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O-GlcNAcylation of BMAL1 regulates circadian rhythms in NIH3T3 fibroblasts

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ABSTRACT

Various physiological processes and behaviors show a circadian rhythm of approximately 24 h, which is crucial in coordinating internal metabolic processes and environmental signals. Post-translational modifications play an important role in regulating circadian core proteins. In this study, we demonstrated that BMAL1 was modified with an O-linked β-N-acetylglucosamine (O-GlcNAc), which stabilized BMAL1 and enhanced its transcriptional activity. Conversely, inhibition of O-GlcNAcylation resulted in inhibition of circadian rhythms of clock gene expression. Because O-GlcNAcylation is sensitive to the glucose level, such a modification may provide a new mechanism connecting metabolism with circadian rhythms.

1. Introduction

Physiology and behavior are subject to daily oscillations driven by an endogenous circadian clock. In mammals, the circadian timing system is composed of a central pacemaker, the suprachiasmatic nucleus (SCN) of the lateral hypothalamus, and subsidiary oscillators in most peripheral tissues [1–3]. The mammalian circadian oscillator is mainly based on a pair of transcriptional activators – BMAL1 and CLOCK, which bind to the so-called E-box motifs and transactivate the expression of *Periods* and *Cryptochromes*. Upon reaching a certain threshold concentration, the PERIOD (PER) proteins together with the CRYPTOCHROME (CRY) proteins repress the BMAL1/CLOCK-mediated transcription, thereby decreasing their own transcription and that of other circadian genes [1,2]. In addition, the expression of BMAL1 is also regulated by an interconnected feedback loop involving orphan nuclear receptors of the REV-ERB and ROR families [2].

The proteins involved in the circadian rhythms undergo extensive post-translational modifications, including phosphorylation, sumoylation, acetylation, and ADP-ribosylation [4]. The first mammalian circadian rhythms mutant, the *tau* hamster, has a mutation in the casein kinase Iε (CKIε), which regulates the degradation of the mammalian PERs [5]. Many other kinases, such as GSK-3β [6] and AMPK [7], are also involved in the phosphorylation of certain clock proteins. Clock proteins BMAL1 [8] and DEC1 [9] were reported to be sumoylated, which plays an important role in the circadian rhythmicity. Furthermore, PER2 [10] and BMAL1 [11] were acetylated under control of the histone deacetylase, SIRT1. Recently, it was reported that poly(ADP-ribosyl)ation of CLOCK [12] integrated the energy metabolism and circadian rhythms.

Attachment with an O-linked β-N-acetylglucosamine (O-GlcNAc) at serine and threonine residues is an important post-translational modification on many nuclear and cytoplasmic proteins involved in cell division, metabolism, transcription and cell signaling [13]. O-GlcNAcylation modification is catalyzed by O-GlcNAc transferase (OGT) and O-GlcNAcase (OGA). Glycosylation with O-GlcNAc influences protein expression, degradation and trafficking [13]. Recently, Kim et al. found that O-GlcNAcylation of the Drosophila PERIOD protein (dPER) contributed to setting the correct pace of the clock by delaying the timing of dPER nuclear entry [14,15]. In this study, we demonstrated that the mammalian clock protein BMAL1 was O-GlcNAcylated, and this modification influenced BMAL1 stability and circadian gene expression.

2. Materials and methods

2.1. Plasmids and animals

A luciferase construct under the control of the *Bmal1* promoter was a kind gift of Dr. Steven A. Brown (University of Zurich, Switzerland). The cDNA constructs of OGA and OGT were kind gifts of Dr. Gerald. W. Hart (Johns Hopkins University, USA). The 3×Flag and 3×HA tags were added at the N-terminus of OGT and OGA, respectively. A luciferase construct under the control of the *prokineticin 2* promoter (PK2.8-Luc) was a kind gift of Dr. Qun-Yong Zhou (University of California, Irvine, USA). C57BL/6 mice were

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purchased from Model Animal Research Center of Nanjing University (www.nicemice.cn). All animal experiments and the care and use of animals were in accordance with institutional guidelines.

