Optimization of chemoenzymatic mass-tagging by strain-promoted cycloaddition (SPAAC) for the determination of O-GlcNAc stoichiometry by Western blotting.

Narek Darabedian,[1*] John W. Thompson,[2] Kelly N. Chuh,[1] Linda C. Hsieh-Wilson,[2] and Matthew R. Pratt[1,3]

Departments of [1]Chemistry and [3]Biological Sciences, University of Southern California, Los Angeles, CA 90089.
[2]Division of Chemistry and Chemical Engineering, California Institute of Technology, Pasadena, CA 91125.
Correspondence should be addressed to matthew.pratt@usc.edu

Table of Contents:

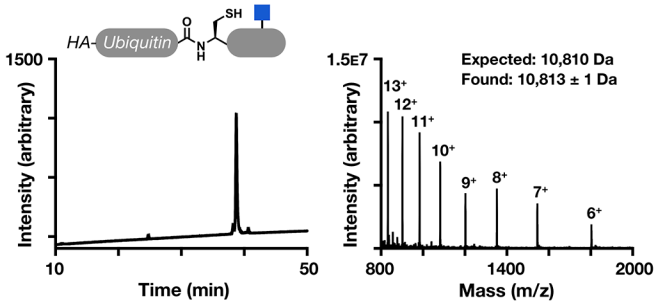

Figure S1. Characterization of O-GlcNAcylated ubiquitin. O-GlcNAcylated ubiquitin was purified by RP-HPLC and characterized by ESI-MS.

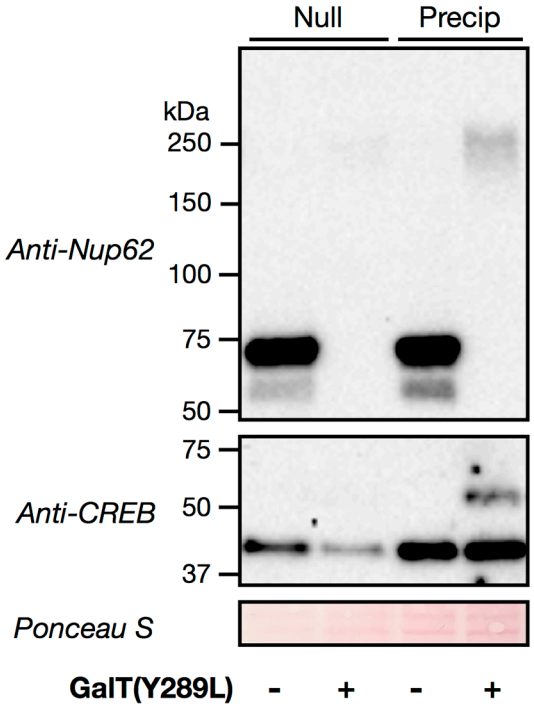

Figure S2. Final protein precipitation improves the detection of mass-tagging. Cell lysates were enzymatically modified by GalNAz and then reacted with DBCO-PEG. The resulting reactions were then terminated by either boiling in SDS-PAGE loading buffer (Null) or by methanol/chloroform/H_2O (Precip). The precipitated proteins were then resuspended, and Nup62 and CREB were visualized by Western blotting.

