Chapter 4: Direct In-Gel Fluorescence Detection and Cellular Imaging of *O*-GlcNAc-Modified Proteins

Portions of this chapter are from Clark, P.M., Dweck, J.F., Mason, D.E., Hart, C.R., Buck, S.B., Peters, E.C., Agnew, B.J. & Hsieh-Wilson, L.C. Direct in-gel fluorescence detection and cellular imaging of *O*-GlcNAc-modified proteins. *J. Am. Chem. Soc.* **130**, 11576-7 (2008).

We report an advanced chemoenzymatic strategy for the direct fluorescence detection, proteomic analysis, and cellular imaging of *O*-GlcNAc-modified proteins. *O*-GlcNAc residues are selectively labeled with fluorescent or biotin tags using an engineered galactosyltransferase enzyme and [3+2] azide-alkyne cycloaddition chemistry. We demonstrate that this approach can be used for direct in-gel detection and mass spectrometric identification of *O*-GlcNAc proteins, identifying 146 novel glycoproteins from the mammalian brain. Furthermore, we show that the method can be exploited to quantify dynamic changes in cellular *O*-GlcNAc levels and to image *O*-GlcNAc glycosylated proteins within cells. As such, this strategy enables studies of *O*-GlcNAc glycosylation that were previously inaccessible and provides a new tool for uncovering the physiological functions of *O*-GlcNAc.

Understanding posttranslational modifications to proteins is critical for elucidating the functional roles of proteins within the dynamic environment of cells. *O*-linked β-*N*-acetylglucosamine (*O*-GlcNAc) glycosylation has emerged as important for the regulation of diverse cellular processes, including transcription, cell division, and glucose homeostasis[1-3]. While new chemical tools have provided rapid, sensitive methods for detecting the modification and enabled better control over the activity of *O*-GlcNAc enzymes[1, 4-10], significant challenges remain with regard to elucidating the functions of *O*-GlcNAc in cells. For instance, a robust method for the direct fluorescence detection of *O*-GlcNAc proteins in gels would permit monitoring of changes in glycosylation levels in response to cellular stimuli and greatly extend the reach of existing technologies. Furthermore, new tools for imaging *O*-GlcNAc glycosylated

proteins would enable the expression and dynamics of the modification to be monitored in cells and tissues. Here, we report an advanced chemoenzymatic labeling strategy that addresses these important needs.

Previous studies have shown that an engineered β-1,4-galactosyltransferase enzyme (Y289L GalT) efficiently transfers a ketogalactose moiety from an unnatural UDP substrate selectively onto *O*-GlcNAc-modified proteins. Treatment with aminooxy-biotin followed by streptavidin capture and elution allowed for identification of *O*-

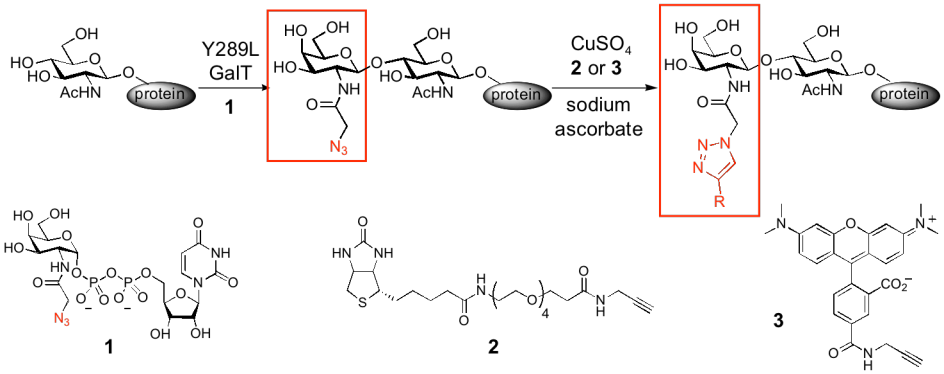

Figure 1: Chemoenzymatic labeling of O-GlcNAc proteins using [3+2] cycloaddition chemistry. R = biotin or TAMRA.

GlcNAc-modified proteins[4, 11]. However when I tried applying this strategy to more in-depth studies of *O*-GlcNAc proteins such as assaying *O*-GlcNAc dynamics across multiple conditions, I found that this was not an ideal system. First, the aminooxy-biotin reagent appeared to have strong nonspecific protein interactions. For example, robust streptavidin signal was detected in the control (- GalT) lane following *in vitro* labeling of α-crystallin even after three days of dialysis to remove the excess aminooxy biotin. Similarly after considerable optimization, I found that quantitative streptavidin capture was achieved only when using a large excess of streptavidin beads, in our case equal to half the reaction volume, to capture the biotin-labeled proteins in the presence of the

excess free biotin, again even after three days of dialysis. Second, the aminooxy–ketone reaction required conditions of pH 4.5 for 24 hours in the presence of 5 M Urea. Yet in many cases, this prolonged reaction with low pH caused up to 50% of the proteins to precipitate out of solution.

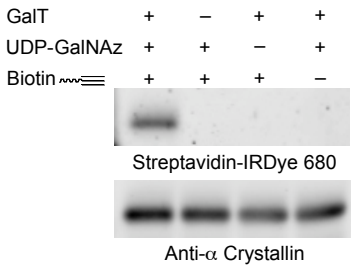

Figure 2: Selective labeling of α-crystallin

We therefore investigated whether Y289L GalT would accept the UDP-azidogalactose substrate **1** (UDP-GalNAz), which would allow for labeling of *O*-GlcNAc proteins using [3+2] azide-alkyne cycloaddition chemistry (**Fig. 1**)[12-14]. In addition to providing alternative dyes to potentially reduce nonspecific interactions, this Cu(I)-catalyzed cycloaddition reaction would have the advantage of being performed more rapidly and at physiological pH.

I tested the approach using α-crystallin, a known *O*-GlcNAc-modified protein with a low extent (~10%) of glycosylation. α-Crystallin was treated with **1** and Y289L GalT, followed by reaction with $CuSO_4$, sodium ascorbate, and the biotin-alkyne derivative **2** for 1 h at 25 °C. Analysis by gel electrophoresis and blotting with streptavidin conjugated to an IR680 dye showed robust, selective labeling of a-crystallin, with no nonspecific labeling in the absence of GalT, **1** or **2** (**Fig. 2**). Notably, as little as 250 fmol of a-crystallin (~25 fmol of glycosylated protein) was detectable, highlighting the sensitivity of the approach. In contrast, other

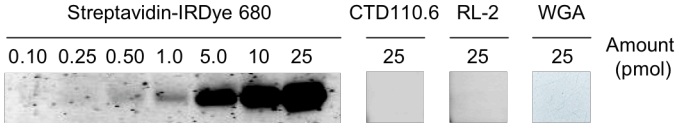

Figure 3: Detection sensitivity of the chemoenzymatic approach

methods such as *O*-GlcNAc antibodies or lectins failed to detect the *O*-GlcNAc modification on a-crystallin (**Fig. 3**)[4].

I next examined whether this approach could be used for direct in-gel fluorescence detection and proteome-wide analyses of *O*-GlcNAc glycosylated proteins. Nuclear and cytosolic protein fractions from rat forebrain were azide-labeled and then

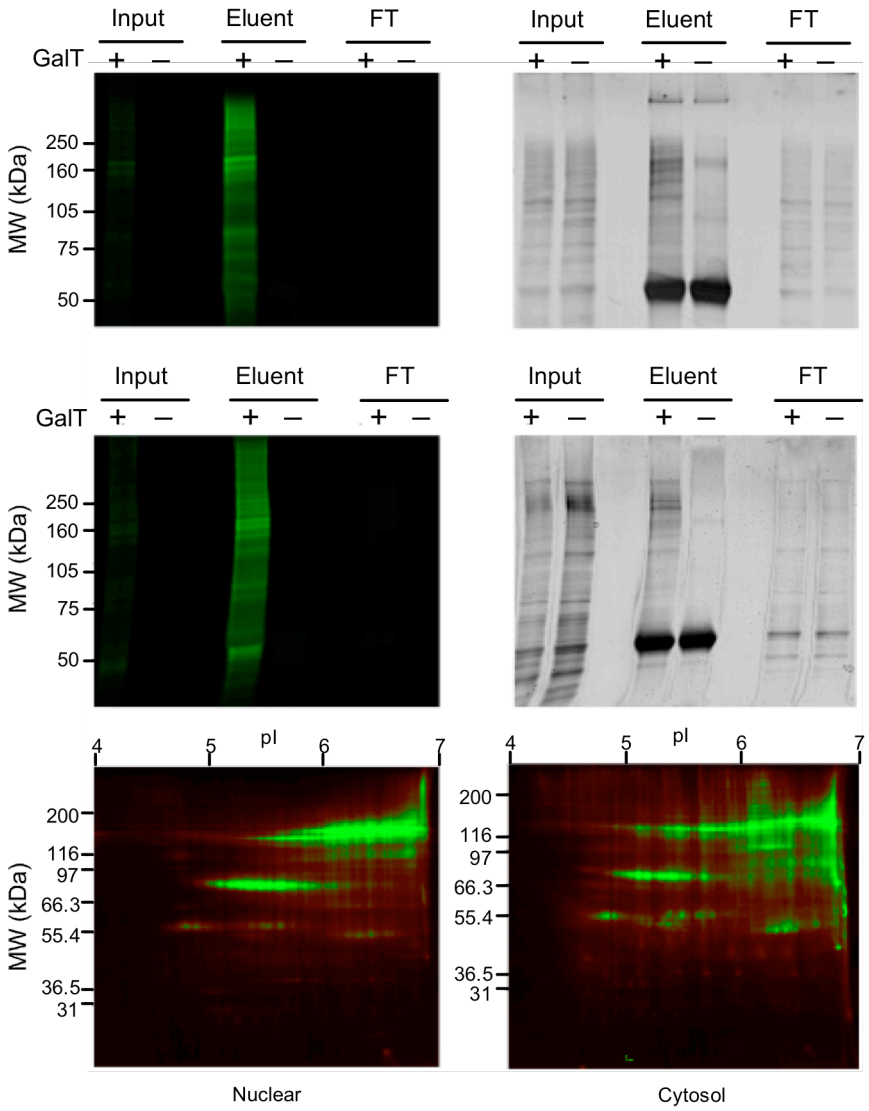

Figure 4: Enrichment and in-gel fluorescence detection of *O*-GlcNAc-modified proteins in 1D (top and middle) and 2D (bottom) gels. For the 1D gels, 15 µg of nuclear (top) or cytoplasmic (middle) protein was loaded in the input and FT lanes; material captured from 470 µg of protein was loaded in the eluent lanes.

reacted with the tetramethyl-6-carboxyrhodamine (TAMRA)-alkyne derivative **3**. The *O*-GlcNAc proteins were immunoprecipitated using an anti-TAMRA antibody to remove non-glycosylated proteins from the lysate, resolved by 1D or 2D gel electrophoresis, and visualized by in-gel fluorescence imaging (**Fig. 4**). Importantly, minimal nonspecific labeling was detected with the TAMRA-alkyne dye (**Fig. 4**, -GalT control lanes), and I observed efficient capture and enrichment of the TAMRA-labeled proteins (+GalT, eluent and flow-through lanes).

