Visualization of *O*-GlcNAc Glycosylation Stoichiometry and Dynamics Using Resolvable Poly(ethylene glycol) Mass Tags

Peter M. Clark,[1,2,3] **Jessica E. Rexach,**[1,2,3] **and Linda C. Hsieh-Wilson**[1,2]

[1]Division of Chemistry and Chemical Engineering, California Institute of Technology, Pasadena, California
[2]Howard Hughes Medical Institute, California Institute of Technology, Pasadena, California
[3]These authors contributed equally to this work

ABSTRACT

O-linked *N*-acetylglucosamine (*O*-GlcNAc) glycosylation is a dynamic protein posttranslational modification with roles in processes such as transcription, cell cycle regulation, and metabolism. Detailed mechanistic studies of *O*-GlcNAc have been hindered by a lack of methods for measuring *O*-GlcNAc stoichiometries and the interplay of glycosylation with other posttranslational modifications. We recently developed a method for labeling *O*-GlcNAc-modified proteins with resolvable poly(ethylene glycol) mass tags. This mass-tagging approach enables the direct measurement of glycosylation stoichiometries and the visualization of distinct *O*-GlcNAc-modified subpopulations. Here, we describe procedures for labeling *O*-GlcNAc glycoproteins in cell lysates with mass tags. *Curr. Protoc. Chem. Biol.* 5:281-302 © 2013 by John Wiley & Sons, Inc.

Keywords: *O*-linked *N*-acetylglucosamine • glycosylation • posttranslational modifications • chemoenzymatic labeling • poly(ethylene glycol) • protein subpopulations

---

INTRODUCTION

O-linked *N*-acetylglucosamine (*O*-GlcNAc) glycosylation (or *O*-GlcNacylation) is the posttranslational modification of serine and threonine residues of proteins with *O*-linked *N*-acetyl-D-glucosamine. The *O*-GlcNAc modification is found on over 800 intracellular proteins and regulates processes such as transcription, apoptosis, glucose sensing, and metabolism (Love and Hanover, 2005; Hart et al., 2007; Rexach et al., 2008). Chemoenzymatic labeling of *O*-GlcNacylated proteins with a resolvable mass tag is a method for quantitatively imaging and analyzing *O*-GlcNAc-modified protein subpopulations. This approach involves attaching a poly(ethylene glycol) (PEG) polymer of defined molecular mass (e.g., 2 kDa or 5 kDa) selectively onto the *O*-GlcNAc sugar and resolving the labeled populations by SDS-PAGE. The polymer shifts the molecular weight of the glycoprotein, which enables visualization of the number of sugars attached, as well as quantification of the glycosylation stoichiometry. The method is performed in three steps: (1) enzymatic labeling of *O*-GlcNAc residues with a mutant galactosyltransferase and an unnatural ketogalactose sugar, (2) conjugation of an aminooxy-functionalized PEG mass tag to the ketogalactose sugar, and (3) SDS-PAGE and immunoblotting for the protein(s) of interest (Fig. 1A; Rexach et al., 2010). A variation on this approach replaces the ketogalactose sugar with an azidogalactose sugar and utilizes [3+2] azide-alkyne cycloaddition chemistry to conjugate an alkyne-functionalized PEG mass tag to the glycoproteins (Fig. 1B; Clark et al., 2008). Notably, this mass-tagging approach can be applied to endogenous proteins in cell lysates and requires no protein purification, radiolabels, or advanced instrumentation.

Current Protocols in Chemical Biology 5:281-302, December 2013
Published online December 2013 in Wiley Online Library (wileyonlinelibrary.com).
DOI: 10.1002/9780470559277.ch130153
Copyright © 2013 John Wiley & Sons, Inc.

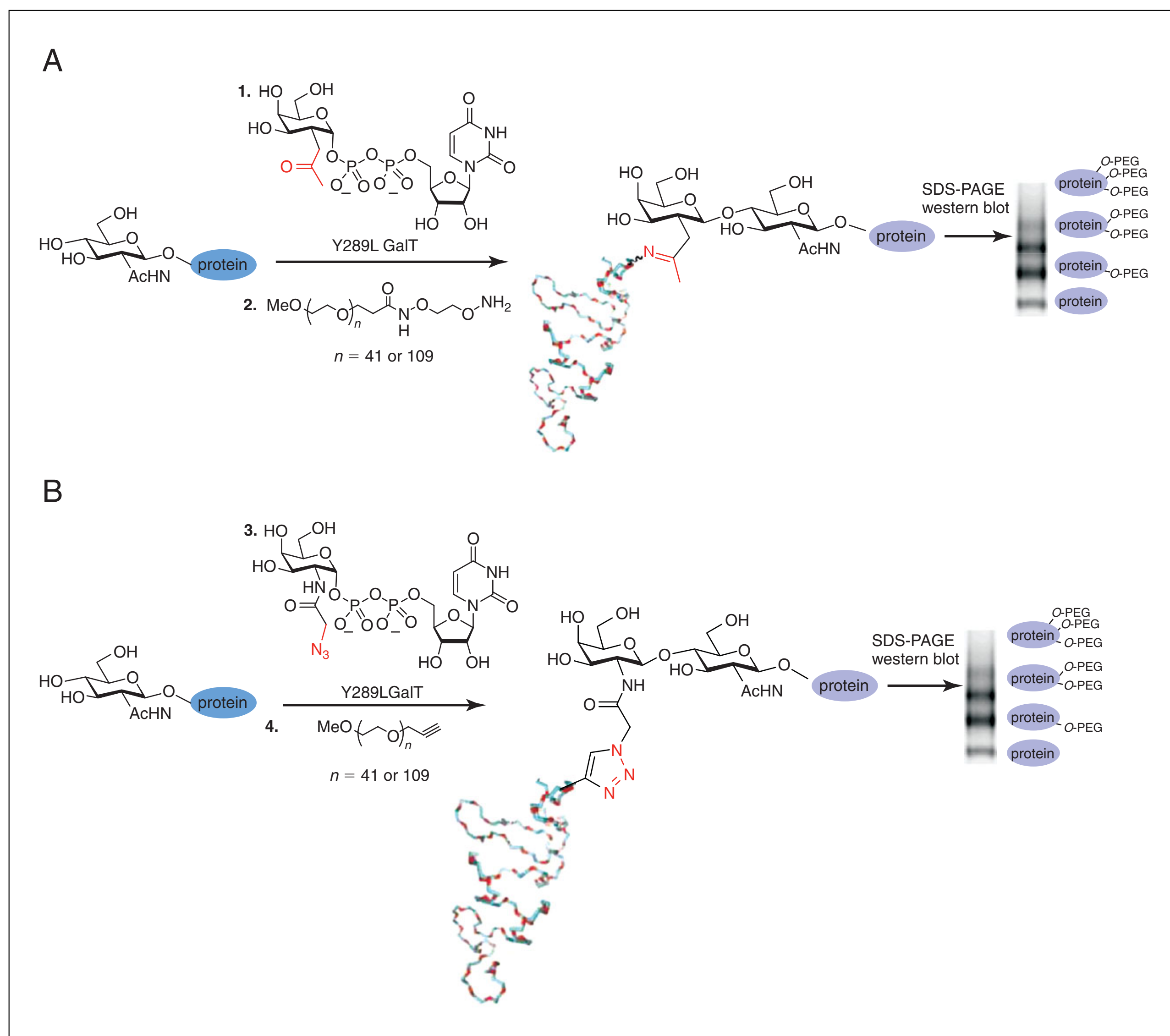

Figure 1 Chemoenzymatic labeling of *O*-GlcNAc residues with poly(ethylene glycol) (PEG) mass tags. (**A**) *O*-GlcNAcylated proteins in cell lysates are labeled with an unnatural ketogalactose sugar (1) and a mutant galactosyltransferase (Y289L GalT). Labeled proteins are reacted with an aminooxy-functionalized 2-kDa or 5-kDa mass tag (2) through a bioorthogonal oxime-forming reaction. Glycosylated proteins labeled with the mass tag are resolved and identified by immunoblotting. The mass tag shifts the mass of the glycosylated subpopulation by intervals of 2 kDa or 5 kDa, depending on the mass tag used and the number of *O*-GlcNAc moieties attached. (**B**) Alternatively, *O*-GlcNacylated proteins in cell lysates are labeled with an unnatural azidogalactose sugar (3) and the mutant GalT. Labeled proteins are reacted with an alkynyl-functionalized 2-kDa or 5-kDa mass tag (4) through a bioorthogonal [3+2] azide-alkyne cycloaddition reaction.

The ability to visualize distinct *O*-GlcNAc-modified subpopulations and quantify in vivo glycosylation stoichiometries (Fig. 2) provides information that is not readily attainable using other methods. *O*-GlcNAc glycosylation stoichiometries can be rapidly measured on endogenous proteins and compared across different cellular conditions (Rexach et al., 2010, 2012; Yi et al., 2012). For example, the mass-tagging approach was used to measure changes in the glycosylation stoichiometry of cyclic-AMP response element–binding protein (CREB) and phosphofructokinase-1 (PFK1) in response to neuronal depolarization and hypoxia, respectively (Rexach et al., 2012; Yi et al., 2012). Additionally, the glycosylation state (e.g., mono-, di-, or tri-glycosylation) of proteins can be rapidly established. A protein that exists in a nonglycosylated, monoglycosylated, and diglycosylated state will resolve into three distinct bands after mass tagging and SDS-PAGE. When used in conjunction with site-directed mutagenesis, the approach can also