2.2. Real-time bioluminescence monitoring

The NIH3T3 fibroblast cell line stably expressing luciferase under the control of the *Bmal1* promoter [16] was maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin, 100 μg/ml streptomycin and 5 μg/ml puromycin at 37 °C in 5% CO_2. A total of 6×10^5 cells were plated in 35-mm dishes and cultured for 5 days. The cells were treated with 0.1 μM dexamethasone (DEX, Sigma, St. Louis, USA) for 2 h, and the medium was changed to phenol red-free DMEM supplemented with 10% FBS, 10 mM HEPES buffer, 0.1 mM luciferin (Promega, Madison, WI, USA), 100 units/ml penicillin and 100 μg/ml streptomycin. Bioluminescence was monitored continuously with a 32-channel LumiCycle (Actimetrics, Wilmette, IL, USA) and was analyzed by LumiCycle analysis software (Actimetrics, Wilmette, IL, USA).

2.3. Real-time quantitative PCR

Confluent NIH3T3 cells were synchronized by treatment with 0.1 μM DEX for 2 h. Cells were harvested every 4 h starting 24 h after synchronization, and total RNA was extracted using the Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Then, 2 μg of total RNA was reverse-transcribed with RevertAid First Strand cDNA Synthesis Kit (Fermentas, Ontario, Canada). cDNA equivalent to 100 ng of total RNA was used for real-time quantitative PCR with 1×SYBR Green PCR master mix (Takara Shuzo Co., Kyoto, Japan). The relative clock gene expression in comparison with the glyceraldehyde-3-phosphate dehydrogenase gene (GAPDH) was calculated using the comparative C_T method [17].

2.4. Analysis of BMAL1 stability

Cells were incubated with 0.1 mg/ml cycloheximide (CHX, Sigma, St Louis, USA) to inhibit protein synthesis. Cells were lysed with 2×SDS lysis buffer containing 1 mM PMSF and protease inhibitor cocktail (Sigma, St Louis, USA) at 0, 2, 4, 8, and 12 h after CHX treatment. Equal amounts of whole cell lysates were analyzed by immunoblot analysis with the anti-BMAL1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for endogenous BMAL1 in NIH3T3 cells or with the anti-FLAG M2 antibody (Sigma, St Louis, USA) for ectopically expressed Flag-tagged BMAL1 in Human Embryonic Kidney (HEK293) cells.

2.5. Immunoprecipitation

HEK293 cells were transfected with indicated plasmids using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Cells were harvested with RIPA buffer (150 mM NaCl, 1% NP-40, 0.1% SDS, 2 mM EDTA, 50 mM Tris, pH 8.0, 1 mM PMSF, and protease inhibitor cocktail) at 48 h after transfection. Approximately 600 μg of whole cell lysates were incubated with 1 μg of anti-FLAG M2 antibody with constant agitation overnight at 4 °C. Then, 30 μl of protein G agarose bead slurry (Sigma, St Louis, USA) was added to pull down the immunocomplexes. Beads were collected by centrifugation and washed extensively with cold phosphate buffered saline (PBS). After boiling with 2×SDS loading buffer, the protein samples were subjected to 8% SDS–PAGE gel and transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA, USA). The membrane was blocked in Tris-Buffered saline supplemented with 5% bovine serum albumin (BSA). O-GlcNAcylation was detected with the CTD110.6 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

2.6. Glutathione S-transferase (GST) pull-down assay

The expression of GST fusion proteins was induced by 100 mM IPTG for 3 h at 37 °C. Recombinant GST fusion proteins were purified by incubating the *Escherichia coli* extracts with a 30 μl slurry of glutathione-Sepharose beads (GE healthcare, UK) in buffer A (PBS, 1% NP-40 with 1 mM EDTA supplemented with 1 mM PMSF and protease inhibitor cocktail) for 3 h at 4 °C with constant agitation. After washing with buffer A, the beads were resuspended in 1 ml of buffer A containing lysates from cells that transiently expressed Flag-tagged OGT, and incubated at 4 °C overnight with constant agitation. The glutathione beads were collected by centrifugation and washed extensively in buffer A; the bound proteins were eluted in 2×SDS loading buffer and subjected to immunoblotting with anti-FLAG M2 antibody.

2.7. Co-immunoprecipitation

HEK293 cells were transfected with plasmids expressing BMAL1 and Flag-tagged OGT and were harvested with the lysis buffer (150 mM NaCl, 1% NP-40, 2 mM EDTA, 50 mM Tris, pH 8.0, 1 mM PMSF, and protease inhibitor cocktail) at 48 h after transfection. Approximately 600 μg of whole cell lysates were incubated with 1 μg of anti-FLAG M2 antibody with constant agitation overnight at 4 °C. Then, 30 μl of a protein G agarose bead slurry (Sigma, St Louis, USA) was added to pull down the immunocomplexes. The beads were collected by centrifugation, washed extensively with PBS, and boiled with 2×SDS loading buffer; the protein samples were then subjected to immunoblot analysis with the anti-BMAL1 antibody.