To identify *O*-GlcNAc proteins, bands from the gel were excised, proteolytically digested, and subjected to nanoLC-MS/MS analysis. The data acquisition and subsequent database searching methodologies employed are detailed in the methods section. In total, Daniel Mason and I identified 213 proteins, representing 67 previously known and 146 novel, putative *O*-GlcNAc modified proteins (**Table 1**). The majority of the proteins identified participate in neuronal signaling and synaptic function, suggesting important functional roles for *O*-GlcNAc in neuronal communication (**Fig. 5**). Surprisingly, in contrast to previous proteomic analyses of brain tissue[5,15,16], we identified many proteins involved in metabolism and biosynthesis, consistent with roles for *O*-GlcNAc in nutrient sensing and cell survival observed in other tissues[1-3]. Interestingly, the metabolic proteins included 9 of the 10 enzymes required for glycolysis, suggesting a previously unidentified level of control by *O*-GlcNAc of this pathway. Thus, the approach enables the identification of a large number of unique *O*-GlcNAc modified proteins and has the advantages of ease and accessibility (e.g., short incubation times, simple gel-based detection and separation versus multiple chromatography steps, high-throughput analyses, commercially available reagents).

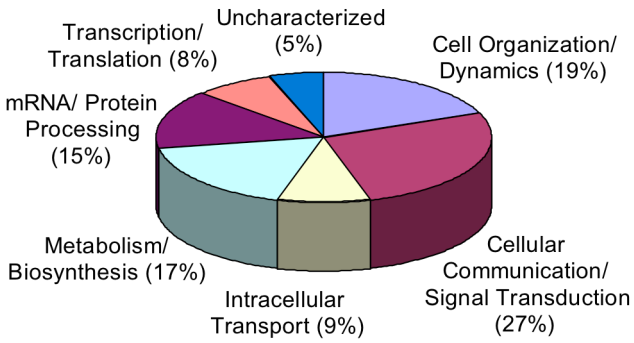

Figure 5: Functional classification of *O*-GlcNAc proteins from rat brain identified by MS

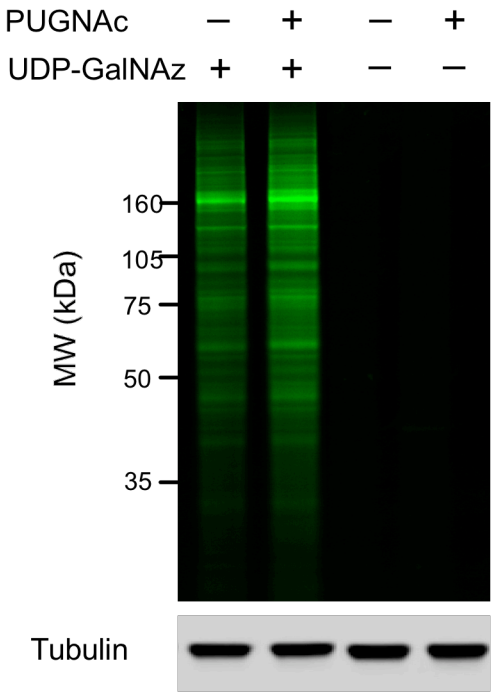

Figure 6: Direct detection of changes in *O*-GlcNAc glycosylation levels upon cellular stimulation. Tubulin controls indicate equal loading of protein in each lane.

Understanding the cellular dynamics of *O*-GlcNAc glycosylation will be critical for elucidating its functional roles in both physiological and diseased states. However, few methods exist for quantifying changes in *O*-GlcNAc glycosylation in response to cellular stimuli. Glycosylation levels are typically monitored by immunoblotting with a general *O*-GlcNAc antibody[17], which detects only a limited number of *O*-GlcNAc proteins and affords no opportunity to identify proteins undergoing changes in glycosylation. We examined whether our chemoenzymatic approach could overcome such limitations.

HeLa cells were stimulated with PUGNAc (*O*-(2-acetamido-2-deoxy-D-glucopyranosylidene)amino-*N*-phenylcarbamate), an inhibitor of the β-*N*-acetylglucosaminidase enzyme that removes *O*-GlcNAc, and the *O*-GlcNAc-modified proteins were labeled and analyzed as before.

PUGNAc treatment resulted in a 163 ± 3% increase in overall *O*-GlcNAc glycosylation levels, and interestingly, ranged from 136–176%, depending on the specific protein (**Fig. 6**). The varying extent to which *O*-GlcNAc is induced upon cellular stimulation may indicate complex regulatory control of the modification. Thus, this approach provides a new method to visualize and quantify dynamic changes in protein *O*-GlcNAc glycosylation which, when coupled with in-gel digestion and MS analyses as described above, will enable the identification of specific proteins undergoing those changes.

O-GlcNAc is known to modify a variety of components of the transcriptional machinery, including RNA polymerase II[18], CREB[19], and histone lysine methyltransferase MLL5[20]. Furthermore proteomic studies have demonstrated that transcription factors are over-represented among identified *O*-GlcNAc proteins[1, 5]. Thus I next examined whether our approach could be used to investigate *O*-GlcNAc on chromatin. DNA and proteins were cross-linked with formaldehyde, the chromatin was fragmented, and the lysate was azide-labeled and then reacted with **3**. The *O*-GlcNAc proteins were immunoprecipitated using an anti-TAMRA antibody, the associated DNA was separated from the proteins, and the DNA was amplified by PCR. I observed an enrichment of eluent signal on the POMC promoter specifically in the presence of GalNAz and TAMRA antibody but no enrichment on the control 18S ribosomal RNA promoter (**Fig. 7**). This suggests that our approach can be used to identify specific gene promoters that are enriched in *O*-GlcNAc levels. To more broadly identify such promoters, in collaboration with Jessica Rexach and Rosemarie Tsoa, immunoprecipitated *O*-GlcNAc-associated chromatin and then assayed the results on a promoter array. Using this approach, we identified 154 promoters in which *O*-GlcNAc

levels were specifically enriched (**Table 2**). These enriched promoters are distributed evenly across the mouse chromosomes (**Fig. 8**) and are over-represented in genes important for neural tube development as well as genes important in cell-cell adhesion and alkali metal ion binding. In particular, *O*-GlcNAc levels were enriched on the promoter of four different potassium channels: Kcnt1, Kcnab1, Kcne3, and Kcnj14. Thus this approach enables the identification of chromatin regions enriched in *O*-GlcNAc levels and could be used to identify dynamic changes in *O*-GlcNAc levels on the chromatin. Furthermore this approach could be expanded upon with a second immunoprecipitation step to determine colocalization of *O*-GlcNAc and specific proteins or other modifications on a specific region of DNA.

Finally, Jessica Dweck examined whether *O*-GlcNAc modified proteins could be

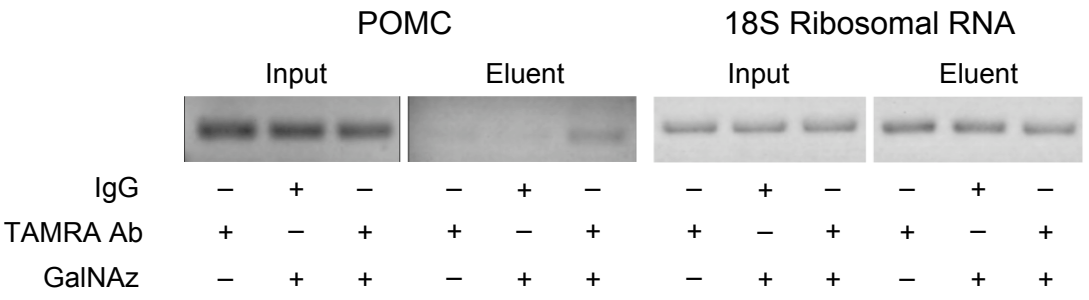

Figure 7: Detection of *O*-GlcNAc levels on chromatin

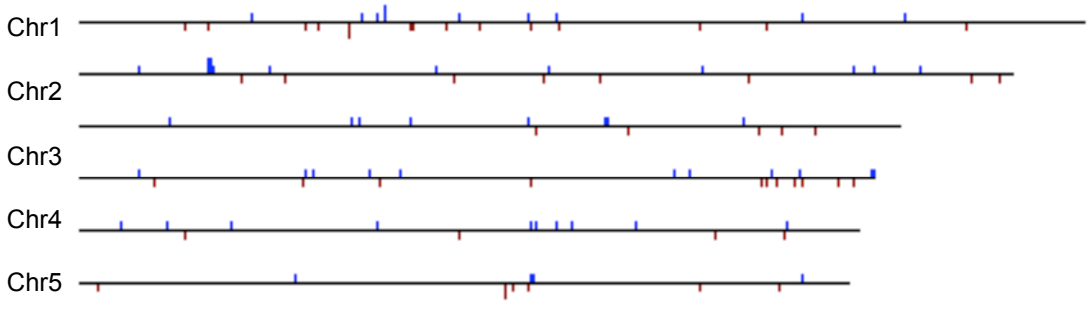

Figure 8: *O*-GlcNAc levels are distributed evenly across the mouse chromosomes.

