Supplementary Methods for

Probing the Dynamics of *O*-GlcNAc Glycosylation in the Brain using Quantitative Proteomics

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Supporting Online Material

General Reagents

Unless otherwise noted, reagents were purchased from the commercial suppliers Fisher (Fairlawn, NJ) and Sigma-Aldrich (St. Louis, MO) and were used without further purification.

General Methods

Preparation of cultured neuronal and brain lysates. Cultured cortical neurons and dissected brain cortices were lysed and separated into nuclear and S100 fractions as described previously[1], except for the following modifications. For the kainic acid samples, crude nuclear pellets were washed (with homogenization buffer not containing PUGNAc or GlcNAc) and lysed directly into boiling 1% SDS by sonication for 3 x 3 sec. After centrifugation at 21,500 xg for 5 min, the supernatant was collected as denatured nuclear extract. Prior to labeling, non-denatured nuclear and cytoplasmic extracts were dialyzed into 20 mM HEPES pH 7.9, 0.1 M KCl, 0.2 mM EDTA, 0.2% Triton X-100, 0.5 mM phenylmethylsulfonyl fluoride (PMSF). Prior to labeling, denatured nuclear extracts were diluted 5-fold with 100 mM HEPES pH 7.9, 5 M NaCl and 20% Triton-X100 to a final concentration of 10 mM HEPES pH 7.9, 100 mM NaCl and 1.8% Triton-X100. Western blots of the kainic acid samples were performed on rat cortices lysed directly into boiling 1% SDS by sonication for 9 x 5 sec. After centrifugation at 21,500 xg for 5 min, the supernatant was collected as denatured cellular extract.

Chemoenzymatic labeling of cultured neuronal and brain lysates. α-Crystallin (6.5-11 μg, ~ 1:1 mixture of A and B crystallin, Sigma) was denatured by boiling for 5 min in 1% SDS containing 25 mM DTT and added to extracts prior to labeling. Similarly, OGT (0.8-2 μg in 50 mM Na_2HPO_4 pH 7.5, 1 mM DTT, 20% glycerol) purified from *Sf9* cells[2] was denatured by boiling for 5 min in 1% SDS containing 25 mM DTT and added to the extracts. These quantities

represent levels of α-crystallin and OGT that are several-fold higher than the endogenous levels. Extracts (0.5-3 mg; 1-3 mg ml^{-1}) containing the α-crystallin and OGT standards were supplemented with 5 mM $MnCl_2$, 1 mM PMSF and Complete™ protease inhibitor tablets (Roche). The extracts were then incubated with the UDP-ketogalactose probe (**1**, 0.5 mM) and mutant Y289L GalT enzyme (60 ng μl^{-1}) for 12-14 h at 4 °C. **1** was synthesized and the mutant GalT was expressed and purified essentially as described[3]. Following enzymatic labeling, extracts were dialyzed into denaturing buffer (5 M urea, 10 mM HEPES pH 7.5; 3 x 3 h). The pH was adjusted with 2.7 M NaOAc pH 3.9 (final concentration 50 mM, pH 4.8), *N*-(aminooxyacetyl)-*N*'-(D-biotinoyl) hydrazine (30 mM aqueous stock, Dojindo) was added to a final concentration of 2.75 mM, and the reactions were incubated for 20-24 h at room temperature. Extracts were dialyzed (2 x 2 h, 1 x 10 h) into 7 M urea, 10 mM HEPES pH 7.5 at room temperature, followed by 2 M urea, 50 mM NH_4HCO_3 pH 8 (3 x 3 h) at 4 ºC.

Proteolytic digestion. Dialyzed extracts were reduced in 10 mM DTT for 1 h at room temperature, alkylated in 20 mM iodoacetamide for 1 h at room temperature, and incubated with 20 mM DTT for 1 h at room temperature to react with excess iodoacetamide. The extract solution was then centrifuged at 15,000 rpm for 5 min to remove any insoluble material. Control and experimental extracts were adjusted to identical protein concentrations with excess 2 M urea, 50 mM NH_4HCO_3 pH 8 and then diluted with 50 mM NH_4HCO_3 pH 8 to a final urea concentration of 1 M. Sequencing-grade trypsin (Promega) was added to provide a final extract to trypsin ratio of 20:1. The trypsin concentration was always maintained above 0.01 mg ml^{-1}, and digestion was carried out in a water bath at 37 ºC for 12-14 h.

