# Supplemental Information

## Validation, identification and biological consequences of the site-specific *O*-GlcNAcylation dynamics of ChREBP

An-Qi Yang,[1] Daoyuan Li,[2] Lianli Chi,[2] Xin-Shan Ye[1,*]

[1]State Key Laboratory of Natural and Biomimetic Drugs, School of Pharmaceutical Sciences, Peking University, Xue Yuan Rd 38, Beijing 100191, China

[2]National Glycoengineering Research Center, and State Key Laboratory of Microbial Technology, Shandong University, Jinan 250100, China

*E-mail: xinshan@bjmu.edu.cn

**Contents:**

**Supplemental Scheme S1.** Schematic diagram of the mammalian hexosamine biosynthetic pathway (HBP) and GlcNAc salvage HBP

**Supplemental Scheme S2.** Schematic diagram of the cell-based validation of ChREBP *O*-GlcNAcylation

**Supplemental Figure S1.** Supplementary evidences for direct *O*-GlcNAcylation on ChREBP and its interactors

**Supplemental Figure S2.** The MS/MS of the $[M+2H]^{2+}$ ion of the *O*-GlcNAcylated peptide $^{513}$ASPPTLASATASPTATATAR$^{532}$ from ChREBP

**Supplemental Figure S3.** The MS/MS of the $[M+3H]^{3+}$ ion of the *O*-GlcNAcylated peptide $^{625}$LSGDLNSIQPSGALSVHLSPPQTVLSR$^{651}$ from ChREBP

**Supplemental Figure S4.** The MS/MS of the $[M+3H]^{3+}$ ion of the *O*-GlcNAcylated peptide $^{625}$LSGDLNSIQPSGALSVHLSPPQTVLSR$^{651}$ from ChREBP

**Supplemental Figure S5.** The MS/MS of the $[M+2H]^{2+}$ ion of the *O*-GlcNAcylated peptide $^{833}$QLSTSTSILTDPSLVPEQATR$^{853}$ from ChREBP

**Supplemental Figure S6.** The MS/MS of the $[M+2H]^{2+}$ ion of the di-*O*-GlcNAcylated peptide $^{513}$ASPPTLASATASPTATATAR$^{532}$ from ChREBP

**Supplemental Figure S7.** The MS/MS of the $[M+2H]^{2+}$ ion of the di-*O*-GlcNAcylated peptide $^{613}$LSPPASSGSER$^{623}$ from ChREBP

**Supplemental Figure S8.** Analysis of ChREBP *O*-GlcNAcylation sites

**Supplemental Figure S9.** Site-mutation analysis of *O*-GlcNAcylation sites on ChREBP.

**Supplemental Figure S10.** Site-specific *O*-GlcNAcylation deficiency on ChREBP affects its transcriptional activity

**Supplemental Figure S11.** *O*-GlcNAcylation deficiency at S839 of ChREBP alters its nuclear export

**Supplemental Figure S12.** Alignment of the *C*-terminal region of the ChREBP and Mlx proteins

**Supplemental Experimental Procedures**

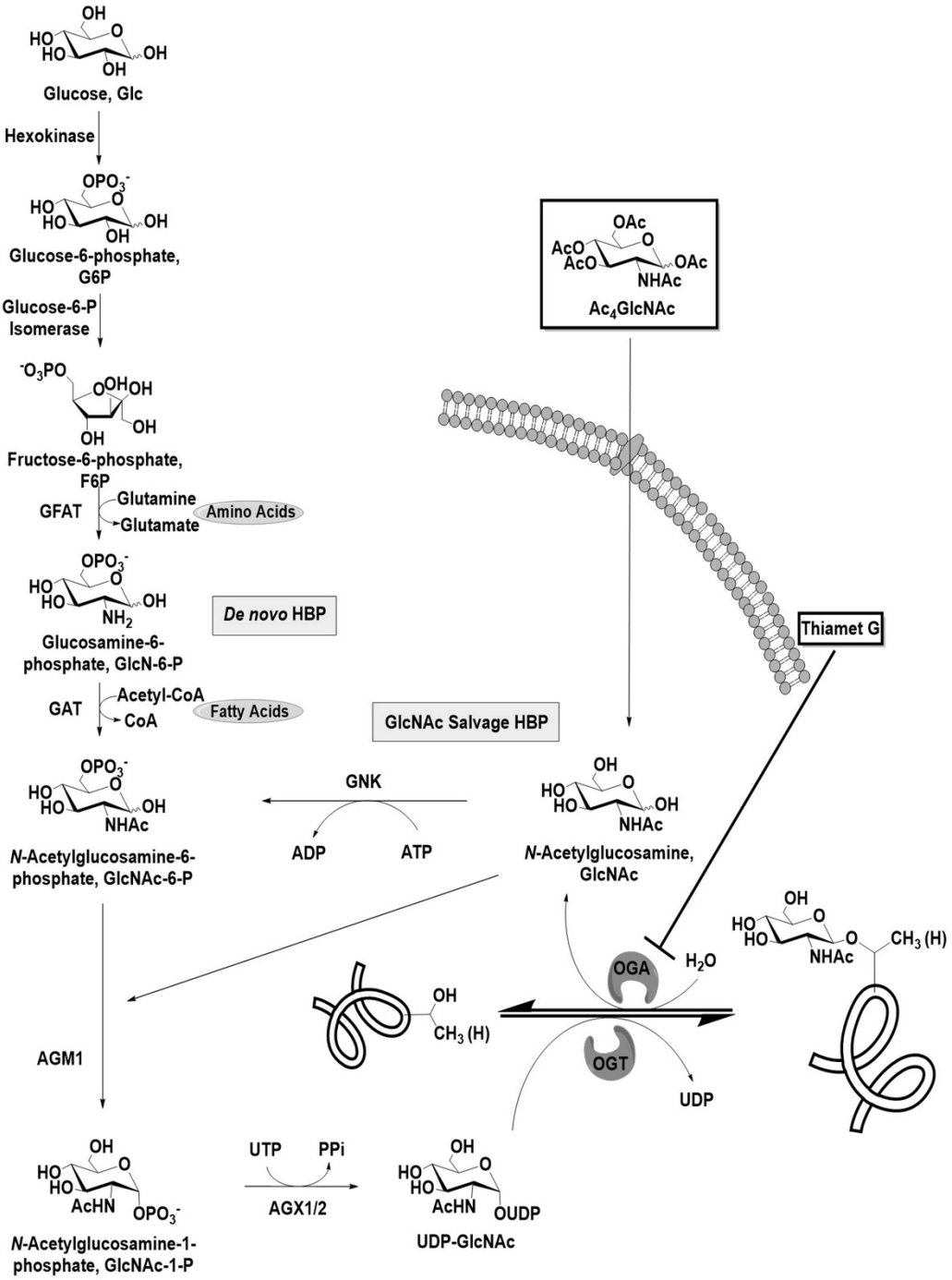
**Supplemental Table S1.** Oligonucleotides used to construct plasmids containing point mutations in the putative