chemoenzymatically tagged and imaged in cells. HeLa cells and cultured cortical neurons were fixed, permeabilized, and labeled with **1** and Y289L GalT, followed by biotin-alkyne **2** or TAMRA-alkyne **3**. The biotin-treated cells were further incubated with a streptavidin-AlexaFluor 488 conjugate. Notably, addition of exogenous GalT and **1** to the cells led to robust labeling of *O*-GlcNAc glycosylated proteins (**Fig. 9**). Although the TAMRA-alkyne **3** produced background labeling in the absence of **1** (data not shown), strong staining and minimal background labeling were observed using biotin-alkyne **2**. Consistent with the reported localization of *O*-GlcNAc enzymes[1,3], *O*-GlcNAc glycosylated proteins were found in both the nucleus and cytoplasm. Moreover, Jessica observed robust staining of proteins along neuronal processes, corroborating our mass spectrometric identification of many *O*-GlcNAc proteins involved in synaptic signaling. This is the first example of exploiting chemical tagging methods to image *O*-GlcNAc-modified proteins within cells. The approach affords high labeling sensitivity without perturbing physiological pathways and should be amenable to tissue samples — features that may complicate other strategies such as metabolic labeling.

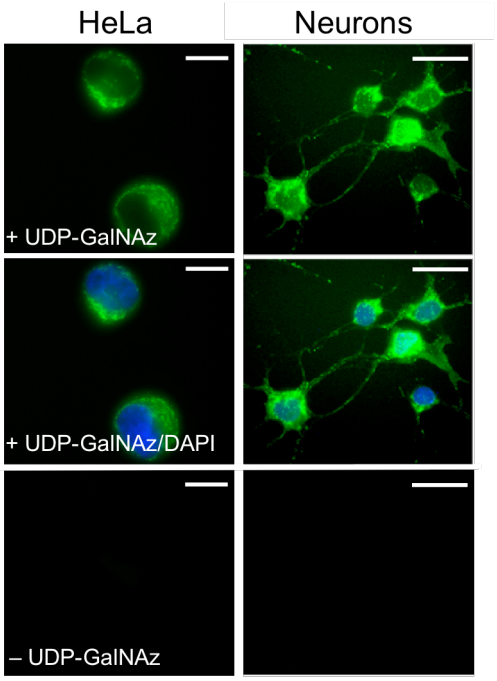

Figure 9: Fluorescence imaging of *O*-GlcNAc proteins (green) in HeLa cells (left) or cortical neurons (right). Nuclei were stained with DAPI (blue). Scale bars = 10 μm (HeLa) and 25 μm

In summary, we describe an advanced chemoenzymatic labeling approach that exploits [3+2] cycloaddition chemistry to attach fluorescent and biotin tags to *O*-GlcNAc residues. This method enables studies of *O*-GlcNAc glycosylation that were previously inaccessible. The ability to label proteins selectively with a fluorescent reporter group permits rapid and direct in-gel detection of *O*-GlcNAc proteins, facilitating proteomic analyses and providing a new method to quantify dynamic changes in glycosylation. Covalent labeling of proteins allows for cellular imaging of *O*-GlcNAc proteins in their native biological environment. Finally, this approach was developed in conjunction with researchers at Invitrogen with the goal of providing commercially available reagents that are now accessible to the wider research community. We anticipate that this new approach will be a powerful tool for advancing our understanding of the physiological functions and dynamic regulation of *O*-GlcNAc glycosylation within cells.

Table 1: *O*-GlcNAc glycosylated proteins identified by mass spectrometry. Proteins are tabulated by function, and the accession number and number of peptides (# Pep.) found for that protein are listed. Previously identified *O*-GlcNAc proteins are indicated. † represents proteins that have been previously identified as *O*-GlcNAc proteins by any method. †† represents proteins that have been previously validated to contain *O*-GlcNAc either by direct identification of the *O*-GlcNAc modification by mass spectrometry or by radioactive GalT labeling.

Protein	Accession Number	# Pep.	Known
Cell Organization / Dynamics			
ACTA2 Actin alpha-2 chain	IPI00008603.1, IPI00021428.1, IPI00023006.1, IPI00025416.3, IPI00110827.1	8	†
ACTB Actin beta chain	IPI00021439.1, IPI00021440.1, IPI00848058.1	27	†
Ank2 Similar to Ankyrin 2 isoform 1	IPI00554111.2	10	
Ank3 Ankyrin 3	IPI00199445.2	19	††
ANXA2 Annexin A2	IPI00797556.1, IPI00848164.1	3	
Anxa6 Annexin A6	IPI00421888.3, IPI00831745.1	7	
ARPC2 Actin-related protein 2/3 complex subunit 2	IPI00005161.3, IPI00661414.2, IPI00764535.2	3	
CAPZB Isoform 1 of F-actin capping protein subunit beta	IPI00026185.5, IPI00191444.3, IPI00218782.2, IPI00269481.7, IPI00365283.1, IPI00406800.4, IPI00474883.2, IPI00642256.1, IPI00776140.1	8	††
Ckap5 Cytoskeleton associated protein 5	IPI00317134.3, IPI00337930.4, IPI00764313.1, IPI00764540.1, IPI00767392.1, IPI00769262.1	4	
Crym Mu-crystallin homolog	IPI00214448.1	14	
Cyln2 CAP-Gly domain-containing linker protein 2	IPI00195929.1	5	
Dnm1 Isoform 1 of Dynamin-1	IPI00272878.6, IPI00331293.3, IPI00413140.3, IPI00657691.2, IPI00816287.2	18	
Dync1h1 Dynein heavy chain, cytosolic	IPI00327630.1	29	†
Epb4.1l1 Isoform S of Band 4.1-like protein 1	IPI00203237.2, IPI00203239.2, IPI00561718.1	19	
Epb4.1l3 Type II brain 4.1 minor isoform	IPI00204503.1, IPI00204506.1, IPI00556956.2, IPI00558692.1, IPI00561669.1, IPI00568756.1	14	†
Fscn1 Fascin	IPI00353563.4, IPI00763106.1, IPI00767873.1	13	†
Ina Alpha-internexin	IPI00135965.2, IPI00211936.2, IPI00848753.1	6	†
LOC367171 Microtubule-associated protein 4 isoform 1	IPI00421342.2	15	††
Map1b similar to Microtubule-associated protein 1B	IPI00372009.3	13	††
Mtap1a Microtubule-associated protein 1A	IPI00199693.2	14	
Mtap2 Isoform MAP2x of Microtubule-associated protein 2	IPI00206171.1, IPI00231051.1, IPI00328017.4	42	††

Mtap6 STOP protein	IPI00210119.1, IPI00734617.2	14	
Myh10 Myosin, heavy polypeptide 10	IPI00338604.4, IPI00391300.3, IPI00397526.2, IPI00479307.3, IPI00515398.1, IPI00757312.1, IPI00790503.2	4	
Myo5a Myosin-Va	IPI00118120.1, IPI00214038.1, IPI00390377.2, IPI00776221.1	5	†
NCKAP1 Nck-associated protein 1	IPI00031982.1, IPI00214442.2, IPI00319320.4, IPI00409684.2, IPI00656204.1, IPI00755241.1, IPI00766452.1	7	
Rad23b UV excision repair protein RAD23 homolog B	IPI00008223.3, IPI00108774.1, IPI00210495.1	23	††
RP1-14N1.3 Ifapsoriasin	IPI00397801.4, IPI00787398.1	3	
Snip SNAP25-interacting protein	IPI00190619.3	9	
Spna2 Spectrin alpha chain, brain	IPI00209258.4	4	
Spnb2 Isoform 1 of Spectrin beta chain, brain 1	IPI00319830.7, IPI00555287.2	51	††
SPTAN1 Spectrin alpha, non-erythrocytic 1	IPI00478292.3, IPI00744706.1, IPI00745092.1, IPI00843765.1, IPI00844215.1	3	
TUBA4A Tubulin alpha-4A chain	IPI00007750.1, IPI00794663.1	18	††
TUBB Tubulin beta chain	IPI00011654.2	16	†
TUBB2A Tubulin beta-2A chain	IPI00013475.1	26	†
TUBB2B Tubulin beta-2B chain	IPI00031370.3	155	
TUBB2C Tubulin beta-2C chain	IPI00007752.1	65	†
TUBB3 Tubulin beta-3 chain	IPI00013683.2	10	
TUBB4 Tubulin beta-4 chain	IPI00023598.2	42	†
Wasf1 WAS protein family member 1	IPI00022007.1, IPI00213598.1, IPI00471372.2	6	
Wdr1_predicted WD repeat protein 1	IPI00215349.5	8	
Cellular Communication / Signal Transduction			
Amph1 Amphiphysin	IPI00196508.1	3	
Ap2a1 Isoform A of AP-2 complex subunit alpha-1	IPI00108780.6, IPI00203346.4, IPI00567919.2, IPI00622911.1, IPI00764057.1, IPI00765430.1, IPI00778656.1	10	††
Ap2a2 AP-2 complex subunit alpha-2	IPI00310131.5, IPI00471901.3, IPI00753468.1	8	
Ap2b1 Isoform 1 of AP-2 complex subunit beta-1	IPI00119689.1, IPI00220991.2, IPI00231502.3, IPI00333383.2, IPI00378063.1, IPI00389753.1, IPI00784156.1, IPI00784366.1, IPI00790702.1	6	
Ap3b2_predicted Similar to Adaptor-related protein complex 3 beta 2 subunit	IPI00368200.2	6	††
Bsn Protein bassoon	IPI00212553.3, IPI00556925.1	98	††
Cadps Calcium-dependent secretion activator 1	IPI00199577.5, IPI00199604.4, IPI00297412.4, IPI00330163.3, IPI00374128.3, IPI00384808.2, IPI00478178.4, IPI00668903.1, IPI00670114.1, IPI00747400.1	13	
CAMK2A Isoform A of Calcium/calmodulin-	IPI00215715.3, IPI00550056.1	51	

