverse physiological pathways including transcription, cell cycle regulation, metabolism, development, neuronal function, and oncogenesis, among others (Rayasam et al., 2009). These diverse functions of GSK3β can be attributed to the large number of substrates it can phosphorylate. GSK3β is a constitutively active Ser/Thr kinase with a preference for primed substrates and is inactivated in response to multiple stimuli by phosphorylation at Ser9 (Cohen and Frame, 2001). GSK3β is also a crucial circadian clock regulator (Iitaka et al., 2005; Martinek et al., 2001). Lithium (a GSK3β inhibitor) treatment lengthens the circadian period and delays the phase of rhythmic clock gene expression (Abe et al., 2000; Iitaka et al., 2005) although a recent report showed that inhibition of GSK3β activity by small molecule inhibitors or siRNAs shortens the circadian period (Hirota et al., 2008). In order to gain further understanding into the effects of GSK3β activity on various biological pathways in general, and circadian regulation in particular, we employed a proteomic approach to elucidate the complexity of the GSK3β circadian phospho-proteome. Interestingly, we identified *O*-GlcNAc transferase (OGT) from the chemical-genetic proteomic screen as a substrate of GSK3β. GSK3β was previously shown to be *O*-GlcNAcylated by OGT *in vitro* (Lubas and Hanover, 2000). Since our data suggests that OGT and GSK3β regulate each other and GSK3β is a critical molecular clock component, we investigated the possibility of *O*-GlcNAcylation as a regulatory post-translational modification in circadian regulation.

O-linked N-acetylglucosamine (*O*-GlcNAc) glycosylation has emerged as one of the most common protein post-translational modifications, with the second most abundant high-energy compound, UDP-GlcNAc, as the direct donor. Two enzymes regulate *O*-GlcNAcylation: the *O*-GlcNAc transferase (OGT) attaches UDP-GlcNAc through a beta-glycosidic *O*-linkage to the serine and threonine residues of proteins while *O*-GlcNAcase (OGA) hydrolyzes *O*-GlcNAc from proteins (Hart et al., 2010). OGT and OGA are highly regulated to prevent unnecessary *O*GlcNAc cycling (Sekine et al., 2010). Here we report that *O*-GlcNAcylation and circadian clock are reciprocally regulated and that *O*-GlcNAcylation modulates CLOCK-dependent transcriptional activity by post-translationally regulating components of the molecular clock. In addition, *O*-GlcNAcylation interplays with phosphorylation on PER2 which likely plays a role in fine-tuning of clock speed.

RESULTS

Rhythmic Phosphorylation of GSK3β Substrates

We developed an ATP analog-specific (AS) chemical genetic approach to identify direct GSK3β substrates that are involved in circadian regulation and coupling of core clock components to input-output pathways. To verify the effect of GSK3β on the circadian clock *in vivo*, we obtained AS-GSK3β knock-in mice, GSK3β$^{AS/AS}$ (Taconic Artemis). Using analogs of the general kinase inhibitor (PP1) that specifically inhibit AS, but not wild type (WT) kinases, kinase activity can be specifically, rapidly, and reversibly inhibited (Bishop et

al., 2000). Wheel-running activity of GSK3β$^{AS/AS}$ mice was analyzed after entrainment in a 12 hour light and 12 hour dark cycle (12L:12D) for one week. These mice showed a statistically significant lengthening of period vs. WT controls [24 vs. 23.7 hours (hrs)] in constant darkness (DD) (Figure 1A), suggesting that the engineered mutation produces an AS-GSK3β kinase with altered enzyme activity. This is consistent with the observation that AS-GSK3β enzyme activity is reduced when compared with WT GSK3β by an *in vitro* kinase assay (Figure S1A and B). AS-GSK3β is inhibited by 1-Na-PP1 inhibitor both *in vitro* in a concentration dependent manner (Figure S1C) and *in vivo* (Figure S1E), whereas WT GSK3β is not affected by 1-Na-PP1. Interestingly, after treatment with the specific AS-GSK3β inhibitor (1-Na-PP1), the period was lengthened further to 24.5 hrs (vs. 24 hrs in GSK3β$^{AS/AS}$ without inhibitor, Figure 1A). This finding is congruent with previous data using lithium (Abe et al., 2000; Duez and Staels, 2008). Since lithium acts on targets other than GSK3β (O'Brien and Klein, 2009), the data from the GSK3β$^{AS/AS}$ mice suggests that specific inhibition of GSK3β leads to lengthening of the circadian period.

GSK3β Ser9 phosphorylation (inactive GSK3β) demonstrates robust circadian oscillation (Iitaka et al., 2005). In order to test the oscillation of GSK3β Ser9 phosphorylation in both brain and peripheral tissues, hippocampus and liver tissues were obtained from WT mice (Figure 1B). Hippocampus was used instead of SCN due to the ease of anatomical dissection and the need to obtain sufficient quantity of tissue for proteomic analysis. Phosphorylation of GSK3β Ser9 in hippocampus peaks at subjective morning (CT0-“lights on” or “dawn” in the light-dark cycle) and is antiphase to liver where it peaks at subjective evening (CT12-“lights off” or “dusk” in the light-dark cycle), consistent with previous findings that kinases demonstrate tissue-specific and time-specific activities (Kategaya et al., 2012). To analyze whether GSK3β activity correlates with the substrates it phosphorylates, we isolated protein extracts from hippocampus and liver of WT mice at CT0 and CT12. Recombinant AS-GSK3β was added to hippocampus and liver protein extracts together with N6-phenethyl ATPγS. AS-GSK3β enzyme prefers ATPγS analogs (N6-benzyl ATPγS and N6-phenethyl ATPγS) as thiophospho-donors, whereas these analogs are not accepted by WT GSK3β (Figure 1C). Thiophosphorylated substrates are then alkylated for recognition by a thiophosphate ester-specific antibody (Figure S2A and B) (Allen et al., 2005). Substrate phosphorylation patterns by AS-GSK3β showed dramatic differences between the two tissues and at different circadian times (CT) when assessed by Western blotting (Figure 1D). The intensity of substrate phosphorylation directly correlated with the GSK3β activity in a circadian manner (the time point with high GSK3β activity in each tissue also showed highest phosphorylation of substrates).

Analog-Specific GSK3β Substrate Identification

We performed kinase reactions of analog-specific substrate labeling by recombinant AS-GSK3β to identify targets from the liver and hippocampus proteomes (at time of peak GSK3β-mediated phosphorylation - CT0 in liver and CT12 in hippocampus) (see Figure 1D). This procedure was performed three times with protein extracts from mouse hippocampus and twice with extracts from mouse liver. In the samples with AS-GSK3β, 343 and 124 potential GSK3β substrates were identified by mass spectrometry (MS) from hippocampus and liver, respectively. Eighty six of these proteins were found in both (Tables S1 & S2). Of the 343 and 124 proteins in these tissues, 145 and 69 of them were found only in samples with AS-GSK3β but not in samples with WTGSK3β, and 30 of them were identified in both hippocampus and liver (Tables S1 & S3). To validate the effectiveness of this approach, we experimentally examined two proteins, Zona occludens protein 1 (ZO1, Figure S2C) and PPP1R9B (Figure 2A and S2D), and confirmed them as substrates of GSK3β. Results from detailed bioinformatic analyses for the proteomic screens can be found in the Supplemental Information (Table S1–3). Proteins identified in the ASGSK3β

hippocampus-positive-only and AS-GSK3β liver-positive-only were further examined in the KEGG database (http://www.genome.jp/kegg) to reveal pathways that are potentially regulated by GSK3β (Figure S7). Many previously known GSK3β involved pathways together with additional pathways were found through this approach, further highlighting the interconnectedness of various regulatory mechanisms. Collectively, these results suggest that daily timing is an important parameter controlling GSK3β substrate specificity and that tissue-specific circadian phospho-regulation of GSK3β substrates may play important roles in the regulation of GSK3β dependent physiological pathways.

