

# Parallel Identification of O-GlcNAc Modified Proteins from Cell Lysates

## - Supporting Information -

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### General Reagents and Methods:

Unless otherwise noted, reagents were purchased from the commercial suppliers Fisher (Fairlawn, NJ) and Sigma-Aldrich (St. Louis, MO), and were used without further purification. Protease inhibitors were purchased from Sigma-Aldrich or Alexis Biochemicals (San Diego, CA). Bovine GalT, ovalbumin and sepharose 6B were obtained from Sigma-Aldrich. Uridine diphospho-D-[6-<sup>3</sup>H]-galactose, Hyperfilm ECL, Hyperfilm MP and Amplify reagent were purchased from Amersham Biosciences (Piscataway, NJ). Peptide N-glycosidase F (PNGase F) was purchased from New England Biolabs (Beverly, MA). Sequencing grade trypsin was from Promega (Madison, WI). Agarose-conjugated protein A, agarose-conjugated streptavidin, SuperSignal West Pico chemiluminescence reagents, horseradish peroxidase (HRP)-conjugated streptavidin and anti-rabbit IgG antibody were from Pierce (Rockford, IL). Nitrocellulose membrane was from Schleicher and Schuell (Keene, NH). Dulbecco's modified Eagle media (DMEM), fetal bovine serum and penicillin/streptomycin were from Gibco (Carlsbad, CA). N-(aminooxyacetyl)-N'-(D-biotinoyl) hydrazine was purchased from Dojindo (Gaithersburg, MD). Anti-CREB, anti-ATF-1 and HRP-conjugated, anti-sheep IgG antibodies were from Upstate (Charlottesville, VA). Anti-PKA catalytic subunit (C-20), anti-c-Fos (4), anti-c-Jun (H-79), and anti-CBP (A-22) antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). CTD 110.6 anti-O-GlcNAc antibody was from Covance (Princeton, NJ). Mutant GalT (Y289L) was kindly expressed and purified by Dr. B. Ramakrishnan as described previously.<sup>1</sup> All protein concentrations were measured using the Bradford assay (Bio-Rad Laboratories, Hercules, CA).

**Preparation of HeLa Cell Extracts.** HeLa (human cervical adenocarcinoma) cells were cultured in 37 °C humidified air with 5% CO<sub>2</sub> in DMEM supplemented with fetal bovine serum (10%), penicillin (100 U/mL) and streptomycin (100 µg/mL). Prior to lysis, HeLa cells were serum starved in serum-free DMEM for 48 h and induced with 20% serum for 2 h.<sup>2</sup> In some experiments, the culture medium was supplemented with 10 mM glucosamine during the last 5 h of serum starvation and throughout serum induction. After induction, cells from a 100 mm dish were trypsinized and pelleted. The pellet was washed with ice-cold TBS (Tris-buffered saline, 50 mM Tris-HCl pH 7.4, 150 mM NaCl), resuspended in 0.5 mL of boiling lysis buffer (20 mM HEPES pH 7.9, 0.5 % SDS, 10 mM DTT), sonicated for 10 s, and boiled for 10 min. After centrifugation at 21,500xg for 15 min, the supernatant was collected as denatured HeLa extract. Denatured extracts were stable when stored at -80 °C for several weeks.

**Labeling and Capturing O-GlcNAc Modified Proteins.** One volume of denatured HeLa extract (typically 700 µg of total protein in 70 µL) was added into four volumes of dilution buffer (6.7 mM HEPES pH 7.9, 1.25% Nonidet P-40 (NP-40), 75 mM NaCl, 1.5 mM DTT)

containing protease inhibitors (15  $\mu\text{g}/\text{mL}$  antipain, 15  $\mu\text{g}/\text{mL}$  leupeptin, 7.5  $\mu\text{g}/\text{mL}$  chymostatin, 7.5  $\mu\text{g}/\text{mL}$  pepstatin, 0.75 mM phenylmethylsulfonyl fluoride). Diluted extract was then supplemented with 5 mM  $\text{MnCl}_2$ , 1.25 mM adenosine 5'-diphosphate, 0.5 mM analogue **1**, 20  $\mu\text{g}/\text{mL}$  mutant GalT and 2500 U/mL PNGase F. The reaction mixture was incubated at 4  $^\circ\text{C}$  for 12 h, and dialyzed into buffer A (8 mM HEPES pH 7.9, 5 M urea, 25 mM NaCl) twice for 4 h at room temperature. Following dialysis, NP-40 and SDS were added to the final concentrations of 0.5% and 0.05%, respectively. The sample was then acidified to pH 4.8 by adding 0.3 M NaOAc pH 3.7 to a final concentration of 1.8 mM and mixed for 10 min. After centrifugation at 21,500xg for 10 min, the supernatant was collected and the aminoxy biotin derivative was added to a final concentration of 3 mM. After incubation at room temperature for 16 h, the sample was neutralized by adding 0.5 M HEPES pH 7.9 to a final concentration of 33 mM, followed by dialysis into buffer B (10 mM HEPES pH 7.9, 6 M urea) three times for 4 h, and into buffer C (10 mM HEPES 7.9, 150 mM NaCl, 1 mM DTT) twice for 3 h. Dialyzed sample was collected and denoted as labeled HeLa extract.