*O*-GlcNAcylation and phosphorylation sites of ChREBP

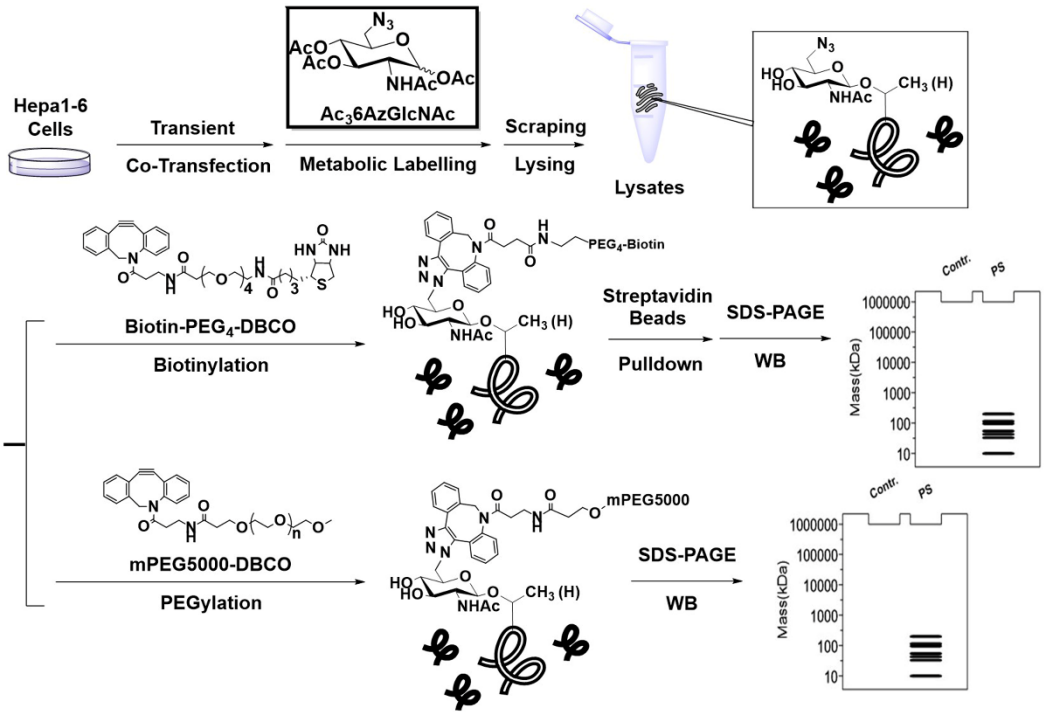
**Appendix: Supplemental Excel File for Supplemental Table S2.** A list of peptide information identified by MS/MS.

**References**

## SUPPLEMENTAL SCHEMES AND FIGURES




**Scheme S1. Schematic diagram of the mammalian hexosamine biosynthetic pathway (HBP) and GlcNAc salvage HBP.** The donor substrate used by OGT, UDP-GlcNAc, is biosynthesized through the action of the enzymes comprising the HBP and the GlcNAc salvage HBP.




**Scheme S2. Schematic diagram of the cell-based validation of ChREBP *O*-GlcNAcylation.**

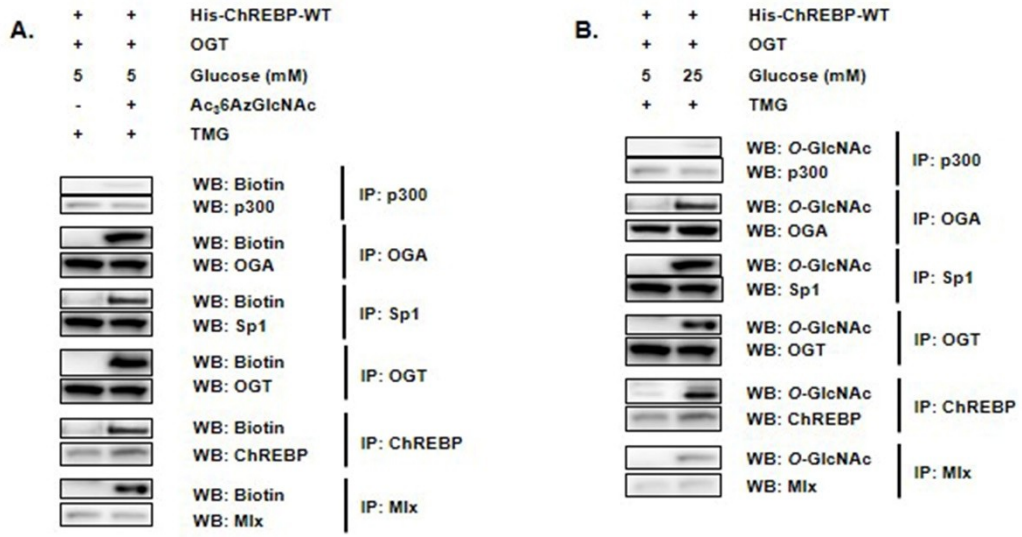



**Figure S1. Supplementary evidences for direct *O*-GlcNAcylation on ChREBP and its interactors. A.** Hepa1-6 cells were transfected and treated as described in Figure 4 legend. The cell lysates were reacted with biotin probe and pulled down by antibodies against different proteins respectively. Eluates from each assay were assessed for biotin by WB using streptavidin-HRP. ChREBP, p300, OGA, OGT, Mlx and SP1 were also detected by WB using corresponding antibodies. **B.** Hepa1-6 cells were co-transfected with OGT and ChREBP and exposed to 5 mM or 25 mM glucose for 24 h with 10 μM TMG treatment for 12 h. The cell lysates were immunoprecipitated with different antibodies against corresponding proteins respectively. Eluates were assessed for *O*-GlcNAc by CTD110.6. Corresponding proteins were also detected by WB. All data are representative of 3 independent experiments.

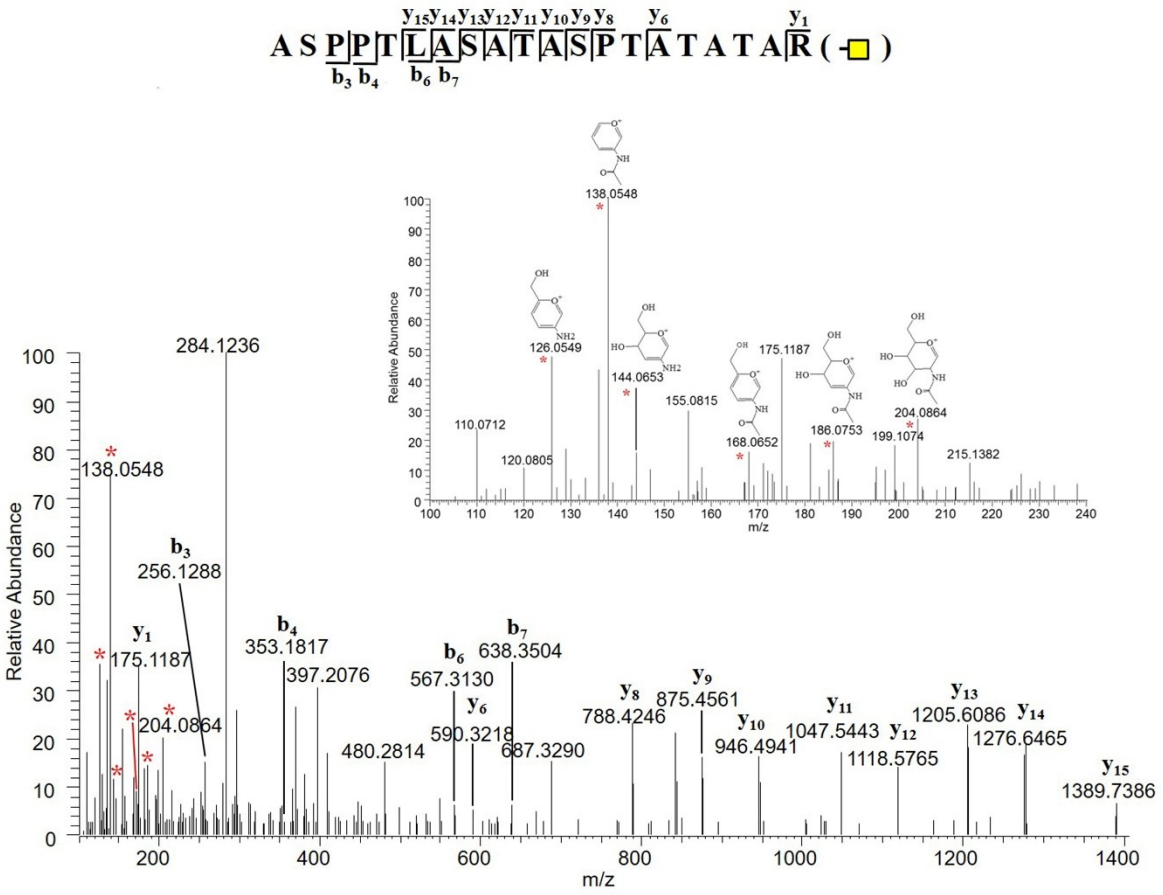



**Figure S2. The MS/MS of the $[M+2H]^{2+}$ ion of the *O*-GlcNAcylated peptide $^{513}$ASPPTLASATASPTATATAR$^{532}$ from ChREBP.** There was one GlcNAc motifs on this peptide (shown in terms of yellow squares). All the serine and threonine residues were likely the *O*-GlcNAcylated site. The GlcNAc fragments were marked with red asterisk.