GSK3β regulates OGT Activity

Intriguingly, *O*-GlcNAc transferase (OGT) was one of the GSK3β substrates that was identified in the chemical-genetic screen (Table S1 and Figure S2E). The covalent dynamic modification of *O*-GlcNAc to proteins by OGT has emerged as a common post-translational modification that is as abundant as phosphorylation within the nucleus and cytoplasm (Torres and Hart, 1984). To validate OGT as an authentic GSK3β substrate, OGT was immunoprecipitated from brain extracts of WT mice using anti-OGT antibody. The precipitants were then subjected to WT- or AS-GSK3β kinase reactions with the ATPγS N6-benzyl analog followed by SDS-PAGE, and Western blots were probed with thiophosphate ester-specific antibody. In the presence of ATPγS N6-benzyl analog, OGT was phosphorylated by AS-GSK3β (Figure 2A). In addition, OGT was phosphorylated by GSK3b using *in vitro* ^{32}P-labeled ATP (Figure 2B), demonstrating that OGT is a GSK3β substrate.

Mass spectrometry was then employed to identify where GSK3β phosphorylates OGT. OGT, in the presence or absence of GSK3β, was digested with trypsin, before the peptides were analyzed by tandem mass spectrometry to identify modified peptides. OGT was found to be phosphorylated on serine 3 (S3) or serine 4 (S4) (data could not distinguish between these potential sites) (Figure 2C, left panel). Extracted ion chromatograms for the intensity of this phosphorylated peptide in the two samples showed a significant increase in the presence of GSK3β, suggesting that this site is modified by GSK3β (Figure 2C, right panel). Interestingly, MS analysis also revealed that both S3 or S4 (but probably not at the same time) of OGT can be *O*-GlcNAc modified (Figure 2D). Hence, phosphorylation by GSK3β and *O*-GlcNAcylation must compete and regulate each other at this N-terminal site of OGT.

OGT activity was next measured in the presence and absence of GSK3β to reveal whether phosphorylation by GSK3β regulates OGT activity. Indeed, OGT activity was enhanced with the presence of GSK3b phosphorylation (Figure 2E, OGT vs pOGT). To test whether phosphoryaltion of S3 and S4 on OGT by GSK3β is responsible for the enhanced activity, we mutated S3 and S4 to either alanine or aspartate (to mimic a constitutively phosphorylated state) before the kinase assays. Increased OGT activity by GSK3β was blocked when S3 and S4 were mutated to alanine, providing evidence that phosphorylation on these two amino acids is necessary for the effect of GSK3β on OGT. Interestingly, when S3 and S4 were mutated to aspartate, OGT activity increased slightly in the absence of GSK3β but significantly in the presence of GSK3β, suggesting that other phosphorylation sites on OGT are needed for the complete activation of OGT activity by GSK3β.

Manipulating *O*-GlcNAcylation Levels Regulates Period Length in both Mice and *Drosophila*

Since cyclic post-translational modifications such as phosphorylation (Chiu et al., 2011; Xu et al., 2007), acetylation (Hirayama et al., 2007), SUMOylation (Cardone et al., 2005), and poly(ADP-ribosyl)ation (Asher et al., 2010) are known to regulate clock proteins for precise timing of circadian progression (Mehra et al., 2009), we investigated whether *O*-

GlcNAcylation affects circadian rhythmicity. We first used primary embryonic fibroblast cells from *Per2*-luciferase mice (Yoo et al., 2005) and synchronized them with glucocorticoids followed by treatment with PUGNAc (OGA inhibitor), Alloxan (OGT inhibitor), or with a pool of 4 siRNAs against *OGT*. Interestingly, OGT inhibitor and *OGT* siRNA (decreased *O*-GlcNAcylation) shortened and OGA inhibitor (increased *O*-GlcNAcylation) lengthened the circadian rhythmicity of *Per2*-Luc oscillation (Figures 3A, B and S3C). We then confirmed period shortening with *OGT* conditional knockout mice (Figures 3C, S3A and B) to reduce *OGT* levels *in vivo* (knockout of *OGT* in mice causes early embryonic lethality (Shafi et al., 2000)).

Next, we asked whether *O*-GlcNAcylation is conserved as a regulatory mechanism in *Drosophila* circadian clock. We crossed *tim*(UAS)-Gal4 (Martinek et al., 2001) with UAS-RNAi*Ogt* or UAS-RNAi*Oga* to downregulate, and with UAS-*Ogt* or UAS-*Oga* to overexpress OGT and OGA respectively in *Drosophila* for behavioral analyses. *Ogt* knockdown and *Oga* overexpression both resulted in period shortening, while *Oga* knockdown and *Ogt* overexpression both led to period lengthening (Figure 3D and S3D). Together, these results suggested that *O*-GlcNAcylation is regulating the circadian clock in mice and flies and raised the possibility that clock proteins may be the direct targets of *O*-GlcNAcylation.

O-GlcNAc modification of *Drosophila* and Mammalian Clock Components

Using *Drosophila* Schneider 2 (S2) cell culture, we identified that dClk and dPer are *O*-GlcNAcylated (Figures 4A and 4B). dClk-V5 co-immunoprecipated with Ogt and Oga (Figure 4C) from S2 cells, supporting the possibility that dClk is a target of Ogt/Oga dependent, reversible *O*-GlcNAcylation. To show that mammalian clock proteins are also *O*-GlcNAc modified, human PER2-His and human OGT-Flag were co-expressed. PER2 was detected by *O*-GlcNAc antibody after anti-His immunoprecipitation whereas *O*-GlcNAcylated PER2 was not detected in the presence of excess GlcNAc in the buffer, suggesting *O*-GlcNAcylation of PER2 is specific (Figure 4D). In the presence of OGA inhibitor (PUGNAc) in the lyses buffer, PER2-His was also detected as *O*-GlcNAc modified without co-transfection with OGT, further supporting that PER2 is modified by *O*-GlcNAc (Figure 4E). Immunoprecipitation was next performed with mouse liver extracts using anti-*O*-GlcNAc antibody, and Western blot analysis indicated *O*-GlcNAcylation of both mouse PER2 and OGT *in vivo* (Figure 4F). Similarly, mouse CLOCK is likely *O*-GlcNAcylated by OGT and de-*O*-GlcNAcylated by OGA in HEK293 cells (Figure 4G). Using mouse liver extracts, OGA co-immunoprecipitated with CLOCK at circadian time CT 8 and CT20 (Figure 4H), indicating that CLOCK interacts with OGA and is likely a target of OGT/OGA dependent *O*-GlcNAcylation.