dependent protein kinase type II alpha chain			
Camkv CaM kinase-like vesicle-associated protein	IPI00205056.1	5	
Coro1a Coronin-1A	IPI00210071.3	13	†
Crmp1 Crmp1 protein	IPI00312527.4, IPI00561065.2	8	
CSNK2A1 Casein kinase 2 alpha 1 polypeptide	IPI00016613.2, IPI00120162.1, IPI00192586.1, IPI00408176.2, IPI00744507.1	14	††
Ctnnd2 Isoform 1 of Catenin delta-2	IPI00136135.1, IPI00228632.1, IPI00553941.3	5	††
Cyfip2 Cytoplasmic FMR1-interacting protein 2	IPI00405625.9, IPI00719600.4, IPI00763802.1, IPI00769269.1, IPI00789699.2	10	
Dclk1 Isoform 1 of Serine/threonine-protein kinase DCLK1	IPI00468380.4, IPI00778626.1	8	
Dctn1 Dynactin subunit 1	IPI00196703.1	8	
Dpysl3 Dihydropyrimidinase-related protein 3	IPI00029111.2, IPI00122349.1, IPI00203250.1, IPI00556970.1	7	
Dpysl4 Similar to Dihydropyrimidinase-related protein 4	IPI00366087.1, IPI00558008.1, IPI00779982.1	9	
Dpysl5 Dihydropyrimidinase-related protein 5	IPI00331981.7	6	
Erc1;LOC100048600 Isoform 1 of ELKS/RAB6-interacting/CAST family member 1	IPI00117731.1, IPI00117733.1, IPI00171230.5, IPI00181684.4, IPI00201791.3, IPI00216719.1, IPI00331792.4, IPI00374976.1, IPI00457547.1, IPI00557326.1, IPI00558224.1	8	
Gdi1 Rab GDP dissociation inhibitor alpha	IPI00324986.1	52	
GIT1 Isoform 1 of ARF GTPase-activating protein GIT1	IPI00384861.3, IPI00470095.1, IPI00649373.1, IPI00795611.1	4	
Gnaq Guanine nucleotide binding protein alpha q polypeptide	IPI00228618.5,IPI00230868.4	4	
GNB1 Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta 1	IPI00026268.3, IPI00120716.3	3	
Gnb2l1 Guanine nucleotide-binding protein subunit beta 2-like 1	IPI00317740.5, IPI00641950.3, IPI00848226.1	5	
Homer1 Isoform 1 of Homer protein homolog 1	IPI00210570.1	6	
Jup Junction plakoglobin	IPI00229475.1, IPI00554711.2, IPI00789324.1	4	†
LOC315676 Similar to Dmx-like 2	IPI00369671.3	9	
LOC681252 Similar to Myristoylated alanine-rich C-kinase substrate	IPI00371946.3, IPI00480687.2	4	
LOC685144;LOC681927 Similar to SEC24 related gene family, member C isoform 3	IPI00365299.2, IPI00388782.2, IPI00763148.1, IPI00767454.1, IPI00769013.1	4	†
Ncdn NORBIN	IPI00205396.1, IPI00331299.9, IPI00549543.1, IPI00555661.1	11	
NSF Vesicle-fusing ATPase	IPI00006451.6, IPI00210635.2, IPI00656325.2	10	
Ogt UDP-N-acetylglucosamine - peptide N-acetylglucosaminyltransferase 110 kDa subunit	IPI00231503.4, IPI00420870.4, IPI00845528.1	10	††
Pacsin1 Protein kinase C and casein kinase substrate in neurons protein 1	IPI00208245.1	7	

Pclo Isoform 1 of Protein piccolo	IPI00203018.1, IPI00231831.1, IPI00758462.1	37	††
Picalm Isoform 2 of Phosphatidylinositol-binding clathrin assembly protein	IPI00194959.5	19	††
Plcb1 1-phosphatidylinositol-4,5-bisphosphate phosphodiesterase beta 1	IPI00192534.1, IPI00468121.1, IPI00558422.1	3	
Ppp1r12a Isoform 1 of Protein phosphatase 1 regulatory subunit 12A	IPI00183002.6, IPI00211695.1, IPI00397730.3, IPI00400680.1, IPI00400681.1, IPI00413191.2, IPI00779684.1	21	
Ppp3ca Isoform 1 of Serine/threonine-protein phosphatase 2B catalytic subunit alpha isoform	IPI00121545.1, IPI00179415.4, IPI00201410.1, IPI00559849.1, IPI00747748.1, IPI00756703.1	17	
Prkacb Isoform 1 of cAMP-dependent protein kinase beta-catalytic subunit	IPI00263822.7, IPI00560492.1, IPI00742329.1, IPI00742400.1, IPI00742438.1	3	
Prkwnk1 Serine/threonine-protein kinase WNK1	IPI00200557.1, IPI00561348.1	8	††
Ptpn23 Protein tyrosine phosphatase non-receptor type 23	IPI00782007.1	23	
Rap1gds1_predicted Similar to RAP1, GTP-GDP dissociation stimulator 1	IPI00369496.3, IPI00763518.1, IPI00777342.1, IPI00778032.1	8	
Rapgef2_predicted Similar to Rap guanine nucleotide exchange factor 2	IPI00368346.3	7	
RGD1562629_predicted Similar to Protein neurobeachin	IPI00567941.2	5	
RGD1563580_predicted Similar to AP2 associated kinase 1	IPI00556943.2, IPI00559288.2, IPI00786812.1	8	
Rims1 Isoform 1 of Regulating synaptic membrane exocytosis protein 1	IPI00200893.1, IPI00206312.1, IPI00568548.2, IPI00780218.1	3	
Rph3a Rabphilin-3A	IPI00189927.1, IPI00389991.3	3	
Sec23ip Similar to Sec23 interacting protein	IPI00359906.2	7	
Sec31l1 Isoform 1 of Protein transport protein Sec31A	IPI00210147.2, IPI00515833.1	15	
Shank2 Isoform 2 of SH3 and multiple ankyrin repeat domains protein 2	IPI00231759.3, IPI00231761.1, IPI00400661.2, IPI00470293.3, IPI00475709.1	17	††
SNAP91 Isoform 1 of Clathrin coat assembly protein AP180	IPI00006612.2, IPI00122409.1, IPI00215134.1, IPI00230165.1, IPI00408269.4, IPI00646376.2, IPI00652215.1, IPI00653617.1	41	††
Syn1 Isoform IA of Synapsin-1	IPI00191335.1	4	††
Synj1 Similar to Synaptojanin-1	IPI00210153.3, IPI00229626.7, IPI00231602.2, IPI00850983.1	12	
Ywhab Isoform Long of 14-3-3 protein beta/alpha	IPI00230837.5, IPI00760126.1	4	
YWHAE 14-3-3 protein epsilon	IPI00000816.1	6	
YWHAG 14-3-3 protein gamma	IPI00220642.7	5	
Ywhaq Isoform 1 of 14-3-3 protein theta	IPI00408378.4, IPI00656269.1	7	†
Intracellular Transport			
ATP2A2 Isoform SERCA2A of Sarcoplasmic/endoplasmic reticulum calcium ATPase 2	IPI00468900.4	9	
ATP6V0A1 Isoform 1 of Vacuolar proton	IPI00465178.5, IPI00743576.1,	15	†

translocating ATPase 116 kDa subunit A isoform 1	IPI00796045.1		
ATP6V1A Vacuolar ATP synthase catalytic subunit A	IPI00007682.2, IPI00373076.1, IPI00407692.3, IPI00844689.1	32	
Atp6v1b2 Vacuolar ATP synthase subunit B brain isoform	IPI00119113.3, IPI00199305.1	39	
Dnm1l Isoform 4 of Dynamin-1-like protein	IPI00193568.3, IPI00208284.3	9	
Gorasp2 Golgi reassembly stacking protein 2	IPI00362488.1	7	††
NAPA Alpha-soluble NSF attachment protein	IPI00009253.2, IPI00189925.1	4	
Nup153 Similar to Nuclear pore complex protein Nup153	IPI00480641.3, IPI00768316.1	5	††
Pacs1 Isoform PACS-1a of Phosphofurin acidic cluster sorting protein 1	IPI00324270.4	4	
SEPT5 Septin-5	IPI00017731.1, IPI00559449.2, IPI00655290.2	4	
Sept6_predicted 49 kDa protein	IPI00363930.4, IPI00420385.4, IPI00454142.5, IPI00454143.3, IPI00780333.1	3	
SEPT7 Isoform 1 of Septin-7	IPI00033025.8, IPI00204899.2, IPI00224626.3, IPI00816201.1	3	
Sept11 Isoform 3 of Septin-11	IPI00420385.4, IPI00454142.5	19	
Slc25a12 Calcium-binding mitochondrial carrier protein Aralar1	IPI00308162.3	15	
Slc25a4 ADP/ATP translocase 1	IPI00115564.5, IPI00231927.11, IPI00676622.1	4	
SLC25A5 ADP/ATP translocase 2	IPI00007188.5, IPI00127841.3, IPI00200466.3, IPI00363182.2, IPI00558425.2, IPI00565507.2	3	
Srprb Signal recognition particle receptor B subunit	IPI00196656.2, IPI00476177.2, IPI00679202.2	6	
VCP Transitional endoplasmic reticulum ATPase	IPI00022774.3, IPI00622235.5, IPI00676914.1	12	†
Vdac2 Voltage-dependent anion-selective channel protein 2	IPI00122547.1, IPI00198327.2	5	
Metabolism / Biosynthesis			
Acot7 Isoform B of Cytosolic acyl coenzyme A thioester hydrolase	IPI00125939.2, IPI00213571.1, IPI00230588.1, IPI00284094.4, IPI00326904.5, IPI00566122.1, IPI00672508.1	3	
Aldoal1 Fructose-bisphosphate aldolase	IPI00195851.1, IPI00221402.7, IPI00231734.5, IPI00465439.5, IPI00796333.1	4	
Aldoc Fructose-bisphosphate aldolase C	IPI00231736.9	15	
Atp5a1 ATP synthase subunit alpha, mitochondrial precursor	IPI00396910.1	11	†
ATP5B ATP synthase subunit beta, mitochondrial precursor	IPI00303476.1, IPI00551812.1	13	
Ctbp1 Isoform 1 of C-terminal-binding protein 1	IPI00128155.2, IPI00392657.1, IPI00754844.1, IPI00780254.1, IPI00845557.1	34	
Dlat Dihydrolipoyllysine-residue acetyltransferase component of pyruvate dehydrogenase complex, mitochondrial precursor	IPI00231714.3, IPI00765153.1	3	

