Electronic Supplementary Material (ESI) for Molecular BioSystems.

Electronic Supporting Information

Comprehensive Mapping of *O*-GlcNAc Modification Sites Using a Chemically Cleavable Tag

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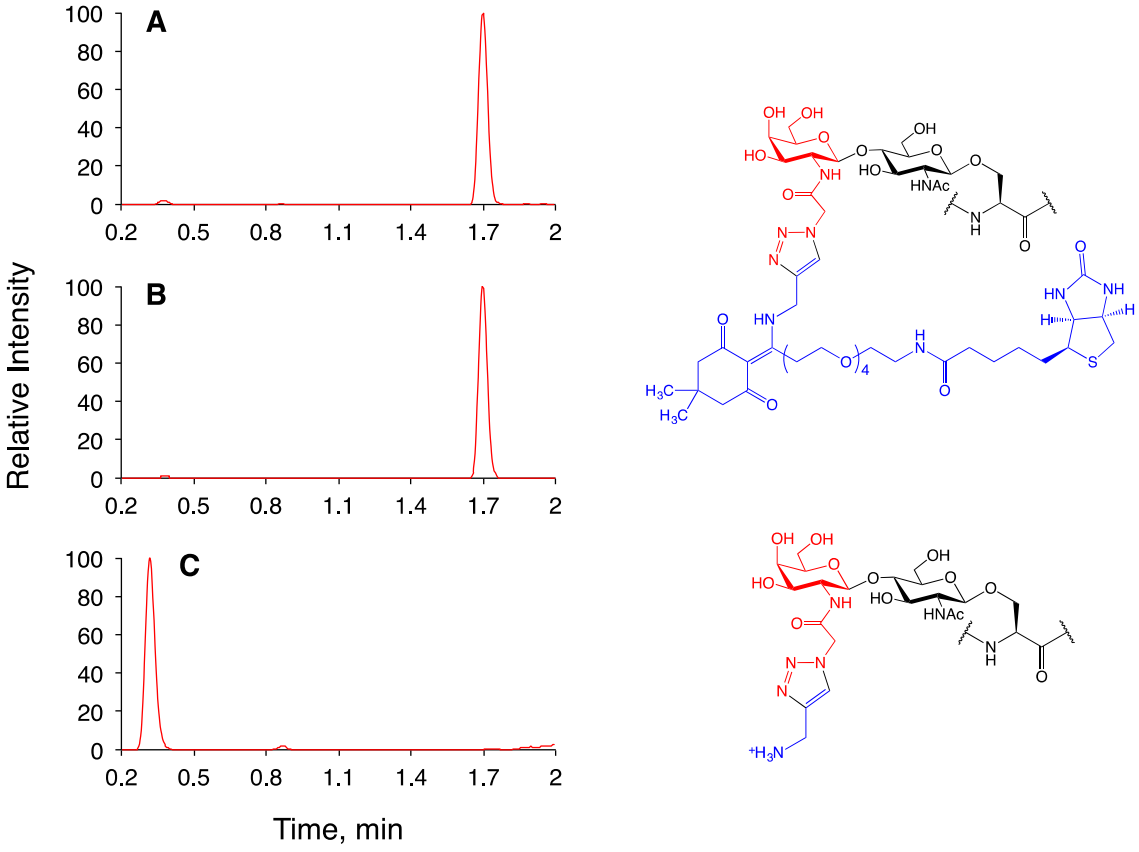
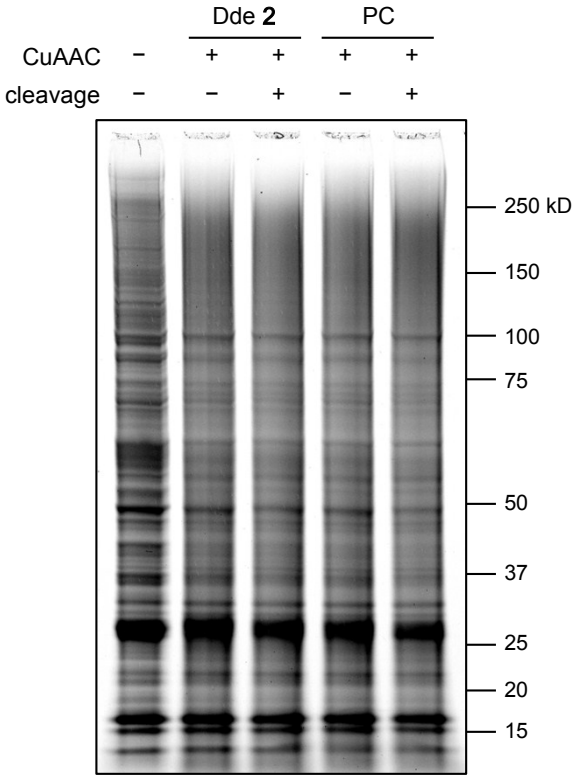