Labeled HeLa extract was supplemented with protease inhibitors (10  $\mu\text{g}/\text{mL}$  antipain, 10  $\mu\text{g}/\text{mL}$  leupeptin, 5  $\mu\text{g}/\text{mL}$  chymostatin, 5  $\mu\text{g}/\text{mL}$  pepstatin, 0.5 mM phenylmethylsulfonyl fluoride), and pre-cleared with sepharose 6B beads (30  $\mu\text{L}/100 \mu\text{g}$  of proteins) for 1 h at 4  $^\circ\text{C}$ . After centrifugation at 5,000xg for 3 min, the supernatant was collected and incubated with agarose-conjugated streptavidin (30  $\mu\text{L}/100 \mu\text{g}$  of proteins) for 2 h at 4  $^\circ\text{C}$ . Following centrifugation at 5,000xg for 3 min, the supernatant was removed, and the beads were washed three times with 8 volumes of low salt wash buffer (0.1 M  $\text{Na}_2\text{HPO}_4$  pH 7.5, 0.15 M NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS) and three times with high salt wash buffer (0.1 M  $\text{Na}_2\text{HPO}_4$  pH 7.5, 0.5 M NaCl, 0.2% Triton X-100). After washing, the beads were boiled for 10 min in 2.5 volumes of elution buffer (50 mM Tris-HCl 6.8, 2.5% SDS, 100 mM DTT, 10% glycerol, 2 mM biotin). After centrifugation at 2,000xg for 1 min, the supernatant was collected as the captured material.

**PNGase F Deglycosylation of Ovalbumin.** Proteins containing *N*-linked glycans with terminal GlcNAc groups can also be labeled by GalT, and, therefore, it is important to remove *N*-linked glycans by PNGase F to ensure labeling specificity.<sup>3,4</sup> Ovalbumin, a glycoprotein with *N*-linked glycans and terminal GlcNAc moieties,<sup>5</sup> was chosen as a positive control to demonstrate that *N*-linked glycans in HeLa extracts can be effectively removed under the specified reaction conditions.

Purified ovalbumin was dissolved in lysis buffer to a final concentration of 2 mg/ml and boiled for 10 min. After denaturation, ovalbumin was diluted and subjected to mutant GalT/PNGase F treatment as described for denatured HeLa extracts. Assuming 10% of HeLa cell proteins were *N*-glycosylated, the amount of ovalbumin treated in parallel represented a 2-fold excess. Following incubation at 4  $^\circ\text{C}$  for 12 h, ovalbumin samples were analyzed by SDS-PAGE and visualized by Coomassie staining.

Supplementary Figure 1A shows that PNGase F-treated ovalbumin has increased gel mobility compared to either denatured ovalbumin (Input) or ovalbumin treated with mutant GalT but not PNGase F. The drastic shift in mobility is due to the removal of *N*-linked glycans by PNGase F. These results confirm the effectiveness of *N*-linked glycan removal under the specified reaction conditions.

**Western Blotting with HRP-Conjugated Streptavidin.** Streptavidin-captured materials from labeled HeLa extracts were resolved by SDS-PAGE and transferred to nitrocellulose membranes. Membranes were blocked with 5% BSA in phosphate-buffered saline (pH 7.4) for 1 h at room temperature, followed by 1 h incubation with HRP-streptavidin in TBS with 0.05% Tween-20 (TBST). After six washes for 10 min in TBST, biotinylated proteins were visualized by chemiluminescence.

**Immunoblotting for the Parallel Identification of *O*-GlcNAc Proteins.** For each immunoblotting analysis, material captured from 20-100  $\mu$ g of HeLa extracts was loaded on the gel, along with 20% of the corresponding input material prior to capture. After SDS-PAGE, proteins were transferred to nitrocellulose membranes. Membranes were blocked with 5% non-fat milk in TBST for 30 min at room temperature, and then incubated with an antibody specific for the protein of interest in blocking buffer for 1-2 h at room temperature. Following three washes for 10 min in TBST, membranes were incubated with the HRP-conjugated secondary antibody in blocking buffer for 1 h at room temperature, and washed three more times. Individual proteins were visualized by chemiluminescence.

**Radiolabeling and Immunoprecipitation of c-Fos.** *O*-GlcNAc glycosylation of c-Fos was confirmed using standard procedures.<sup>4</sup> HeLa cell extract was prepared as described above, except that the lysis buffer contained 50 mM Tris-HCl pH 7.5 instead of HEPES. One volume of HeLa extract was added to four volumes of dilution buffer (10 mM Tris-HCl 7.5, 1.25% NP-40, 2.5 mM CHAPS) with protease inhibitors (10  $\mu$ g/mL antipain, 10  $\mu$ g/mL leupeptin, 5  $\mu$ g/mL chymostatin, 5  $\mu$ g/mL pepstatin, 0.5 mM phenylmethylsulfonyl fluoride). Diluted extract was then supplemented with 5 mM MnCl<sub>2</sub>, 1.25 mM adenosine 5'-diphosphate, 625 mU/mL bovine GalT and 67  $\mu$ Ci/mL UDP-[<sup>3</sup>H]galactose. After incubation at 4 °C for 12 h, the radiolabeling reaction was quenched by the addition of EDTA to a final concentration of 10 mM.