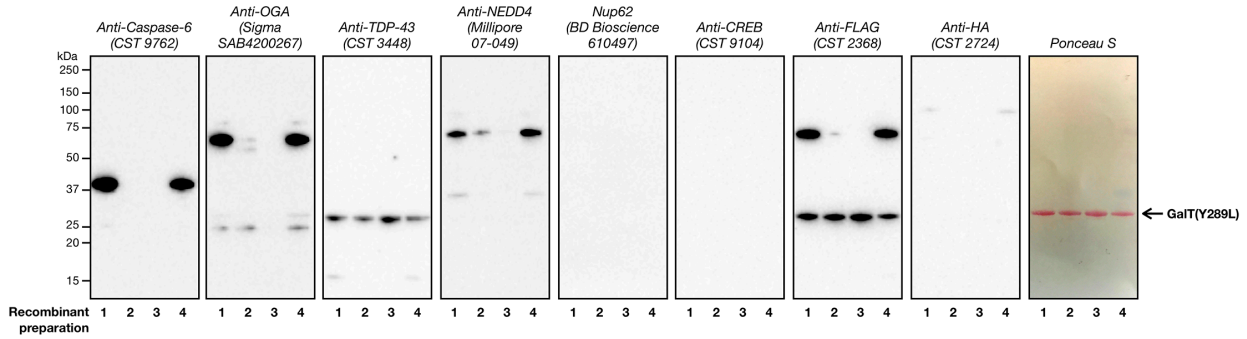

Figure S3. Several antibodies recognize bacterial proteins in recombinant GalT preparations. GalT(Y289L) was recombinantly expressed in E. coli and purified in four independent experiments. These different recombinant preparations (1-4) of GalT(Y289L) were individually subjected to SDS-PAG and Western blotting with the indicated antibodies against various proteins or commonly used affinity tags. The purity of the GalT(Y289L) preps was visualized by Ponceau S staining.

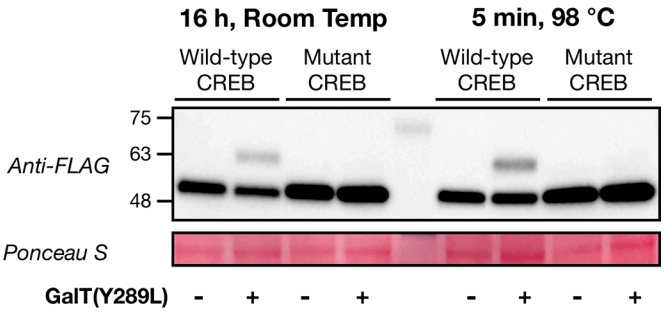

Figure S4. An O-GlcNAc-deficient mutant of CREB is not mass-shifted. H1299 cells were transiently transfected with either FLAG-tagged wild-type CREB or a multiple mutant (S40, T227, T228, T259, S260, T261 to A) that is not O-GlcNAcylated. The corresponding cell lysates were enzymatically modified by GalNAz and then reacted with DBCO-PEG before visualization by Western blotting.

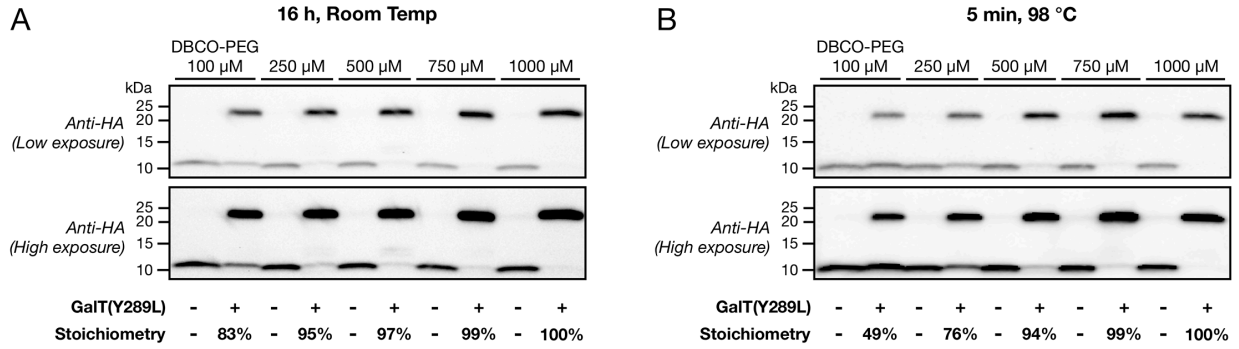

Figure S5. DMSO concentration is not a major driver of SPAAC efficiency. (A) O-GlcNAcylated ubiquitin was added to cell lysates before enzymatic modification with GalNAz and reaction with the indicated concentrations of DBCO-PEG (10% final volume of DMSO) for 16 h at room temperature and analysis by Western blotting. (B) The mass-shifting reactions were performed as in (A) but with SPAAC at 98 °C for 5 min.