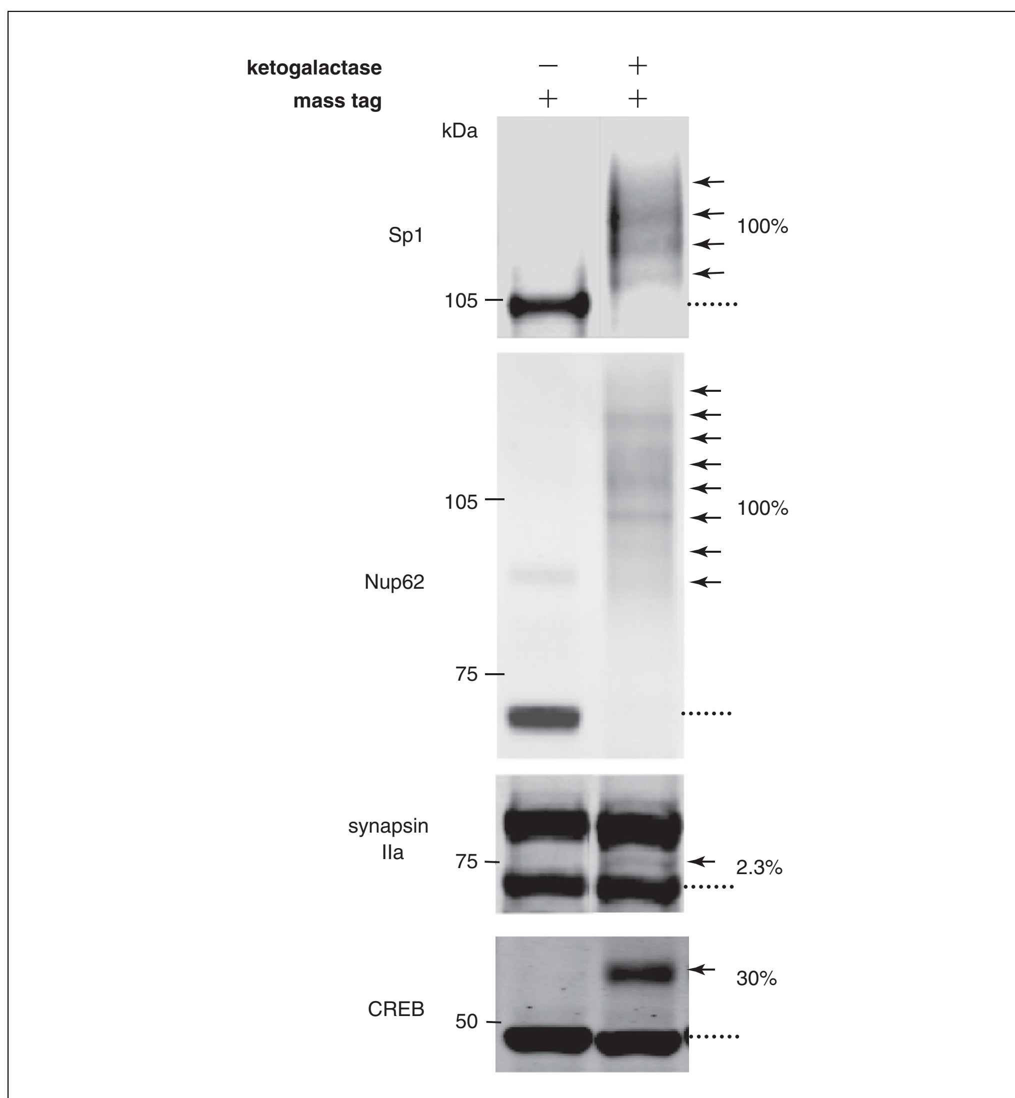

Figure 2 *O*-GlcNAc stoichiometries of Sp1, Nup62, synapsin IIa, and cyclic-AMP response element—binding protein (CREB). Cell lysates were not labeled (−) or labeled (+) with the unnatural ketogalactose sugar, reacted (+) with a 2-kDa or 5-kDa mass tag, resolved by SDS-PAGE, and identified by immunoblotting. A 2-kDa mass tag was used to label CREB and a 5-kDa mass tag was used to label Sp1, Nup62, and synapsin Ia (upper band) and synapsin IIa (lower band). (. . .) denotes the nonglycosylated protein fraction; (←) denotes shifted protein fraction. This figure was adapted from Rexach et al. (2010).

determine the occupancy of the *O*-GlcNAc sugar at specific amino acid sites. Finally, the mass-tagging approach can be used to monitor the interplay between *O*-GlcNacylation and other posttranslational modifications (Rexach et al., 2010, 2012). In previous work, neurons were activated by KCl-induced depolarization and, at defined time points, the cell lysates were mass tagged and immunoblotted with both a total CREB antibody and a phospho-Ser133-specific CREB antibody. This enabled visualization of four distinct posttranslationally-modified subpopulations of CREB and was used to probe how glycosylation affected the kinetics of CREB phosphorylation and vice versa (Rexach et al., 2012).

The Basic Protocol below describes the labeling of *O*-GlcNAc residues with a mass tag, using oxime chemistry. The Alternate Protocol gives a detailed procedure for labeling *O*-GlcNAc residues with [3+2] azide-alkyne cycloaddition chemistry. The Basic Protocol is better characterized than the Alternate Protocol but requires chemical synthesis. Support Protocol 1 describes the expression and isolation of Y289L GalT, a mutant Y289L

galactosyltransferase, for use in the Basic and Alternate Protocols. Support Protocol 2 explains how to calculate protein glycosylation stoichiometries.

BASIC PROTOCOL

LABELING *O*-GlcNAc RESIDUES ON PROTEINS WITH A RESOLVABLE MASS TAG USING OXIME CHEMISTRY

The Basic Protocol describes labeling of *O*-GlcNAc residues on proteins with a resolvable mass tag by reacting a ketone moiety with an aminooxy PEG reagent. This is the best characterized system but requires chemical synthesis of the UDP-ketogalactose and aminooxy-functionalized PEG reagents.

Materials

Cell or tissue pellet
Lysis buffer (see recipe) *or* nondenaturing buffer supplemented with β-*N*-acetylglucosaminidase inhibitor, e.g., 10 μM *O*-(2-acetamindo-2-deoxy-D-glucopyranosylidene)amino *N*-phenylcarbamate (PUGNAc; Toronto Research Chemicals) and phosphatase inhibitors, e.g., 1 mM $NaVO_3$, 29 mM, NaF, and 0.5 mM Na_2MoO_4
Methanol
Chloroform
Water: Milli-Q purified, or equivalent
Ketone labeling resuspension buffer (see recipe)
20% (v/v) Triton X-100
100 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), pH 7.9
100 mM $MnCl_2$
5 mM NaCl
50× complete protease inhibitor cocktail: dissolve one Complete Protease Inhibitor Tablet, EDTA-free (Roche) to 50× concentration in 1 ml water
10 mM uridine 5′-diphospho-2-acetonyl-2-deoxy-α-D-galactopyranose diammonium salt (UDP-ketogalactose; Khidekel et al., 2003)
2 mg/ml mutant Y289L galactosyltransferase (Y289L GalT; Support Protocol 1)
Mass tag resuspension buffer (see recipe)
60 mM aminooxy-functionalized poly(ethylene glycol) (aminooxy-functionalized PEG; Rexach et al., 2010), checked for quality by mass spectrometry for the presence of a single collection of peaks centered around 2 kDa or 5 kDa, respectively
1.8 M sodium acetate buffer, pH 3.89
Neutralization buffer: 1% (w/v) SDS/100 mM HEPES, pH 7.9
1% (w/v) SDS
4% to 12% Bis-Tris NuPAGE gradient gel (Invitrogen)
Positive control lysate (e.g., adult rat brain or 293T cell lysate)

Sonicator (Vibra-Cell 130 watts; Sonics)
Refrigerated centrifuge
1.5-ml microcentrifuge tubes
Test tube inverter (Fisher Scientific)
Table-top test tube rocker

Additional reagents and equipment for determining protein concentration (Olson and Markwell, 2007), carrying out SDS-PAGE (Gallagher, 2012), and performing immunoblottimg (Gallagher, 2001, Ursitti et al., 2001)

Lyse the cells and measure protein concentration

1. Prepare a cell or tissue pellet using standard techniques.

2. Lyse the pellet by adding an equal volume of boiling lysis buffer to resuspend the sample, and sonicate the cell lysate until the solution is no longer viscous (e.g., three times for 5 sec at 30% amplitude).

   When using nondenaturing buffers, prepare the sample on ice using ice-cold buffers and supplement each buffer with a β-N-acetylglucosaminidase inhibitor such as 10 μM O-(2-acetamindo-2-deoxy-D-glucopyranosylidene)amino N-phenylcarbamate (PUGNAc) and the phosphatase inhibitors $NaVO_3$ (1 mM), NaF (20 mM), and Na_2MoO_4 (0.5 mM).

   Lysing the cell or tissue sample quickly under denaturing conditions or on ice with a β-N-acetylglucosaminidase inhibitor is critical to maintaining the protein glycosylation states. Occasionally it may be desirable or advantageous to perform a subcellular fractionation (see Troubleshooting).

   If the sample was lysed in >1% SDS, boil it for 5 min.

3. Centrifuge the sample 10 min at 22,000 × *g*, 4°C.

4. Promptly remove the sample from the centrifuge and place at room temperature to prevent SDS precipitation.

5. Transfer the solubilized lysate in the supernatant into a fresh 1.5-ml microcentrifuge tube, and discard any remaining pellet.

6. Measure the protein concentration of the sample.

   We generally prefer measuring protein concentrations using the BCA protein detection assay due to its compatibility with high concentrations of SDS. However, other protein detection assays that are compatible with the chosen lysis buffer may also be acceptable.

Precipitate the protein

7. Dispense 100 μg of protein into a 1.5-ml microcentrifuge tube. If the volume of the 100 μg protein solution is less than 100 μl, add lysis buffer to 100 μl. If the volume of the 100 μg protein solution is greater than 100 μl, then scale the volume of the following solutions accordingly. Include negative (without Y289L GalT enzyme) and positive (rat brain or T293 cell lysates) control tubes.

   For each set of experimental conditions, it is important to prepare a separate negative control sample that will be incubated with everything except for the Y289L GalT enzyme. It is also advisable to include a positive control sample using lysate from 293T cells (to detect glycosylated Sp1) or rat brain (to detect glycosylated Nup62).