Eno1 Alpha-enolase	IPI00462072.3, IPI00464815.11	40	†
Eno2 Gamma-enolase	IPI00326412.4	7	†
Fasn Fatty acid synthase	IPI00200661.1	4	†
Gda Guanine deaminase	IPI00325884.5, IPI00851130.1	16	
Glud1 Glutamate dehydrogenase 1, mitochondrial precursor	IPI00016801.1, IPI00027146.1, IPI00114209.1, IPI00324633.2, IPI00753095.1	8	
Glul Glutamine synthetase	IPI00324020.6, IPI00626790.2	10	
Got1 Aspartate aminotransferase, cytoplasmic	IPI00421513.8	11	
Got2 Aspartate aminotransferase, mitochondrial precursor	IPI00210920.1	17	
Gpi Glucose-6-phosphate isomerase	IPI00364311.1	3	
Hk1 Hexokinase-1	IPI00202543.1	22	
Hmgcs1 Hydroxymethylglutaryl-CoA synthase, cytoplasmic	IPI00188158.1	4	
IDH3A Isoform 1 of Isocitrate dehydrogenase [NAD] subunit alpha, mitochondrial precursor	IPI00030702.1, IPI00198720.1, IPI00459725.2	3	†
LOC316632 NADH dehydrogenase 1 alpha subcomplex 10-like protein	IPI00189759.1, IPI00561513.1	3	
LOC360975 2-oxoglutarate dehydrogenase E1 component, mitochondrial precursor	IPI00215093.1, IPI00390995.2, IPI00782594.1	3	
Ndufs1 NADH-ubiquinone oxidoreductase 75 kDa subunit, mitochondrial precursor	IPI00358033.1	56	
Ndufs2 NADH dehydrogenase [ubiquinone] iron-sulfur protein 2, mitochondrial precursor	IPI00128023.3, IPI00471647.1, IPI00830766.1	3	
Oxr1 Similar to Oxidation resistance 1	IPI00199013.7, IPI00764149.1	3	
Pdha1 Pyruvate dehydrogenase E1 component alpha subunit somatic form, mitochondrial precursor	IPI00191707.4, IPI00337893.2, IPI00393034.3, IPI00764176.1, IPI00768086.2	3	
Pdhb Pyruvate dehydrogenase E1 component subunit beta, mitochondrial precursor	IPI00194324.2	7	
Pfkm 6-phosphofructokinase muscle type	IPI00331541.5	12	
Pfkp 6-phosphofructokinase type C	IPI00231954.5	4	
Pgam1 Phosphoglycerate mutase 1	IPI00421428.9, IPI00453476.2, IPI00457898.3, IPI00549725.6, IPI00740800.1	9	†
Pgk1 Phosphoglycerate kinase 1	IPI00231426.6, IPI00372910.2, IPI00555069.3	9	†
Phgdh D-3-phosphoglycerate dehydrogenase	IPI00225961.5, IPI00475835.3	3	†
Pkm2 Isoform M1 of Pyruvate kinase isozymes M1/M2	IPI00231929.6	11	†
Psat1 Phosphoserine aminotransferase	IPI00331919.5	6	
Pygb Glycogen phosphorylase brain form	IPI00229796.3, IPI00357945.1	6	
Taldo1 Transaldolase	IPI00124692.1, IPI00190377.2	3	
Tpi1 Triosephosphate isomerase	IPI00231767.5,IPI00339162.1	7	†
Tst Thiosulfate sulfurtransferase	IPI00366293.3, IPI00566218.1	3	
mRNA / Protein Processing			
Carm1 Isoform 1 of Histone-arginine methyltransferase CARM1	IPI00125950.2, IPI00279931.1, IPI00366497.3, IPI00412880.2, IPI00568674.2, IPI00639957.2, IPI00650083.2, IPI00655258.2,	5	

	IPI00830611.1		
Cct2 T-complex protein 1 subunit beta	IPI00366218.3	3	
Cct8_predicted Similar to T-complex protein 1 subunit theta	IPI00370815.3	28	†
Fbxo2 F-box only protein 2	IPI00153176.2, IPI00209303.1	3	
Fkbp4 Similar to FK506-binding protein 4	IPI00358443.3, IPI00767393.1	3	
HNRPA1 Isoform A1-B of Heterogeneous nuclear ribonucleoprotein A1	IPI00215965.2, IPI00224251.5, IPI00465365.4, IPI00553777.2, IPI00748262.1, IPI00797148.1	9	†
Hnrpa2b1_predicted Heterogeneous nuclear ribonucleoproteins A2/B1	IPI00212969.2, IPI00358211.3, IPI00396378.3, IPI00405058.6, IPI00414696.1, IPI00622847.2, IPI00828488.1, IPI00853914.1	7	†
Hnrpa3 Isoform 1 of Heterogeneous nuclear ribonucleoprotein A3	IPI00269661.1, IPI00269662.1, IPI00419373.1, IPI00455134.1, IPI00459722.2, IPI00461800.1, IPI00466185.3, IPI00470076.5, IPI00623731.1, IPI00660502.1, IPI00664047.1, IPI00664791.1	12	†
Hnrpc Heterogeneous nuclear ribonucleoprotein C	IPI00130343.2, IPI00187860.3, IPI00216592.2, IPI00223443.1, IPI00223444.1, IPI00477313.3, IPI00759596.1, IPI00759870.1, IPI00759886.1, IPI00781839.1	4	
Hnrpk Hnrpk protein	IPI00194974.2, IPI00216049.1, IPI00216746.1, IPI00223253.1, IPI00224575.1, IPI00514561.1, IPI00777007.1, IPI00780608.1, IPI00807545.1	37	†
Hnrpul2 Heterogeneous nuclear ribonucleoprotein U-like protein 2	IPI00222208.2, IPI00360386.3, IPI00561756.2, IPI00565127.2, IPI00756515.1, IPI00849047.1	4	
Hsp110 Isoform HSP105-alpha of Heat shock protein 105 kDa	IPI00123802.5, IPI00224109.2, IPI00471835.1, IPI00568014.2, IPI00778569.1, IPI00779326.1, IPI00830204.1	3	
Hspa12a_predicted Similar to Heat shock protein 12A	IPI00358537.2	6	
Hspa4 Heat shock 70 kDa protein 4	IPI00387868.2	10	††
Hspd1 Isoform 1 of 60 kDa heat shock protein, mitochondrial precursor	IPI00308885.6, IPI00339148.2, IPI00472102.3, IPI00763910.1, IPI00784154.1, IPI00790763.1	36	††
Hsph1 Heat shock protein 105 kDa	IPI00218993.1, IPI00471835.1, IPI00513743.1, IPI00514983.3	8	
Huwe1 HECT, UBA and WWE domain containing 1	IPI00463909.3, IPI00655012.2	37	
NPEPPS Puromycin-sensitive aminopeptidase	IPI00026216.4, IPI00130000.1, IPI00372700.1, IPI00608097.1, IPI00767572.1, IPI00768609.1	8	†
Otub1;LOC100046081 Ubiquitin thioesterase OTUB1	IPI00154004.1, IPI00371462.3, IPI00755837.1	4	
PABPC1 Isoform 1 of Polyadenylate-binding protein 1	IPI00008524.1, IPI00124287.1, IPI00189074.3, IPI00331552.4,	6	†