Radiolabeled extract (150  $\mu$ g) was pre-cleared by incubation with 10  $\mu$ L of protein A-agarose beads at 4 °C for 1 h. Following centrifugation at 2,000xg for 20 s, the supernatant was collected and incubated with 20  $\mu$ L of protein A-agarose beads that had been pre-incubated with 2  $\mu$ g of anti-c-Fos antibody. After 4 h incubation at 4 °C, the beads were washed twice with wash buffer (20 mM Tris-HCl pH 7.5, 1% NP-40, 0.1% SDS, 2 mM CHAPS). Immunoprecipitated material was eluted by boiling for 10 min with 50  $\mu$ L of elution buffer (1% SDS, 1% 2-mercaptoethanol). After centrifugation at 2,000xg for 1 min, the supernatant was collected and diluted into 50  $\mu$ L of PNGase F buffer (0.15 M Na<sub>2</sub>HPO<sub>4</sub> pH 8.6, 15 mM EDTA, 5% NP-40). 1250 U of PNGase F was then added to the sample, followed by 12 h incubation at 37 °C and SDS-PAGE analysis. After Coomassie staining and destaining, the gel was immersed in 2% glycerol for 30 min, followed by Amplify reagent for 30 min, and dried under vacuum. Tritium-labeled proteins were detected by autoradiography.

As shown in Supplementary Figure 1B, immunoprecipitated c-Fos was detected by autoradiography after 1000 h. Importantly, PNGase F treatment removed *N*-linked glycans from the IgG heavy chain as expected,<sup>6</sup> but c-Fos radioactivity remained unaffected. These results confirm that c-Fos is *O*-GlcNAc glycosylated.

**Labeling of CREB and *O*-GlcNAc transferase (OGT) for mass spectrometry.** Baculovirus preparation and protein expression were performed as described previously.<sup>7</sup> CREB (2  $\mu$ g) or

OGT (10  $\mu$ g) in 20 mM HEPES pH 7.9, 100 mM KCl, 0.2 mM EDTA, 15% glycerol were supplemented with 5 mM MnCl<sub>2</sub>. Analogue **1** and Y289L GalT were added to final concentrations of 750  $\mu$ M and 40 ng/ $\mu$ L, respectively. Control reactions without enzyme or analogue **1** were treated identically. Following incubation at 12 h at 4 °C, the reactions were diluted 2-fold with saturated urea. 2.7 M NaOAc pH 3.9 was added to a final concentration of 50 mM and a final pH of 4.8. Aminooxy biotin derivative was added to a final concentration of 5 mM, and the biotinylation reactions were incubated with gentle shaking for 20-24 h at 23 °C. Reactions were aliquoted for analysis by Western blotting or mass spectrometry and stopped by boiling in SDS-PAGE loading dye. Proteins were resolved by 10% SDS-PAGE and either electrophoretically transferred to nitrocellulose or stained with Coomassie Brilliant Blue. Western blotting with streptavidin-HRP was performed as described above to confirm successful labeling (data not shown).

**In-Gel Trypsin Digestion, Avidin Enrichment and MALDI-TOF Analysis of Labeled CREB and OGT.** CREB and OGT bands were excised from Coomassie-stained gels and treated essentially as described by Shevchenko *et al.*<sup>8</sup> Briefly, excised bands were destained overnight in 50% MeOH, 5% AcOH. Destained bands were dehydrated in CH<sub>3</sub>CN, dried by vacuum, and rehydrated in 10 mM DTT. After 30 min reduction at room temperature, excess DTT was removed, and proteins were alkylated in 50 mM iodoacetamide for 30 min at room temperature in the dark. After alkylation, excess iodoacetamide was removed and protein bands were washed in 100 mM NH<sub>4</sub>HCO<sub>3</sub> pH 8.0 for 10 min, followed by two successive dehydrations in CH<sub>3</sub>CN. Wash and dehydration steps were repeated once more, and excess CH<sub>3</sub>CN was removed under vacuum. Protein bands were rehydrated in 15 ng/ $\mu$ L trypsin in 50 mM NH<sub>4</sub>HCO<sub>3</sub> pH 8.0. Excess trypsin solution was removed after rehydration, and 20-30  $\mu$ L of 50 mM NH<sub>4</sub>HCO<sub>3</sub> pH 8.0 was then added to cover the gel slices. Proteins were digested overnight at 37 °C. Following digestion, peptides were extracted with successive washes of water followed by 50% acetonitrile/5% formic acid in water, and dried by vacuum centrifugation.

A small portion of each sample was saved prior to affinity chromatography for matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). The remainder was subjected to avidin affinity chromatography (Applied Biosystems, Foster City, CA). Chromatography was performed essentially as described by the manufacturer except that the volume of washes was doubled. Eluted peptides were partially dried by vacuum centrifugation, and a small portion of the eluted peptides was analyzed by MALDI-TOF MS. For the analysis, peptide samples were concentrated on C18 zip tips (Millipore, Bedford, MA) and combined with the MALDI matrix (2,5-dihydroxybenzoic acid in 20% CH<sub>3</sub>CN, 0.1% TFA in water). Spectra were acquired on a PerSeptive Biosystems Voyager-DE Pro at 20,000 kV in the reflector mode.