8. To the 100 μl protein lysate, add 300 μl methanol, and vortex the solution. Next, add 100 μl chloroform, and vortex the solution again. Finally, add 225 μl water and vortex. Vortex ∼10 sec each time.

   The solution should quickly separate into two phases, with a clear bottom phase and a cloudy top phase.

9. Centrifuge the samples 10 min at 22,000 × *g*, 4°C.

   Two separate phases should appear after centrifuging the samples, and a white pellet (the precipitated protein) should reside at the interface of those phases.

10. Immediately remove and discard most of the top phase without disturbing the pellet.

   The top layer can usually be removed down to within a millimeter or two of the pellet without disturbing it. These steps should be done at room temperature.

11. Add 225 µl methanol, and gently disrupt the protein pellet by briefly vortexing or inverting the tube several times until the top and bottom phases are fully mixed.

    Be careful not to lose the pellet in the tube cap in this step.

12. Centrifuge the sample 10 min at 22,000 × *g*, 4°C.

    After centrifuging the sample, the solution should be in only one phase and the white pellet should now reside at the bottom of the tube.

13. Remove the liquid phase, and allow the pellet to air dry, typically ~30 min.

    It is important that the solution phase fully evaporates without overdrying the pellet. Over- or underdried pellets will be difficult to resuspend. An ideal point is typically when the pellet is still somewhat translucent and no liquid puddle remains. If all of the supernatant has been carefully removed from the pellet, the pellet will usually dry within 30 min.

Label samples with ketogalactose and mutant Y289L galactosyltransferase

14. Resuspend the pellet in 20 µl of ketone labeling resuspension buffer.

    Resuspending the protein sample after precipitation is often difficult, particularly if it is over- or underdried. Vortexing the sample (being careful not to cause foam), sonicating the sample, boiling the sample, and agitating the sample by pipetting may all improve the speed and efficiency of protein resuspension.

15. For each sample, place the 20 µl of resuspended protein (5 mg/ml) on ice and add in the following order:

    9 µl of 20% (v/v) Triton X-100
    38 µl of H_2O
    10 µl of 100 mM HEPES, pH 7.9
    10 µl of 100 mM $MnCl_2$
    2 µl of 5 M NaCl
    2 µl of 50× complete protease inhibitor cocktail.

    Mix by pipetting up and down. Then add 5 µl of 10 mM UDP-ketogalactose substrate and mix. To all samples except the negative control reaction, add 4 µl of 2 mg/ml Y289L GalT. To the negative control reaction, add 4 µl water.

    In those situations where a significant number of samples will be labeled, a master mix containing water, HEPES, $MnCl_2$, NaCl, and protease inhibitor cocktail can be prepared. In that case, add the Triton X-100 to the sample and pipet to fully mix, followed by the master mix, UDP-ketogalactose, and Y289L GalT (in that order).

    The addition of 4 µl of 2 mg/ml Y289L assumes that the Y289L GalT is sufficiently active to completely label a model glycosylated peptide at a concentration of 50 ng/µl (see Support Protocol 1). If the Y289L GalT is less active, then adjust the enzyme concentration to ensure complete reaction.

16. Incubate the reaction overnight (12 to 16 hr) at 4°C, mixing by inversion of the tube end-over-end.

17. Precipitate the protein as described in steps 7 to 13.

Label samples with aminooxy-functionalized PEG mass tag

18. Resuspend the precipitated protein in 43.6 µl of mass tag resuspension buffer.

    As before, this step is often difficult if the pellet is not ideally dried. It may take up to an hour for the precipitated protein to redissolve.

19. Add 6 µl of 60 mM aminooxy-functionalized PEG and 1.422 µl of 1.8 M sodium acetate buffer, pH 3.89, and mix. Check the final pH of the solution on pH paper to ensure that it is 4.5.

    The aminooxy reaction requires a pH of 4.5.

    Before using any new batch of PEG, it is important to check the quality by mass spectrometry. The spectrum of high-quality PEG has a single collection of peaks centered on the correct mass (2 kDa or 5 kDa). The spectrum of low-quality PEG has multiple collections of peaks, usually centered at intervals of the correct mass (2 kDa or 5 kDa).

20. Incubate at room temperature for 20 to 24 hr. Gently rock the samples by placing them in a tube rack on a table-top rocker.

21. After the 20 to 24 hr incubation, add 50 µl of neutralization buffer, mix, and precipitate the reaction as described in steps 7 to 13.

22. Resuspend the pellet in 30 µl of 1% (w/v) SDS to a final concentration of 3.3 mg/ml.

23. Carry out gel electrophoresis (typically using a 4% to 12% Bis-Tris NuPAGE gradient gel and positive controls, e.g., labeled adult rat brain or 293T cell lysate) and immunoblotting, using standard techniques.

    During initial experiments, we typically run the entire sample in a single gel lane.

    Best results are generally achieved by resolving 50% to 75% of the labeled protein dissolved in SDS-PAGE loading dye on a 4% to 12% Bis-Tris NuPAGE gradient gel. To adequately resolve the shifted from the nonshifted protein subpopulations, the gel is typically run until the protein of interest has migrated through at least 50% of the gel.

    The control lysate can be blotted with an antibody against Nup62 (rat brain lysate) or Sp1 (293T cell lysate). As Nup62 and Sp1 are heavily O-GlcNAc glycosylated, they serve as excellent positive controls for the labeling experiments (Fig. 2).

    See Support Protocol 2 to calculate protein glycosylation stoichiometries.

LABELING *O*-GlcNAc RESIDUES IN CELL LYSATES WITH A RESOLVABLE MASS TAG USING [3+2] AZIDE-ALKYNE CYCLOADDITION CHEMISTRY

ALTERNATE PROTOCOL

An alternative to the *O*-GlcNAc labeling approach described in the Basic Protocol relies on [3+2] azide-alkyne cycloaddition (Click) chemistry, which can be performed with commercially available materials (Fig. 1B). The Click protocol uses a Click-iT *O*-GlcNAc Enzymatic Labeling System for the first set of reactions, and a Click-iT Protein Reaction Buffer Kit and alkynyl-functionalized PEG for the second set of reactions. Whereas both methods apply the same principles, it is important to note that the Click protocol may require some validation. For example, it is essential to confirm that the cycloaddition reaction proceeds to completion under your conditions and verify the results using *O*-GlcNacylated proteins of known stoichiometry.

Additional Materials *(also see Basic Protocol)*

Azide labeling resuspension buffer (see recipe)
10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), pH 7.9
Click-iT *O*-GlcNAc Enzymatic Labeling System (Life Technologies), including
- UDP-GalNAz (component A)
- Y289L GalT, labeled as Gal-T1 (Y289L) (component B)
- Click-iT labeling buffer (component C)

Click-iT Protein Reaction Buffer Kit (Life Technologies), including
- Click-iT reaction buffer (component A)
- Click-iT reaction component B ($CuSO_4$)

Click-iT reaction buffer additive 1 (component C)
Click-iT reaction buffer additive 2 (component D)

50× complete protease inhibitor cocktail (EDTA-free): dissolve one Complete Protease Inhibitor Tablet, EDTA-free (Roche) to 50× concentration in 1 ml water

Alkynyl labeling resuspension buffer (see recipe)

10 mM alkynyl-functionalized poly(ethylene glycol) in DMSO: (alkynyl-functionalized PEG; mPEG-alkyne, 2-kDa or 5-kDa; Creative PEGWorks), checked for quality by mass spectrometry for presence of a single collection of peaks centered around 2 kDa or 5 kDa, respectively

Test tube rocker

1. Follow steps 1 to 13 of the Basic Protocol.

Label samples with azidogalactose and mutant Y289L galactosyltransferase

The following set of steps is modified from the Click-iT Enzymatic Labeling System manual.

2. Resuspend the pellet in 20 µl of azide labeling resuspension buffer.

   Resuspending the protein sample after precipitation is often difficult, particularly if it is over- or underdried. Vortexing the sample (being careful not to cause foam), sonicating the sample, boiling the sample, and agitating the sample by pipetting may all improve the speed and efficiency of protein resuspension.

3. Add 144 µl of 10 mM HEPES, pH 7.9, to the UDP-GalNAz (component A of the Click-iT *O*-GlcNAc Enzymatic Labeling System) and mix well.

   Keep on ice. Dispense aliquots of any remaining UDP-GalNAz and store at −80°C until further use.

4. For each resuspended protein sample (step 2), place the 20 µl of resuspended protein on ice and add, in the following order:

   22.5 µl of water
   40 µl of Click-iT labeling buffer (component C of the Click-iT *O*-GlcNAc Enzymatic Labeling System)
   5.5 µl of 100 mM $MnCl_2$
   2 µl of 50× Roche Complete Protease Inhibitor Cocktail.

   Mix by pipetting up and down.

5. Then add 5 µl of 0.5 mM UDP-GalNAz (from step 3) and mix. Finally, to each sample except the negative control reaction, add 5 µl of Y289L GalT, labeled as Gal-T1 (Y289L) (component B of the Click-iT *O*-GlcNAc Enzymatic Labeling System). To the control reaction, add 5 µl of water.

6. Incubate the reaction overnight (12 to 16 hr) at 4°C, mixing by inversion of the tube end-over-end.

7. Precipitate the protein as described in steps 7 to 13 of the Basic Protocol.

Label samples with the alkynyl-functionalized PEG mass tag

The following set of steps is modified from the Click-iT Protein Reaction Buffer Kit manual.

8. Resuspend the precipitated protein in 25 µl of alkynyl labeling resuspension buffer.

   This step is often difficult. It may take up to an hour for the precipitated protein to redissolve.

9. Add 10 mM alkynyl-functionalized PEG to the Click-iT reaction buffer (component A of the Click-iT Protein Reaction Buffer Kit) to a final concentration of 1 mM.

   Store any unused reaction buffer at −20°C for up to a year.

   Before using any new batch of PEG, it is important to check the quality by mass spectrometry. The spectrum of high-quality PEG has one collection of peaks centered on the correct mass (2 kDa or 5 kDa). The spectrum of low-quality PEG has multiple collections of peaks, usually centered at intervals of the correct mass (2 kDa or 5 kDa).