2.8. Luciferase reporter assay

HEK293 cells were transfected with the indicated plasmids using Lipofectamine 2000 reagent. Then, 5 ng of reporter plasmids were mixed with 20 ng of expression constructs for BMAL1 and CLOCK in the presence or absence of OGA expression construct. Equal amounts of DNA were used for all of the transfection combinations by adding the appropriate vector DNA. At 48 h after transfection, the cells were washed with PBS and lysed with Reporter Lysis Buffer (Promega, Madison, WI, USA). The extracts were assayed for luciferase activity using the Luciferase Assay System (Promega, Madison, WI, USA) according to the manufacturer's protocol. All reporter assays were conducted at least three times and performed in triplicate on different days using different batches of cells.

2.9. Succinylated wheat germ agglutinin enrichment of O-GlcNAcylated proteins

Cells were lysed in lysis buffer (10 mM HEPES, 150 mM NaCl, 1 mM $CaCl_2$, 1% NP-40, 1 mM PMSF, and protease inhibitor cocktail), and the O-GlcNAcylated proteins were enriched using succinylated wheat germ agglutinin (sWGA)-agarose beads (Vector Laboratories, Burlingame, CA). Then, 500 μg of whole cell lysates were incubated with 30 μl of the sWGA-agarose bead slurry with constant agitation overnight at 4 °C. To demonstrate the binding specificity, N-acetyl-D-glucosamine (N-GlcNAc, Sigma, St Louis, USA) was added to the cell lysates to a final concentration of 0.12 g/ml. sWGA beads were collected by centrifugation and washed extensively with cold PBS; the proteins were eluted in

2×SDS loading buffer and subjected to immunoblotting with the indicated antibodies.

3. Results

3.1. Inhibition of O-GlcNAcylation affected circadian rhythms

To study the effects of O-GlcNAcylation on the circadian rhythms, we set up a bioluminescence monitoring system by stably transfecting a luciferase construct under the control of the *Bmal1* promoter (Bmal1-luc) into NIH3T3 cells. As shown in Fig. 1A, following synchronization with dexamethasone, the bioluminescence observed in these cells showed a circadian rhythm. However, when an O-GlcNAcylation inhibitor, 6-diazo-5-oxo-L-norleucine (DON), was added into the culture medium at a concentration of 100 μM, the circadian rhythm was significantly dampened. As shown in Fig. 1B, DON significantly decreased the O-GlcNAcylation level of proteins in these cells.

To further investigate the circadian gene expression after DON treatment, NIH3T3 fibroblast cells were synchronized and then harvested every 4 h beginning 24 h after synchronization. The clock gene mRNA levels were measured by real-time quantitative PCR. As shown in Fig. 1C–F, the expression of clock genes *Bmal1*, *Dbp*, *Per2*, and *Rev-erba* was significantly downregulated.

3.2. The O-GlcNAcylation of BMAL1 showed circadian rhythms

To identify the O-GlcNAcylated clock proteins, we used a GlcNAc-recognition lectin and succinylated wheat germ agglutinin (sWGA), to pull down ectopically expressed BMAL1, CLOCK, PER1, PER2, CRY1 and detected the eluted proteins with respective antibodies. However, only BMAL1 was pulled down by sWGA (Fig. 2A). The sWGA-BMAL1 interaction was specifically mediated by the glycosylation, as N-acetyl-D-glucosamine (N-GlcNAc) was able to competitively eliminate the binding. Endogenous BMAL1 from the NIH3T3 cells was also pulled down by sWGA, which was also inhibited in the presence of N-GlcNAc (Fig. 2A).