As shown in Supplementary Figure 2A, a number of CREB tryptic peptides were observed prior to affinity chromatography. The expected *O*-GlcNAc peptide <sup>256</sup>TAPTSTIAPGVVMASSPALPTQPAEEAAR<sup>284</sup>,<sup>7</sup> which had been labeled with a ketone-biotin moiety, was present in low abundance (*m/z* 3539.55). Following avidin chromatography, selective enrichment of this peptide was clearly observed (Supplementary Figure 2B). Two additional variants corresponding to multiply oxidized forms of this peptide were also detected. These results demonstrate that *O*-GlcNAc peptides that are labeled using our chemoenzymatic strategy can be selectively captured for MS analysis.

We next examined the MALDI-TOF MS spectra of the peptides corresponding to OGT. Prior to avidin chromatography, a number of tryptic peptides of OGT were observed (Supplementary Figure 3A). Notably, however, no labeled glycopeptides were detected. Following avidin chromatography, significant enrichment of a peak ( $m/z$  2548.16) corresponding to the OGT sequence  $^{390}$ ISPTFADAYSNMGLK $^{406}$  plus the ketone-biotin moiety was obtained (Supplementary Figure 3B). As in the case of CREB, two additional multiply oxidized variants of the captured peptide were observed.

**LC-MS/MS Analysis of Avidin-Enriched CREB and OGT Peptides.** Having confirmed the efficacy of the enrichment procedures using MALDI-TOF MS, subsequent analyses were performed directly using LC-MS/MS. Automated nanoscale liquid chromatography and tandem mass spectrometry (LC-MS/MS) were conducted using a ThermoFinnigan Surveyor HPLC and LTQ ion trap mass spectrometer along with a variation of the “vented column” approach described by Licklider *et al.*<sup>9</sup> Avidin-enriched peptides were loaded onto a 5 cm-long X 75  $\mu$ m i.d. precolumn packed with 5  $\mu$ m C-18 silica (Monitor 100 Å) retained by a Kiesel frit. After thorough washing, the vent was closed and the sample was transferred to a 12 cm-long X 75  $\mu$ m i.d. column with a pulled 5  $\mu$ m tip packed with the same material. The chromatographic profile was from 100% solvent A (0.1% aqueous AcOH) to 50% solvent B (0.1% AcOH in CH<sub>3</sub>CN) in 30 min at approximately 200 nL/min (manual split from 300  $\mu$ L/min). Additional time was allotted for column washing and reequilibration. The LTQ was operated in automated mode using Xcalibur<sup>TM</sup> software. The acquisition method during MS/MS analysis involved one MS precursor ion scan followed by five data-dependent MS/MS scans. Higher order MS analyses involved an MS precursor scan followed by targeted MS<sup>4</sup> scans of those masses that specifically demonstrated loss of the ketone-biotin moiety and ketone-biotin-GlcNAc moiety in the MS/MS analysis. In the case of the OGT sample peptides, MS<sup>4</sup> data was used to search against an OGT sequence database using SEQUEST.<sup>10</sup> All potential peptide identifications were manually verified. In the case of the CREB sample, the acquisition method involved targeted MS/MS analysis of the presumptive ketone-biotin-GlcNAc modified peptide at  $m/z$  1181.2, with simultaneous targeted MS<sup>3</sup> analysis of the GlcNAc modified peptide at  $m/z$  1513.6 and MS<sup>4</sup> analysis of the unmodified peptide at  $m/z$  1412.1.

The electrospray voltage was set at 1.6 kV and the heated capillary was set at 250 °C. The ion selection window was set at 500-2000  $m/z$  for all experiments. For MS/MS and higher order MS analyses, the relative collision energy for collision-induced dissociation (CID) was preset to 35% and a default charge state of +2 was selected to calculate the scan range for acquiring tandem MS spectra. The precursor ion isolation window was set at 3.5 for maximum sensitivity.