O-GlcNAcylation Modulates dClk and dPer Transcriptional Activities

To gain further insight into the effect of *O*-GlcNAcylation on dClk in circadian regulation, we employed a luciferase assay to measure dClk-dependent E-box activation of the *per* promoter in the presence of Ogt or Oga (Figure 5A) and demonstrated that de-*O*-GlcNAcylated dClk activates and *O*-GlcNAcylated dClk represses E-box dependent *per*-luc activation more than basal dClk (i.e. without exogenous Ogt or Oga). Interestingly, the transcriptional activity of dClk when dClk and dPer are both de-*O*-GlcNAcylated is similar to the activity of dClk without dPer, and the repressive effect of dPer is further enhanced when dClk and dPer are both *O*-GlcNAcylated, implying Ogt enhances and Oga relieves dPer-dependent *per*-luc inhibition through dClk. These results demonstrate that *O*-GlcNAc modification of dClk transcriptional activity is integral for regulation of the molecular clock.

Since *O*-GlcNAcylation modulates dClk transcriptional activity, we next measured dTim and dPer levels. When *Ogt* is overexpressed (i.e. reduced dClk transcriptional activity), both dTim and dPer levels were reduced compared to control flies and their rhythm phases showed a subtle trend of delay in clock neurons (Figures 5B and S4B). On the other hand, *Ogt* RNAi (i.e. increased dClk transcriptional activity) resulted in an increased d*Tim* and d*Per* protein (Figures 5C) and RNA levels (Figure 5D) with clear advance of the phases. Taken together, these results point to the necessity for balanced OGA and OGT activities and tightly controlled levels of *O*-GlcNAc modified clock components for proper clock function.

Competition of *O*-GlcNAcylation and Phosphorylation for the PER2 S662–S674 Region

Human PER2 S662-S674 is a critical site for regulating clock speed by serial phosphorylation of multiple residues; a Serine 662 to Glycine mutation leads to hypophosphorylation of this region and causes Familial Advanced Sleep Phase Disorder (Toh et al., 2001). We hence investigated the possibility that *O*-GlcNAcylation interplays with phosphorylation at S662-S674 and MS-MS analyses were carried out to identify *O*-GlcNAc sites on PER2 focusing on this region (Figure 6A). *O*-GlcNAcylation can occur on S662 and this only occurs together with *O*-GlcNAcylation on S671 (Figure 6A), implying a potential antagonism of *O*-GlcNAcylation with phosphorylation in this region. Interestingly, *O*-GlcNAc sites found with our method concentrate in the CK1 binding domain (S566, S580, S653, S662, S668, S671, T734) and in the C-terminus (T965, S983, T1180) of PER2 (Figure 6B). To investigate the possible interplay between phosphorylation and *O*-GlcNAcylation on PER2 S662-S674, HEK293 cells were transfected with PER2-His alone or co-transfected with either OGT or OGA, followed by immunoprecipitation with His antibody. Western blot analysis using an antibody specific for phospho-S662 PER2 revealed that phosphorylated S662 PER2 level was reduced in cells cotransfected with PER2 and OGT while *O*-GlcNAcylation was increased on PER2, suggesting that *O*-GlcNAcylation can block S662 phosphorylation (Figure 6C). However, neither OGT nor OGA interfered with the binding of CK1 to PER2. PER2 peptides (Xu et al., 2007) containing either S662 or pS662 were then used in an *O*-GlcNAcylation assay and pS662 peptide significantly reduced the capacity for *O*-GlcNAcylation (Figure 6D), supporting the competitive interplay between phosphorylation and *O*-GlcNAcylation at S662. Consistent with the finding for dClk and dPer transcriptional activity (Figure 5A), OGT inhibited CLK-BMAL1 transcription and further enhanced PER2 repressor activity (Figure S5). This is congruent with the previous finding that PER2-S662G is a stronger repressor than WT PER2 (Xu et al., 2007), and as expected, repressor activity of PER2-S662G is not modulated by OGT (Figure S5). Taken together, these data strongly suggest that *O*-GlcNAcylation and phosphorylation compete at S662 of PER2 to fine tune its activity.

Since *O*-GlcNAcylation is the direct readout for the Hexosamine Biosynthetic Pathway (HBP, converting glucose to UDP-GlcNAc), a nutrient sensing pathway through glucose, we further investigated the impact of glucose on *O*-GlcNAcylation and phosphorylation of PER2 S662, S665, S668. We employed an *in vitro* system with glucose levels that were established previously for this analysis (Lamia et al., 2009). In the presence of high glucose (but not low glucose), OGT was able to block the phosphorylation by CK1δ of this region (Figure 6E, right panel, lanes 3&4), suggesting that high glucose levels likely increase *O*-GlcNAcylation which blocks phosphorylation of the PER2 S662–S668 region. Intriguingly, high glucose can significantly prevent phosphorylation even in the presence of OGA (Figure 6E, right panel, lane 6). Given the role of PER2 S662 phosphorylation in regulating human clock (Xu et al., 2007), these results suggest that glucose metabolism and *O*-GlcNAcylation modulate the circadian clock partially through its competition with phosphorylation in the PER2 S662 region (Figure 6F).

O-GlcNAcylation is Regulated by Circadian Clock

Since phosphorylation and *O*-GlcNAcylation both modify Ser and Thr residues and multiple phosphorylation events are known to exhibit circadian oscillation, we set out to determine whether *O*-GlcNAc modification on clock proteins also oscillates. dClk was pulled down from the heads of yw;;dClk-V5 flies (Menet et al., 2010) at different circadian time points and then immunoblotted with *O*-GlcNAc antibody. Endogenous dClk protein underwent *O*-GlcNAc modification in a time-dependent manner peaking at ZT10 (Figures 7A and B) though total dClk protein levels oscillate in the opposite phase. dPer co-immunoprecipitated with dClk (Menet et al., 2010) and exhibited a similar rhythmic *O*-GlcNAc modification pattern (Figure 7A and B). Similarly, dClk and dPer *O*-GlcNAcylation peaked in constant conditions at CT 10–14 (Figure S6A and B). In addition, like what was found for mammalian CLOCK (Figure 4G), Oga protein co-immuoprecipitated with the dClk/dPer complex *in vivo* (Figure S6B). These results indicate that *O*-GlcNAc modification is likely under circadian clock regulation. We next analyzed OGT and OGA protein expression levels from liver tissues of WT mice over circadian time. Expression levels of OGT (110 kDa) did not oscillated. However, OGA protein exhibited an oscillating expression pattern that peaks around CT 8–12 (Figure 7C) (*OGA* mRNA expression also oscillates with a peak at CT 2.3, http://bioinf.itmat.upenn.edu/circa). Ogt and Oga protein levels of WT fly heads showed similar expression patterns to mouse liver (Figures 7D and E). Therefore, while OGA protein levels (and presumably total OGA activity) oscillate in a circadian manner, OGT protein levels are constant. But OGT activity is modulated by GSK3β (Figure 2) and GSK3β activity is known to oscillate through Ser9 phoshorylation. This would imply that OGT acitivity could also oscillate. Interestingly, we found that the *O*-GlcNAc on OGT itself showed a possible oscillating modification pattern through time (Figure S6E) which supports the likelyhood that OGT activity oscillates. Taken together, these data suggest that both OGT incorporation of donor sugar nucleotides to proteins, and OGA removal of these sugar nucleotides, occur in a circadian time-dependent manner.