	IPI00410017.1, IPI00478522.1, IPI00796945.1		
PCBP2 Poly(rC)-binding protein 2 isoform b	IPI00012066.2, IPI00127707.1, IPI00216689.2, IPI00221796.1, IPI00221799.1, IPI00470509.2, IPI00796337.1	4	†
Pdia3 Protein disulfide-isomerase A3 precursor	IPI00324741.2	9	
Rbm12 Swan	IPI00421433.1, IPI00560597.1	12	
Rbmx Heterogeneous nuclear ribonucleoprotein G	IPI00124979.2, IPI00304692.1, IPI00370207.3, IPI00474144.1, IPI00559910.1, IPI00604873.2, IPI00663587.1, IPI00763272.1, IPI00766882.1, IPI00775821.1, IPI00775899.1	6	
Sf3a1_predicted Similar to Splicing factor 3 subunit 1	IPI00215030.1, IPI00408796.3	5	
SFPQ Isoform Long of Splicing factor, proline- and glutamine-rich	IPI00010740.1, IPI00129430.1, IPI00627068.1, IPI00752791.1, IPI00755611.1, IPI00767277.1, IPI00849080.1	3	†
Thop1 Thimet oligopeptidase 1	IPI00564198.2	4	
Ubqln2_predicted Similar to ubiquilin 2	IPI00362791.3	66	
Uqcrc1 Ubiquinol-cytochrome-c reductase complex core protein 1, mitochondrial precursor	IPI00471577.1	6	
Uqcrc2 Ubiquinol-cytochrome-c reductase complex core protein 2, mitochondrial precursor	IPI00188924.4	3	
USP5 Isoform Long of Ubiquitin carboxyl-terminal hydrolase 5	IPI00024664.1, IPI00207657.1, IPI00375145.1, IPI00767186.1, IPI00768802.1	4	
Transcription / Translation			
Hcfc1_predicted Similar to Host cell factor C1	IPI00367724.3, IPI00765252.1	13	††
CAND1 Isoform 1 of Cullin-associated NEDD8-dissociated protein 1	IPI00100160.3, IPI00205466.1, IPI00420562.5, IPI00746694.1, IPI00753059.1	3	
CNOT1 CCR4-NOT transcription complex subunit 1 isoform A	IPI00166010.6, IPI00359049.4, IPI00673465.1, IPI00674283.1, IPI00752506.1, IPI00757812.1	17	††
DDX17 DEAD box polypeptide 17 isoform 1	IPI00023785.6, IPI00396797.2, IPI00651653.1, IPI00651677.1, IPI00653307.1	4	
DDX5 Probable ATP-dependent RNA helicase DDX5	IPI00017617.1, IPI00420363.2, IPI00464718.1	3	
Eef1a1 Elongation factor 1-alpha 1	IPI00195372.1, IPI00307837.5, IPI00396485.3, IPI00472724.1, IPI00551729.1	5	†
EEF2 Elongation factor 2	IPI00186290.6, IPI00203214.6, IPI00466069.3, IPI00849291.1	4	††
EG268795 Similar to 60S ribosomal protein L7a (Surfeit locus protein 3) isoform 1	IPI00265107.4, IPI00299573.12, IPI00330363.8, IPI00354363.3, IPI00397676.4, IPI00462006.3,	3	

	IPI00462453.4, IPI00478896.2, IPI00479315.2, IPI00622160.3		
Eif4a2 Eukaryotic initiation factor 4A-II	IPI00193595.3, IPI00328328.3, IPI00400432.2, IPI00409717.1, IPI00409918.1	3	
Eif4g3_predicted Similar to Eukaryotic translation initiation factor 4 gamma 3	IPI00365284.3, IPI00767350.1	6	
pur-beta Transcriptional activator protein Pur-beta	IPI00189358.2	3	
RGD1560833_predicted Similar to MKL/myocardin-like 2	IPI00765655.1	2	
Ripx Protein RUFY3	IPI00204065.1, IPI00206350.3	3	
RPS3 40S ribosomal protein S3	IPI00011253.3, IPI00134599.1, IPI00212776.1	4	†
RPS8 40S ribosomal protein S8	IPI00216587.9, IPI00231202.6, IPI00274175.1, IPI00466820.4, IPI00475203.1, IPI00621229.1, IPI00645201.1, IPI00671398.1, IPI00756488.1, IPI00756959.1, IPI00828628.1, IPI00849948.1	3	†
Vezf1_predicted 22 kDa protein	IPI00780927.1	4	
Zfr Similar to Zinc finger RNA binding protein	IPI00367952.3, IPI00765814.1	8	††
Uncharacterized			
Hnrpul2 Heterogeneous nuclear ribonucleoprotein U-like protein 2	IPI00222208.2, IPI00360386.3, IPI00561756.2, IPI00565127.2, IPI00756515.1, IPI00849047.1	4	
Immt 82 kDa protein	IPI00364895.4, IPI00566985.1, IPI00777695.1	8	
LOC314432 Similar to Ubiquitin-protein ligase (EC 6.3.2.19) E1-mouse	IPI00368347.2	7	
LOC501546 LOC501546 protein	IPI00201213.3	3	
MGC93707 Mitochondrial antiviral-signaling protein	IPI00364200.1	3	
RGD1562348_predicted Similar to Ankyrin repeat domain protein 17 isoform B	IPI00361795.2, IPI00388314.3	9	
RGD1563977_predicted Similar to Protein 4.1G	IPI00191995.2, IPI00192909.2, IPI00368431.2, IPI00388101.1, IPI00393242.1	3	
RGD1566064_predicted Similar to HBxAg transactivated protein	IPI00363856.3	13	
SH3GLB2 Isoform 1 of SH3 domain GRB2-like protein B2	IPI00024540.3, IPI00153832.1, IPI00398828.1, IPI00626834.2, IPI00756786.1, IPI00776533.1, IPI00779094.1, IPI00828453.1	16	
Ubap2_predicted Similar to Ubiquitin-associated protein 2	IPI00190431.4	13	
Ubap2l Isoform 5 of Ubiquitin-associated protein 2-like	IPI00407835.1, IPI00412535.2, IPI00514856.4, IPI00761937.1	6	

Table 2: Promoters enriched in *O*-GlcNAc levels. The corresponding gene name and description for each promoter are listed. The location of the promoter and the log ratio of the chromatin immunoprecipitation eluent over input are also listed.

Gene Name	Description	Location	LogRatio
Rfwd2	Ring finger and WD repeat domain 2	chr1:161066116-161066175	1.12E+00
Arl6ip1	ADP-ribosylation factor-like 6 interacting protein 1	chr7:117911348-117911397	1.05E+00
Gpr21	G protein-coupled receptor 21	chr2:037340628-037340687	1.01E+00
Bhlhb5	Basic helix-loop-helix family, member e22	chr3:018243837-018243896	9.98E-01
Pde6b	Phosphodiesterase 6B, cGMP, rod receptor, beta polypeptide	chr5:108630597-108630649	9.94E-01
Gapdh	Glyceraldehyde-3-phosphate dehydrogenase	chr6:125131691-125131742	9.51E-01
Slc4a4	Solute carrier family 4 (anion exchanger), member 4	chr5:090007481-090007540	9.05E-01
Kcnab1	Potassium voltage-gated channel, shaker-related subfamily, beta member 1	chr3:065195669-065195719	8.92E-01
Hspb7	Heat shock protein family, member 7 (cardiovascular)	chr4:140694790-140694840	8.88E-01
Phf13	PHD finger protein 13	chr4:150833827-150833886	8.73E-01
Hivep3	Human immunodeficiency virus type I enhancer binding protein 3	chr4:119307364-119307417	8.58E-01
Ifnb1	Interferon beta 1, fibroblast	chr4:087991861-087991916	8.49E-01
Spg20	Spastic paraplegia 20 (Troyer syndrome)	chr3:055222441-055222497	8.46E-01
Pin1	Peptidylprolyl cis/trans isomerase, NIMA-interacting 1	chr9:020401004-020401061	8.46E-01
Tmc7	Transmembrane channel-like gene family 7	chr7:118377551-118377610	8.31E-01
Trp53inp2	Tumor protein p53 inducible nuclear protein 2	chr2:155073250-155073294	8.30E-01
Sgcb	Sarcoglycan, beta (dystrophin-associated glycoprotein)	chr5:073912548-073912601	8.27E-01
NC2_00099332	Unknown	NC2_00099332	8.24E-01
Mmp8	Matrix metallopeptidase 8	chr9:007558381-007558437	8.19E-01
Kcnj14	Potassium inwardly-rectifying channel, subfamily J, member 14	chr7:045690923-045690982	8.11E-01
Pde3a	Phosphodiesterase 3A, cGMP inhibited	chr6:141210944-141210995	8.07E-01
Ccdc106	Coiled-coil domain containing 106	chr7:004654767-004654811	8.00E-01
Gpsm1	G-protein signaling modulator 1 (AGS3-like, C. elegans)	chr2:026137783-026137827	7.86E-01
NC2_00099332	Unknown	NC2_00099332	7.81E-01
Snrp70	Small nuclear ribonucleoprotein 70	chr7:045263625-045263669	7.72E-01
Aspm	Asp (abnormal spindle)-like, microcephaly	chr1:141271216-	7.69E-01

	associated	141271266	
NC2_00099332	Unknown	NC2_00099332	7.58E-01
Ltb4dh	Prostaglandin reductase 1	chr4:059078901-059078954	7.55E-01
NC2_00099332	Unknown	NC2_00099332	7.50E-01
Rexo4-Adamts13	Unknown	chr2:026795163-026795217	7.49E-01
Aox3	Aldehyde oxidase 3	chr1:058058797-058058847	7.48E-01
NC2_00099332	Unknown	NC2_00099332	7.40E-01
Sema3c	Semaphorin 3c	chr5:017086294-017086338	7.31E-01
Gucy2c	Guanylate cyclase 2c	chr6:136750955-136751011	7.28E-01
Krtap5-5	Keratin associated protein 5-5	chr7:142043215-142043274	7.20E-01
Itm2c	Integral membrane protein 2C	chr1:087720489-087720548	7.12E-01
Kcnt1	Potassium channel, subfamily T, member 1	chr2:025700956-025701003	7.10E-01
Fbxl13	F-box and leucine-rich repeat protein 13	chr5:021054005-021054064	7.04E-01
Snrpn	Small nuclear ribonucleoprotein N	chr7:059883275-059883319	6.96E-01
2600010E01Rik	Proline rich 5 like	chr2:101598189-101598234	6.95E-01
Myo3b	Myosin IIIB	chr2:070085726-070085785	6.74E-01
Lrch4	Leucine-rich repeats and calponin homology (CH) domain	chr5:137860009-137860054	6.71E-01
Bai3	Brain-specific angiogenesis inhibitor 3	chr1:025776261-025776316	6.67E-01
Il11	Interleukin 11	chr7:004383274-004383318	6.61E-01
Gm1040	Nucleolar protein with MIF4G domain 1	chr5:029766349-029766408	6.61E-01
1200015F23Rik-Gchfr	Unknown	chr2:118854850-118854901	6.59E-01
Coq10b	Coenzyme Q10 homolog B	chr1:054999769-054999828	6.50E-01
Tas2r139	Taste receptor, type 2, member 139	chr6:042071031-042071090	6.47E-01
Rab23	RAB23, member RAS oncogene family	chr1:033664505-033664560	6.47E-01
Ralb	V-ral simian leukemia viral oncogene homolog B	chr1:121334415-121334464	6.44E-01
BC026590	Unknown	chr4:056900096-056900155	6.43E-01
Ndufs2	NADH dehydrogenase (ubiquinone) Fe-S protein 2	chr1:173071744-173071801	6.43E-01
chr6:054357273-054357332	Unknown	chr6:054357273-054357332	6.40E-01
Klk1b11	Kallikrein 1-related peptidase b11	chr7:043860709-043860757	6.40E-01
Chn1	Chimerin (chimaerin) 1	chr2:073460953-	6.38E-01