10. Add 500 µl of water to Click-iT reaction buffer additive 1 (component C of the Click-iT Protein Reaction Buffer Kit), and mix thoroughly.

    Store any unused reagent at −20°C for up to a year. Discard if the reagent turns brown.

11. Add 540 µl of water to Click-iT reaction buffer additive 2 (component D of the Click-iT Protein Reaction Buffer Kit), and mix thoroughly.

    Store any unused reagent at 4°C for up to a year.

12. To the resuspended protein (step 8), add the following:

    50 µl of the alkynyl-poly(ethylene glycol) in Click-iT reaction buffer (step 9)
    2 µl of 50× complete protease inhibitor cocktail (EDTA-free)
    3 µl of water.

    Vortex for 5 sec.

13. Add 5 µl of $CuSO_4$. Vortex for 5 sec.

14. Add 5 µl of Click-iT reaction buffer additive 1 solution (step 10). Vortex for 5 sec, and wait 3 min (but not longer than 5 min).

15. Add 10 µl of Click-iT reaction buffer additive 2 (step 11). Vortex for 5 sec.

    The solution should turn orange at this point.

16. Incubate at room temperature overnight (12 to 16 hr). Gently rock the samples by placing them in a tube rack on a table-top rocker.

17. After the 12- to 16-hr incubation, add 50 µl of neutralization buffer, and precipitate the reaction as described in steps 7 to 13 of the Basic Protocol.

18. Resuspend the pellet in 30 µl of 1% (w/v) SDS.

19. Carry out gel electrophoresis and immunoblotting using standard techniques.

    During initial experiments, we typically run the entire sample in a single gel lane.

    Best results are generally achieved by resolving 50% to 75% of the labeled protein on a 4% to 12% Bis-Tris NuPAGE gradient gel. To adequately resolve the shifted from the nonshifted protein subpopulations, the gel is typically run until the protein of interest has migrated through at least 50% of the gel.

    As Nup62 and Sp1 are heavily O-GlcNAc glycosylated in rat brain and 293T cell lysates, respectively, they serve as excellent positive controls for the labeling experiments.

    See Support Protocol 2 to calculate protein glycosylation stoichiometries.

SUPPORT PROTOCOL 1

EXPRESSION AND ISOLATION OF Y289L GalT

Active Y289L GalT can be expressed and isolated from an *E. coli* expression system (Ramakrishnan and Qasba, 2002). Upon overexpressing Y289L GalT, *E. coli* sequester the protein in inclusion bodies. This protocol describes the isolation and lysis of these inclusion bodies, followed by refolding of the Y289L GalT protein to its active conformation. Y289L GalT is also available commercially as part of the Click-iT *O*-GlcNAc Enzymatic Labeling System.

NOTE: All solutions and equipment coming into contact with cells must be sterile, and proper aseptic technique should be used accordingly.

Materials

BL21(DE3) cells (Lucigen)
289L GalT cDNA in a pET23a plasmid backbone (Ramakrishnan and Qasba, 2002)
Luria-Bentani (LB) agar plates containing 100 μg/ml ampicillin (e.g., see Stevenson, 2000)
LB broth containing 100 μg/ml of ampicillin
Isopropylthio-β-galactoside (IPTG, Sigma-Aldrich)
Phosphate-buffered saline (PBS, Sigma-Aldrich)
25% (w/v) sucrose in PBS
Y289L resuspension buffer (5 M guanidine hydrochloride/0.3 M sodium sulfite): store up to 1 day at 4°C
2-nitro-5-(sulfothio)-benzoate (NSTB), made fresh (Thannhauser et al., 1984)
5 M guanidine hydrochloride
Refolding solution (see recipe)
Dialysis solutions (see recipe)
10-kDa nominal molecular weight limit (NMWL) dialysis tubing (Spectrum Labs)
Coomassie blue stain (see recipe)
5 mM $MnCl_2$
10 mM 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), pH 7.9
1.5 μl of 10 mM UDP-ketogalactose (Khidekel et al., 2003)
100 pmol/μl Click-iT *O*-GlcNAc Peptide LC/MS Standard (Life Technologies)
Dihydroxybenzoic acid (DHB) matrix (see recipe)

37°C incubator with shaker
Spectrophotometer
Refrigerated centrifuge
Sonicator (Vibra-Cell 130 watts; Sonics)
10-kDa NMWL Centricon centrifugal filter unit (Millipore)

Additional reagents and equipment for carrying out electroporation (Seidman et al., 2001), protein quantification (Olson and Markwell, 2007), SDS-PAGE (Gallagher, 2012), and mass spectrometry (Dave et al., 2011)

Induce protein expression

1. Electroporate BL21(DE3) cells with 0.25 μg of Y289L GalT DNA in a pET23a plasmid, and plate the bacteria on an agar plate containing 100 μg/ml of ampicillin. Incubate the plate overnight at 37°C.

2. Pick three colonies and place each separately into 100 ml of LB broth containing 100 μg/ml of ampicillin. Incubate the LB broth overnight at 37°C, with shaking at 235 RPM.

3. Choose the densest culture and dilute it to 1 liter with LB broth containing 100 μg/ml of ampicillin. Incubate the bacteria culture at 37°C, with shaking at 235 RPM. Periodically measure the absorbance of the culture at 600 nm.

We generally measure the absorbance of the culture every 45 min after diluting with LB.

4. When the absorbance of the culture at 600 nm has reached 0.7 compared to an LB control, add IPTG to a final concentration of 1 mM. Return the bacteria culture to 37°C, with shaking at 235 RPM for an additional 4 hr.

5. After the 4 hr, centrifuge the bacteria for 10 min at 600 × *g*, 4°C. Discard the supernatant and save the pellet.

   After the supernatant has been removed, the bacteria can be frozen at −20°C for up to 6 months.

Isolate the Y289L GalT enzyme

6. Resuspend the bacteria by pipetting in 10 ml of ice-cold PBS.

   Unless otherwise noted, throughout the GalT isolation and refolding procedure, the GalT protein is kept on ice.

   It is important to ensure that all the bacteria have been resuspended before moving on to the sonication step.

7. Sonicate thoroughly on ice to lyse the bacteria.

   The time and intensity of sonication will depend on the type of sonicator used. We generally sonicate 10 times for 30 sec at 40% amplitude with 30-sec pauses in between to keep the sample from heating.

8. Add 70 ml of ice-cold PBS and centrifuge 30 min at 14,000 × *g*, 4°C. Discard the supernatant.

9. Resuspend the pellet by pipetting in 50 ml of ice-cold 25% (*w/v*) sucrose in PBS. Centrifuge 30 min at 14,000 × *g*, 4°C.

   Resuspending the pellet in this and subsequent steps is often difficult and laborious. However, it is critical to resuspend the pellet fully at each step to ensure adequate purity of the Y289L GalT.

10. Repeat step 9 three more times.

    With each subsequent wash, the pellet color should change from the straw color of LB to a more ivory color.

    The pellet can be stored after the final wash in 5 ml of 25% sucrose in PBS overnight at 4°C. Be certain to remove the 25% sucrose in PBS before the next step.

Refold the Y289L GalT

11. Resuspend the pellet in 20 ml of ice-cold Y289L resuspension buffer.

12. Add 2 ml of NTSB, and shake vigorously at room temperature for ∼45 min.

    The NTSB should initially be a pale yellow color. When added to the solution, the NTSB should turn the solution red. The reaction is complete when the solution turns from red back to pale yellow. This usually takes ∼45 min.

13. Add 180 ml of ice-cold water to precipitate the protein and centrifuge the sample 10 min at 10,000 × *g*, 4°C.

14. Discard the supernatant and resuspend the pellet in 10 ml of ice-cold water. Centrifuge the sample 10 min at 10,000 × *g*, 4°C.

15. Repeat step 14 two more times.

16. Resuspend the pellet in 5 M guanidine hydrochloride to a protein concentration of 1 mg/ml.

    1 mg/ml of Y289L GalT has an absorbance of 1.9 to 2.0 at 275 nm.

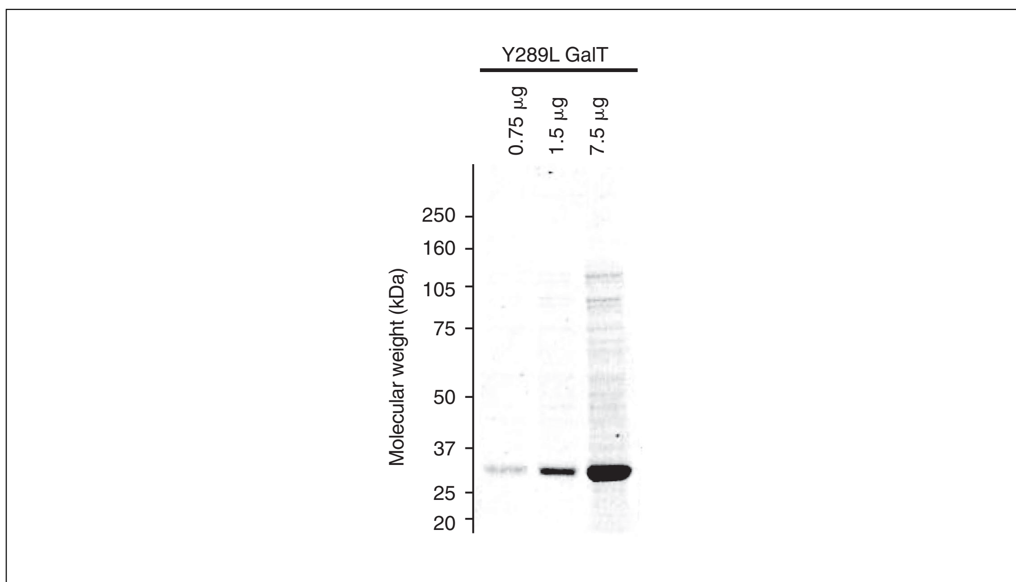

Figure 3 Y289L GalT. 0.75, 1.5, and 7.5 µg of expressed and refolded Y289L GalT was resolved on a 4% to 12% Bis-Tris NuPAGE gel and stained with Coomassie brilliant blue.