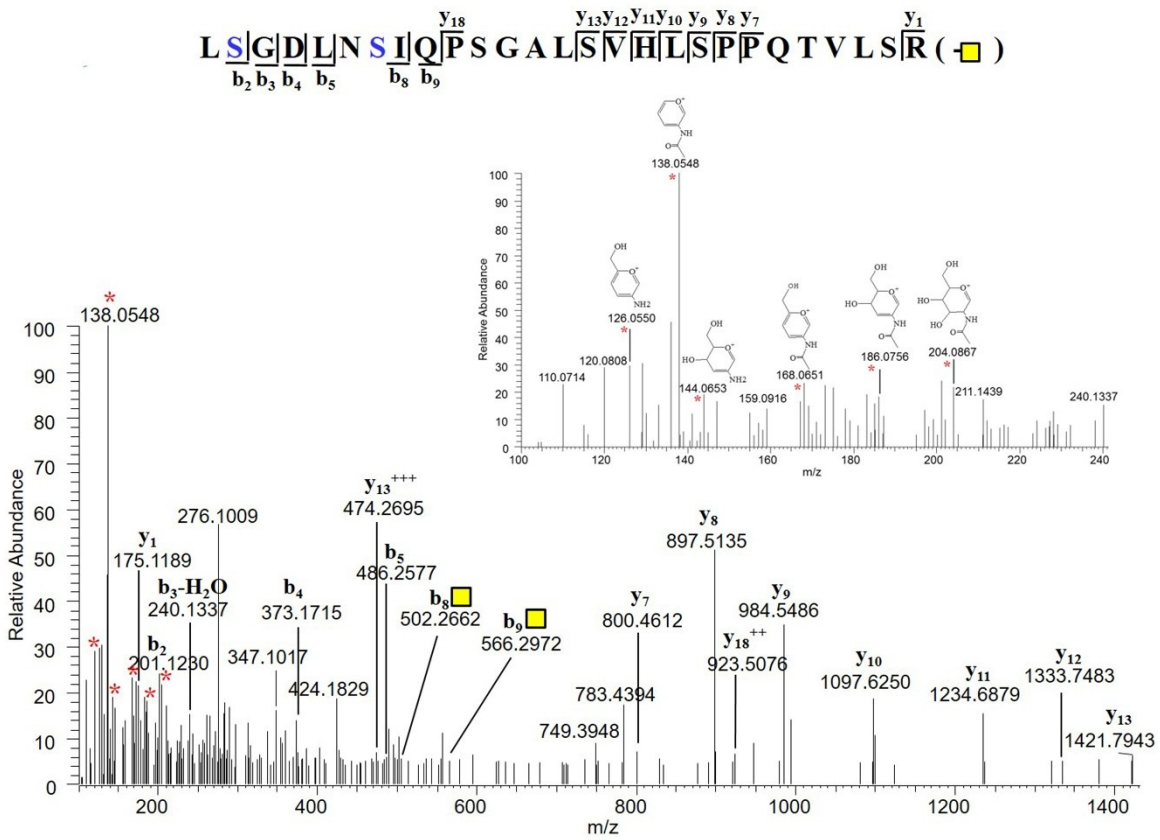



**Figure S3. The MS/MS of the $[M+3H]^{3+}$ ion of the *O*-GlcNAcylated peptide $^{625}$LSGDLNSIQPSGALSVHLSPPQTVLSR$^{651}$ from ChREBP.** There was one GlcNAc motifs on this peptide (shown in terms of yellow squares). The *O*-GlcNAcylated serine residue was one of serine residues written in blue. The GlcNAc fragments were marked with red asterisk.

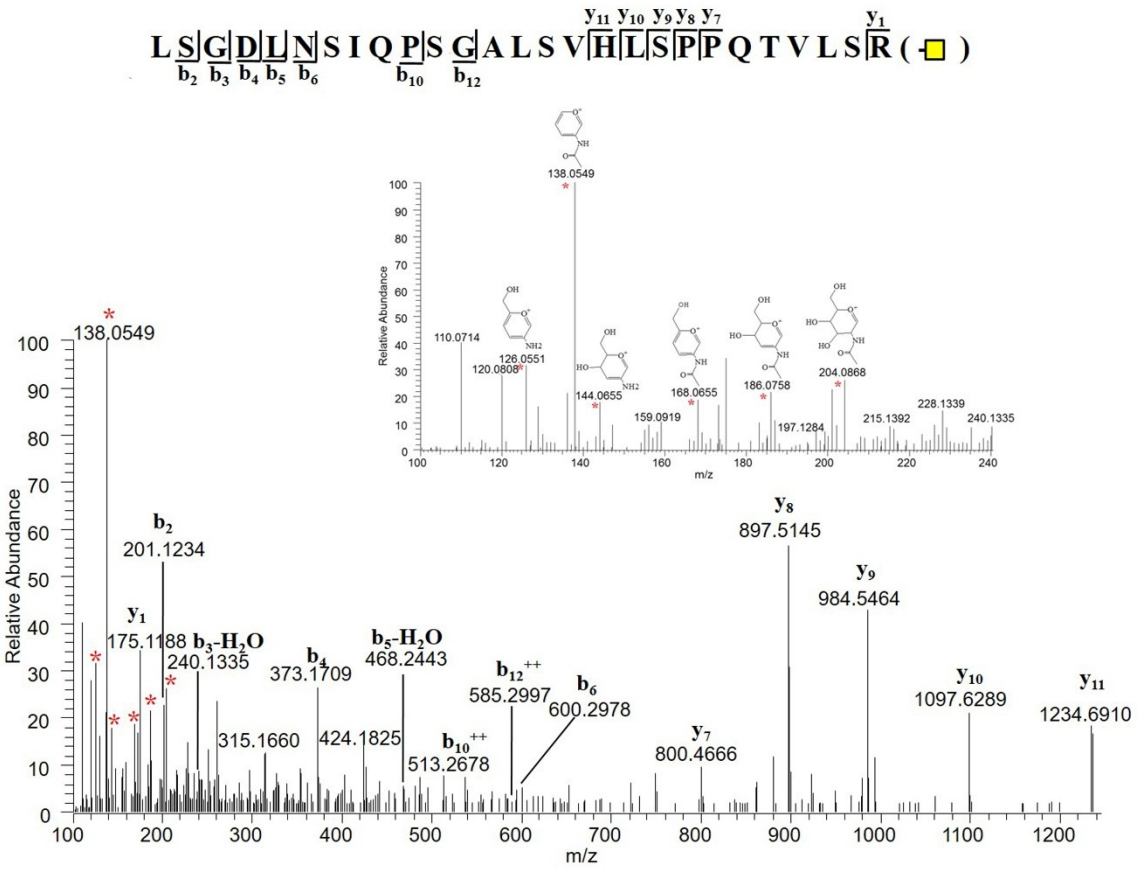



**Figure S4. The MS/MS of the $[M+3H]^{3+}$ ion of the *O*-GlcNAcylated peptide $^{625}$LSGDLNSIQPSGALSVHLSPPQTVLSR$^{651}$ from ChREBP.** There was one GlcNAc motifs on this peptide (shown in terms of yellow squares). All the serine and threonine residues were likely the *O*-GlcNAcylated site. The GlcNAc fragments were marked with red asterisk.