DISCUSSION

We developed an analog-specific chemical-genetic proteomic approach to characterize the GSK3β-dependent circadian phospho-proteome and identified OGT as a GSK3β substrate. Here, we found that OGT is phosphorylated at serines 3 or 4 by GSK3β and that *O*-GlcNAcylation of OGT also occurs on the same or neighboring serine residues, suggesting interacting phosphorylation and *O*-GlcNAcylation events on OGT itself.

OGT is expressed constitutively while OGA levels oscillate with a peak around CT8–12, and phosphorylation of OGT by GSK3β increases OGT activity. Given that GSK3β activity oscillates through circadian time, it implies that the activity of OGT oscillates. OGT activity is likely further regulated by additional modulations including auto-*O*-GlcNAcylation. Intriguingly, the *O*-GlcNAc modification patterns of OGT support a possibility for oscillating OGT activity. Moreover, OGA activity may also be regulated by other factors and post-translational modifications. Interestingly, OGA was also identified in our GSK3β chemical genetics screen (Table S1). Together, all these pathways contribute to the determination of *O*-GlcNAcylation oscillation patterns of clock components. Hence, it is possible that the peak of *O*-GlcNAc modification of clock proteins may not be the same or anti-phase to the peak of OGA expression. This intricate regulatory system is therefore multi-layered and consistent with the notion that delicately modulated mechanisms are required for circadian regulation at different levels. Recently, *O*-GlcNAcylation was shown to modulate *Drosophila* Per (Kim et al., 2012) and mammalian BMAL1 (Durgan et al., 2011) in regulating circadian clock. Though some discrepancies exist among different reports, one consistent finding is the modulation of circadian period length by the level of *O*-GlcNAcylation. In addition, OGT overexpression leading to reduction in Period protein

levels was found by two independent studies (we and the Drugan group). This modulation of PER levels by *O*-GlcNAcylation could be one of the major reasons for the observed period changes, since it has been shown that PER rhythmic abundance is the driving force for the clock oscillation (Chen et al., 2009; Yu et al., 2006). Additional work is needed to identify site-specific roles of *O*-GlcNAcylation first and then their interplay with phosphorylation and other post-translational modifications in clock proteins in order to further reveal the complex regulatory mechanisms of the circadian rhythms.

Protein *O*-GlcNAcylation has been shown to regulate transcriptional machinery (Ozcan et al., 2010). For example, the RNA pol II C-terminal domain is modified by both phosphorylation and *O*-GlcNAcylation in a mutually exclusive manner. It was proposed that transcriptionally inactive *O*-GlcNAcylated RNA pol II holoenzyme localizes to promoters in a poised state but can only effect transcriptional elongation when the C-terminal domain *O*-GlcNAc is removed and becomes hyperphosphorylated upon gene activation (Comer and Hart, 2001). It is possible that a similar mechanism is utilized to fine-tune the activity of Clock transcriptional function. Another possibility is that the balance between *O*-GlcNAcylation and phosphorylation of Clock modulates interactions between Clock and its binding partners (such as repressors) in a similar manner to what has been shown for Myc (Kamemura et al., 2002). Our finding that *O*-GlcNAcylation of the PER2 S662 regulatory region blocks CK1 dependent PER2 phosphorylation also supports the latter hypothesis. The phospho-*O*-GlcNAc switch therefore provides a possible mechanism for tight control of the molecular clock to maintain precise daily rhythms. Many questions remain, such as how *O*-GlcNAcylation and other post-translational modifications (in addition to phosphorylation) synergistically regulate the intricate circadian clock? Clock has acetyltransferase activity and regulates chromatin remodeling through the acetylation of Bmal1 (Hirayama et al., 2007), and Ogt has been identified as a repressor from the polycomb complex (Gambetta et al., 2009; Myers et al., 2011). Thus, the fact that clock proteins are modified by OGT/OGA connects the HBP and epigenetics (Polycomb as a protein complex for chromatin modulation) to the circadian core-clock loop. Since protein *O*-GlcNAcylation is regulated principally by substrate UDP-GlcNAc availability, and HBP (a nutrient sensor) flux is known to parallel substrate (glucose) availability, our results showing high glucose blocks CK1-dependent PER2 phosphorylation (Figure 6E) raise the intriguing possibility that the HBP and *O*-GlcNAc turnover represent a glucose dependent mechanism for regulating circadian clocks and supports the connection for metabolic pathways and molecular clock. Interestingly, circadian misalignment was shown to increase blood sugar concentrations in human (Buxton et al., 2012; Scheer et al., 2009), indicating the timing of nutrient intake needs to be correlated with the timing of circadian expression of genes responsible for metabolism. Our finding that glucose levels through *O*-GlcNAcylation can modulate circadian clock protein further provides evidence that nutrient intake (and its timing) and circadian clock have a closely maintained cooperative relationship. In total, our data represents a further step toward a greater understanding of the complex mechanisms that control our bodily circadian functions in a highly coordinated manner.

EXPERIMENTAL PROCEDURES

Plasmid Constructs

See Supplemental Information.

Kinase Assay

Non-Radioactive—Glutathione S-transferase (GST) fusion of either WT-GSK3β or AS-GSK3β protein was expressed in *E.coli* and purified by glutathione sepharose 4B (GE Healthcare) (Kosuga et al., 2005). GST tag was then removed by PreScission Protease

digestion (GE Healthcare). AS-GSK3β kinase activity was comparable to commercial GSK3β kinase activity (New England Biolabs). 100nM of recombinant WT-GSK3β or AS-GSK3β were used for kinase assay (Allen et al., 2007). Kinase assay buffer contains 20 mM HEPES pH 7.5, 10 mM $MgCl_2$, 0.01 mM DTT, 0.2 mM ATPγS (or ATPγS analogs), 1mM GTP, and 1mM ATP. 2 μg MBP or 5 μg GSK3β was used as AS-GSK3β substrate. Reactions were incubated for 10 min at room temperature (RT) and terminated by 2.5 mM EDTA. Alkylating agent, 2mM p-nitrobenzylmesylate (PNBM), in DMSO was added and reactions were incubated for 1.5 h at room temperature. Laemli buffer was added and the samples were analyzed by Western blotting.

Radioactive—350 ng of recombinant human OGT protein (OriGene) was phosphorylated by 1000 U GSK3β (NEB) for 15 min at 30°**C** in 1× GSK3 buffer (NEB) and supplemented with 0.5μl [^{32}P]ATP (3,000 Ci/mmol) and 200μM of cold ATP. For negative control reactions, OGT was incubated without GSK3β. Proteins were separated by 4–12% Tris-HCl gels (BioRad) and transferred to nitrocellulose membrane for autoradiography or Western blot.