The ectopically expressed BMAL1 was also immunoprecipitated by the M2 antibody, which recognizes the FLAG tag located at the C-terminus of BMAL1, while the O-GlcNAcylation was detected by

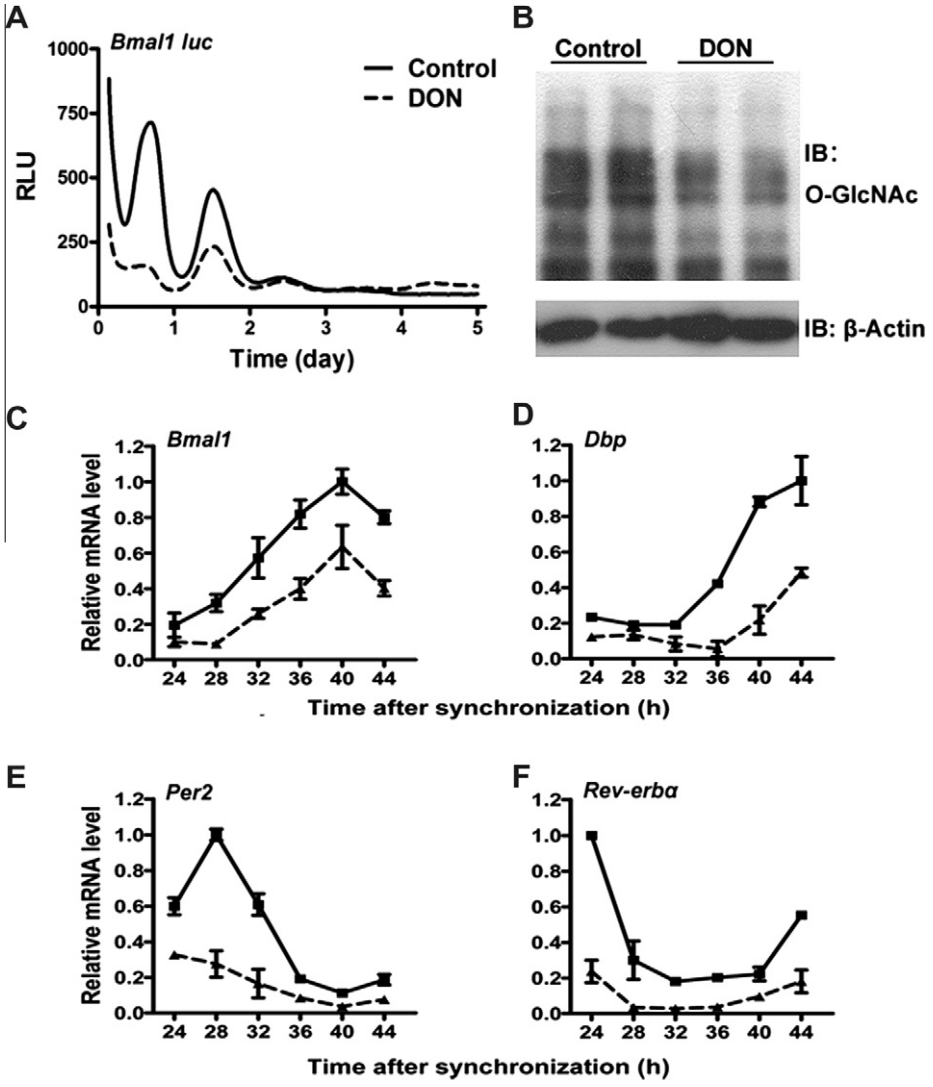

Fig. 1. Inhibition of O-GlcNAcylation dampened the circadian rhythms. (A) O-GlcNAcylation inhibitor, DON, dampened the circadian rhythm. A luciferase construct under the control of *Bmal1* promoter was stably transfected into NIH3T3 cells. The bioluminescence from the synchronized NIH3T3 cells was monitored on a LumiCycle, and 100 μM DON was added to the monitoring medium as indicated. RLU, relative light unit. (B) DON decreased the global O-GlcNAcylation of proteins in NIH3T3 cells, as detected by the CTD110.6 antibody. β-Actin was used as protein loading control. (C–F) DON treatment resulted in decreased clock gene expression in synchronized NIH3T3 cells, and 100 μM DON was added into the culture medium 24 h before synchronization and maintained till the cells were harvested. The data are the means ± SEM of a representative experiment analyzed in triplicate. Solid lines, control; dashed lines, DON.