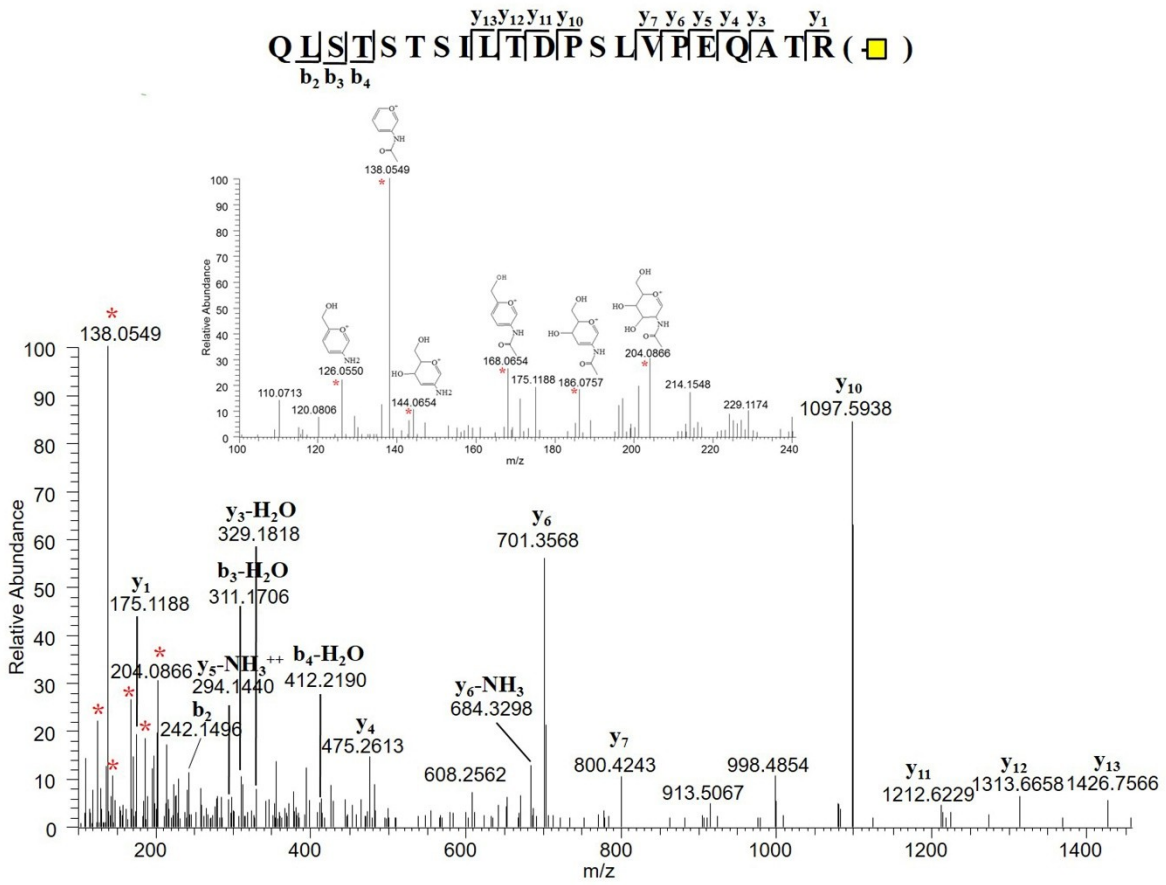



**Figure S5. The MS/MS of the $[M+2H]^{2+}$ ion of the *O*-GlcNAcylated peptide $^{833}$QLSTSTSILTDPSLVPEQATR$^{853}$ from ChREBP.** There was one GlcNAc motifs on this peptide (shown in terms of yellow squares). All the serine and threonine residues were likely the *O*-GlcNAcylated site. The GlcNAc fragments were marked with red asterisk.

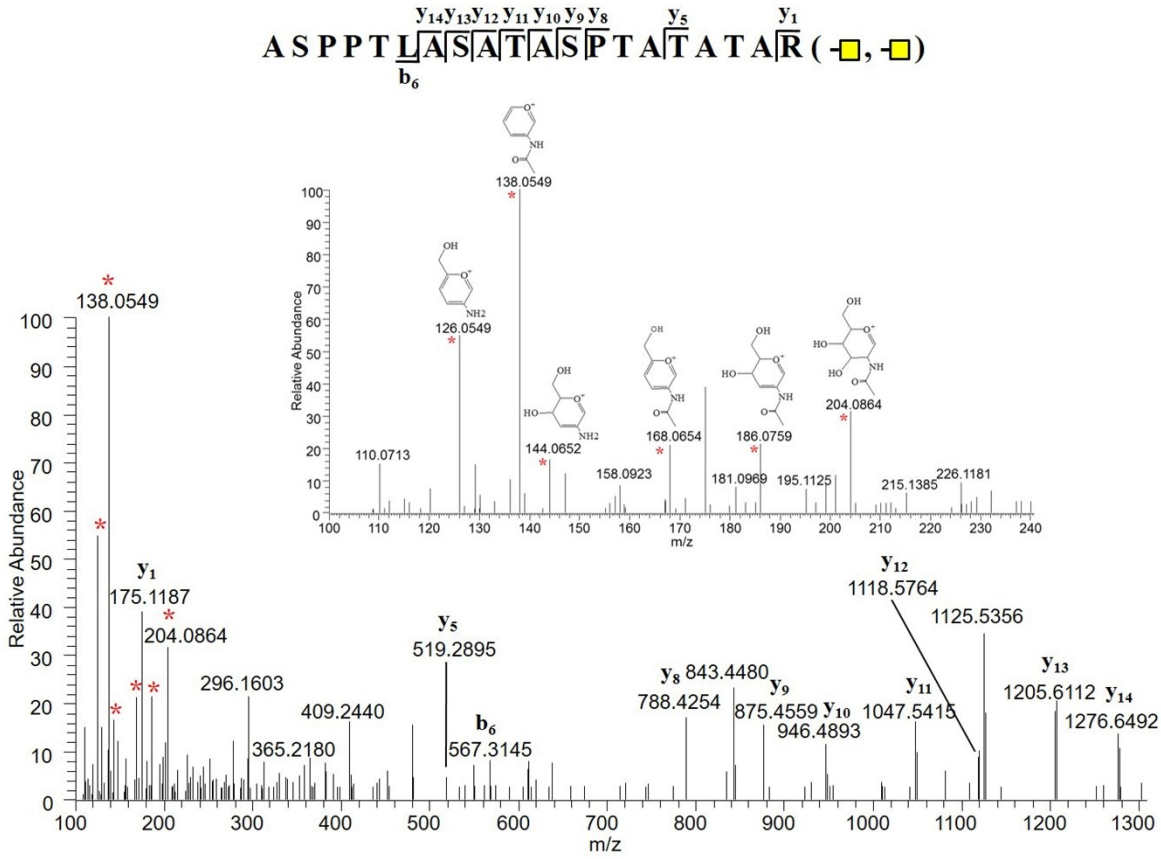



**Figure S6. The MS/MS of the $[M+2H]^{2+}$ ion of the di-*O*-GlcNAcylated peptide $^{513}$ASPPTLASATASPTATATAR$^{532}$ from ChREBP.** There were two GlcNAc motifs on this peptide (shown in terms of yellow squares). All the serine and threonine residues were likely the *O*-GlcNAcylated site. The GlcNAc fragments were marked with red asterisk.