Assay for OGT Activity

100 ng of recombinant human OGT-V5, S3AS4A Ogt-V5 or S3DS4D Ogt-V5 was purified from HEK293 cells. OGT was eluted with V5 peptide from the agarose beads and was phosphorylated by GSK3β (NEB) for 1 h at RT in the presence of ATP in 1 × GSK3β buffer with 200μM of cold ATP. For the unphosphorylated OGT, the reactions were carried out without ATP. For the OGT activity assay, pOGT and OGT were incubated with 500 μM of CKII peptide (^{340}PGGSTPVSSANMM^{352}) in the presence of 0.02 μCi of UDP(^{3}H)GlcNAc (NEN Life Science Products) in 25 mM 5'AMP, 500mM sodium cacodylate (pH6.0), and 10 mM 1-amino-GlcNAc (Sigma). Reactions were incubated for 30 min at RT and stopped by 50mM formic acid. Reactions were purified with Sep-Pak C18 cartridges (Waters), peptides were eluted by methanol, and ^{3}H incorporation to the peptide was measured by scintillation counter. Reactions were performed in triplicates. Phospho-662 PER2 and PER2 peptides were described previously (Xu et al., 2007). Vangl1 peptide ^{517}RLQSETSV^{524} was used as the negative control.

Cell Culture

HEK293 cells were transfected with h*PER2*-His, hS662G *PER2*-His or h*PER2*-Flag, m*CLOCK*-Flag, m*BMAL1*, h*OGT*-V5, h*OGA*-Flag, *CK1delta*-myc using FuGENE HD according to manufacturer's protocol (Roche). For IP TAP buffer was used (Angers et al., 2006) and 100μM PUGNAc was included in the experiments when used. pGL3-TAT (Meijsing et al., 2009) and per2-luc plasmid (Kaasik and Lee, 2004) were used for luciferase assay. Anti-Flag M2 affinity gel (Sigma) or His antibody (Sigma) was used for pulldown.

Mouse Models

All experiments with mice were conducted according to protocols approved by the Institutional Animal Care and Use Committee at University of California San Francisco. GSK3β$^{AS/AS}$ homozygous knock-in mice was obtained from Taconic, Inc. To specifically inhibit GSK3β$^{AS/AS}$ with AS kinase inhibitors such as 1-Na-PP1, GSK3β$^{AS/AS}$ knock-in mice in wheel-running cages were entrained in a light-dark cycle (12hrs Light: 12 hrs Dark) for 1 week. Mice were then released into constant darkness for 2 weeks. Intraperitoneal injection of 1mM 1-Na-PP1 (100 μM per 25g body weight) was given at CT12 every day for a week beginning after one week in constant darkness. Ogt$^{tm1Gwh/Y}$ mice were a gift from Dr. J. Marth (Shafi et al., 2000), tetO-cre (Perl et al., 2002), actin rtTA (Sarin et al., 2005) mice were obtained from the Jackson Laboratory. 5 OGT$^{F/Y}$/ tetO-cre/ actin rtTA

mice and 5 $OGT^{F/Y}$/ tetO-cre mice in wheel-running cages were entrained in a light-dark cycle (12hrs Light: 12 hrs Dark) for 1 week. Mice were then released into constant darkness for 3 weeks and doxycycline hydrochloride (Sigma-Aldrich) was supplied in the drinking water at a concentration of 2 mg/ml. The Doxycyline containing water was renewed every three days. Wheel running activity was recorded and analyzed by ClockLab.

Fly Cell Culture, Transgenic Fly, and Locomotor Behavior Analysis

Drosophila luciferase assay was carried out as described previously (Kivimae et al., 2008). Knockdown and overexpression of Ogt (CG10392) and Oga (CG5871) was performed using Drosophila. For knockdown, UAS-RNAi lines of Ogt (18610, 18611) and Oga (41823, 41822) were obtained from the Vienna Drosophila RNAi Center (Dietzl et al., 2007). To enhance the knockdown effect, transgenic flies were crossed with UAS-Dicer2. The Tim-UAS-GAL4 driver was used to knockdown or overexpress Ogt and Oga (Blau and Young, 1999). UAS-HA-Ogt (or UAS-Ogt) and UAS-HA-Oga (or UAS-Oga) flies were generated to overexpress Ogt and Oga using the GAL4 system (Genetic Services, Inc). Analysis of locomotor activity of individually housed male flies (or virgin females) was performed in constant darkness at 25°C using the *Drosophila* Activity Monitoring System (Trikinetics). Per^0 flies were obtained from the Drosophila Stock Center (Bloomington, IN), yw;;Clk-V5 flies were obtained from a previous report (Menet et al., 2010).

Lumicycle Analysis

Primary fibroblasts from *Per2*-luc mice (Yoo et al., 2004) at E13–14 were extracted, cultured and treated with 0.1μM dexamethasone (Sigma) to reset circadian clock. PUGNAc (Toronto Research Chemicals, Inc) was used to inhibit OGA and Alloxan (Sigma) was used to inhibit OGT. Pools of 4 siRNAs of *OGT* were used and delivered with Accell siRNA reagent to primary *Per2*-luc fibroblasts (Dharmacon). Luciferase reporter activity was measured in the Lumicycler (Actimerics).

Statistical Analysis

Significant differences were determined by applying one-tailed or two-tailed Student's t tests, depending on the sample types. Wheel-running activity was compared between groups using repeated standard t-tests for pairwise comparisons. qRT-PCR results were analyzed by Student's t test. Error bars indicate the mean ± standard error.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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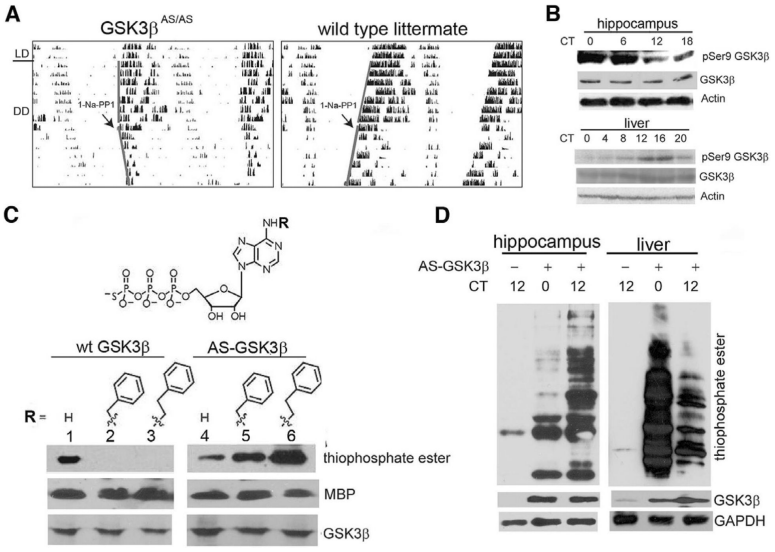

Figure 1. Characterization of GSK3β by ATP analog-specific chemical genetic method