17. Dilute the protein tenfold in ice-cold refolding solution at 4°C over the course of 15 min with slow mixing.

    It is important to add the refolding solution slowly with shaking. We usually place the protein solution on an orbital shaker and add one-fifteenth of the refolding solution each minute over the course of 15 min.

    Some protein typically precipitates as a white precipitate during the refolding.

18. (Optional) Remove the precipitate by centrifuging10 min at 10,000 × g, 4°C. Collect the supernatant with a pipet.

    Alternatively, the precipitate can be carried on to step 19 and removed at step 20.

19. Dialyze the refolded protein three times for 4 hr with 4 liters of dialysis solution at 4°C using 10-kDa NMWL dialysis tubing.

    Here again, protein will precipitate. Precipitation should not be interpreted as a failed isolation.

20. Collect the dialyzed solution, and centrifuge 10 min at 10,000 × *g*, 4°C.

21. Retain the supernatant and concentrate it using a 10-kDa NMWL Centricon centrifugal filter unit.

    We usually concentrate the GalT until it has reached a concentration of 2 mg/ml, which usually requires ∼100-fold concentration.

22. Measure the protein concentration of the GalT. Check the purity of the GalT by carrying out SDS-PAGE with 7.5 µg of the concentrated protein and by staining the gel with Coomassie blue stain.

    The approximate molecular weight of Y289L GalT is 32 kDa. We regularly obtain Y289L GalT in high purity (Fig. 3).

23. Check the activity of the Y289L GalT in an *O*-GlcNAc peptide labeling reaction. Prepare separate 30-µl reactions containing

    0, 10, 25, 50, or 100 ng/µl of Y289L GalT

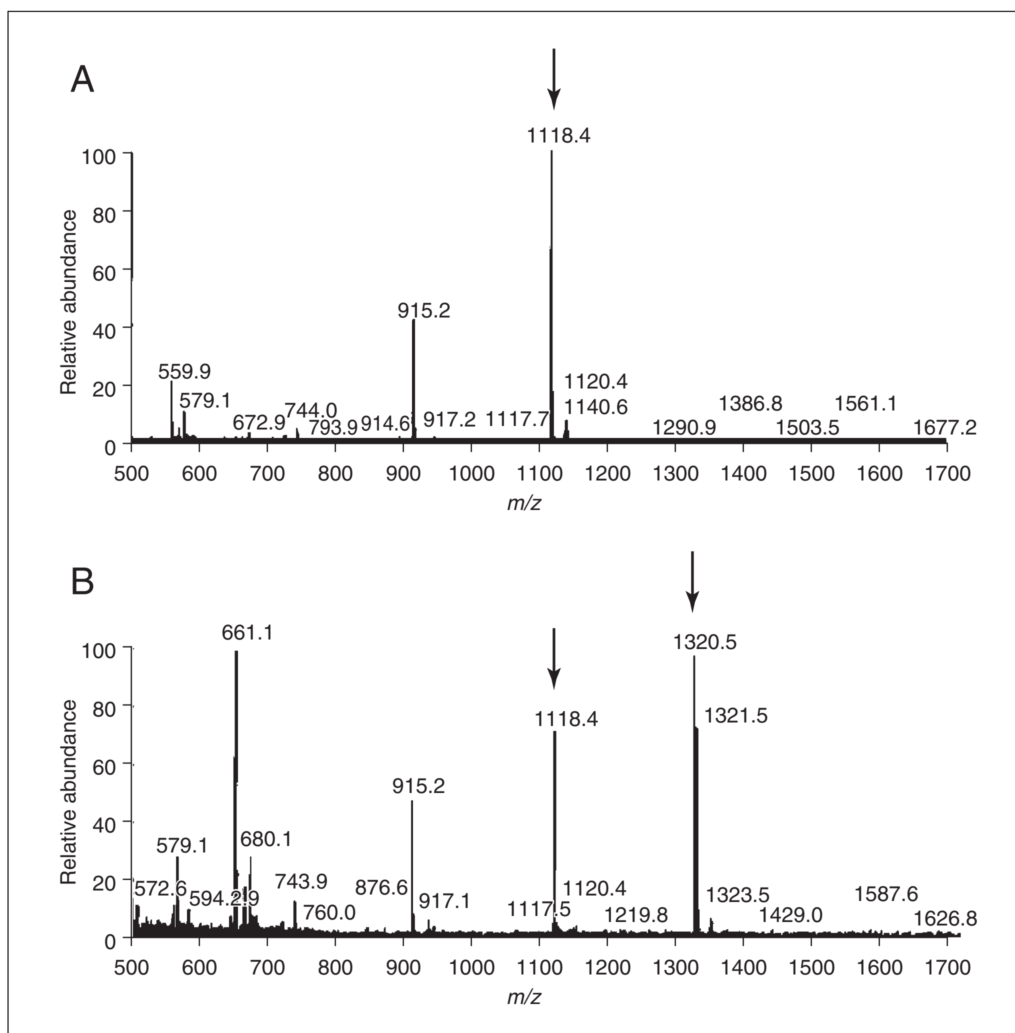

Figure 4 Mass spectrometry analysis of the ketogalactose labeling of an *O*-GlcNAc glycosylated peptide. (**A**) Control reaction (no GalT). The unlabeled peptide has a molecular weight of 1118.4 daltons. (**B**) Labeling reaction. The product of the ketogalactose labeling has a molecular weight of 1320.5 daltons. The presence of unlabeled peptide at *m/z* 1118.4 suggests that the reaction did not go to completion and that a higher concentration of GalT should be used for labeling reactions. This figure was adapted from Khidekel et al. (2003).

5 mM $MnCl_2$
10 mM HEPES, pH 7.9
1.5 µl of 10 mM UDP-ketogalactose
4 µl of 100 pmol/µl Click-iT *O*-GlcNAc Peptide LC/MS Standard.

Incubate the reactions overnight at 4°C.

24. Monitor the addition of the ketogalactose to the *O*-GlcNAc residue on the peptide by mass spectrometric analysis (Fig. 4), e.g., using a dihydroxybenzoic acid (DHB) matrix.

We usually analyze the reactions with matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry using a dihydroxybenzoic acid (DHB) matrix, although other mass spectrometry methods also work. The unmodified peptide has a molecular weight of 1118.5 Da. The ketogalactose adds 202 Da to the molecular weight of the peptide. Typically, complete labeling of the peptide occurs using 50 ng/µl of GalT.

Once isolated, the Y289L GalT is stable for ∼1 year at 4°C.

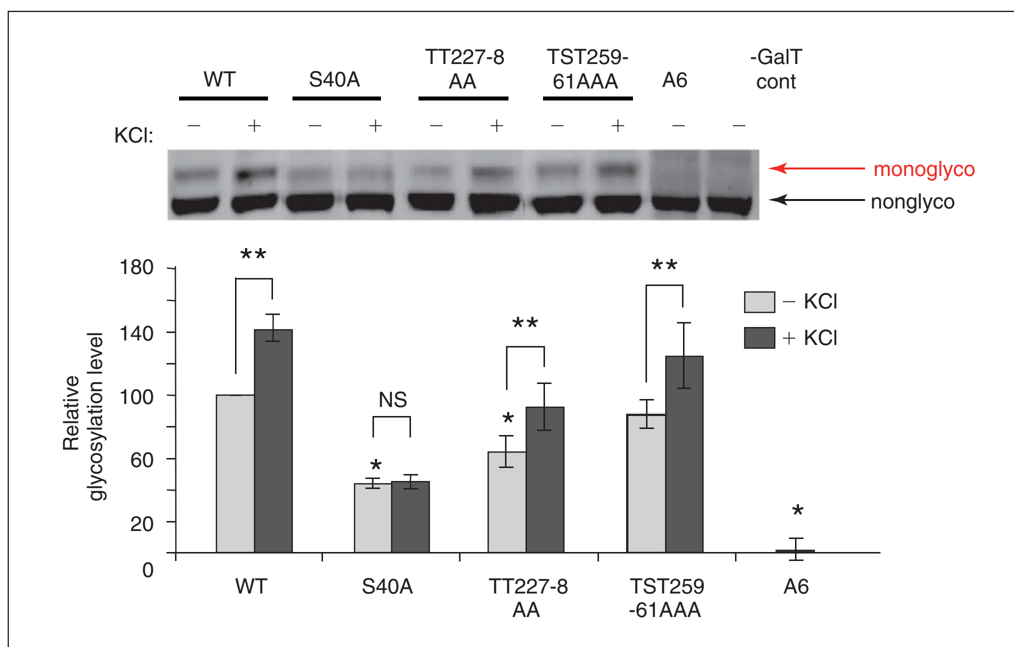

Figure 5 The mass-tagging approach was used to determine that (cyclic-AMP response element—binding protein) CREB is primarily glycosylated at Ser40 and that glycosylation is specifically induced by neuronal depolarization with KCl at Ser40. Neurons were electroporated with wild-type (WT) or mutant CREB constructs, in which the six potential glycosylation sites were mutated in various combinations. Neurons were then depolarized with 55 mM KCl, as indicated, for 2 hr. Cell lysates were labeled with the mass-tagging approach and visualized by immunoblotting for FLAG-tagged CREB. Error bars, mean ± s.e.m. $^{*}P<0.01$ compared to WT, −KCl, $^{**}P<0.05$; NS, not significant. This figure was adapted from Rexach et al. (2012).