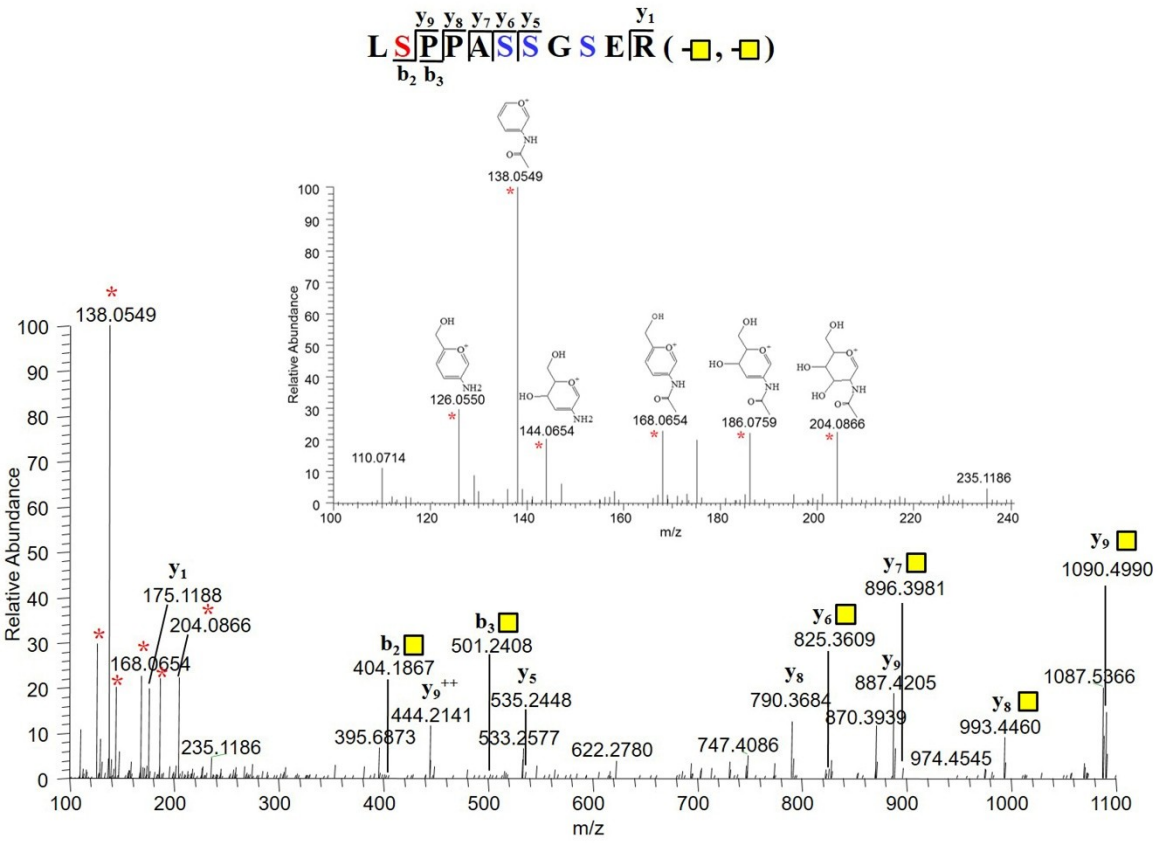



**Figure S7. The MS/MS of the $[M+2H]^{2+}$ ion of the di-*O*-GlcNAcylated peptide $^{613}$LSPPASSGSER$^{623}$ from ChREBP.** The identified *O*-GlcNAcylated serine residue was written in red and the GlcNAc motifs were shown in terms of yellow squares. The other *O*-GlcNAcylated serine residue was one of serine residues written in blue. The GlcNAc fragments were marked with red asterisk.


**Figure S8. Identification of ChREBP *O*-GlcNAcylation sites by MS/MS. A.** From all of the MS/MS analyses performed, ~56% total coverage was achieved for ChREBP. Peptides generated using trypsin were shaded grey, and *O*-GlcNAcylated peptides were shaded yellow. The *O*-GlcNAcylation site identified by MS was written in red and all sites studied in this study was written in green. **B.** Multiple protein sequence alignment of the *C*-terminal region of ChREBP from different species (up), as well as between ChREBP and MondoA (down) using conserved domain and local sequence similarity information via NCBI BLAST+ 2.5.0. The red color indicated residues highly conserved and blue indicated less conserved. *O*-GlcNAcylated serine and threonine residues of ChREBP were shaded yellow.

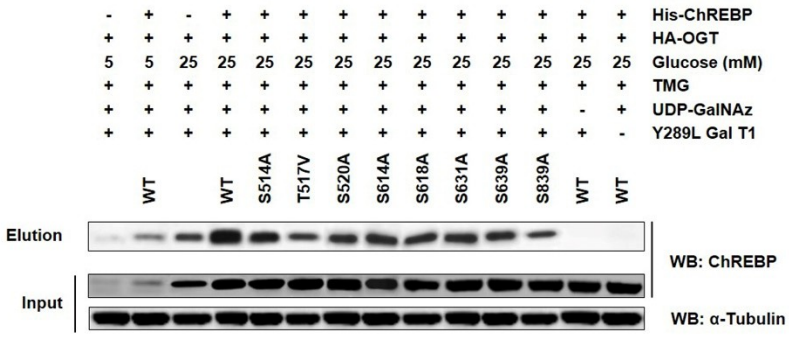



**Figure S9. Site-Mutation analysis of *O*-GlcNAcylation sites on ChREBP.** Each ChREBP construct was co-transfected with OGT into HEK293T cells and the cells were incubated under the indicated conditions with TMG treatment for 24 h. *O*-GlcNAcylated proteins in cell lysates were chemoenzymatically tagged with an azido-galactose sugar using a mutant galactosyltransferase (GalT1, Y289L) and the non-natural nucleotide sugar analogue UDP-GalNAz, and then biotinylated by reaction of the azido-galactose sugar with an alkyne-functionalized biotin molecule. The biotinylated proteins were pulled down using streptavidin beads, and eluted with SDS. Lysates prior to pull down (input) and the captured proteins (elution) were immunoblotted with an antibody towards ChREBP. *O*-GlcNAcylation levels of WT ChREBP were much stronger compared with putative *O*-GlcNAcylation site deficient mutants expressed in HEK293T cells. Control experiments in the absence of GalT1 or UDP-GalNAz demonstrated selective labelling of the *O*-GlcNAcylation on ChREBP.

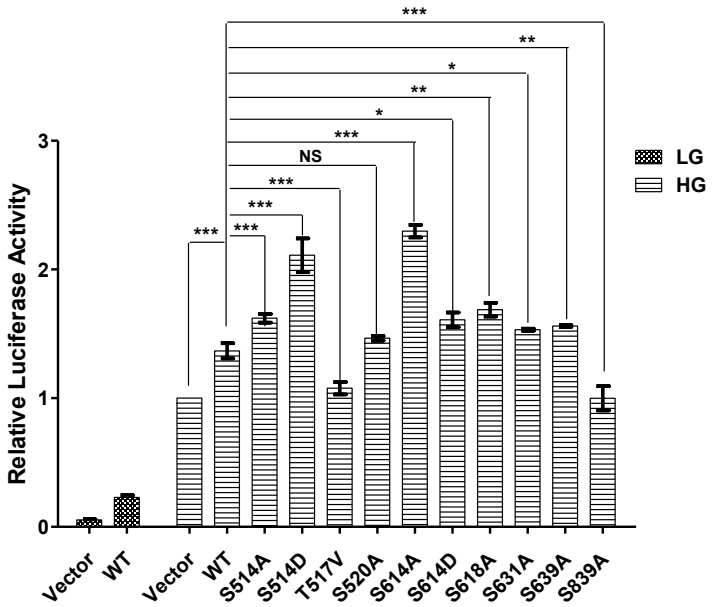



**Figure S10. Site-Specific *O*-GlcNAcylation deficiency on ChREBP affects its transcriptional activity.** Transcriptional reporter gene assay was performed as described in the supplementary method. Values were shown as relative light units to that of cells transfected with vector and cultured in HG conditions. Error bars denote the mean ± SEM, n = 7. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; NS: no significance.