(A) Representative double-plotted actograms of daily wheel running activity for GSK3β$^{AS/AS}$ and WT littermate mice. 1-Na-PP1 treatment was initiated after one week in constant darkness and administered daily for a week. The arrow on each actogram indicates the beginning of inhibitor treatment. 1-Na-PP1 treatment lengthens the circadian period of GSK3β$^{AS/AS}$ mice in constant darkness from τ= 24.0+/−0.12hrs (no inhibitor, n=10) to t=24.5+/−0.1hrs (with inhibitor, n=6, $p < 0.01$). 1-Na-PP1 treatment does not affect circadian period length in WT littermates (τ=23.7+/−0.15hrs, n=10 without inhibitor vs. τ=23.5+/−0.07hrs, n=6 with inhibitor; $p < 0.02$). (B) Western blot demonstrates that pSer9 of GSK3β oscillates at different phases in different tissues with the phase of hippocampus antiphase to the phase of liver. (C) *In vitro* kinase reactions show that WT-GSK3β is unable to utilize N-6 modified ATPγS analogs (lanes 2 and 3) as phosphodonors, but utilizes ATPγS (lane 1). AS-GSK3β (L132G) efficiently utilizes both unmodified (lane 4) and N-6 modified ATPγS analogs (lanes 5 and 6) and prefers N-6-phenethyl ATPγS (lane 6). Loading controls for the substrate Myelin Basic Protein (MBP) and GSK3β are included. (D) GSK3β demonstrates antiphase phosphorylation of substrates in hippocampal vs. liver tissue. AS-GSK3β kinase was added to the protein extracts and kinase assays were performed in the presence of N-6-phenethyl ATPγS. After PNBM alkylation, Western blot analysis was performed using antibodies indicated. Blots for Glyceraldehyde Phosphate Dehydrogenase (GAPDH) demonstrate equal loading.

See also Figure S1, S2, S7, Tables S1, S2, S3.

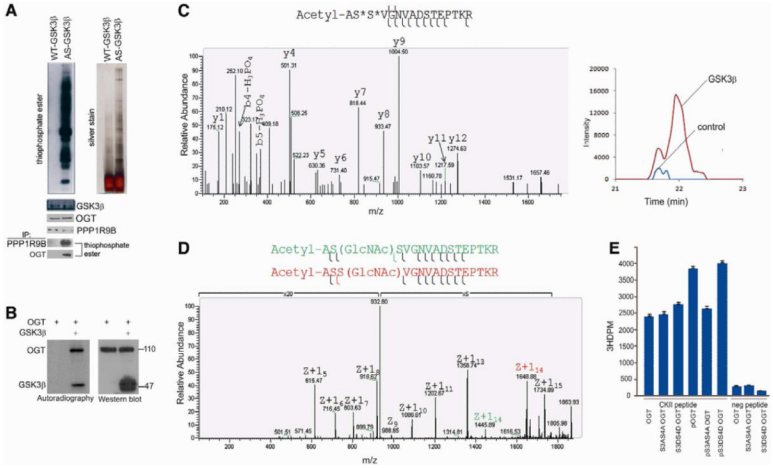

Figure 2. OGT activity is regulated by GSK3β

(A) Top panels: Kinase assays for mouse brain extracts from WT-GSK3β or AS-GSK3β were visualized by silver staining (right panel) or by Western blot against thiophosphate ester antibody (left panel). Middle panels: Western blots of the same brain extracts probed with antibodies against GSK3β, OGT and PPP1R9B. Bottom panels: Kinase reactions of brain extracts were immunoprecipitated by antibodies for PPP1R9B or OGT, followed by Western blot against thiophosphate ester antibody. PPP1R9B and OGT were specifically phosphorylated by AS-GSK3β but not by WT GSK3β. (B) *In vitro* phosphorylation of OGT by GSK3β in the presence of radioactive ATP (left panel). Proteins used were detected by Western blotting probed with anti-OGT and anti-GSK3β antibodies (right panel). (C) Increased phosphorylation of OGT N-terminal peptide by GSK3β. Extracted ion chromatograms of the m/z of the phosphorylated version of the tryptic peptide spanning residues 2–17 of the protein OGT shows a significantly increased level upon GSK3β treatment (right panel). CID fragmentation spectrum determines the phosphorylation to be present on either serine 2 or serine 3 of the peptide (i.e. serine 3 or serine 4 of the OGT protein) (left panel). (D) OGT can be *O*-GlcNAc modified on serine 2 or serine 3 of the peptide. ETD Fragmentation spectrum of a singly GlcNAc-modified version of the tryptic peptide spanning residues 2–17 of the protein OGT shows a mixture spectrum, demonstrating modified versions where serine 2 and serine 3 of the peptide are both present. (E) *O*-GlcNAcylation *in vitro* assay. *O*-GlcNAc transferase activity was measured using affinity-purified OGT, in the presence or absence of GSK3β, and UDP-[^{3}H]GlcNAc. OGT activity was enhanced by the presence of GSK3β (pOGT). S3 and S4 of OGT were mutated to either alanine (S3AS4A) or aspartate (S3DS4D) for the assay. CKII peptide and control peptide were used as substrates in the assay. 3HDPM: ^{3}H Distintegrations per minute. Error bars indicate the mean ± standard error.

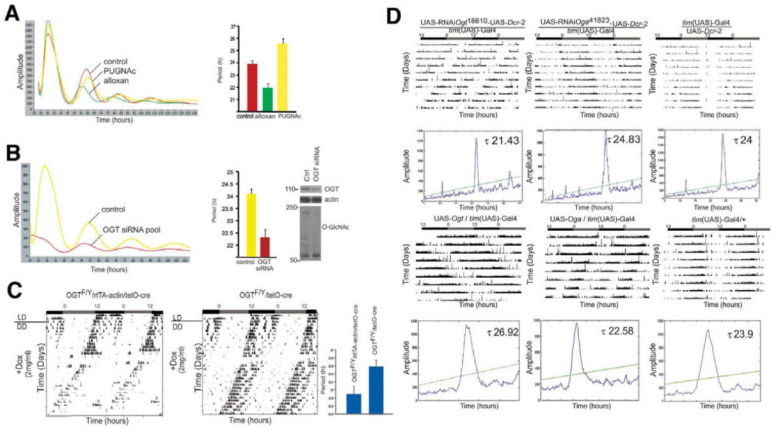

Figure 3. *O*-GlcNAcylation levels regulate period length

(A) Alloxan 1mM (OGT inhibitor) shortens (22.2h+/−19min) and PUGNAc 100μM (OGA inhibitor) lengthens (25.6h+/−15min) the circadian period of primary fibroblasts extracted from *Per2*-Luc reporter mice. For control, the periods of *Per2*-Luc fibroblasts were measured as 24.2h+/−21min. Concentration of Alloxan and PUGNAc were experimentally tested in pilot experiments (data not shown). Illustrated is a representative of experiments repeated in triplicate. (B) A pool of 4 *OGT* siRNAs shortens *Per2*-Luc fibroblast periods to 22.5h+/−20min compared to control siRNAs pool at 24.4h+/−30 min. A representative of experiments repeated in triplicate is illustrated. OGT and *O*-GlcNAc levels in the control and siRNA cells were determined by Western blotting shown to the right. (C) $OGT^{F/Y}$-rtTA-actin/tetO-cre (conditional knock out) and $OGT^{F/Y}$/tetO-cre (control) mice were entrained in 12hD:12hL cycle. The mice were released to constant darkness before doxycycline (2mg/ml) was supplemented into drinking water. Representative locomotor activity recordings of indicated mouse genotypes are presented. For visualization, 2 days are plotted per row. Quantifications of period length by hours are shown in the right panel. N=8 for both mice genotype. (D) Representative locomotor activity recordings and periodograms of indicated fly genotypes over circadian time. For visualization, 2 days are plotted per row. On top of the panels, the subjective light (grey bar) and dark (black bar) phases are indicated. Numbers in the oscillation graphs indicate tau(τ), the expressed period of the free running rhythm in constant darkness, of flies with the indicated genotype. Details and quantifications are specified in Table S4. Controls for (A), (C), and (D) are shown in Figure S3. Error bars represent SEM.