		073461012	
Fn1	Fibronectin 1	chr1:071585152-071585211	6.37E-01
Hrh3	Histamine receptor H3	chr2:180037363-180037418	6.37E-01
Arid1a	AT rich interactive domain 1A (SWI-like)	chr4:133027660-133027704	6.37E-01
Sepn1	Selenoprotein N, 1	chr4:133817519-133817563	6.36E-01
4931406C07Rik	Unknown	chr9:015049879-015049938	6.35E-01
Kcne3	Potassium voltage-gated channel, Isk-related subfamily, gene 3	chr7:100047744-100047792	6.34E-01
Prss3-EG436523	Unknown	chr6:041317008-041317067	6.32E-01
Sult2b1	Sulfotransferase family, cytosolic, 2B, member 1	chr7:045626168-045626227	6.30E-01
Nanos2	Nanos homolog 2 (Drosophila)	chr7:018146282-018146328	6.25E-01
Nadk	NAD kinase	chr4:154404524-154404575	6.24E-01
Scly	Selenocysteine lyase	chr1:093150219-093150267	6.20E-01
EG229862	Unknown	chr3:137487520-137487579	6.17E-01
Olfr666	Olfactory receptor 666	chr7:104767985-104768044	6.14E-01
D430042O09Rik	Unknown	chr7:125502534-125502585	6.12E-01
Lmo4	LIM domain only 4	chr3:144147353-144147412	6.00E-01
Iqsec3	IQ motif and Sec7 domain 3	chr6:121437055-121437114	5.99E-01
chr7:062089379-062089438	Unknown	chr7:062089379-062089438	5.91E-01
9130221D24Rik	Unknown	chr3:133170194-133170253	5.91E-01
Sh2d2a	SH2 domain protein 2A	chr3:087934416-087934460	5.90E-01
Tmcc2	Transmembrane and coiled-coil domains 2	chr1:134217724-134217783	5.88E-01
Ccdc9	Coiled-coil domain containing 9	chr7:015445399-015445449	5.86E-01
Capn5	Calpain 5	chr7:098018304-098018355	5.80E-01
Rab43	RAB43, member RAS oncogene family	chr6:087781399-087781454	5.73E-01
2610209A20Rik	Lipoyl(octanoyl) transferase 2 (putative)	chr7:100034032-100034090	5.71E-01
Csn1s2b	Casein alpha s2-like B	chr5:088895459-088895518	5.68E-01
Mag	Myelin-associated glycoprotein	chr7:030626049-030626105	5.68E-01
Cort	Cortistatin	chr4:147968893-147968944	5.66E-01

F2rl3	Coagulation factor II (thrombin) receptor-like 3	chr8:075653052-075653097	5.64E-01
Tlx2	T-cell leukemia, homeobox 2	chr6:083034418-083034462	5.60E-01
2310038H17Rik	Unknown	chr1:064549300-064549347	5.59E-01
C1qc	Complement component 1, q subcomponent, C chain	chr4:136162907-136162966	5.58E-01
Madd	MAP-kinase activating death domain	chr2:090983205-090983264	5.57E-01
Kdelc1	KDEL (Lys-Asp-Glu-Leu) containing 1	chr1:044046571-044046615	5.55E-01
Inpp1	Inositol polyphosphate-1-phosphatase	chr1:052761859-052761903	5.54E-01
Gm973	Predicted gene 973	chr1:059459724-059459783	5.53E-01
Fhod1	Formin homology 2 domain containing 1	chr8:108237017-108237065	5.51E-01
Gpr109a	G protein-coupled receptor 109A	chr5:124125079-124125129	5.51E-01
Cnr2	Cannabinoid receptor 2 (macrophage)	chr4:135181859-135181915	5.50E-01
Syt6	Synaptotagmin VI	chr3:103706243-103706302	5.47E-01
Rpn1	Ribophorin I	chr6:088050301-088050345	5.47E-01
Stoml3	Stomatin (Epb7.2)-like 3	chr3:053572971-053573030	5.46E-01
Hdac4	Histone deacetylase 4	chr1:093978609-093978663	5.45E-01
Tesk2	Testis-specific kinase 2	chr4:116220557-116220616	5.44E-01
Isg20l1	Apoptosis enhancing nuclease	chr7:078761115-078761174	5.42E-01
Il8rb	Interleukin 8 receptor, beta	chr1:074089611-074089670	5.42E-01
Cpsf3l	Cleavage and polyadenylation specific factor 3-like	chr4:154732636-154732695	5.41E-01
Dbndd2	Dysbindin (dystrobrevin binding protein 1) domain containing 2	chr2:164183228-164183287	5.39E-01
Yipf7-Guf1	Unknown	chr5:069830810-069830869	5.37E-01
2010315L10Rik	Vesicle transport protein USE1 isoform 3	chr8:074298265-074298309	5.37E-01
Ank1	Ankyrin 1, erythroid	chr8:024519526-024519585	5.37E-01
Igsf21	Immunoglobin superfamily, member 21	chr4:139519948-139519992	5.36E-01
Calcr	Calcitonin receptor	chr6:003719766-003719819	5.35E-01
Siglec1	Sialic acid binding Ig-like lectin 1, sialoadhesin	chr2:130780557-130780616	5.33E-01
Nmur1	Neuromedin U receptor 1	chr1:088217036-088217084	5.32E-01
Shroom3	Shroom family member 3	chr5:093758832-	5.30E-01

		093758876	
Clic3	Chloride intracellular channel 3	chr2:025276329-025276385	5.29E-01
Lim2	Lens intrinsic membrane protein 2	chr7:043296801-043296855	5.29E-01
Otud6b	OTU domain containing 6B	chr4:014753291-014753341	5.29E-01
A930008G19Rik	Family with sequence similarity 53, member B	chr7:132651633-132651677	5.29E-01
Chrm4	Cholinergic receptor, muscarinic 4	chr2:091728313-091728369	5.27E-01
chr4:102789696-102789755	Unknown	chr4:102789696-102789755	5.27E-01
Cblc	Casitas B-lineage lymphoma c	chr7:018955195-018955239	5.24E-01
Gem	GTP binding protein (gene overexpressed in skeletal muscle)	chr4:011628685-011628729	5.24E-01
Cryge	Crystallin, gamma E	chr1:064986389-064986435	5.21E-01
Cyp26b1	Cytochrome P450, family 26, subfamily b, polypeptide 1	chr6:084559974-084560027	5.21E-01
Ceacam9	Carcinoembryonic antigen-related cell adhesion molecule 9	chr7:015875265-015875309	5.20E-01
Mcm3	Minichromosome maintenance complex component 3	chr1:020804983-020805042	5.20E-01
Il15ra	Interleukin 15 receptor, alpha	chr2:011624220-011624274	5.19E-01
Slmo2	Slowmo homolog 2	chr2:174114425-174114484	5.17E-01
Calr3	Calreticulin 3	chr8:075372337-075372395	5.17E-01
Slc6a17	Solute carrier family 6, member 17	chr3:107651018-107651077	5.14E-01
Gatad2a	GATA zinc finger domain containing 2A	chr8:072924369-072924421	5.14E-01
Pax3	Paired box gene 3	chr1:078083580-078083635	5.13E-01
Aldh1b1	Aldehyde dehydrogenase 1 family, member B1	chr4:045817830-045817881	5.12E-01
Col27a1	Collagen, type XXVII, alpha 1	chr4:062702912-062702961	5.12E-01
Casp6	Caspase 6, apoptosis-related cysteine peptidase	chr3:129888382-129888441	5.11E-01
Tspan2	Tetraspanin 2	chr3:102864758-102864809	5.11E-01
Wdr54	WD repeat domain 54	chr6:083121238-083121294	5.10E-01
Defb9	Defensin beta 9	chr8:023352585-023352644	5.10E-01
Slc39a10	Solute carrier family 39 (zinc transporter), member 10	chr1:046797981-046798031	5.09E-01
Fzd7	Frizzled homolog 7	chr1:059426879-059426925	5.09E-01
Cyb5r2	Cytochrome b5 reductase 2	chr7:107550066-107550125	5.08E-01

Lrp1b	Low density lipoprotein receptor-related protein 1B	chr2:042476511-042476570	5.08E-01
Lcn8	Lipocalin 8	chr2:025476940-025476998	5.07E-01
Pcdh7	Protocadherin 7	chr5:058004012-058004063	5.07E-01
Smad1	SMAD family member 1	chr8:082300818-082300874	5.06E-01
Gnb2	Guanine nucleotide binding protein (G protein), beta 2	chr5:137761013-137761064	5.06E-01
Slc25a34	Solute carrier family 25, member 34	chr4:140895902-140895946	5.06E-01
Trim46	Tripartite motif-containing 46	chr3:089329908-089329967	5.06E-01
Melk	Maternal embryonic leucine zipper kinase	chr4:044321192-044321251	5.05E-01
Olfr1336	Olfactory receptor 1336	chr7:006059882-006059930	5.04E-01
Olfr71	Olfactory receptor 71	chr4:043732351-043732410	5.04E-01
Casc4	Cancer susceptibility candidate 4	chr2:121559691-121559750	5.04E-01
Slc25a40	Solute carrier family 25, member 40	chr5:008432144-008432203	5.03E-01
Gpr175	G protein-coupled receptor 175	chr6:088869789-088869848	5.03E-01
C430003P19Rik	BRISC complex subunit Abro1	chr7:132714914-132714973	5.02E-01
Qrfp	Pyroglutamylated RFamide peptide	chr2:031634928-031634987	5.01E-01
Abca2	ATP-binding cassette, sub-family A (ABC1), member 2	chr2:025251853-025251912	5.00E-01