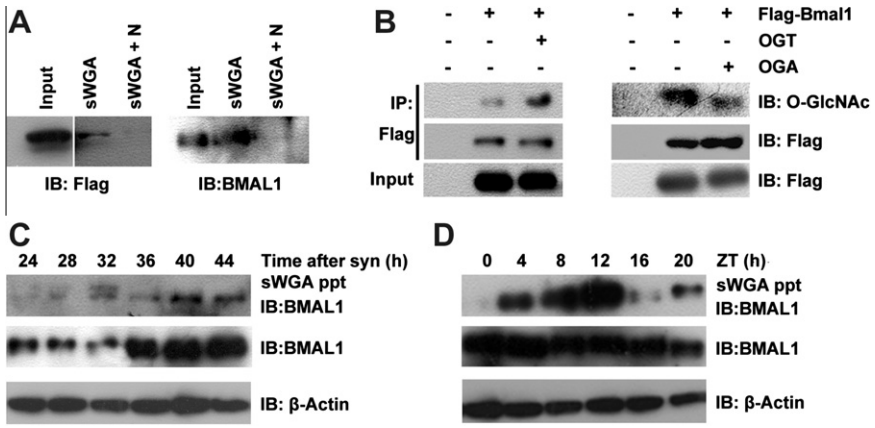

Fig. 2. The O-GlcNAcylation of BMAL1 showed circadian rhythms. (A) Ectopically expressed Flag-BMAL1 was pulled down with sWGA-agarose beads, and the protein was detected with the anti-FLAG M2 antibody. N-GlcNAc was included in the pull-down buffer to competitively inhibit the interaction between sWGA and O-GlcNAcylated protein. Proteins from the NIH3T3 cells were pulled down with sWGA-agarose and were detected with the anti-BMAL1 antibody. N-GlcNAc was included in the pull-down buffer to competitively inhibit the interaction between sWGA and O-GlcNAcylated protein, (B) Ectopically expressed Flag-BMAL1 was immunoprecipitated with the anti-FLAG M2 antibody, and O-GlcNAcylation was detected with the CTD110.6 antibody. OGT increased, whereas OGA decreased the O-GlcNAcylation level of BMAL1, (C) NIH3T3 cells were synchronized with DEX and harvested at indicated time points; the O-GlcNAcylated proteins were enriched by sWGA-agarose, and detected with the anti-BMAL1 antibody and (D) Mouse livers were collected at indicated time points. The liver proteins were extracted and pulled down with sWGA-agarose and analyzed by immunoblot with anti-BMAL1.

an O-GlcNAcylation specific antibody, CTD110.6 (Fig. 2B). As expected, OGT increased, whereas OGA decreased, the O-GlcNAcylation of BMAL1.

Furthermore, the O-GlcNAcylation level of BMAL1 was measured in synchronized NIH3T3 cells and mouse livers harvested around a circadian cycle. As shown in Fig. 2C and D, the protein levels of BMAL1 in the synchronized cells and mouse livers showed a circadian rhythm, and the O-GlcNAcylation of BMAL1 also showed a circadian rhythm in both synchronized NIH3T3 cells and mouse livers.

3.3. BMAL1 interacted with OGT

Next, we investigated whether BMAL1 could interact specifically with OGT by co-immunoprecipitation and a glutathione S-transferase (GST) pull-down assay. As shown in Fig. 3A, the antibody that specifically recognized Flag-tagged OGT was able to pull down BMAL1 when BMAL1 and Flag-OGT were co-expressed, but not when BMAL1 was expressed alone. Further, GST-fused BMAL1 protein, but not GST, was able to pull down Flag-tagged OGT (Fig. 3B). We thus demonstrated specific binding between OGT and BMAL1 *in vitro*.

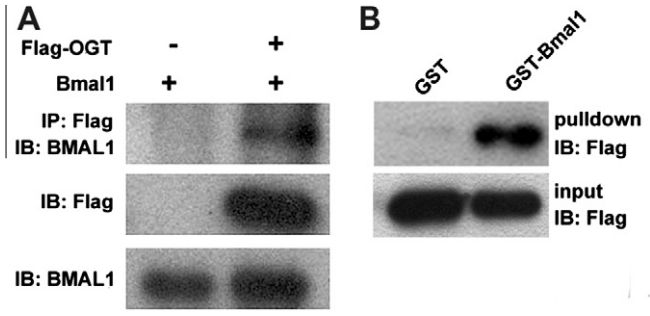

Fig. 3. BMAL1 interacted with OGT as demonstrated by co-immunoprecipitation (A) or GST pull-down (B). (A) The protein immunoprecipitated (IP) by the anti-FLAG M2 antibody was immunoblotted (IB) with anti-BMAL1 antibody. (B) Immobilized GST or GST-BMAL1 was used to pull-down the ectopically expressed Flag-OGT protein, which is detected with the anti-FLAG M2 antibody.