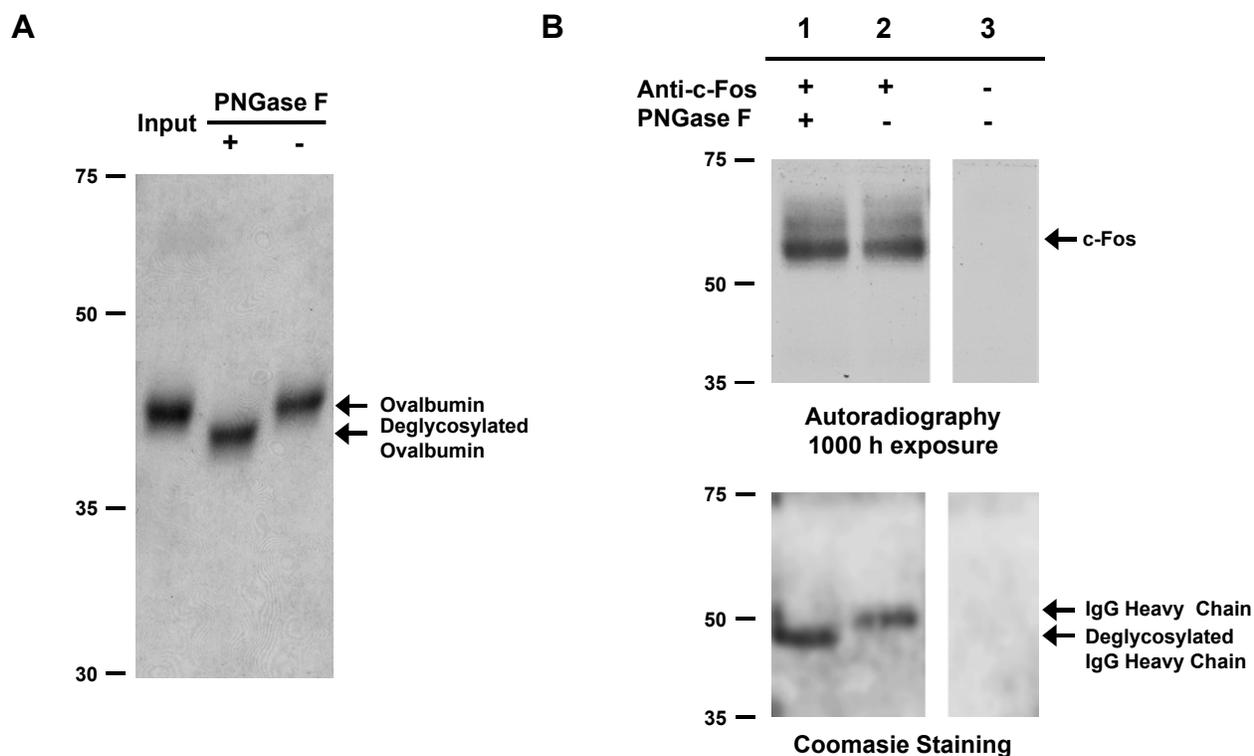
Avidin affinity capture of tryptic peptides from 250 ng of CREB protein identified the expected *O*-GlcNAc peptide  $^{256}$ TAPTSTIAPGVVMASPPALPTQPAEEAAR $^{284}$ .<sup>7</sup> Supplementary Figure 4 (see also Figure 3A, main text) shows the expected doubly charged ion labeled with the biotin-ketone moiety ( $m/z$  1181.37). Upon tandem MS, loss of the ketone-biotin moiety ( $m/z$  1512.97) as well as the ketone-biotin-GlcNAc moiety ( $m/z$  1411.49) were observed. Targeted MS<sup>4</sup> analysis of the unmodified peptide yielded a number of y and b ions that verified the identification of this peptide.

Experiments with avidin affinity captured OGT peptides identified a number of candidate *O*-GlcNAc peptides. Tandem MS of these peptides revealed characteristic charge losses corresponding to loss of the ketone-biotin moiety and ketone-biotin-GlcNAc moiety,

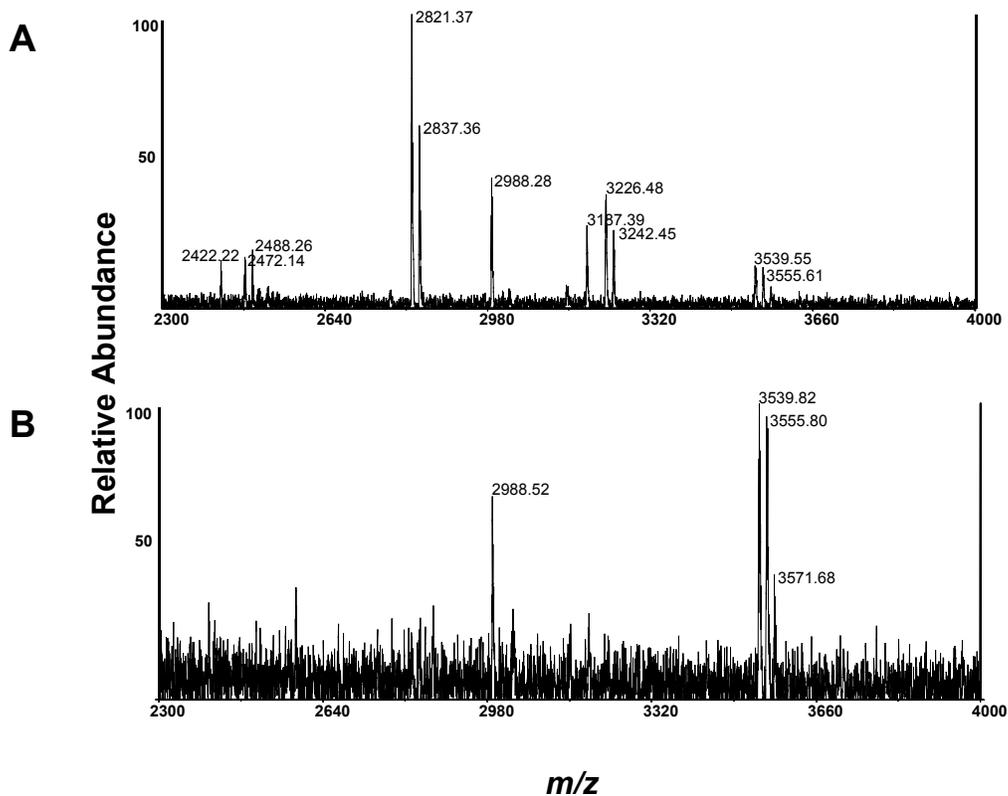
which served to unambiguously identify the peptides as *O*-GlcNAc modified (Supplementary Figure 5).