Experimental Methods

General

Unless otherwise indicated, all solvents and reagents were purchased from commercial sources (Sigma-Aldrich, Fluka, MilliporeSigma, Novagen, etc.) and used without any further purification. Iodoacetamide was purchased from VWR, TCEP (tris(2-carboxyethyl)phosphine) was purchased from ThermoFisher Scientific, the Click-iT O-GlcNAc Enzymatic Labeling System (C33368) was purchased from ThermoFisher Scientific. Alkyne-PEG_{5000}, DBCO-PEG_{5000}, and THPTA (tris-hydroxypropyltriazolylmethylamine), were purchased from Click Chemistry Tools, Aminooxy-PEG_{5000} was purchased from Nanocs Inc. UDP-2-keto-galactose was synthesized following published procedures.[1] UDP-GalNAz was also synthesized in house following published procedures.[2] All aqueous solutions were prepared using ultrapure laboratory grade water (deionized, filtered, sterilized) obtained from an in-house ELGA water purification system and filter sterilized with 0.45 μm syringe filters (VWR) before use. Growth media (LB broth, Miller, Novagen and TB broth, Sigma) were prepared, sterilized, stored, and used according to the manufacturer. Antibiotics were prepared as stock solutions at a working concentration of 1000 X (ampicillin sodium salt, MilliporeSigma 100 mg mL^{-1}; kanamycin sulfate, MilliporeSigma, 50 mg mL^{-1}) and stored at -20 °C. All bacterial growth media and cultures were handled using sterile conditions under open flame. All silica gel column chromatography was performed using 60 Å silica gel (MilliporeSigma) and all thin-layer chromatography performed using 60 Å, F254 silica gel plates (MilliporeSigma) with detection by ceric ammonium molybdate (CAM) and/or UV light. Reversed phase high-performance liquid chromatography (RP-HPLC) was performed using an Agilent Technologies 1200 Series HPLC with Diode Array Detector. Unless otherwise stated the HPLC buffers used were buffer A: 0.1% trifluoroacetic acid (TFA) in H_2O, buffer B: 0.1% TFA, 90% acetonitrile in H_2O. Mass spectra were acquired on an API 3000 LC/MS-MS System (Applied Biosystems/MDS SCIEX).

Peptide Synthesis

Solid-phase peptide syntheses were conducted manually on unprotected Rink amide ChemMatrix resin (PCAS BioMatrix) with an estimated loading of 0.6 mmol g^{-1}. Protected O-GlcNAcylated serine was prepared as previously described.[3] Commercially available N-Fmoc and side-chain-protected amino acids (5 equiv, Anaspec) were activated for 20 min with HBTU (5 equiv, Novabiochem) and N,N-diisopropylethylamine (DIEA) (10 equiv, Sigma) and then coupled to the resin for 1.25 h followed by 4 x 1 min DMF washes. After coupling, the terminal Fmoc group was removed with 2 x 20% v/v piperidine in DMF for 20 min and then followed by 4 x 1 min DMF washes. For activated glycosylated amino acids, 2 equivalents of the pentafluorophenyl activated-ester in DMF was coupled overnight. When peptides were completed, the final Fmoc group was removed as described above, and the N-terminal amine was acetylated with 5 equivalents each of pyridine and acetic anhydride in DMF and then washed 3 x DMF. The acetyl groups on glycosylated amino acids were deprotected with 2 x MeOH for 1 min followed by 3 x 20% hydrazine monohydrate in MeOH twice for 30 min. The peptide was then cleaved from the resin by incubating in cleavage cocktail (95:2.5:2.5 TFA/H_2O/triisopropylsilane) for 3.5 h at room temperature. The peptides were then diluted approximately 1/10 in cold diethyl ether and precipitated overnight (-80 °C). The resulting suspensions were centrifuged (30 min, 5,000 x g, 4 °C). The pellet was then resuspended in H_2O, flash frozen, and lyophilized. Crude lyophilized material was purified using an 8% to 17.5% B gradient over 16 min. Calculated = 1179.64, observed = 1179.04. Yield, 10.2 mg.