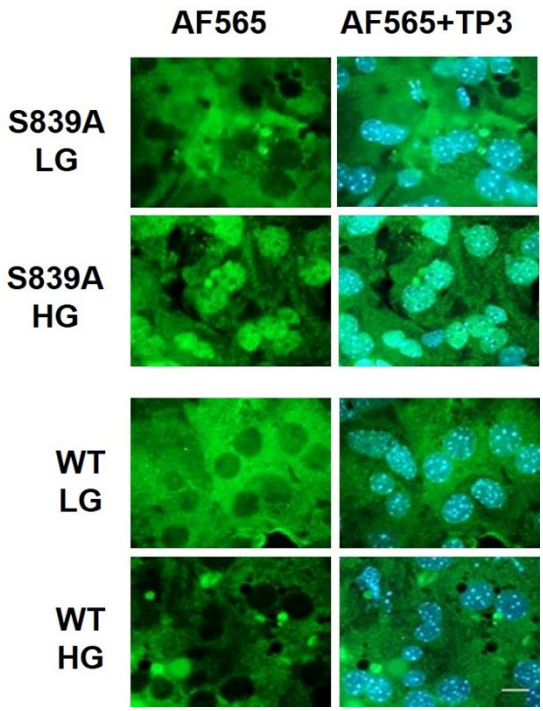



**Figure S11. *O*-GlcNAcylation deficiency at S839 of ChREBP alters its nuclear export.** Hepa1-6 cells were transfected with His-tagged WT ChREBP or the S839A mutant and incubated for 24 h. Cells were then treated with low or high glucose and 50 μM TMG for 6 h before fixation. Cells were PFA fixed and stained with a monoclonal antibody against His-tag. Immunofluorescence was detected with an Alexa Fluor 565 (AF565)-conjugated secondary antibody (Thermo Scientific) using confocal microscopy and shown in green. Nuclei were stained with TO-PRO®-3 (TP3, Thermo Scientific Molecular Probes™) and shown in blue. The AF565+TP3 panels showed the Alexa Fluor 565 and nuclear images merged. Scale bar, 20 μm. More S839A ChREBP was arrested in the nucleus under the HG condition compared with WT ChREBP.


**Figure S12. Alignment of the *C*-terminal region of the ChREBP and Mlx proteins.** Multiple protein sequence alignment of the *C*-terminal region of ChREBP (Q99MZ3) with Mlx (O08609) using conserved domain and local sequence similarity information via NCBI BLAST+ 2.5.0. The red color indicated residues highly conserved and blue indicated less conserved.

## SUPPLEMENTAL EXPERIMENTAL PROCEDURES

**Materials and General Methods.** All reagents used for chemical synthesis were purchased from Sigma-Aldrich, Alfa Aesar or EMD Millipore unless otherwise specified and used without further purification. DBCO-$PEG_4$-Biotin and DBCO-mPEG5000 were purchased from Click Chemistry Tools. Western blot reagents were purchased from Bio-Rad and Life Technologies. All anhydrous reactions were performed under argon atmosphere. Analytical TLC was conducted on EMD Silica Gel 60 $F_{254}$ plates with detection by ceric ammonium molybdate (CAM), anisaldehyde, or UV. $^1$H NMR spectra were obtained on a Bruker AVANCE III 400 MHz spectrometer in $CD_3OD$ or $CDCl_3$ using tetramethylsilane (TMS) as an internal standard.

**Isolation and Culture of Mouse Primary Hepatocytes.** Mouse primary hepatocytes were isolated from male C57BL/6 mice, 6-8 week old, according to M. N. Berry's method (11) and plated at a density of $5 \times 10^6$ cells /100-mm culture dish (Corning). After 12 h attachment period in modified Williams E media (Gibco, Life Technologies) supplemented with 23 mM HEPES, 26 mM sodium bicarbonate, 2 mM glutamine, 0.617 pM insulin, 0.01 mM dexamethasone, and 10 mM lactate, the medium was replaced with glucose-free Dulbecco's modified Eagle's medium (Gibco, Life Technologies) supplemented with 10% fetal bovine serum (Gibco, Life Technologies), 100 units/mL penicillin and 100 μg/mL streptomycin (Hyclone, GE), and indicated concentrations of glucose (Sigma). Hepatocytes were cultured an additional 24 h before harvested for western blot.

**Molecular Biology.** A synthetic ORF of Mouse ChREBP (Q99MZ3.1) with an *N*-terminus $His_6$-tag (Life Technologies) was cloned into expression construct in pCMTNT™ Vector (Promega). A synthetic ORF of Mouse Mlx (O08609.1) with an *N*-terminus $His_6$-tag (Life Technologies) was cloned into expression construct in pcDNA3.1 (Invitrogen). The promoter region between positions -206 and -7 of the L-PK gene was ligated into the firefly luciferase reporter plasmid, pGL-3 basic vector (Promega), as described previously (12). Point mutations in the ChREBP clones were created in the putative *O*-GlcNAcylation and phosphorylation sites using QuikChange® site-directed mutagenesis kit (Stratagene) according to the manufacturer's instruction. Oligonucleotides used to introduce the desired point mutations are listed in **Table S1.** The double-point-mutated plasmids were constructed by using the same method as that used for making single-point mutants. The mutations and coding sequences were confirmed by DNA sequencing. All constructions were transformed into DH5α (Biomed), amplified and prepared using PureYield™ Plasmid Midiprep System according to manufacturer's instruction.

**Cell Culture and Transfections.** The cell lines HEK293T and Hepa1-6 were obtained from ATCC and cultured according to ATCC protocols. Mouse primary hepatocytes were isolated according to the detailed method described above. All cells were maintained in a 5% $CO_2$, water-saturated atmosphere at 37 °C. Transiently transfections of cells with indicated expression constructs were performed using Opti-MEM (Life Technologies) and Lipofectinamine™2000 (Life Technologies) according to the manufacturer's instructions. The DNA/Liposome mixture was allowed to remain on the cells overnight and then was replaced with experimental media. Cells were cultured an additional 24 h before harvested.

**Transcriptional Reporter Gene Assay.** Hepa1-6 cells were seeded in six-well plates (Corning) and cultured for 12 h before transfection. Cells were transfected as method described above. A mixture of firefly luciferase reporter plasmid (2 μg) and a renilla luciferase control plasmid pRL-CMV (Promega) (25 ng) were co-transfected with 2 μg of each expression plasmid for ChREBP mutants. Cells were cultured in the low glucose condition (LG, 5 mM) for 12 h to allow expression of recombinant proteins. Subsequently, cells were maintained in LG medium or switched to the high glucose condition (HG, 25 mM) with Thiamet G (10 μM) for 12 h, and cell lysates were harvested the following day in Passive Lysis Buffer (Promega). Dual-luciferase assays were performed following the manufacturer's instructions.

**Modulation of Cellular *O*-GlcNAc Levels.** For OGT overexpression experiments, HEK293T cells or Hepa1-6 cells were transiently transfected with a pDEST26-HA-OGT vector (provided by Professor G.-J. Boons) using Lipofectamine™ 2000 reagent (Invitrogen) according to the manufacturer's protocol. Cells were collected 24 h after transfection, lysed in RIPA lysis buffer containing 10 μM *O*-(2-acetamido-2-deoxy-D-glucopyranosylidene)amino *N*-phenylcarbamate (PUGNAc; Santa Cruz Biotechnology), and immunoblotted for *O*-GlcNAc levels. For Thiamet G treatments to enhance *O*-GlcNAc levels, cells cultured in DMEM high or low glucose media (with 10% FBS, 100 U/mL penicillin/streptomycin) were treated with Thiamet G (10 μM, Sigma) for 12 h and then immunoblotted for *O*-GlcNAc levels. OGT overexpression and Thiamet G treatment did not affect cell viability, as determined by trypan blue exclusion.