SUPPORT PROTOCOL 2

DATA ANALYSIS FOR CALCULATING PROTEIN GLYCOSYLATION STOICHIOMETRIES

The number of extra bands in the experimental lane represents the different glycosylated subpopulations of the protein. A single band shifted higher by 2 kDa or 5 kDa suggests that the protein is predominantly monoglycosylated. The presence of two bands, one shifted higher by 2 kDa or 5 kDa and the other by 4 kDa or 10 kDa, suggests that the protein is both mono- and diglycosylated. It is important to note that although a protein may be monoglycosylated, it can still have multiple sites of glycosylation. These cases can be distinguished by combining this approach with site-directed mutagenesis (e.g., see Fig. 5 in the Commentary) to show the contributions of each individual site to the stoichiometry of glycosylation. The number of mass-shifted bands only describes the distinct glycosylated subpopulations that have a total of one, two, three, etc. *O*-GlcNAc sugars per protein. As such, the monoglycosylated subpopulation may consist of multiple different glycoforms, as in the case of CREB (Rexach et al., 2012).

O-GlcNacylation stoichiometries must be carefully calculated to obtain accurate, reproducible results. To determine the stoichiometry of a specific glycosylated subpopulation, the ratio of the intensity of that mass-shifted band to the intensity of all the protein bands (shifted and nonshifted) is taken, accounting for background signal in the control lane. Specifically, in the experimental lane, the intensity of the nonshifted band (e_n) and each mass-shifted band should be individually quantified (e_{s1}, e_{s2}, and so on). In the control lane, the intensity of the background signal corresponding to each of the mass-shifted bands should be individually quantified (c_{s1}, c_{s2}, and so on). The following formula can then be used to calculate the total protein glycosylation stoichiometry:

Total protein glycosylation stoichiometry: $\Sigma\,(e_{si} - c_{si})/[e_n + \Sigma\,(e_{si} - c_{si})]$

Alternatively, the glycosylation stoichiometry of a specific glycosylated subpopulation si can be calculated as:

Glycosylation stoichiometry of subpopulation, si: $(e_{si} - c_{si})/[e_n + \Sigma\,(e_{si} - c_{si})]$

When quantifying immunoblots, care should be taken to use a quantitative immunoblotting system (e.g., an Odyssey imaging system; LI-COR) or enhanced chemiluminescence (ECL) film exposures in which the intensity of each band is within the linear range.

REAGENTS AND SOLUTIONS

Use ultrapure (Milli-Q) grade water for all solutions and protocol steps.

Alkynyl labeling resuspension buffer

1% (w/v) SDS in 50 mM Tris·Cl, pH 7.5
1× Roche Complete Protease Inhibitor Cocktail (EDTA free)
Once the protease inhibitors have been added, store up to 2 weeks at 4°C

Storage at 4°C may cause precipitation of the SDS. If this occurs, be certain to redissolve it by warming the buffer to room temperature before use.

Azide labeling resuspension buffer

1% (w/v) SDS in 20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), pH 7.9
1× complete protease inhibitor cocktail: one tablet Complete Protease Inhibitor (Roche) in 50 ml buffer
Once the protease inhibitors have been added, store up to 2 weeks at 4°C

Storage at 4°C may cause precipitation of the SDS. If this occurs, be certain to redissolve it by warming the buffer to room temperature before use.

Coomassie brilliant blue stain

0.1% (w/v) Coomassie brilliant blue R-250
50% (v/v) methanol
7.5% (v/v) acetic acid
42.5% water
Store up to 6 months at 25°C

DHB matrix

Solution 1: Prepare a 20 mg/ml solution of 2,5-dihydroxybenzoic acid in an 80% (v/v) acetonitrile solution containing 0.1% (v/v) trifluoroacetic acid.

Solution 2: Prepare a 20 mg/ml solution of 2-hydroxy-5-methoxybenzoic acid in an 80% (v/v) acetonitrile solution containing 0.1% (v/v) trifluoroacetic acid.

Prepare a 9:1 mixture of solution 1/solution 2.

Dialysis solution

50 mM Tris·Cl, pH 8.0
5 mM EDTA
4 mM cysteamine
2 mM cystamine
Store up to 1 day at 4°C.

Ketone labeling resuspension buffer

1% (w/v) SDS in water
1× complete protease inhibitor cocktail: one tablet Complete Protease Inhibitor (Roche) in 50 ml buffer
Once the protease inhibitors have been added, store up to 2 weeks at 4°C

Storage at 4°C may cause precipitation of the SDS. If this occurs, be certain to redissolve it by warming the buffer to room temperature before use.

Lysis buffer

2% (w/v) SDS in water
1× complete protease inhibitor cocktail: one tablet Complete Protease Inhibitor (Roche) in 50 ml buffer
Once the protease inhibitors have been added, store the solution up to 2 weeks at 4°C

Storage at 4°C may cause the SDS to precipitate. If this occurs, be certain to redissolve by warming the sample to room temperature before use.

Mass tag resuspension buffer

7 M urea (ultra pure; MP Biomedicals)
10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), pH 7.9
2% (w/v) 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS, Sigma-Aldrich)
1 mM DTT
1× complete protease inhibitor cocktail: one tablet Complete Protease Inhibitor (Roche) in 50 ml buffer
Prepare fresh each time

Freshly dissolved, ultrapure urea is critical in this recipe. A urea breakdown product is isocyanate, which may inhibit the aminooxy reaction and is found as an impurity in urea preparations.

Refolding solution

0.5 M L-arginine
50 mM Tris·Cl, pH 8.0
5 mM EDTA
4 mM cysteamine
2 mM cystamine
Store up to 1 day at 4°C

COMMENTARY

Background Information

Selective and quantitative labeling of *O*-GlcNAc residues with the poly(ethylene glycol) mass tag is critical to measuring accurate *O*-GlcNAc stoichiometries using this chemoenzymatic approach. Previous studies have shown that GalT demonstrates >11-fold selectivity for *N*-acetylglucosamine over glucose and glucosamine (Ramakrishnan and Qasba, 2002). The unnatural UDP-ketogalactose sugar is a selective substrate for the Y289L GalT mutant and is a poor substrate for endogenous GalT (Khidekel et al., 2003). Thus, the enzymatic labeling step allows for selective labeling of *O*-GlcNAc residues only in those reactions in which both the Y289L GalT and UDP-ketogalactose sugar have been included. Moreover, the oxime formation reaction between aminooxy and ketone groups is considered bioorthogonal, as ketones and aldehydes are generally not found on proteins. At pH 4.5, only those proteins labeled with the unnatural ketogalactose sugar will be further elaborated with the aminooxy-functionalized PEG tag. Finally, and most importantly, both the GalT reaction and subsequent oxime formation reaction proceed quantitatively. The mutant GalT quantitatively labels a model

O-GlcNacylated peptide within 6 hr at 4°C (Khidekel et al., 2003), and the aminooxy reaction goes to completion on model peptides and proteins within 20 hr at room temperature (Khidekel et al., 2003; Rexach et al., 2010). The quantitative yield for each reaction is critical to ensure accurate calculation of glycosylation stoichiometries.

Most other methods for *O*-GlcNAc detection focus on relative changes in *O*-GlcNAc levels, rather than absolute values (Zachara, 2009). In contrast, the mass-tagging approach allows for direct measurement of in vivo *O*-GlcNAc stoichiometries. This enables a more comprehensive analysis of the role of *O*-GlcNAc glycosylation in specific protein contexts. For example, we found that glucosamine stimulation of rat embryonic neurons induced a 1.4- and 3.1-fold increase in *O*-GlcNacylation of CREB and Golgi reassembly-stacking protein of 55 kDa (GRASP55), respectively, suggesting that GRASP55 might be more responsive than CREB to glucosamine. However, further examination using the mass-tagging approach showed that both proteins underwent a similar change in glycosylation stoichiometry (13.1 ± 0.2% and 10.2 ± 0.5%, respectively), suggesting instead that glucosamine may uniformly induce *O*-GlcNAc transferase (OGT) activity toward these substrates (Rexach et al., 2010). In another example, the mass-tagging approach showed that CREB is predominantly monoglycosylated in neurons, despite having at least three distinct glycosylation sites. By combining the approach with site-directed mutagenesis, we found that CREB is glycosylated primarily at a single site, Ser40, and that glycosylation is induced by neuronal activity specifically at this site (Fig. 5; Rexach et al., 2012). Thus, the mass-tagging approach provides a direct readout of the glycosylation state and stoichiometry, and when applied in conjunction with site-directed mutagenesis, it has the power to reveal the sugar occupancy at specific glycosylation sites. In contrast, mass spectrometry methods are valuable for identifying the *O*-GlcNAc sites, but they cannot readily determine their relative occupancy or interrelationship within the same molecule. This highlights the complementary information obtained using the mass-tagging approach and mass spectrometric analyses.

Knowing the glycosylation stoichiometry of a protein may provide evidence of the modification's significance or may suggest how best to study it. For example, if the stoichiometry is low, glycosylation may be inducible, and further tests can be performed to examine the glycosylation dynamics in response to cellular stimuli. It is also worth noting that the same chemoenzymatic strategy can be used to attach other reporter groups, e.g., a biotin moiety. The biotin handle provides a sensitive method for the enrichment and detection of *O*-GlcNacylated peptides/proteins (Tai et al., 2004), and it can facilitate the identification of *O*-GlcNAc modification sites by mass spectrometry (Khidekel et al., 2004, 2007).

In addition to quantifying glycosylation stoichiometries, the mass-tagging approach can provide insights into the complex interplay between *O*-GlcNAc and other posttranslational modifications. By immunoblotting the mass tagged lysate with both a phospho-specific antibody and an antibody against the total protein, four different subpopulations are resolved: the (1) nonglycosylated, (2) glycosylated, (3) phosphorylated, nonglycosylated, and (4) simultaneously phosphorylated and glycosylated forms (Fig. 6). This provides a direct readout of whether the two modifications are mutually exclusive on proteins of interest or whether they can coexist on the same molecule. The strategy also enables dynamic changes in glycosylation to be monitored specifically on the phosphorylated subpopulation and vice versa. For example, we determined that phosphorylation precedes glycosylation of CREB in response to KCl-induced neuronal depolarization (Rexach et al., 2012). In other studies, traditional *O*-GlcNAc detection methods showed that total *O*-GlcNacylation levels increase on the transcriptional repressor methyl-CpG-binding protein 2 (MeCP2) in response to glucosamine (GlcN), while total Ser80 phosphorylation levels decrease, suggestive of an opposing, yin-yang relationship between glycosylation and phosphorylation. However, closer inspection using the mass-tagging approach revealed that glycosylation was induced preferentially on the Ser80 phosphorylated subpopulation of MeCP2. Moreover, despite overall decreases in Ser80 phosphorylation in response to GlcN, Ser80 phosphorylation increased specifically on the glycosylated MeCP2 subpopulation. These results suggest instead a cooperative relationship and highlight the complex, potentially synergistic relationship between glycosylation and phosphorylation on MeCP2 (Rexach et al., 2010).