Cloning of pTXB1-HA-Ubiquitin-AvaDnaE-6XHis

The plasmid was prepared by following standard molecular cloning techniques. pTXB1-Ubiquitin-AvaDnaE-6XHis was generated as previously described.[4,5] HA-Ubiquitin insert was

generated by cloning ubiquitin to include 5' NdeI digestion site followed by a HA tag, and a '3 Bpu10I digestion site from pTXB1-Ubiquitin-AvaDnaE-6XHis. The resulting PCR product was cloned into the pTXB1-Ubiquitin-AvaDnaE-6XHis vector between the Ndel and Bpu10I sites. Plasmid is available upon request.

HA-Ubiquitin-MESNa Expression and Purification.
pTXB1-HA-Ubiquitin-AvaDnaE-6XHis was subsequently transformed into BL21 *E. coli* (Novagen). Terrific broth (4 x 300 mL) containing ampicillin (100 μg mL^{-1}) was inoculated with 3 mL of starter culture grown overnight at 37 °C. The 300-mL culture was grown at 37 °C until an OD (A600) of 0.60, at which time expression was induced with 0.5 mM isopropyl β-D-1-thiogalacto-pyranoside (IPTG) for 16 h at 25 °C. Cells were harvested by centrifugation (10 min, 6,000 x g, 4 °C). The pooled pellets were suspended in 40 mL Buffer A (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, pH 8) and sonicated for 8 min (30 s on, 30 s off). The resulting lysate was then centrifuged for 1 h at 6,000 x g, 4 °C. The supernatant was transferred into a new tube and 2.5 mL of prewashed Ni-NTA Agarose (Qiagen) was added and placed onto a rotator for 1 h, 4 °C. The solution was then transferred to a gravity-flow column and allowed to drain, and washed with an addition 100 mL of Buffer A. HA-Ubiquitin-AvaDnaE-6XHis was eluted with Buffer B (Buffer A with 250 mM imizozole). Eluted fractions were dialyzed against 1 L dialysis buffer (100 mM NaH_2PO_4, 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), and 1 mM TCEP HCl, pH 7.75). To the dialyzed protein, an equal volume of sodium mercaptoethanesulfonate (MESNa) buffer (100mM NaH_2PO_4, 150 mM NaCl, 1 mM EDTA, 2 mM TCEP HCl, and 400 mM MESNa, pH 7.75) was added, and the mixture was incubated overnight to generate the protein thioester. Crude material was purified using a 30% to 60% B gradient over 20 min. Calculated = 9773 Da, observed = 9775 ± 2 Da. Yield, 10 mg L^{-1} of TB

Expressed Protein Ligation.
To a tube containing 11 mg of HA-Ubiquitin-MESNa (1 eq) in 4.4 mL of ligation buffer (3 M guanidine HCl, 300 mM NaH_2PO_4, 30 mM MPAA, 30 mM TCEP, pH 7.5) was added 2.8 mg of the O-GlcNAc Peptide (2 eq). The reaction was covered in foil and left on a rocker for 24 h. Crude material was purified by 38% to 42% B gradient over 15 min. Calculated = 10,810 Da, observed = 10,813 ± 1 Da. Yield, 5.1 mg.

Antibodies
Anti-HA-Tag (3724S) was purchased from Cell Signaling Technology, and the blocking solution was 5% BSA in Cell Signaling Technology TBST. Anti-CREB1 (9104S) was purchased from Cell Signaling Technology, and the blocking solution was 5% non-fat milk in Cell Signaling Technology TBST. Anti-Nup62 (610497) was purchased from BD Biosciences, and the blocking solution was 5% non-fat milk in ThermoFisher Scientific TBST. Anti-FLAG-Tag (2368S) and Anti-TDP43 (3448S) were purchased from Cell Signaling Technology, and the blocking solution was 5% BSA in Cell Signaling Technology TBST. Anti-caspase-6 (9104S) was purchased from Cell Signaling Technology, and the blocking solution was 5% non-fat milk in Cell Signaling Technology TBST. Anti-OGA was purchased from Sigma Aldrich, and the blocking solution was 5% non-fat milk in ThermoFisher Scientific TBST. Anti-NEDD4 (07-049) was purchased from MilliporeSigma, and the blocking solution was 5% non-fat milk in ThermoFisher Scientific TBST. Horseradish peroxidase (HRP)-conjugated secondary antibody was purchased from Jackson ImmunoResearch and incubated in the same TBST as primary antibodies.