3.4. O-GlcNAcylation enhanced the stability of BMAL1

O-GlcNAcylation may protect proteins against proteasomal degradation by modifying the target proteins or the proteasome itself [18,19]. To explore whether O-GlcNAcylation modification is able to regulate circadian rhythms in this manner, we compared the stability of endogenous BMAL1 in the presence or absence of DON. As shown in Fig. 4A and B, BMAL1 is quite stable after treatment with a protein synthesis inhibitor, CHX. However, addition of DON promoted the degradation of BMAL1 after CHX treatment. Consistent with these data, co-expression of OGA resulted in faster degradation of ectopically expressed BMAL1 (Fig. 4C). Our results thus demonstrated that O-GlcNAcylation might regulate circadian rhythms by affecting BMAL1 stability.

3.5. O-GlcNAcylation regulated BMAL1 transcriptional activity

To study the functional consequences of BMAL1 O-GlcNAcylation, we utilized a luciferase assay to measure the transcriptional activity of BMAL1. A 2.8-kb fragment of *prokineticin 2* gene promoter, containing four E-box elements, was fused to the luciferase gene (pk2.8-luc). As shown in Fig. 4D, the BMAL1/CLOCK heterodimer was able to activate pk2.8-luc, while co-expression of the OGA gene resulted in dose-dependent inhibition of BMAL1/CLOCK transcriptional activity. Moreover, DON also inhibited BMAL1/CLOCK transcriptional activity in a dose-dependent manner (Fig. 4E).

4. Discussion

O-GlcNAcylation on serine or threonine residues of nucleocytoplasmic proteins is a ubiquitous post-translational modification. A number of nutrient- and stress-responsive transcription factors including SP1 [20], p53 [21], NF-κB [22], CREB [23], CRTC2 [24] and FOXO1 [25] have been found to be O-GlcNAcylated. O-GlcNAcylation regulates the functions of these transcription factors by various mechanisms including altering localization, stability, protein–protein interactions, phosphorylation, DNA binding and transcriptional activity [26]. In this study, we demonstrated that O-GlcNAcylation of BMAL1, a transcription factor involved in circadian rhythms, stabilized BMAL1. Interestingly, O-GlcNAcylation of