**Cell Extracts and Protein Preparation.** Cells were treated as indicated, collected by scraping, pelleted by centrifugation at 4 °C for 5 min at 500 × g, and washed twice with ice-cold PBS (1 mL).

For the preparation of Non-idet P-40 (NP-40)-soluble lysates, cell pellets were resuspended in 1% NP-40 Lysis Buffer [1% NP-40, 150 mM NaCl, 50 mM triethanolamine (TEA), pH 7.4] with Halt™ Protease and Phosphatase Inhibitor Cocktail (100X) (Thermo Fisher Scientific) on ice for 30 min. Then the supernatant (soluble cell lysate) was collected by centrifuging at 4 °C for 20 min at 16000 × g. The protein concentration was determined by bicinchoninic acid (BCA) assay (Thermo Fisher Scientific).

For the preparation of nuclear and cytoplasmic extracts, cell pellets were treated with NE-PER® Nuclear and Cytoplasmic Extraction Reagents (Thermo Fisher Scientific) according to the manufacturer's instructions. The protein concentration was determined by BCA assay.

For the preparation of RIPA total cell lysis, cell pellets were resuspended in RIPA Lysis Buffer [50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% NP-40, 0.1% SDS, 0.5% sodium deoxycholate] with Halt™ Protease and Phosphatase Inhibitor Cocktail (100X) on ice for 30 min. Then the supernatant (soluble cell lysate) was collected by centrifuging at 4 °C for 20 min at 16000 × g. The protein concentration was determined by BCA assay.

**Chemical Synthesis and Labeling Using Copper-free Click Chemistry.** $Ac_3$6AzGlcNAc was synthesized as described in the literature report (13). For biotinylation, proteins at ~1 mg/mL in labeling buffer (8 M urea, 10 mM HEPES, pH 7.9, Halt™ protease and phosphatase inhibitor cocktail, EDTA-free, 1 mM PMSF) were labeled by the addition of DBCO-$PEG_4$-biotin to a final concentration of 50 μM and agitated mildly at 37 °C for 2 h protecting from light, followed by precipitation and further analysis. To quantitatively visualize the *O*-GlcNAc-modified proteins, protein samples labeled with the azide at ~1 mg/mL in labeling buffer (8 M urea, 10 mM HEPES, pH 7.9, 2% (w/v) CHAPS, 1 mM DTT, Halt™ protease and phosphatase inhibitor cocktail, 1 mM PMSF) were reacted with 1 mM DBCO-mPEG5000 at r.t. for 16 h, before further analysis.

**Protein Precipitation Using Chloroform ($CHCl_3$)/Methanol (MeOH) Precipitation Method.** Protein concentration was normalized by BCA assay to 1 mg/mL and 200 μL of protein was precipitated by sequential mixing with 600 μL of MeOH, 200 μL of $CHCl_3$ and 450 μL $H_2O$ on ice. It was centrifuged at 22,000 × g for 15 min, and then as much of the upper aqueous phase as possible was carefully discarded while leaving the interface layer containing the protein precipitate intact. Precipitated proteins were washed with 450 μL of MeOH and centrifuged at 22,500g for 15 min. The pellet was then air-dried for 1 h and the air-dried protein pellet was resuspended in different buffers by bath sonication for further use.

**Biotin Pull-down Assay.** 500 μg of precipitated biotinylated proteins were resolubilized in Resolubilization Buffer (1% SDS, 50 mM $Na_2HPO_4$, pH 8.0, 100 mM NaCl) and neutralized with a twice volume of Neutralization Buffer (6% NP-40, 50 mM $Na_2HPO_4$, pH 8.0, 100 mM NaCl) to a final protein concentration of 1 mg/mL. Proteins were then incubated with 30 μL of Strepavidin-Beads (BioVision) with

end-to-end rotation at 4 °C overnight. Beads were then washed five times with 1 mL of Low-Salt Buffer (50 mM $Na_2HPO_4$, pH 8.0, 100 mM NaCl, 0.1% SDS, 1% Triton X-100, 0.5% sodium deoxycholate) and five times with 1 mL of High-Salt Buffer (50 mM $Na_2HPO_4$, pH 8.0, 500 mM NaCl, 1% Triton X-100). Bound proteins were eluted by boiling the agarose in 2× Loading Buffer with 20 mM free biotin for 10 min. Western blot analysis was carried out with anti-ChREBP antibody (Abcam). To quantify the level of glycosylation, the intensity of the total ChREBP band (Input) and the glycosylated ChREBP band (Elution) were measured, and the ratio of the intensity of the glycosylated protein versus the intensity of the total protein was taken as the level of glycosylation.

**DNA Probe Pull-down Assay.** Cells were treated as indicated, collected by scraping, pelleted by centrifugation at 4 °C for 5 min at 500 × g, and washed twice with ice-cold PBS (1 mL). Then cells were lysed in RIPA Lysis Buffer and the protein concentration was normalized by BCA assay to 1.5 mg/mL. 500 μg of cell lysate was neutralized with a twice volume of Neutralization Buffer (6% NP-40, 50 mM $Na_2HPO_4$, pH 8.0, 100 mM NaCl) to a final protein concentration of 1 mg/mL. Proteins were then incubated with 20 nM DNA probe with end-to-end rotation at 4 °C overnight, after which 30 μL of Strepavidin-Beads (BioVision) were added and incubated for another 4 h. Beads were then washed five times with 1 mL of Low-Salt Buffer (50 mM $Na_2HPO_4$, pH 8.0, 100 mM NaCl, 1% Triton X-100) and five times with 1 mL of High-Salt Buffer (50 mM $Na_2HPO_4$, pH 8.0, 500 mM NaCl, 1% Triton X-100). Bound proteins were eluted by boiling the agarose in 2× Loading Buffer with 20 mM free biotin for 10 min. Western blot analysis was carried out with anti-ChREBP antibody (Abcam).

**Immunoprecipitation.** 500 μg of the obtained cell lysates were incubated with indicated amount of anti-ChREBP (abcam) or anti-His-tag (MBL) antibody as given in antibody datasheets for 4 h at 4 °C. 20 μL of 50% slurry of protein G-agarose (Santa Cruz Biotechnology) was added and the protein mixture was incubated at 4 °C overnight. After 4 washes with lysis buffer, proteins were eluted from the beads in 2× Loading Buffer, separated by SDS-PAGE and revealed by western blot.

**Western Blot**. Following lysates preparation, protein concentration normalization and Click Reaction (where indicated), 30 μg of proteins of each sample were separated by SDS-PAGE at 130V before being transferred to PVDF membrane (0.45 μm, Merck Millipore) at 300 mA for 50 min. Blots were blocked by 5% BSA in TBST (25mM Tris-HCl, pH7.4, 150 mM NaCl, 0.1% Tween-20) at room temperature for 1 h, then incubated with corresponding primary antibody at 4°C overnight. After washing with TBST for 5 × 5 min, blots were incubated with HRP-conjugated anti-mouse or anti-rabbit antibodies (Jackson ImmunoResearch) at 1：10,000 dilutions for 1 h at room temperature, then washed with TBST for 5 × 5 min again. The blots were developed using ECL reagents (Thermo Fisher Scientific) and the ChemiDoc XRS+ molecular imager (Bio-Rad). All western blot results were repeated in three independent experiments. Antibodies used in this part were obtained from the following sources: *O*-GlcNAc antibody (CTD110.6, Sigma-Aldrich, 1:2000 working dilution), Ubiquitin antibody (abcam, 1:2000 working dilution), ChREBP antibody (abcam, 1:1500 working dilution), His-tag antibody (MBL, 1:5000 working dilution), OGT antibody (DM-17, Sigma-Aldrich, 1:1000 working dilution), OGA antibody (Sigma-Aldrich, 1:1000 working dilution), Mlx antibody (Sigma-Aldrich, 1:1000 working dilution), p300 antibody (Santa Cruz Biotechnology, 1:1000 working dilution), sp1 antibody (Santa Cruz Biotechnology, 1:200 working dilution), β-Actin antibody (MBL, 1:2000 working dilution), α-Tubulin antibody (MBL, 1:2000 working dilution), and Streptavidin-HRP (CST, 1:2000 working dilution).