General Procedure for Western Blotting
After proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE, Criterion TGX 4-20% Gel, Bio-Rad), proteins were transferred to a PVDF membrane (Bio-Rad) using manufacturer's protocols on a Trans-Blot SD Semi-Dry Transfer Cell. All Western blots were blocked for

1 h at room temperature in appropriate blocking buffer. The blots were then incubated with the primary antibody in appropriate blocking buffer overnight at 4 °C. All primary antibodies were used at 1:1,000. The blots were then washed three times in TBST for 10 min and incubated with the horseradish peroxidase (HRP)-conjugated secondary antibody for 1 h in appropriate blocking buffer at room temperature. HRP-conjugated secondary antibodies were used at 1:10,000 dilutions. After being washed three more times with TBST for 10 min, the blots were developed using ECL reagents (Bio-Rad) and the ChemiDoc XRS+ molecular imager (Bio-Rad).

Cell Culture

H1299 were grown in RPMI media (Corning) supplement with 10% Fetal Bovine Serum (Altanta Biologicals). Cells were grown at 37 °C and 5.0% CO_2.

Expression and Purification of GalT(Y289L)

pET23a GalT(Y289L) plasmid, provided by P. Qasba, National Cancer Institute, was transformed into BL21 *E. coli* (Novagen). Terrific broth (300 mL) containing ampicillin (100 μg mL^{-1}) was inoculated with 2.5 mL of starter culture grown overnight at 37 °C. The 300-mL culture was grown at 37 °C until an OD (A600) of 0.70, at which time expression was induced with 1 mM IPTG for 4 h at 37 °C. Cells were harvested by centrifugation (10 min, 6,000 x g, 4 °C) and resuspended in 10 mL of suspension buffer (25% sucrose w/v in 1 x DPBS). Cells were lysed using sonication (30 s pulse, 30 s rest, 12 min at 4 °C). The resulting lysate was diluted to 80 mL in cold suspension buffer, and inclusion bodies were harvested by centrifugation (30 min, 15,000 x g, 4 °C) and washed 8 times by resuspension in 80 mL of suspension buffer followed by centrifugation. Inclusion bodies were then washed once with 80 mL of 10 mM phosphate (pH 7.0) and harvested by centrifugation. Inclusion bodies were then resuspended in 14 mL of cold H_2O, poured over a mixture of 12 g solid guanidine HCl (GuHCl) and 944 mg Na_2SO_3, resulting in a final concentration of 5 M GuHCl and 300 mM Na_2SO_3, and then vortexed vigorously before adjusting the volume to 24 mL with cold H_2O. Freshly made NTSB solution (50 mM (5,5-dithio-bis-(2-nitrobenzoic acid) (DTNB), 1 M Na_2SO_3 in water pH 7.5) was then added with vigorous vortexing to sulfonate free thiols. Completion of the reaction is indicated by a color change from dark orange to pale yellow. Protein was then precipitated with cold H_2O (250 mL) and centrifuged immediately (30 min, 15,000 x g, 4 °C). The protein pellet was washed three times by resuspension in 250 mL cold H_2O and centrifugation. The protein pellet was finally resuspended in 14 mL of cold H_2O, poured over 12 g solid GuHCl, vortexed, and then diluted to 25 mL with H_2O (resulting in a 5 M final concentration). The protein solution was diluted to a final concentration of 1 mg mL^{-1} with 5 M GuHCl solution (OD (A275) ≈ 2.0). This solution was diluted 10-fold into cold refolding buffer (5 mM EDTA, 4 mM cysteamine, 2 mM cystamine, 100 mM Tris base, pH 8.0) with gentle stirring. Protein was allowed to refold for 48 h at 4 °C without agitation. The still refolding protein was then dialyzed two times into cold H_2O for 24 h and concentrated using stirred cells (Amicon, MilliporeSigma) and centrifugal filters (10-kDa cutoff, Amicon Ultra, MilliporeSigma) to 3 mL. Buffer was exchanged once with 10 mL of reaction buffer (10 mM Tris base, pH 8.0), and final protein concentration was determined (OD (A275) ≈ 2). Purified protein was stored at 4 °C.