LC-MS analysis. Approximately 1/5 of the avidin-enriched peptides from each cation exchange fraction was loaded onto a 360 μm O.D. X 75 μm I.D. precolumn packed with 4 cm of 5 μm

Monitor C18 particles (Column Engineering) at a flow rate of 4 μl min^{-1}. After desalting, the vent was closed and peptides eluted to a 360 μm O.D. X 75 μm I.D. analytical column with integrated emitter tip (10 cm of 5 μm C18, ca. 5 μm tip). The chromatographic profile was from 100% solvent A (0.1% aqueous AcOH) to 50% solvent B (0.1% AcOH in CH_3CN) in 30 min. The flow rate through the analytical column was approximately 100 nl min^{-1}.

Chemoenzymatic labeling and streptavidin capture of *O*-GlcNAc proteins. Chemoenzymatic labeling was performed on neuronal lysates as described above. After reaction with the aminooxy biotin derivative, proteins were dialyzed (1 x 10 h, 2 x 3 h) into 7 M urea, 10 mM HEPES, pH 7.5 at room temperature followed by 10 mM HEPES pH 7.5, 100 mM NaCl, 0.2% Triton-X 100 (2 x 2 h, 1 x 10 h) at 4 °C. Fresh PMSF (1 mM) was added at each stage of dialysis. Proteins were captured on streptavidin beads as previously described[4] and probed by immunoblotting.

Western blotting. Lysates were resolved by SDS-PAGE, transferred to nitrocellulose membranes and immunoblotted as described previously[4]. Total *O*-GlcNAc levels were monitored using the anti-*O*-GlcNAc antibody CTD110.6 (Covance, 1:5000). The following primary antibodies were also used: EGR-1 (Upstate Biotechnology, 1:1000), GRASP-55 (BD Transduction Laboratories, 1:1000), eIF4G (Santa Cruz, 1:100), OGA (a kind gift from Prof. Sidney Whiteheart, University of Kentucky, 1:1000), p66β (Upstate, 1:500) and SRC-1 (Santa Cruz, 1:100). After incubation with the secondary antibodies IRDye 800 goat anti-rabbit (Rockland Immunochemicals) or Alexa Fluor 680 goat anti-mouse (Molecular Probes), proteins were visualized and quantified using the Odyssey infrared imaging system (LI-COR Biosciences). To quantify differences in *O*-GlcNAc levels, we measured the relative intensities of the input bands (lysate prior to streptavidin capture) and eluent bands (lysate after streptavidin

capture) using Odyssey imaging software (Version 2.1). For each sample, the ratio of the eluent signal to the corresponding signal was calculated. The resulting values were corrected for non-specific background by subtracting the corresponding ratios from control reactions performed in the absence of GalT. The corrected intensity for the PUGNAc-treated sample was then divided by the corrected intensity for the untreated sample to determine the fold increase. In Figure 5b, the fold-change for the untreated sample was normalized to 1.0 for each protein.

Statistical analysis. Quantification was conducted by generating single ion chromatograms from the orbitrap MS scans for candidate *O*-GlcNAc peptides. Peak areas of isotopic clusters were derived using Xcalibur 1.4 software. Mean values, standard deviations and confidence intervals were calculated using the program Excel on log-transformed ratios and reported in the original scale as previously described[5,6]. We used the geometric standard deviation (g.s.d.) to calculate maximum absolute standard deviations and reported these values in Supplementary Tables 1 and 2. Standard peptide ratios were tested for goodness of fit to the log-normal distribution via the D'Agostino-Pearson omnibus test and were used to determine the confidence with which changes in experimental peptides could be detected. Experimental peptide ratios were normalized against the slope of the linear regression produced by the heavy versus light forms of standard peptides within experiments.

References

1. Khidekel, N., Ficarro, S.B., Peters, E.C. & Hsieh-Wilson, L.C. Exploring the *O*-GlcNAc proteome: direct identification of *O*-GlcNAc-modified proteins from the brain. *Proc. Natl. Acad. Sci. USA* **101**, 13132-13137 (2004).

2. Lamarre-Vincent, N. & Hsieh-Wilson, L.C. Dynamic glycosylation of the transcription factor CREB: a potential role in gene regulation. *J. Am. Chem. Soc.* **125**, 6612-6613 (2003).

3. Khidekel, N. *et al.* A chemoenzymatic approach toward the rapid and sensitive detection of *O*-GlcNAc posttranslational modifications. *J. Am. Chem. Soc.* **125**, 16162-16163 (2003).

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

5. Molina, H., Parmigiani, G., and Pandey, A. Assessing reproducibility of a protein dynamics study using *in vivo* labeling and liquid chromatography tandem mass spectrometry. *Anal. Chem.* **77**, 2739 -2744 (2005).

6. Wodicka, L., Dong, H., Mittmann, M., Ho, M-H., Lockhart, D.J. Genome-wide expression monitoring in *Saccharomyces cerevisiae*. *Nat. Biotechnol.* **15**, 1359 - 1367 (1997).