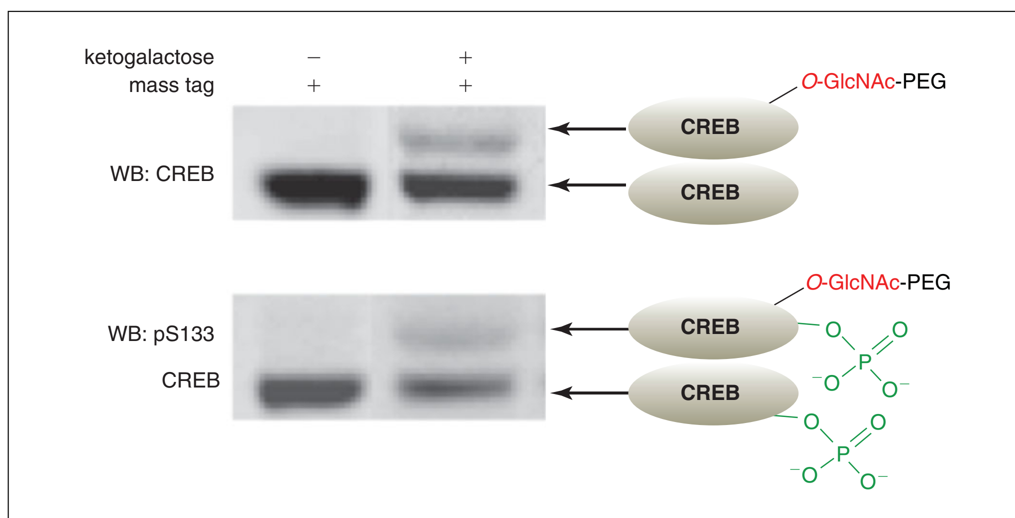

Figure 6 The mass-tagging approach can be used to identify different posttranslationally modified protein subpopulations. Cell lysates were labeled with the PEG mass-tagging approach, resolved by SDS-PAGE, and immunoblotted with an antibody against cyclic-AMP response element–binding protein (CREB) or Ser133-phosphorylated CREB. This figure was adapted from Rexach et al. (2010).

Critical Parameters and Considerations

The main choice to be made during this protocol is whether to use a 2-kDa or 5-kDa PEG mass tag in step 2. The selection depends on the size of the protein of interest. In our experience, proteins smaller than 50 kDa are better visualized using a 2-kDa mass tag, whereas proteins larger than 50 kDa are better visualized using a 5-kDa mass tag. Glycosylation of the 110-kDa subunit of OGT was readily visualized with a 5-kDa mass tag, whereas a 2-kDa mass tag was used for the 43-kDa protein CREB (Rexach et al., 2010). Although our studies have focused on proteins within this molecular weight range (40 to 110 kDa), the mass-tagging approach should be amenable in principle to proteins outside of this range.

Different antibodies display different background signals. Additionally, the mass tag could limit antibody recognition of the protein, although we have never directly encountered this situation. For initial experiments, we recommend immunoblotting mass tag–labeled lysate with at least two independent antibodies for the protein of interest to ensure minimal background and consistent detection of the mass tag–labeled samples (Fig. 7).

Good positive control proteins for this reaction include specificity protein 1 (Sp1) and nucleoporin 62-kDa (Nup62). Sp1 from 293T cells and Nup62 from adult rat brain are 100% glycosylated and should resolve as a ladder of four and eight shifted bands, respectively (Fig. 2). Appropriate antibodies for detecting these proteins include Millipore 07-645 for Sp1 and BD Biosciences 610497 for Nup62. An incomplete shift in Sp1 and Nup62 would suggest that the labeling reaction did not go to completion. If this is the case, longer protein transfer times, longer incubation times, or more enzyme and substrate may be required (see Troubleshooting).

Chemoenzymatic labeling with mass tags is a quantitative approach for measuring the *O*-GlcNacylation stoichiometry on a protein. In our experience, many proteins are *O*-GlcNAcylated at low stoichiometry (<10%). We have been able to detect as little as 0.8% of a glycosylated OGT standard and 2.3% of endogenous glycosylated synapsin from the adult rat brain cortex, highlighting the sensitivity of the mass-tagging approach (Figs. 2 and 8). As traditional enhanced ECL-based methods provide a limited linear detection range, we recommend analyzing the immunoblots using a more quantitative detection method such as the Odyssey Imaging System.

We developed the mass-tagging approach to measure the stoichiometry of intracellular *O*-GlcNAc glycosylation. The method works by selectively labeling terminal GlcNAc residues. Terminal GlcNAc residues are characteristic of intracellular *O*-GlcNAc glycosylation but may also be found on complex extracellular glycans. In most cases, proteins are known to be intracellular or extracellular, making it easy to determine whether the

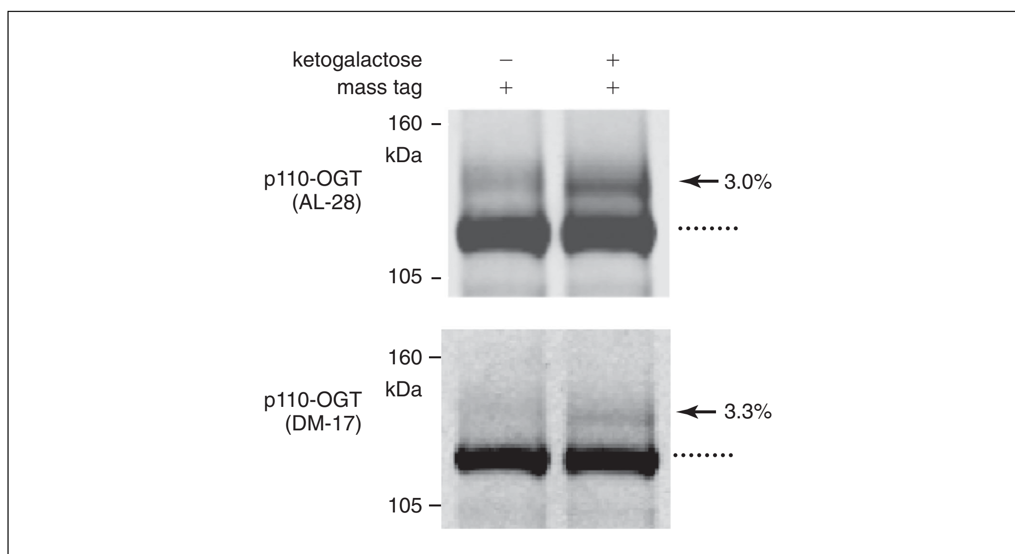

Figure 7 Immunoblotting with at least two independent antibodies for a specific protein helps confirm that the mass tag does not affect antibody recognition. In this example, cell lysate was labeled using the mass-tagging approach, resolved by SDS-PAGE, and immunoblotted with two different antibodies against *O*-GlcNAc transferase (OGT). This figure was adapted from Rexach et al. (2010).

terminal GlcNAc comprises the intracellular *O*-GlcNAc modification or a larger extracellular glycan. However, proteins with extracellular *O*-GlcNAc glycosylation have recently been identified (Matsuura et al., 2008). In those cases where the assignment is less clear, additional methods such as subcellular fractionation or mass spectrometry may be required to carefully characterize the type of glycosylation. For example, chemoenzymatic labeling

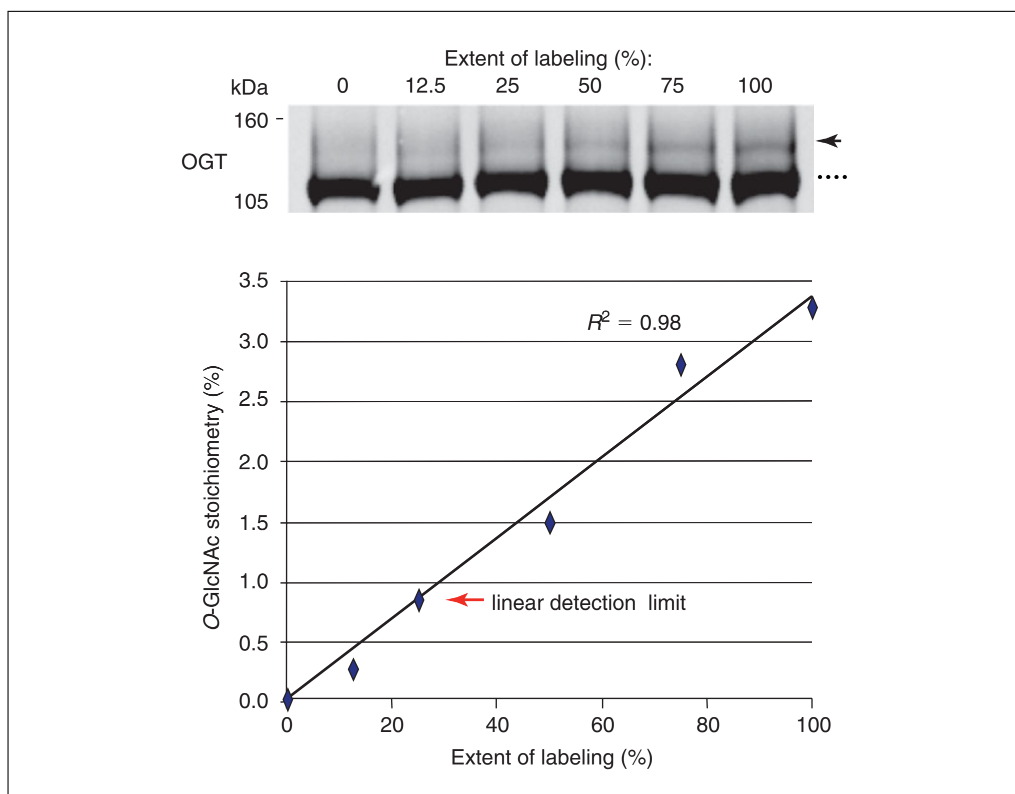

Figure 8 The mass-tagging approach can detect as little as 0.8% of glycosylated *O*-GlcNAc transferase (OGT). Labeled and unlabeled cell lysate was combined to mimic different OGT glycosylation stoichiometries. The limit of detection was determined as the lowest stoichiometry within 10% of the linear fit. This figure was adapted from Rexach et al. (2010).