Supplemental Figure 1. Labelled peptide is stable to wash conditions. Reverse-phase LC-MS analysis of alkyne-Dde-biotin-labelled peptide after 1 h incubation with (A) 6 M urea, (B) 1% RapiGest, or (C) 2% hydrazine. All graphs show extracted ion chromatograms of the starting material and possible product within ± 1 *m/z*.



Supplemental Figure 2. Full Coomassie-stained gel for Figure 3. 20 μg of protein from each sample condition was resolved by SDS-PAGE and visualized by Coomassie staining. Coomassie staining confirms equal protein loading in each lane.

Experimental Methods

Reagents and materials. All proteins, chemicals, and reagents were of analytical grade, obtained from Sigma Aldrich (St. Louis, MO), and used without further purification unless specified. The *O*-GlcNAcylated peptide TAPT(gS)TIAPG, high capacity Neutravidin agarose resin, spin columns, and C18 desalting tips were purchased from ThermoFisher Scientific (Waltham, MA). Thiamet G was received from Tocris Biosciences (Avonmouth, Bristol, UK). cOmplete™ protease inhibitor cocktail without EDTA (PIC-EDTA) was purchased from Roche Diagnostics Corp. (Indianapolis, IN). Baculovirus preparation and protein expression of short-form OGT (sOGT) in *Spodoptera frugiperda* (Sf9) cells was performed as previously described.[1] Cerebral cortices were obtained from adult C57BL/6 mice bred in house. All protein concentrations were measured using the BCA assay (ThermoFisher Scientific). RapiGest and UDP-GalNAz **1** were synthesized as referenced.[2, 3] Y289L GalT was expressed and purified as described previously. Tris(3-hydroxypropyltriazolylmethyl)amine (THPTA), alkyne-Dde-biotin **2**, and the alkyne-photocleavable-biotin linker (alkyne-PC-biotin) were purchased from Click Chemistry Tools (Scottsdale, AZ).

Peptide labelling. The labelling protocol was adapted from a previously reported method.[4] The peptide TAPT(gS)TIAPG (20 μM final) was dissolved in a 200 μL solution of 10 mM HEPES pH 7.9, 5.5 mM $MnCl_2$, 1 mM UDP-GalNAz **1**, and 100 ng/μL Y289L GalT and rotated end-over-end overnight at 4 °C. Prior to enzyme addition, an aliquot was removed as an initial time point for LC-MS analysis. The reaction was acidified to 0.1% TFA, desalted using a C18 tip, and an aliquot was saved for analysis. The labelled peptide (10 μM final) was diluted into a 400 μL solution of 10 mM sodium phosphate pH 7.6, 100 μM alkyne-Dde-biotin **2**, 2 mM sodium ascorbate, and 100 μM THPTA. $CuSO_4$ was added (1 mM final), and the reaction was incubated while rotating end-over-end at RT for 1 h. After removing a sample, the reaction was acidified and desalted again. The peptide (10 μM) was then split into fractions of 50 μL containing 25 mM sodium phosphate pH 7.6 and either 1% RapiGest, 6 M urea, or 2% hydrazine monohydrate and incubated for 1 h at RT. Samples were acidified, desalted, and subjected to LC-MS analysis.

LC-MS analysis of O-GlcNAc peptide labelling. Liquid chromatography and mass spectrometry (LC-MS) were performed using an LTQ linear ion trap mass spectrometer combined with an Accela LC and PAL autosampler (Thermo Scientific, Waltham, MA). Approximately 10 pmol peptide from each sample was injected onto a CORTECS UPLC C18+ column (2.1 x 50 mm, Waters Corp., Milford, MA). Flow rate was set at 0.4 mL/min. Solvent A (ddH_2O, 1% formic acid) and Solvent B (acetonitrile, 1% formic acid) were used to create a gradient. The gradient consisted of 0-0.2 min, 5% B; 0.2-3.5 min 5-65% B, 3.5-4.0 min 65% B with injection into the MS starting at 0.2 min to avoid salt contamination. All peptide products were found to elute during the linear gradient between 0.2 and 2.0 min. For the biotinylated and cleaved peptide, alkyne reagent **2** was not sufficiently removed by the C18 tips. Therefore, the reaction was monitored using an extracted ion chromatogram by extracting all ions within ± 1 *m/z* of the calculated masses.