Supplementary Figure 5A shows a peptide corresponding to the sequence <sup>390</sup>ISPTFADAYSNM<sub>ox</sub>GNTLK<sup>406</sup> labeled with the ketone-biotin moiety ( $m/z= 856.02$ ). Upon tandem MS, loss of the ketone-biotin moiety ( $m/z 1025.00$ ) followed by loss of the GlcNAc sugar ( $m/z 923.56$ ) was observed. Similarly, Figure 5B shows a peptide corresponding to the sequence <sup>1037</sup>IKPVEVTESA<sup>1046</sup> of OGT labeled with the ketone-biotin moiety ( $m/z 895.96$ ). Upon tandem MS, loss of the ketone-biotin moiety ( $m/z 1275.03$ ) followed by loss of the GlcNAc sugar ( $m/z 1072.03$ ) was observed. Notably, three other peptides also displayed the characteristic loss signatures. Their masses, ( $m/z 1209.05$ ), ( $m/z 946.20$ ), and ( $m/z 769.56$ ) corresponded to the labeled OGT peptides <sup>407</sup>EMQDVQGALQCYTR<sup>420</sup>, <sup>421</sup>AIQINPAFADHSNLSIHK<sup>440</sup>, and <sup>826</sup>TIIVTTR<sup>832</sup> (with an oxidized biotin moiety) respectively.

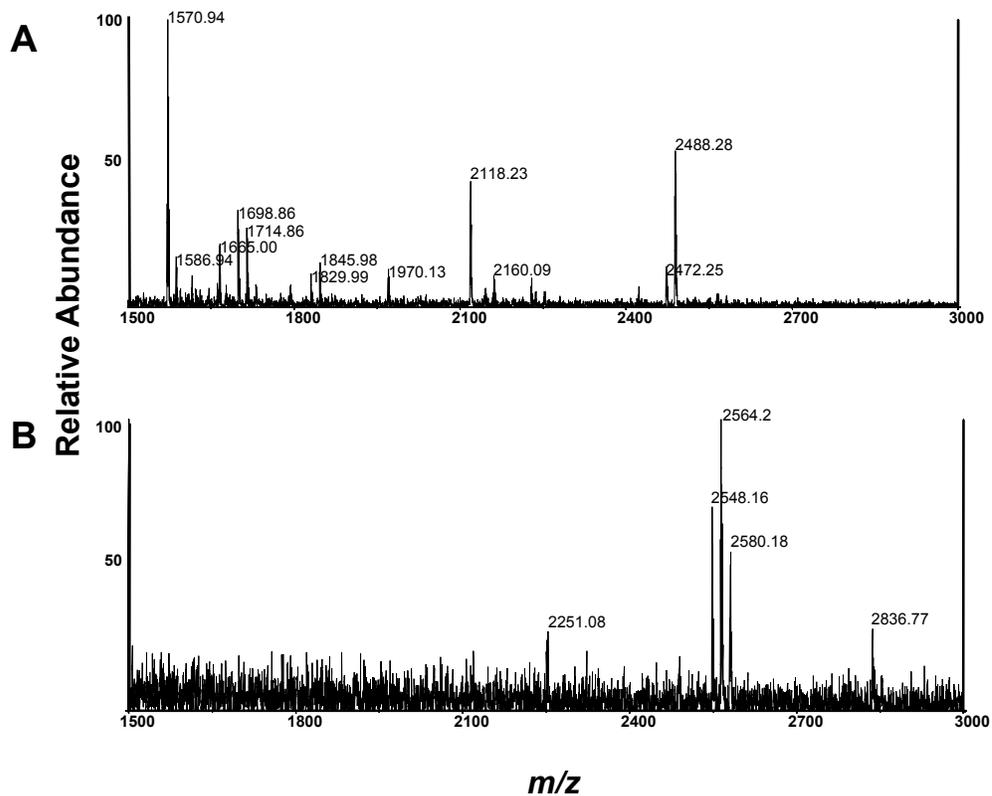
To confirm the sequences of the modified peptides, we conducted targeted higher order mass spectrometry on the candidate species. As depicted in Supplementary Figure 5, the peptides corresponding to  $m/z 856.02$  and  $m/z 895.96$  were successfully sequenced by MS<sup>4</sup> analyses. Resultant y and b ions from the MS<sup>4</sup> spectra allowed identification of the peptides as <sup>390</sup>ISPTFADAYSNM<sub>ox</sub>GNTLK<sup>406</sup> and <sup>1037</sup>IKPVEVTESA<sup>1046</sup>, respectively. Internal fragment ions in the MS<sup>4</sup> spectrum of the latter helped to conclusively identify this peptide.



**Supplementary Figure 1.** (A) Removal of *N*-linked glycans from ovalbumin using PNGase F. Denatured ovalbumin (left lane), PNGase F/GalT treated (middle lane) and GalT treated (right lane) ovalbumin were analyzed by SDS-PAGE and visualized by Coomassie staining. Increased gel mobility of PNGase F-treated ovalbumin indicates removal of *N*-linked glycans under the GalT labeling conditions. (B) Immunoprecipitation of radiolabeled c-Fos. HeLa extracts were first labeled with bovine GalT and UDP-[<sup>3</sup>H]galactose, and c-Fos was immunoprecipitated in the presence (lanes 1 and 2) or absence (lane 3) of anti-c-Fos antibody, followed by incubation with PNGase F (lane 1). After SDS-PAGE analysis, gels were Coomassie stained (lower panel), dried, and subjected to autoradiography (upper panel). Radiolabeled c-Fos was specifically pulled-down by the anti-c-Fos antibody. Removal of *N*-linked glycans with PNGase F enhanced the mobility of IgG heavy chain (lower panel, lanes 1 and 2) but did not affect the tritium labeling of c-Fos (upper panel, lanes 1 and 2), indicating that c-Fos is *O*-GlcNAc glycosylated.