Table 1 Troubleshooting Mass-Tagging of *O*-GlcNAc

Problem	Possible cause	Solution
No mass tag shift is detected even though other *O*-GlcNAc detection methods suggest that the protein is glycosylated, and a control *O*-GlcNAc protein (e.g., Nup62 or Sp1) in the lysate is showing the anticipated mass shift	The protein glycosylation stoichiometry level is below the detection limit of the antibody	Increase the amount of the protein of interest on the SDS-PAGE gel, either by labeling a greater amount of lysate or by enriching for the protein through subcellular fractionation or immunoprecipitation
No or low levels of glycosylation are detected on positive control proteins (e.g., Nup62 or Sp1), despite verified reagents and good quality sample	The mass-tagged proteins are difficult to transfer to the immunoblotting membrane and have failed to fully transfer	Increase the transfer time by 33%
	The labeling reaction did not proceed quantitatively	Increase enzyme and substrate concentrations and/or reaction times
The glycosylated protein fraction was not adequately separated from the nonglycosylated protein fraction	The protein was not sufficiently resolved	Run the protein of interest further through the gel to allow for adequate subpopulation separation. Consider a gradient gel or altering the acrylamide percentage.
	The size of the mass tag chosen was too small	Choose a larger size mass tag (e.g., 5 kDa instead of 2 kDa). We usually use a 2-kDa PEG mass tag for proteins smaller than 50 kDa and a 5-kDa PEG mass tag for proteins larger than 50 kDa.
Multiple bands are seen in both the control and experimental lanes of the immunoblot	The antibody chosen for analyzing the protein of interest identifies multiple proteins and is obscuring the mass shift	Probe the blot with a different antibody against the protein of interest
	The protein is modified by other posttranslational modifications (e.g., ubiquitinylation) that cause a shift in the protein's apparent molecular weight	Pretreat the samples to remove these other posttranslational modifications

of *O*-GlcNAc-modified proteins with a biotin tag provides a unique signature upon MS/MS analysis that allows for unambiguous assignment of the *O*-GlcNAc modification (Khidekel et al., 2004).

We have studied the [3+2] azide-alkyne cycloaddition labeling method in less detail than the ketone-aminoxy oxime labeling method. The Y289L GalT/UDP-GalNAz step goes to completion on a model peptide within 12 hr at 4°C. When using this approach, it will be important to determine conditions that lead to complete reaction between the azido-galactose-labeled proteins and alkynyl-functionalized PEG. *O*-GlcNacylation stoichiometries should be interpreted carefully until the approach is fully validated with proteins of known stoichiometry (Rexach et al., 2010).

Troubleshooting

Table 1 describes some problems that may be encountered using the mass-tagging methodology, along with explanations of possible causes and recommendations for overcoming or avoiding these problems.

Anticipated Results

Chemoenzymatic labeling with mass tags can provide information on the *O*-GlcNAc

modification state of a protein (e.g., whether the protein is predominantly mono-, di-, or triglycosylated), as well as its in vivo glycosylation stoichiometry. The expected result from a successful labeling reaction is an immunoblot containing a negative control lane (e.g., no GalT added) with a band for the protein of interest and an experimental lane containing this band shifted (either entirely or in part) by 2- or 5-kDa mass increments, depending on the mass tag used (Fig. 2).

Time Considerations

The entire labeling experiment can be completed in 2 to 3 days. Both the ketogalactose labeling step and the aminooxy PEG labeling steps require ~ 90 min of hands-on time. Cell lysis, protein concentration measurements, SDS-PAGE, and immunoblotting procedures can take variable amounts of hands-on time, depending on individual approaches to each step.

Acknowledgements

We thank P. Qasba (National Cancer Institute at Frederick) for helpful discussions on GalT and for generously providing the Y298L GalT construct. This work was supported by the National Institutes of Health (R01 GM084724 to L.C.H.-W., F31 NS056525 to J.E.R., and 5T32 GM07737 to P.M.C.).

Literature Cited

Aniento, F. and Gruenberg, J. 2004. Subcellular fractionation of tissue culture cells. *Curr. Protoc. Prot. Sci.* 32:4.3.1-4.3.21.

Clark, P.M., Dweck, J.F., Mason, D.E., Hart, C.R., Buck, S.B., Peters, E.C., Agnew, B.J., and Hsieh-Wilson, L.C. 2008. Direct in-gel fluorescence detection and cellular imaging of *O*-GlcNAc-modified proteins. *J. Am. Chem. Soc.* 130:11576-11577.

Dave, K.A., Headlam, M.J., Wallis, T.P., and Gorman, J.J. 2011. Preparation and analysis of proteins and peptides using MALDI TOF/TOF mass spectrometry. *Curr. Protoc. Protein Sci.* 63:16.13.1-16.13.21.

Gallagher, S. 2001. Immunoblot detection. *Curr. Protoc. Prot. Sci.* 4:10.10.1-10.10.12.

Gallagher, S.R. 2012. One-dimensional SDS gel electrophoresis of proteins. *Curr. Protoc. Prot. Sci.* 68:10.1.1-10.1.44.

Hart, G.W., Housley, M.P., and Slawson, C. 2007. Cycling of *O*-linked beta-*N*-acetylglucosamine on nucleocytoplasmic proteins. *Nature* 446:1017-1022.

Khidekel, N., Arndt, S., Lamarre-Vincent, N., Lippert, A., Poulin-Kerstien, K.G., Ramakrishnan, B., Qasba, P.K., and Hsieh-Wilson, L.C. 2003. A chemoenzymatic approach toward the rapid and sensitive detection of *O*-GlcNAc posttranslational modifications. *J. Am. Chem. Soc.* 125:16162-16163.

Khidekel, N., Ficarro, S.B., Peters, E.C., and Hsieh-Wilson, L.C. 2004. Exploring the *O*-GlcNAc proteome: Direct identification of *O*-GlcNAc-modified proteins from the brain. *Proc. Natl. Acad. Sci. U.S.A.* 101:13132-13137.

Khidekel, N., Ficarro, S.B., Clark, P.M., Bryan, M.C., Swaney, D.L., Rexach, J.E., Sun, Y.E., Coon, J.J., Peters, E.C., and Hsieh-Wilson, L.C. 2007. Probing the dynamics of *O*-GlcNAc glycosylation in the brain using quantitative proteomics. *Nat. Chem. Biol.* 3:339-348.

Love, D.C. and Hanover, J.A. 2005. The hexosamine signaling pathway: Deciphering the "*O*-GlcNAc code". *Sci. STKE* 2005:re13.

Matsuura, A., Ito, M., Sakaidani, Y., Kondo, T., Murakami, K., Furukawa, K., Nadano, D., Matsuda, T., and Okajima, T. 2008. *O*-linked *N*-acetylglucosamine is present on the extracellular domain of notch receptors. *J. Biol. Chem.* 283:35486-35495.

Olson, B.J. and Markwell, J. 2007. Assays for determination of protein concentration. *Curr. Protoc. Prot. Sci.* 48:3.4.1-3.4.29.

Phelan, M.C. 2006. Techniques for mammalian cell tissue culture. *Curr. Protoc. Molec. Biol.* 74:A.3F.1-A.3F.18.

Ramakrishnan, B. and Qasba, P.K. 2002. Structure-based design of beta 1,4-galactosyltransferase I (beta 4Gal-T1) with equally efficient N-acetylgalactosaminyltransferase activity: Point mutation broadens beta 4Gal-T1 donor specificity. *J. Biol. Chem.* 277:20833-20839.

Rexach, J.E., Clark, P.M., and Hsieh-Wilson, L.C. 2008. Chemical approaches to understanding *O*-GlcNAc glycosylation in the brain. *Nat. Chem. Biol.* 4:97-106.

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.

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.

Seidman, C.E., Struhl, K., Sheen, J., and Jessen, T. 2001. Introduction of plasmid DNA into cells. *Curr. Protoc. Mol. Biol.* 37:1.8.1-1.8.10.

Stevenson, B. 2000. Commonly used methods for cell culture. *Curr. Protoc. Microbiol.* 00:A.4A.1-A.4A.8.

Tai, H.C., Khidekel, N., Ficarro, S.B., Peters, E.C., and Hsieh-Wilson, L.C. 2004. Parallel identification of *O*-GlcNAc-modified proteins from cell lysates. *J. Am. Chem. Soc.* 126:10500-10501.

Thannhauser, T.W., Konishi, Y., and Scheraga, H.A. 1984. Sensitive quantitative analysis of disulfide bonds in polypeptides and proteins. *Anal. Biochem.* 138:181-188.

Ursitti, J. A., Mozdzanowski, J. and Speicher, D. W. 2001. Electroblotting from polyacrylamide gels. *Curr. Protoc. Prot. Sci.* 00:10.7.1-10.7.14.

Yi, W., Clark, P.M., Mason, D.E., Keenan, M.C., Hill, C., Goddard, W.A., 3rd, Peters, E.C., Driggers, E.M., and Hsieh-Wilson, L.C. 2012. Phosphofructokinase 1 glycosylation regulates cell growth and metabolism. *Science* 337:975-980.

Zachara, N.E. 2009. Detecting the "*O*-GlcNAc-ome": Detection, purification, and analysis of *O*-GlcNAc modified proteins. *Methods Mol. Biol.* 534:251-279.