Linker comparison by protein labelling. Labelling with Y289L GalT and UDP-GalNAz **1** was conducted as previously described.[5] Briefly, 500 µg of HEK-293T cell lysate in 1% SDS, 1x PBS pH 7.4 (10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl), 10 µM Thiamet G, and 1x PIC-EDTA was diluted to a protein concentration of 1 mg/mL using 1% SDS, 1x PBS pH 7.4 and precipitated by adding three volumes of methanol, one volume of chloroform, and two volumes of ddH_2O with vortex mixing after each addition. Protein was pelleted at the aqueous-organic interface by centrifuging at 21,000 x *g* for 5 min. The top, aqueous layer was removed, and one volume of methanol was added with mixing. The protein was pelleted again, and all liquid was removed. After the pellets were air-dried, samples were redissolved at 5 mg/mL (100 µL) in 1% SDS, 20 mM HEPES pH 7.9 by sonication. To each sample, the following were added in the given order: 10 µL of 50x PIC-EDTA, 112.5 µL of ddH_2O, 200 µL of 2.5x labelling buffer (50 mM HEPES pH 7.9, 125 mM NaCl, 5% NP-40), and 27.5 µL of 100 mM $MnCl_2$. Samples were briefly mixed by pipetting and placed on ice. Next, 25 µL of 0.5 mM UDP-GalNAz **1** was added followed by pipetting to mix. Finally, 25 µL of 2 mg/mL Y289L GalT was added, and samples were rotated end-over-end for 16 h at 4 °C. Proteins were then precipitated again, and pellets were air dried. Pellets were next dissolved at 4 mg/mL (125 µL) in 1% SDS, 20 mM HEPES pH 7.9. An aliquot of the GalT-labeled sample was removed (25 µL), and the remaining sample was split into two equal portions (50 µL each) to be labelled with either alkyne-Dde-biotin **2** or alkyne-PC-biotin. All manipulations with the photocleavable

linker were performed in the dark. To each sample, the following were added in the given order with mixing: 4 µL of 50x PIC-EDTA, 78 µL of ddH_2O, and 10 µL of 20x PBS pH 7.4. Next, the CuAAC reagents were added with vortex mixing after each addition: 4 µL of 5 mM alkyne-dde-biotin **2** or alkyne-PC-biotin (stock in DMSO), 4 µL of 100 mM sodium ascorbate (freshly prepared), 10 µL of 2 mM THPTA (stock in 4:1 *t*BuOH/DMSO), and 4 µL of 50 mM $CuSO_4$ (freshly prepared). Samples were rotated end-over-end for 1 h at RT, and the reaction was halted by the addition of 25 µL EDTA pH 8.0. Samples were acetone precipitated, and the pellet was washed once with 1 mL MeOH to remove residual, unreacted linker. The pellet was then air-dried and redissolved at 4 mg/mL (50 µL) in 1% SDS, 20 mM HEPES pH 7.9. An aliquot of each sample was reserved (25 µL), and the remaining sample was cleaved. For the alkyne-Dde-biotin sample, the mixture was diluted to 1 mg/mL with 2% hydrazine monohydrate, and the sample was rotated end-over-end for 1 h at RT. For the alkyne-PC-biotin sample, the protein was diluted to 1 mg/mL with ddH_2O, and the liquid was irradiated from the open top of the tube (2 cm distance) with 365 nm UV light (UVGL-25 handheld UV lamp, 1.5 mW/cm^2) for 1 h at RT with mixing every 10 min. Both samples were then precipitated by addition of four volumes of -20 °C acetone and storage at -20 °C for 1 h. Samples were air-dried and then redissolved at 4 mg/mL (25 µL) in 1% SDS, 20 mM HEPES pH 7.9.