UDP-GalNAz Enzymatic Labeling for Strain-Promoted Azide-Alkyne Cycloaddition (SPAAC)

H1299 cells grown to 80% confluency in a 10-cm dish were collected by trypsinization and washed two times with PBS (2 min, 2,000 x g, 4 °C). The resulting cell-pellets were resuspended in 26 μL H_2O containing 10X of c0mplete mini protease inhibitor cocktail (Sigma Aldrich), and 50 μL of 0.05% SDS buffer (0.05% SDS, 10 mM TEA pH 7.4, 150 mM NaCl) was added. To this, 1 μL benzonase (MilliporeSigma) was added, and the cells were incubated on ice for 30 min. Then, 200 μL of 4% SDS buffer (4% SDS, 10 mM TEA pH 7.4, 150 mM NaCl) was added, and the cells were briefly sonicated followed by centrifugation (10,000 x g for 10 min at 15 °C). Soluble protein concentration was

normalized by BCA assay (Pierce, ThermoFisher Scientific) to 1 mg mL^{-1}. Proteins were precipitated by adding 3 x volume of methanol, 0.75 x volume of chloroform, and 2 x volume of H_2O followed by vortexing and centrifugation (5 min, 5,000 x g). The aqueous phase was discarded without disturbing the interface layer before adding a 2.5 x volume of methanol, vortexing, and pelleting the protein (10 min, 5,000 x g). The resulting protein pellet was allowed to air dry for 5-10 min before being resuspended in a fourth of the original volume using 1% SDS GalT buffer (1% SDS, 20 mM HEPES, pH 7.9). Protein concentration was again normalized using the BCA Assay and diluted to 2.5 mg mL^{-1} in 1% SDS GalT buffer. To 40 μL of resuspended protein (100 μg) was added 49 μL of H_2O, 80 μL of 2.5 X labeling buffer (5% IGEPAL CA-630, 125 mM NaCl, 50 mM HEPES, pH 7.9), 11 μL of $MnCl_2$ (100 mM in H_2O), and 10 μL of UDP-GalNAz (0.5 mM in 10 mM HEPES, pH 7.9) and vortexed. Finally, 10 μL of purified GalT(Y289L) or H_2O was added, and the reaction mixture was incubated for 20 h at 4 °C.

UDP-GalNAz Enzymatic Labeling for Cu(I)-Catalyzed [3 + 2] Azide−Alkyne Cycloaddition (CuAAC)

After protein lysis and precipitation which was described previously, soluble protein concentration was normalized by BCA assay to 2 mg mL^{-1}. Dithiothreitol (DTT) was added (1 M stock, 25 mM final) and allowed to react at 60 °C for 45 min; afterwards, iodoacetamide (500 mM stock, 50 mM final) was added and allowed to react for 45 min in the dark. Unreacted DTT and iodoacetamide were then removed by methanol/chloroform/H_2O precipitation as described previously, and pellets were washed 5 times with 500 μL methanol. The resulting protein pellet was allowed to air dry for 5-10 min before being resuspended at 2.5 mg mL^{-1} using 1% SDS GalT buffer. To 40 μL of resuspended protein (100 μg) was added 49 μL of H_2O, 80 μL of 2.5 X labeling buffer (5% IGEPAL CA-630, 125 mM NaCl, 50 mM HEPES, pH 7.9), 11 μL of $MnCl_2$ (100 mM in H_2O), and 10 μL of UDP-GalNAz (0.5 mM in 10 mM HEPES, pH 7.9) and vortexed. Finally, 10 μL of purified GalT Y289L or H_2O was added, and the reaction mixture was incubated for 20 h at 4 °C.