**Expression and Purification of *O*-GlcNAcylated ChREBP.** ChREBP and OGT expression constructs were transiently co-transfected into HEK293T cells and allowed for expression in DMEM high-glucose media (with 10% FBS, 100 U/mL penicillin/streptomycin) for 36 h with 10 μM Thiamet G treatment for 12 h before cells harvested. To purify $His_6$-tagged ChREBP, cells were lysed in Urea Lysis Buffer (50 mM $Na_2HPO_4$, pH 8.0, 500 mM NaCl, 8 M Urea). The lysate (300 mg) was incubated with 10 mL prewashed Ni-NTA Agarose (Qiagen)

at 4 °C overnight with end-to-end rotation, and then the beads were collected, washed, and eluted according to the manufacturer's instruction. The eluent was further purified and concentrated using an Amicon Ultra Centrifugal Filter (10 kDa molecular weight cutoff, Millipore) in a buffer containing 8 M Urea, 50 mM $Na_2HPO_4$, pH 7.4, and 500 mM NaCl.

**Table S1. Oligonucleotides used to construct plasmids containing point mutations in the putative *O*-GlcNAcylation and phosphorylation sites of ChREBP.**

| Mutant | Orientation | Sequence |
|---|---|---|
| S514A | Forward | 5'-GGACAAAAAGCAGCTCCACCCACCTTG-3' |
| | Reverse | 5'-CAAGGTGGGTGGAGCTGCTTTTTGTCC-3' |
| S514D | Forward | 5’-GGGACAAAAAGCAGATCCACCCACCTTG-3’ |
| | Reverse | 5’-CAAGGTGGGTGGATCTGCTTTTTGTCCC-3’ |
| T517V | Forward | 5’-CAAAAAGCAAGTCCACCCGTCTTGGCCTCTGCCACCG-3’ |
| | Reverse | 5’-CGGTGGCAGAGGCCAAGACGGGTGGACTTGCTTTTTG-3’ |
| S520A | Forward | 5’-GTCCACCCACCTTGGCCGCTGCCACCGCCAGTCCC-3’ |
| | Reverse | 5’-GGGACTGGCGGTGGCAGCGGCCAAGGTGGGTGGAC-3’ |
| S614A | Forward | 5’-GCAGAGCGGCTCGCACCTCCAGCCTC-3’ |
| | Reverse | 5’-GAGGCTGGAGGTGCGAGCCGCTCTGC-3’ |
| S614D | Forward | 5’-GCAGAGCGGCTCGATCCTCCAGCCTC-3’ |
| | Reverse | 5’-GAGGCTGGAGGATCGAGCCGCTCTGC-3’ |
| S618A | Forward | 5’- GGCTCTCGCCTCCAGCCGCCAGCGGCAGTGAGCGG-3’ |
| | Reverse | 5’-CCGCTCACTGCCGCTGGCGGCTGGAGGCGAGAGCC-3’ |
| S631A | Forward | 5’-GGGGATCTCAACGCCATACAACCCTC-3’ |
| | Reverse | 5’-GAGGGTTGTATGGCGTTGAGATCCCC-3’ |
| S639A | Forward | 5’-CTCGGGGGCACTGGCAGTCCACCTGTCTC-3’ |
| | Reverse | 5’-GAGACAGGTGGACTGCCAGTGCCCCCGAG-3’ |
| S839A | Forward | 5’-CAGCACTTCCACAGCCATCCTGACTGAC-3’ |
| | Reverse | 5’-GTCAGTCAGGATGGCTGTGGAAGTGCTG-3’ |

The mutated nucleotides are underlined.

**Appendix: Supplemental Excel File for Table S2.** A list of peptide information identified by MS/MS.

## REFERENCES

(1) Cairo, S., Merla, G., Urbinati, F., Ballabio, A., and Reymond, A. (2001) WBSCR14, a gene mapping to the Williams--Beuren syndrome deleted region, is a new member of the Mlx transcription factor network. *Hum. Mol. Genet. 10*, 617-627.

(2) Stoeckman, A. K., Ma, L., and Towle, H. C. (2004) Mlx is the functional heteromeric partner of the

carbohydrate response element-binding protein in glucose regulation of lipogenic enzyme genes. *J. Biol. Chem. 279*, 15662-15669.
(3) Li, M. V., Chang, B., Imamura, M., Poungvarin, N., and Chan, L. (2006) Glucose-dependent transcriptional regulation by an evolutionarily conserved glucose-sensing module. *Diabetes 55*, 1179-1189.
(4) Davies, M. N., O'Callaghan, B. L., and Towle, H. C. (2010) Activation and repression of glucose-stimulated ChREBP requires the concerted action of multiple domains within the MondoA conserved region. *Am. J. Physiol. Endocrinol. Metab. 299*, E665-674.
(5) Merla, G., Howald, C., Antonarakis, S. E., and Reymond, A. (2004) The subcellular localization of the ChoRE-binding protein, encoded by the Williams-Beuren syndrome critical region gene 14, is regulated by 14-3-3. *Hum. Mol. Genet. 13*, 1505-1514.
(6) Fukasawa, M., Ge, Q., Wynn, R. M., Ishii, S., and Uyeda, K. (2010) Coordinate regulation/localization of the carbohydrate responsive binding protein (ChREBP) by two nuclear export signal sites: discovery of a new leucine-rich nuclear export signal site. *Biochem. Biophys. Res. Commun. 391*, 1166-1169.
(7) de Luis, O., Valero, M. C., and Jurado, L. A. (2000) WBSCR14, a putative transcription factor gene deleted in Williams-Beuren syndrome: complete characterisation of the human gene and the mouse ortholog. *Eur. J. Hum. Genet. 8*, 215-222.
(8) Ge, Q., Nakagawa, T., Wynn, R. M., Chook, Y. M., Miller, B. C., and Uyeda, K. (2011) Importin-α protein binding to a nuclear localization signal of carbohydrate response element-binding protein (ChREBP). *J. Biol. Chem. 286*, 28119-28127.
(9) Li, M. V., Chen, W., Poungvarin, N., Imamura, M., and Chan, L. (2008) Glucose-mediated transactivation of carbohydrate response element-binding protein requires cooperative actions from Mondo conserved regions and essential trans-acting factor 14-3-3. *Mol. Endocrinol. 22*, 1658-1672.
(10) Sakiyama, H., Wynn, R. M., Lee, W. R., Fukasawa, M., Mizuguchi, H., Gardner, K. H., Repa, J. J., and Uyeda, K. (2008) Regulation of nuclear import/export of carbohydrate response element-binding protein (ChREBP): interaction of an α-helix of ChREBP with the 14-3-3 proteins and regulation by phosphorylation. *J. Biol. Chem. 283*, 24899-24908.
(11) Berry, M. N., and Friend, D. S. (1969) High-yield preparation of isolated rat liver parenchymal cells: a biochemical and fine structural study. *J. Cell Biol. 43*, 506-520.
(12) Hasegawa, J., Osatomi, K., Wu, R. F., and Uyeda, K. (1999) A novel factor binding to the glucose response elements of liver pyruvate kinase and fatty acid synthase genes. *J. Biol. Chem. 274*, 1100-1107.
(13) Chuh, K. N., Zaro, B. W., Piller, F., Piller, V., and Pratt, M. R. (2014) Changes in metabolic chemical reporter structure yield a selective probe of *O*-GlcNAc modification. *J. Am. Chem. Soc. 136*, 12283-12295.