Coomassie staining and streptavidin blotting. Aliquots corresponding to 20 µg of protein (5 µL) for each sample were resolved by SDS-PAGE as follows. Samples were added to 10 µL ddH_2O and 5 µL 4x SDS-PAGE loading buffer (200 mM Tris pH 6.8, 400 mM DTT, 8% SDS, 0.4% bromophenol blue, 40% glycerol) and were then used directly without boiling to avoid cleaving the linkers. The mixtures were loaded in duplicate on NuPAGE™ Novex™ 4-12% Bis-Tris protein gels (ThermoFisher Scientific, 1.0 mm, 10-well). One duplicate was then stained with Imperial™ protein stain (ThermoFisher Scientific) according to the manufacturer's specifications and imaged by an Odyssey scanner (LI-COR Biosciences). The other set of samples was transferred onto an Immobilon-FL PVDF membrane (EMD Millipore, 0.45 µM) and blocked for 1 h at RT with 5% bovine serum albumin (BSA) in 1x TBST (19 mM Tris pH 7.4, 137 mM NaCl, 2.7 mM KCl, 0.1% Tween 20). The blot was then incubated with 1:20,000 AlexaFluor-680-conjugated streptavidin (ThermoFisher Scientific) in 5% BSA/TBST for 1 h at RT, washed three times with TBST for 5 min, and imaged with an Odyssey scanner.

α-Crystallin and sOGT labelling. Labelling was conducted as described above using 200 µg of cortical lysate, 20 µg of α-crystallin, and 5 µg of sOGT.

Enrichment and elution of labelled proteins. Labelled samples were diluted to 1 mL using 1x PBS pH 7.4, 1x PIC-EDTA. For each sample, 25 µL (settled volume) of high-capacity Neutravidin agarose was washed twice with 500 µL of 1x PBS in spin columns, and samples were added to the washed beads. Mixtures were rotated end-over-end for 1 h at RT. Lysate was removed by centrifugation at 2,000 x *g* for 30 s. The beads were washed with 1% SDS (5 x 0.5 mL), 6 M urea (5 x 0.5 mL), and 1x PBS pH 7.4 (5 x 0.5 mL). Beads were then resuspended in 50 µL of 2% hydrazine monohydrate in ddH_2O and rotated end-over-end for 1 h at RT. The elution volume was removed, and beads were washed with 50 µL of 1x PBS pH 7.4. The wash volume was combined with the elution volume, and samples were flash-frozen and stored at -80 °C prior to analysis.

Sample processing for MS analysis. Samples were thawed and precipitated by addition of four volumes of -20 °C acetone. Samples were stored at -20 °C for 1 h and centrifuged at 21,000 x *g* for 5 min. Pellets were redissolved in 20 µL of 8 M urea, 100 mM Tris pH 8.0, 10 mM DTT and incubated at 60 °C with shaking for 20 min. Cysteine residues were blocked by addition of 25 mM iodoacetamide for 45 min. Samples were diluted four-fold with 100 mM Tris pH 8.0. Samples were split in two and digested with 0.01 mg/mL trypsin or chymotrypsin for 4-16 h at 37 °C. A portion of the trypsin digests was further digested with 7 µg/mL AspN for 6 h at 37 °C. Digests were acidified to a final concentration of 0.5% formic acid, 0.05% TFA.

LC separation and MS analysis. The digests were analyzed by nanoLC/MS on the LTQ-Velos with a 0 to 30% B in 120 min gradient with top 5 MS/MS (A: ddH_2O, 0.1% formic acid; B: acetonitrile, 0.1% formic acid). Samples were desalted on a 360 x 100 µm Kasil fritted pre-column (2 cm Monitor C18) prior to separation on a 360 x 75 µm (10 cm BEH130 C18, 1.7 µm) analytical column/tip. Full scan MS was acquired at 60,000 resolution followed by top 5 tandem MS in the linear ion trap alternating between ETD

and CID modes of the same precursor. The ETD reaction time was 100 ms with supplemental activation. RAW files were converted to MGF files for Mascot searching using Proteome Discoverer with CID and ETD spectra extracted to separate MFG files. Data was searched against a custom database with fixed modifications of carbamidomethyl (C) and variable mods of oxidation (M) and a custom modification for the tagged *O*-GlcNAc. The custom modification was defined as addition of C(19) H(30) N(6) O(10) to Ser or Thr (net addition of 502.202341 Da). For CID, a scoring neutral loss of C(19) H(30) N(6) O(10) was included, but this was omitted for ETD. Enzyme specificity was trypsin (KR), chymotrypsin (FLYW), or trypsin-AspN_ND (cleave C-term KR and N-term ND). Mass tolerances were 25 ppm and 0.8 Da for precursor and fragments ions, respectively. The instrument type was chosen as either ESI-TRAP or ETD-TRAP. Search results were combined in Scaffold 4.4, filtered for 80% peptide confidence and modifications manually evaluated.

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