See also Figure S3.

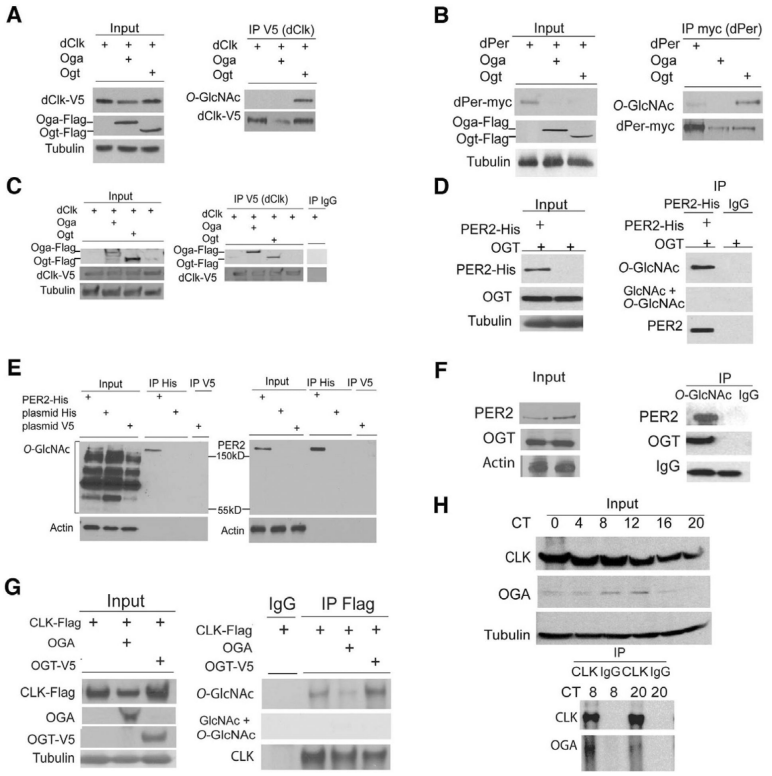

Figure 4. *O*-GlcNAcylation of clock proteins

dClk-V5 (A) or dPer-myc (B) was transfected into S2 cells alone or together with Ogt-Flag or Oga-Flag followed by dClk-V5 or dPer-myc pulldown and *O*-GlcNAc Western analysis. (C) dClk-V5 was transfected into S2 cells alone or together with Ogt-Flag or Oga-Flag, and dClk-V5 pulldown was performed using V5. Western blot was performed with Flag antibody. IgG was used as negative control for pulldown. (D) Human PER2 (PER2) is *O*-GlcNAcylated in HEK293 cells (upper panel). GlcNAc in the Western buffer blocks the interaction of *O*-GlcNAc antibody with PER2 indicating PER2 is *O*-GlcNAcylated (middle panel). (E) hPER2-His or empty plasmids was transfected into HEK293 cells and pulldown was performed with His or V5 antibodies. The OGA inhibitor PUGNAc was included in the lysis buffer and Western blot was probed with *O*-GlcNAc (left panel) or PER2 (right panel) antibodies. In the presence of PUGNAc, PER2 is *O*-GlcNAcylated without additional OGT (left panel). Cells transfected with empty plasmids were included as negative controls. (F) Western analysis for immunoprecipitants using anti-*O*-GlcNAc from mouse liver extracts with anti-PER2 (mouse PER2) and anti-OGT antibodies demonstrate that PER2 is *O*-GlcNAc modified. OGT is auto-*O*-GlcNAcylated and serves as a positive control. IP with IgG was used as a negative control. (G) mCLOCK (CLK) is *O*-GlcNAcylated by OGT and de-*O*-GlcNAcylated by OGA. CLK-Flag was transfected into HEK293 cells either alone or together with OGA or OGT-V5 followed by Flag pulldown and *O*-GlcNAc Western analysis. (H) CLOCK protein interacts with OGA in the mouse liver. CLOCK protein was immunoprecipitated from the mouse liver at CT8 and CT20. Western blot of the CLK pulldown was probed with CLK and OGA antibodies (right panel). Inputs are shown over the time course including CT8 and CT20 (left panel).

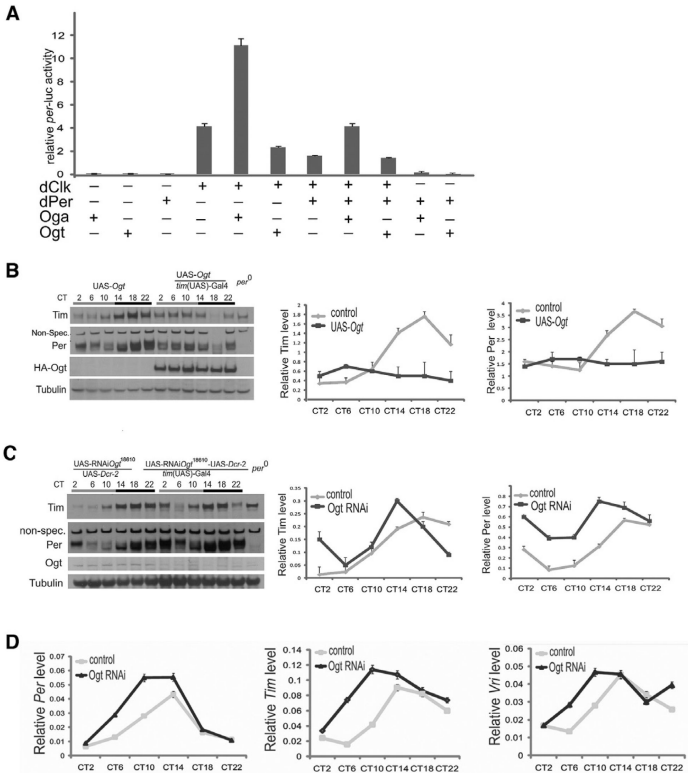

Figure 5. Functional effects of *O*-GlcNAcylation on dClk

(A) The effects of *Oga* and *Ogt* on dClk-dependent *per*-luc activity. Luciferase assays were performed in triplicate on at least three independent occasions. (B) dTim, dPer and dOgt protein levels under *Ogt* overexpression (B) and *Ogt* RNAi (C) as illustrated by Western analyses (left panels) and quantifications (right panels). (D) Quantification of *Per*, *Tim* and *Vri* mRNA levels from *Ogt* RNAi flies by RT-qPCR. Three independent experiments were used for each circadian time point. Error bars represent SEM.