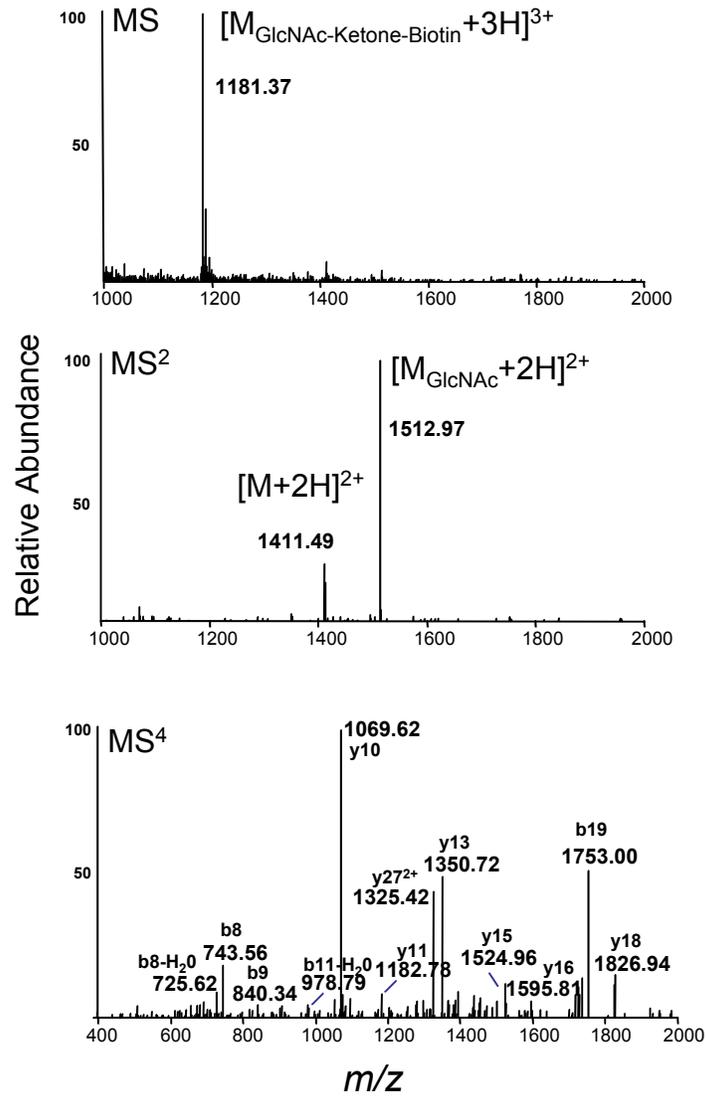


**Supplementary Figure 2.** Enrichment of CREB *O*-GlcNAc peptides via the chemoenzymatic strategy. (A) MALDI-TOF spectrum of CREB tryptic peptides prior to avidin chromatography. The peak at  $m/z$  3539.55 corresponds to the mass of the *O*-GlcNAc glycosylated peptide labeled with the ketone-biotin moiety. (B) MALDI-TOF spectrum of the eluent following avidin affinity capture of CREB peptides. The spectrum reveals enrichment of the labeled CREB peptide at  $m/z$  3539.82 as well as two peaks at  $m/z$  3555.80 and 3571.68 that correspond to oxidized forms of this peptide. The peptide at  $m/z$  2988.52 displays some nonspecific interaction with the avidin column and can be readily discerned as unlabeled by LC-MS/MS.