ThermoFisher Scientific Enzymatic Labeling

Click-iT protein analysis was conducted as described by manufacture (ThermoFisher Scientific, Click-IT O-GlcNAc Enzymatic Labeling System). Sample preparation was conducted as above, but to 40 μL of resuspended protein (100 μg) was added 49 μL of H_2O, 80 μL of labeling buffer, 11 μL of $MnCl_2$, and 10 μL of UDP-GalNAz and vortexed. Finally, 10 μL of GalT Y289L or H_2O was added, and the reaction mixture was incubated for 20 h at 4 °C.

UDP-2-keto-galactose Enzymatic Labeling

Sample preparation was conducted as above, but to 40 uL of resuspended protein (100 μg) was added 49 μL of H_2O, 80 μL of labeling buffer, 11 μL of $MnCl_2$, and 10 μL of UDP-2-keto-galactose (10 mM in 10 mM HEPES, pH 7.9) and vortexed. Finally, 10 μL of GalT Y289L or H_2O was added, and the reaction mixture was incubated for 20 h at 4 °C.

Alkylation of Cysteines and SPAAC

After chemoenzymatic labeling as described previously, 7.5 μL of freshly made iodoacetamide (600 mM) in H_2O was added directly for a final concentration of 22.6 mM, and the mixture was incubated for 30 min in the dark. Unreacted UDP-GalNAz and iodoacetamide were then removed by methanol/chloroform/H_2O precipitation as described previously. Air-dried protein pellets were resuspended in 90 μL 1% SDS (1% SDS, 150 mM NaCl, 50 mM TEA, pH 7.4). Then, 10 μL of 10 mM DBCO-PEG_{5000} in DMSO was added to a final concentration of 1 mM, and the mixture was incubated for 16 h in the dark or for 5 min at 98 °C. Unless otherwise noted, unreacted DBCO-PEG_{5000} was then removed by precipitation as described previously. The pellet was allowed to air dry for 5-10 min, and then 25 μL 4% SDS buffer was added to each sample. The mixture was sonicated in a bath sonicator to ensure

complete dissolution, and 25 μL of 2 x loading buffer (20% glycerol, 0.2% bromophenol blue, 1.4% β-mercaptoethanol, pH 6.8) was then added. The samples were boiled for 5 min at 98 °C, and ~40 μg (or ~18.75 μg) of protein was then loaded per lane for SDS-PAGE separation.

Cu(I)-Catalyzed [3 + 2] Azide−Alkyne Cycloaddition (CuAAC)

Chemoenzymatic labeling was conducted as described above, and CuAAC was performed as described previously.[6] Unreacted UDP-GalNAz was then removed by precipitation as described. Air-dried protein pellets were resuspended in 50 μL 1% SDS-Tris (1% SDS, 50 mM Tris, pH 8) and 46 μL of H_2O was added. In a separate tube, 2 μL of THPTA (500 mM THPTA in DMSO), 20 μL of $CuSO_4 \cdot 5H_2O$ (50 mM in H_2O), and 400 μL of alkyne-PEG_{5000} (10 mM in 200 mM Na_2HPO_4 pH 8) were mixed (volumes indicated are for a total of 4 reactions). To each resuspended pellet, 100 μL of the above THPTA/$CuSO_4$/PEG solution was added, and the solution was vortexed briefly before adding 4 μL of TCEP (100 mM in H_2O). The reaction mixture was then vortexed gently and allowed to incubate for 24 h at 37 °C. Unreacted click reagents were removed by methanol/chloroform/H_2O precipitation as described previously, and pellets were washed 5 times with 500 μL of MeOH. The pellets were allowed to air dry for 5-10 min, and 25 μL of 4% SDS buffer was added to each sample. The mixture was sonicated in a bath sonicator to ensure complete dissolution, and 25 μL of 2 x loading buffer was then added. The samples were boiled for 5 min at 98 °C, and 40 μg of protein was loaded per lane for SDS-PAGE.