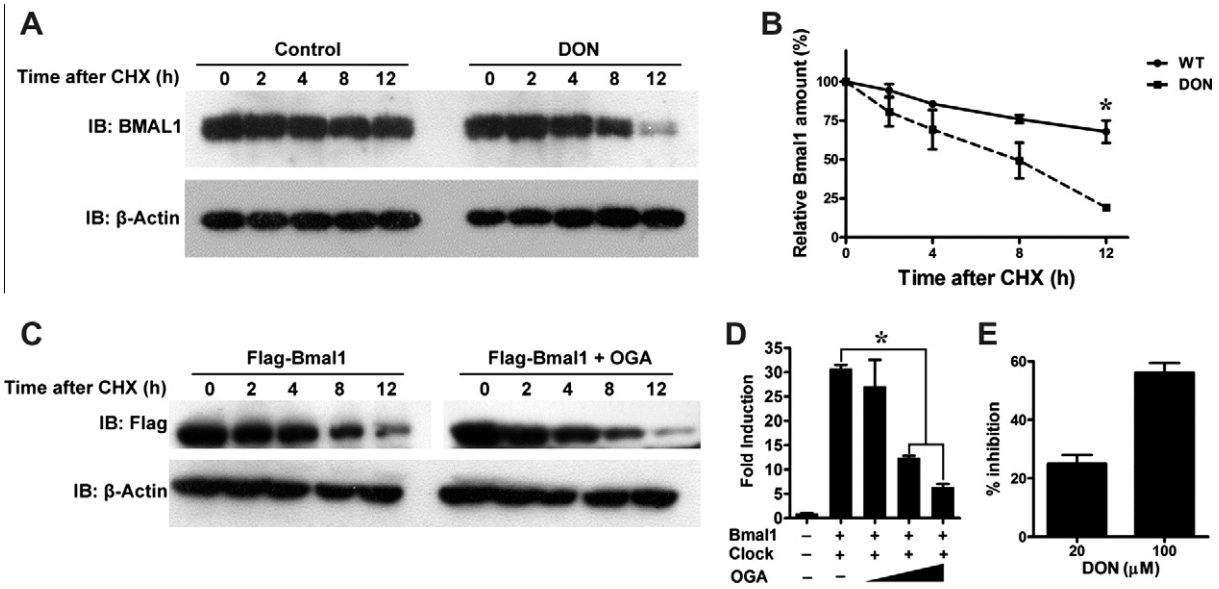

Fig. 4. O-GlcNAcylation regulated BMAL1 stability and transcriptional activity. (A) NIH3T3 cells were cultured in a medium containing 100 μM DON or vehicle for 24 h, and then treated with a protein synthesis inhibitor CHX. The BMAL1 protein levels were detected by immunoblotting at various time points after CHX treatment, (B) Line graph showing quantitation of BMAL1 levels normalized to the β-actin levels. The data are the means ± SEM of triplicate experiments, $^{*}P < 0.05$ by two-way ANOVA with Bonferroni tests, (C) HEK293 cells were transiently transfected with Flag-BMAL1, alone or together with OGA. CHX was added to inhibit the protein synthesis at 48 h after transfection, and BMAL1 protein levels were detected with the anti-FLAG M2 antibody at various time points after CHX treatment, (D) Co-expression of OGA (20, 40 and 80 ng) inhibited the transcriptional activity of BMAL1/CLOCK heterodimer. The data are means ± SEM; $^{*}P < 0.05$ by one-way ANOVA with Dunnett's multiple comparison tests and (E) DON inhibited the transcriptional activity of the BMAL1/CLOCK heterodimer.

dPER, a protein involved in circadian rhythms in drosophila, also influences its stability along with nuclear entry [14].

The dynamic cross-talk between O-GlcNAcylation and phosphorylation is extensive [27]. A recent study found that elevated GlcNAcylation decreased phosphorylation at 280 sites and increased phosphorylation at 148 sites in the 711 phosphopeptides examined [28]. O-GlcNAcylation and phosphorylation may modify the same or proximal Ser/Thr residues, and therefore a competition between O-GlcNAcylation and phosphorylation for occupancy of the Ser/Thr sites may occur [13]. BMAL1 undergoes diverse post-translational modifications, including phosphorylation [29,30], acetylation [11], sumoylation [8] and ubiquitylation [31]. Tamaru et al. demonstrate the phosphorylation of BMAL1 by casein kinase (CK)-2α [29]. Mutation of the highly conserved CK2-phosphorylation site in BMAL1, Ser90, results in impaired nuclear accumulation and disruption of the clock function of BMAL1. GSK3β, a ubiquitous kinase that regulates various cellular functions, has been reported to phosphorylate BMAL1 as well [30]. Ser17 and Thr21 are critical sites of GSK3β-mediated phosphorylation, and inhibition of GSK3β or S17A/T21A mutation enhances BMAL1 stability. Thus, we speculate that O-GlcNAcylation of BMAL1 may enhance protein stability by suppressing phosphorylation of BMAL1 at certain sites. It will be intriguing to decipher the O-GlcNAcylation sites on BMAl1 and investigate the relationship between O-GlcNAcylation and phosphorylation on BMAL1.

While light–dark cycles are the predominant Zeitgebers (timing cues) for the SCN pacemaker, cyclic feeding behavior is a strong Zeitgeber for clocks operating in many peripheral tissues [1,2,32–34]. Recent studies indicate that genetic alteration in the core molecular clock machinery can have pronounced effects on both peripheral and central metabolic regulatory signals [35,36]. Remarkably, CLOCK mutant mice (*Clk*/*Clk*) develop obesity and have impaired glucose homeostasis [37]. Many metabolic systems may consequently affect the function of clock genes and circadian systems [38]. However, the underlying mechanism is not well understood. Our data indicated that O-GlcNAcylation influences the circadian rhythms by modification of BMAL1. O-GlcNAc modification of many transcription factors is tightly regulated, particularly in a glucose-dependent manner. Actually, UDP-GlcNAc, the donor of the O-GlcNAc moiety, is synthesized *de novo* from glucose via the hexosamine biosynthetic pathway (HBP). Approximately 2–5% of glucose entering the cell is diverted into the HBP [13]. Inhibition of O-GlcNAcylation resulted in diminished circadian rhythms and clock gene expression. Our data imply that O-GlcNAcylation might serve as a link between circadian rhythms and energy metabolism.

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