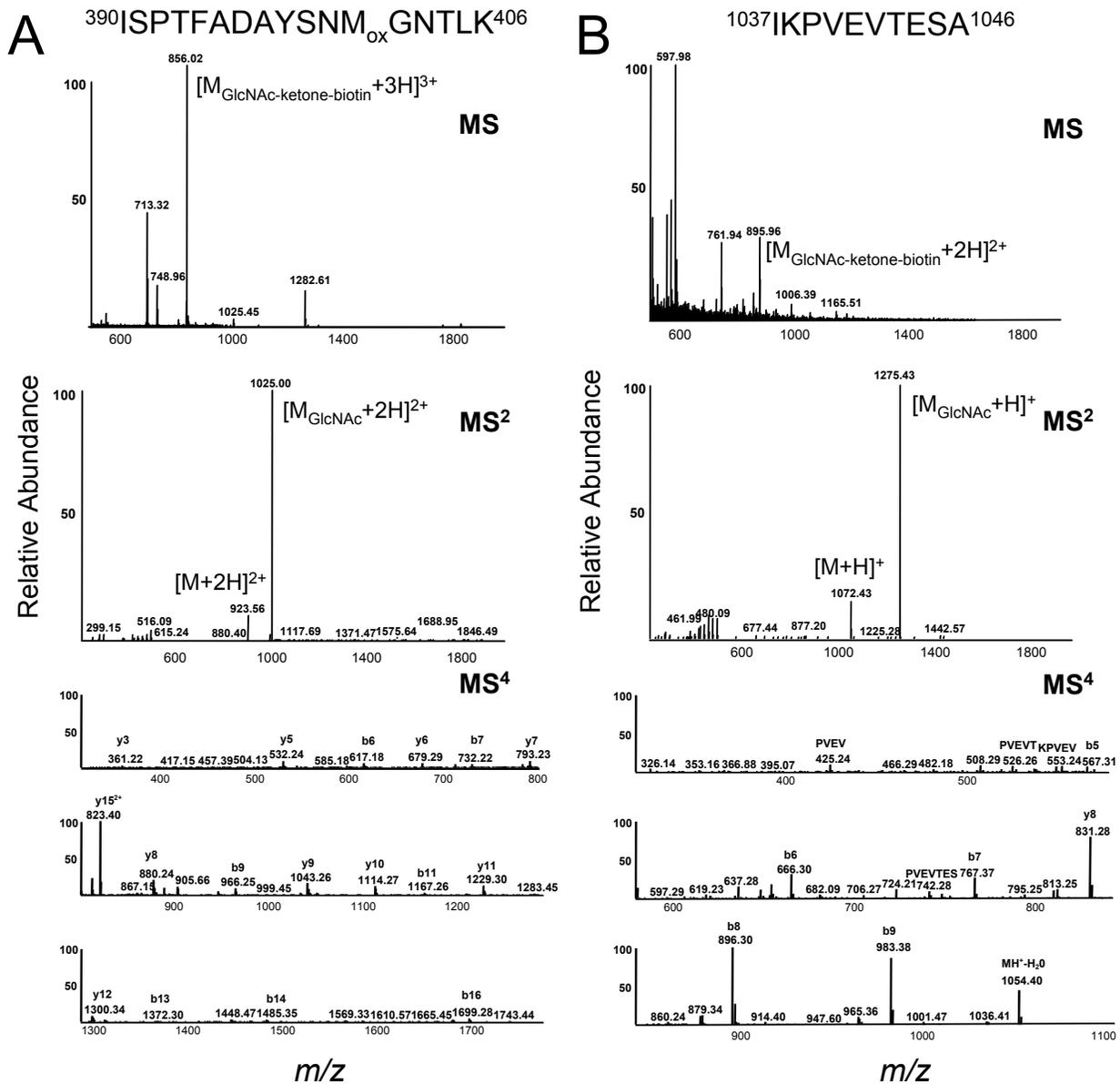


**Supplementary Figure 3.** Enrichment of OGT *O*-GlcNAc peptides via the chemoenzymatic labeling strategy. (A) MALDI-TOF spectrum of OGT tryptic peptides prior to avidin chromatography reveals a number of OGT peptides while no labeled *O*-GlcNAc modified peptides are visible. (B) MALDI-TOF spectrum of eluted peptides following avidin affinity chromatography reveals enrichment of a peak at  $m/z$  2548.16 and two oxidized forms of the same peptide. This mass corresponds to the labeled *O*-GlcNAc peptide  $^{390}\text{ISPTFADAYSNMGNTLK}^{406}$ , whose sequence was confirmed by LC-MS/MS. The mass at  $m/z$  2836.77 may correspond to the labeled *O*-GlcNAc form of the OGT tryptic peptide  $^{421}\text{AIQINPAFADAHSNLSIHK}^{440}$ . However, tandem MS analysis was inconclusive. The mass at  $m/z$  2251.08 does not correspond to theoretical OGT tryptic modified or unmodified peptides and may be a contaminant.

256 TAPTSTIAPGVMASSPALPTQPAEEAAR 284



**Supplementary Figure 4.** Identification of the *O*-GlcNAc modified peptide on CREB by LC-MS/MS. Tandem mass spectra of the labeled *O*-GlcNAc peptide <sup>256</sup>TAPTSTIAPGVMASSPALPTQPAEEAAR ( $m/z$  1181.37). CID revealed signature losses of the ketone-biotin moiety ( $m/z$  1512.97) and the GlcNAc moiety ( $m/z$  1411.49). Higher order MS analysis verified the identification of this peptide from the resultant y and b ions.



**Supplementary Figure 5.** Identification of *O*-GlcNAc modified peptides on OGT by LC-MS/MS. (A) Tandem mass spectra of the labeled *O*-GlcNAc peptide  $^{390}\text{ISPTFADAYSNM}_{\text{ox}}\text{GNTLK}^{406}$  ( $m/z$  856.02). CID revealed signature losses of the ketone-biotin moiety ( $m/z$  1025.00) and the GlcNAc moiety ( $m/z$  923.56). Higher order MS analysis provided conclusive identification of this peptide from the resultant y and b ions. (B) Tandem mass spectra of the labeled *O*-GlcNAc peptide  $^{1037}\text{IKPVEVTESA}^{1046}$  ( $m/z$  895.96). CID revealed signature losses of the ketone-biotin moiety ( $m/z$  1275.43) and the GlcNAc moiety ( $m/z$  1072.43). Higher order MS analysis provided conclusive identification of this peptide from the resultant y and b ions as well as internal fragment ions.

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