Oxime Formation

After chemoenzymatic labeling, unreacted UDP-2-keto-galactose was removed by precipitation as described previously.[1] Air-dried protein pellets were resuspended in 169.6 μL of 7 M urea in 10 mM HEPES, 2% CHAPS, 1 mM DTT, pH 7.9. Then 11.4 μL of 1.8 M sodium acetate (pH 3.89) was added followed by 24 μL of aminoxy-PEG_{5000} (60 mM in DMSO, Nanocs Inc.). 5 μL of the reaction was used to check the pH (pH-indicator strips pH 4.0 - 7.0, MilliporeSigma); the pH was further adjusted to 4.5 with 1.8 M sodium acetate (pH 3.89) if necessary. The reaction was then incubated for 24 h at 25 °C before unreacted aminoxy-PEG_{5000} was removed by methanol/chloroform/H_2O precipitation as described previously for CuAAC. The pellet was allowed to air dry for 5-10 min, and then 25 μL of 4% SDS buffer was added to each sample. The mixture was sonicated in a bath sonicator to ensure complete dissolution, and 25 μL of 2 x loading buffer was then added. The samples were boiled for 5 min at 98 °C, and 40 μg of protein was loaded per lane for SDS-PAGE.

GalT(Y289L) Background Testing and Antibodies

2.5 μg of GalT(Y298L) (to mimic amount loaded for a Chemoenzymatic Labeling) from four different batches of purified GalT(Y289L) was loaded into each well. Western blotting was performed as described above.

Transient CREB Transfection

H1299 cells were grown to 80% confluency and the media was exchanged with fresh media. The cells were transiently transfected with pLEMPRA-CREB-FLAG or pLEMPRA-CREB(6A)-FLAG plasmids[7] using Lipofectamine 2000 (ThermoFisher Scientific) according to the manufacturer's protocol. Twenty-four hours post-transfection, cells were harvested and then sample preparation was conducted as above.

References

(1) Rexach, J. E., Rogers, C. J., Yu, S.-H., Tao, J., Sun, Y. E., and Hsieh-Wilson, L. C. (2010) Quantification of O-glycosylation stoichiometry and dynamics using resolvable mass tags. *Nat Chem Biol 6*, 645–651.

(2) Hang, H. C., Yu, C., Kato, D. L., and Bertozzi, C. R. (2003) A metabolic labeling approach toward proteomic analysis of mucin-type O-linked glycosylation. *Proc Natl Acad Sci USA 100*, 14846–14851.

(3) Marotta, N. P., Cherwien, C. A., Abeywardana, T., and Pratt, M. R. (2012) O-GlcNAc Modification Prevents Peptide-Dependent Acceleration of α-Synuclein Aggregation. *ChemBioChem 13*, 2665–2670.

(4) Shah, N. H., Dann, G. P., Vila-Perelló, M., Liu, Z., and Muir, T. W. (2012) Ultrafast Protein Splicing is Common among Cyanobacterial Split Inteins: Implications for Protein Engineering. *J Am Chem Soc 134*, 11338–11341.

(5) Abeywardana, T., and Pratt, M. R. (2015) Extent of inhibition of α-synuclein aggregation in vitro by SUMOylation is conjugation site- and SUMO isoform-selective. *Biochemistry 54*, 959–961.

(6) Thompson, J. W., Griffin, M. E., and Hsieh-Wilson, L. C. (2018) Methods for the Detection, Study, and Dynamic Profiling of O-GlcNAc Glycosylation. *Meth Enzymol 598*, 101–135.

(7) Rexach, J. E., Clark, P. M., Mason, D. E., Neve, R. L., Peters, E. C., and Hsieh-Wilson, L. C. (2012) Dynamic O-GlcNAc modification regulates CREB-mediated gene expression and memory formation. *Nat Chem Biol 8*, 253–261.