See also Figure S4.

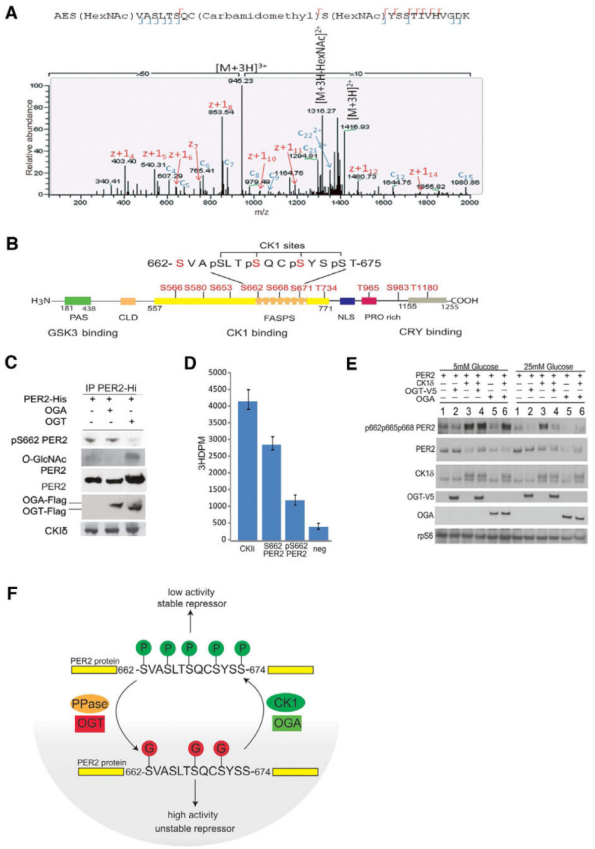

Figure 6. Competition of *O*-GlcNAcylation and phosphorylation for residues in the PER2 S662-S674 region

(A) MS-MS spectrum of doubly *O*-GlcNAc modified PER2 S660-S682 region. Full length human PER2 was purified using FLAG affinity tag from the HEK293 cells before being subjected to MS-MS analysis. Modified c_4 and c_5 ions localize one of the modifications sites to S662, while the mass shift between unmodified $z+1_{11}$ and modified $z+1_{12}$ pinpoints the second modification site to S671. (B) MS-MS identified *O*-GlcNAc sites S566, S580, S653, S662, S668, S671, T734, T965, S983 and T1180 (labeled in red) for PER2 in the CK1 binding domain (amino acids 557–771) and C-terminus. The identified S662, S668 and S671 *O*-GlcNAc sites are also CK1 phosphorylation sites. (C) Western blots performed with antibodies indicated on the left demonstrating that the presence of OGT inhibits PER2 S662 phosphorylation, but the presence of either OGT or OGA does not block CK1 binding to PER2. (D) *In vitro O*-GlcNAcylation assay using affinity-purified OGT peptides and UDP-[^{3}H]GlcNAc. *O*-GlcNAcylation of pS662 PER2 peptide is significantly reduced compared to nonphosphorylated S662 PER2 peptide. CKII peptide served as positive control and Vangl1 peptide as negative control. Error bars indicate the mean ± standard error. (E) The PER2 S662 region is regulated by both *O*-GlcNAcylation and phosphorylation. PER2 was transfected alone (lane 1) or with OGT (lane2), CK1 (lane 3), OGT and CK1 (lane4), OGA (lane 5), OGA and CK1 (lane 6) in the presence of low (left panel) or high (right panel) glucose into HEK293 cells. Western blots of protein extracts from these cells were probed with antibodies indicated on the left of the panels. (F) Proposed model for competitive regulation by *O*-GlcNAcylation and phosphorylation of the PER2 S662-S674 region. When PER2 S662-S674 is hypo-phosphorylated (ie *O*-GlcNAcylated), it is known to be a stronger repressor with lower protein stability; whereas when this region is hyper-phosphorylated, it is a weaker repressor with higher stability (Xu et al., 2007).

See also Figure S5.

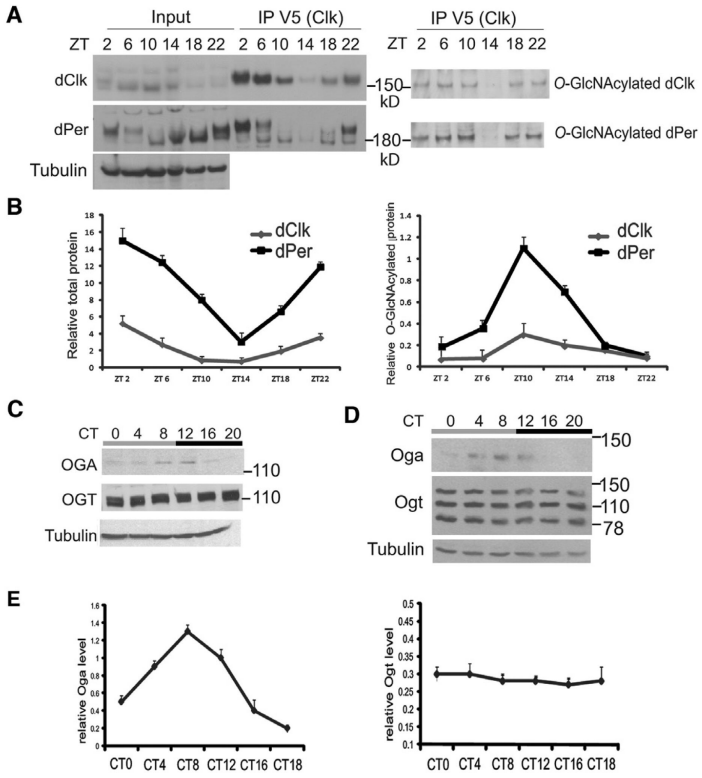

Figure 7. *O*-GlcNAcylation is regulated by circadian clock

(A) dClk was pulled-down from yw;;dClk-V5 flies (one extra copy of Clk) at different Zeitgeber Times (ZT) prior to being immunoprecipitated by V5 antibody. Immunoprecipitaed preparations were run on identical gels. Western blots of these gels were then immunoblotted by dClk, dPer, Tubulin (left panels) and *O*-GlcNAc (right panels) antibodies separately. (B) Quantification of immunoprecipitated proteins (left panel) and *O*-GlcNAcylated proteins normalized with immunoprecipitated protein levels (right panel). (C) Immunoblotting indicates that OGA protein levels oscillate over 24 hrs and OGT expression levels (110kDa band) are constant over 24 hrs in extracts of mouse liver tissue. (D) Oga shows oscillation over 24 hrs and Ogt expression is constant in protein extracts from WT fly heads. (E) Quantification of (D). Error bars represent SEM.

See also Figure S6.