Methods

General Reagents and Methods:

Unless otherwise noted, reagents were purchased from the commercial suppliers Fisher (Fairlawn, NJ) or Sigma-Aldrich (St. Louis, MO). Protease inhibitors were purchased from Roche Applied Sciences (Indianapolis, IN), sequencing grade trypsin was from Promega (Madison, WI), agarose-conjugated protein G was from Pierce (Rockford, IL), and Immobilon-FL PDVF membrane was from Millipore (Billerica, MA). Dulbecco's modified Eagle media (DMEM), B27, fetal bovine serum (FBS) and penicillin/ streptomycin were from Gibco (Carlsbad, CA). The anti-α-crystallin (ab5595) and anti-β-tubulin antibodies were from Abcam and Sigma, respectively. Click-It™ *O*-GlcNAc Enzymatic Labeling System, Click-It™ Biotin Glycoprotein Detection Kit, Click-It™ Tetramethylrhodamine (TAMRA) Glycoprotein Detection Kit, anti-TAMRA antibody, 4-12% NuPAGE® Bis-Tris Mini gels, pH 4-7 Zoom IPG strips, and lithium dodecyl sulfate (LDS) buffer were from Invitrogen (Carlsbad, CA). Peptide N-glycosidase F (PNGase F) was purchased from New England Biolabs (Beverly, MA). The anti-*O*-GlcNAc antibodies CTD110.6 and RL-2 were from Covance (Princeton, NJ) and Affinity Bioreagents (Golden, CO), respectively. Wheat germ agglutinin (WGA) lectin was from EY laboratories (San Mateo, CA). The secondary goat anti-rabbit antibody conjugated to IRDye800 was from Rockland Immunochemicals (Gilbertsville, PA), and the streptavidin-IR680 conjugate was from Li-COR Bioscences (Lincoln, NE). Sprague-

Dawley and Long Evans rats were from Charles River Laboratories (Wilmington, MA). All protein concentrations were measured using the BCA protein assay (Pierce). Western blots were visualized and quantified using an Odyssey infrared imaging system (LI-COR Biosciences). In-gel fluorescence detection was performed using a FujiFilm FLA-3000 or FLA-5100 scanner, and the fluorescence was displayed in green pseudocolor.

Chemoenzymatic Labeling of α-Crystallin. α-Crystallin from the Click-It™ *O*-GlcNAc Enzymatic Labeling System (20 μg) was labeled with UDP-GalNAz **1** and biotin alkyne **2** as per the Click-It™ *O*-GlcNAc Enzymatic Labeling System and Click-It™ Biotin Glycoprotein Detection Kit instructions. Negative controls were performed under identical conditions, except GalT, **1**, or **2** were left out of the reactions. α-Crystallin (10 pmol from each reaction) was resolved on a 1.5 mm, 10-well NuPAGE 4-12% Bis-Tris gel and transferred to PDVF. The membrane was blocked with 5% BSA in 50 mM Tris-HCl pH 7.4, 150 mM NaCl containing 0.1% Tween (TBST) for 1 h at RT followed by 1 h incubation with a streptavidin-IR680 conjugate (1:10,000) in TBST. After four washes for 15 min in TBST, the membrane was visualized using an Odyssey imaging system. The same membrane was then blotted with an anti-α-crystallin antibody (1:1000) in 5% nonfat milk / TBST for 1 h at RT. Following three washes in TBST for 5 min, the membrane was incubated with a goat anti-rabbit antibody conjugated to IRDye800 (1:10,000) in the same buffer for 1 h at RT, washed three more times for 10 min, and then visualized using an Odyssey imaging system. To examine the detection sensitivity, 25, 10, 5, 1, 0.5, and 0.25 pmol of labeled α-crystallin were resolved by SDS-PAGE,

transferred to PDVF, and immunoblotted with a streptavidin-IR680 conjugate (1:10,000). For comparison, unlabeled α-crystallin (25 pmol) was resolved by SDS-PAGE, transferred to PDVF, and immunoblotted with the CTD110.6 antibody (1:1000), RL-2 antibody (1:1000), or WGA lectin (10 μg/mL) for 1 h at RT.

Chemoenzymatic Labeling of Rat Forebrain Extracts. The forebrains from three adult rats (~150 g, male Sprague Dawley) were extracted and fractionated using the Qproteome Cell Compartment Kit (Qiagen). Fractions 1 (the cytoplasmic fraction) and 3 (the nuclear fraction) were precipitated with 4 volumes ice-cold acetone followed by overnight incubation at -20 ºC and redissolved in 2% SDS plus Complete™ protease inhibitors. Protein from each fraction (1.5 mg for the 2D gel labeling, 0.5 mg for the 1D gel labeling) was precipitated and labeled at 1 mg/mL as per the Click-It™ *O*-GlcNAc Enzymatic Labeling System instructions except that Complete™ protease inhibitors were added during the labeling reaction, followed by labeling with the TAMRA-alkyne dye **3** as per the Click-It™ TAMRA Glycoprotein Detection Kit instructions except that EDTA-free Complete™ protease inhibitors were added during the TAMRA labeling reaction. For the 1D gels, negative controls were performed under identical conditions except that GalT was omitted from the labeling reaction.

Immunoprecipitation of TAMRA-Labeled *O*-GlcNAc Proteins. Labeled samples were precipitated using methanol/chloroform/water, brought up to a concentration of 2 mg/mL in 1% SDS plus Complete™ protease inhibitors, and boiled. The SDS was then quenched

with 1 volume of NETFD buffer (100 mM NaCl, 50 mM Tris-HCl pH 7.4, 5 mM EDTA, 6% NP-40) plus protease inhibitors, and the lysate was precleared against washed protein G sepharose beads (1 mL/1.5 mg of protein) at 4 ºC for 1 h. After centrifugation, the supernatant was collected and incubated with an anti-TAMRA antibody (100 μg/1.5 mg of protein) at 4 ºC for 4 h. The samples were then added to pre-washed protein G sepharose beads (1 mL/1.5 mg of protein) at 4 ºC for 1.5 h. Following centrifugation, the beads were washed once with 4 column volumes of NETFD buffer and three times with 4 column volumes of NETF buffer (100 mM NaCl, 50 mM Tris-HCl pH 7.4, 5 mM EDTA). After washing, the beads were boiled in elution buffer (200 mM Tris pH 6.8, 400 mM DTT, 8% SDS, 50 μL buffer/100 μL beads). The supernatant was collected after centrifugation and precipitated by adding 4 volumes of ice-cold acetone and incubating at -20 ºC for 16 h.

1D Gel Electrophoresis and Silver Staining. The precipitated eluents (cytoplasmic, nuclear, and controls) from above, along with the input (before immunoprecipitation) and flow-through fractions (15 μg) were separated on a 1.5 mm, 10-well NuPAGE 4–12% Bis-Tris gel. The gels were imaged using a FujiFilm FLA-3000 or FLA-5100 scanner and silver stained using a protocol adapted from Blum, Shevchenko, and co-workers[21,22]. Briefly, the gels were fixed in an aqueous solution of 50% MeOH, 10% acetic acid for 30 min and then again in 5% MeOH, 1% acetic acid for 15 min. The gels were then washed 3 x 10 min with H_2O and sensitized for 90 s with $Na_2S_2O_3 \cdot 5H_2O$ (20 mg/100 mL). After rinsing for 3 x 30 sec with H_2O, the gels were exposed to $AgNO_3$ (200 mg/100 mL) for 30

min and rinsed for 3 x 60 s with H_2O. Finally, the gels were developed for 2.5 min in a solution containing Na_2CO_3 (6 g/100 mL), 37% formaldehyde (50 μL/100 mL), $Na_2S_2O_3 \cdot 5H_2O$ (0.4 mg/100 mL). The reaction was stopped with 6% acetic acid. Twelve equally-spaced gel pieces were excised from each of the eluent lanes (cytoplasmic, nuclear, and –GalT controls), spanning the full height of the gel. Individual gel pieces were destained in a solution containing 0.4 g $K_3Fe(CN)_6$ in 200 mL of an aqueous sodium thiosulphate solution (0.2 g $Na_2S_2O_3 \cdot 5H_2O$ in 1L of H_2O) for 15 min, and washed 4 times for 15 min and 1 time for 16 h with H_2O.

2D Gel Electrophoresis. Precipitated eluents (cytoplasmic, nuclear) were resuspended in 100 mM Tris, pH 8.0, 1% SDS, and then reduced and alkylated with tributyl phosphine (200 mM) and *N,N*-Dimethylacrylamide (0.5%) by heating at 65° C for 10 min, followed by rotation end-over-end at RT for 20 min. The samples were precipitated with methanol/chloroform/water and resuspended in 7 M urea, 2 M thiourea, 2% CHAPS, 2% ASB-14 (Sigma) buffer (173.5 μL), and 2 M DTT (5.5 μL) plus pH 4–7 ampholytes (1 μL) were added. The samples were centrifuged at 20,000 rpm for 3 min, the supernatant was loaded onto pH 4–7 strips, and the sample was rehydrated for 90 min. The strips were focused for 20 min at 200V, 25 min at 450V, 20 min at 700V, and 55 min at 2000V, after which they were incubated in 1x LDS sample buffer plus 50 mM DTT, and resolved on a NuPAGE 4–12% Bis-Tris gel. The gel was imaged using a fluorescence scanner, and the fluorescent spots were excised from the gel and fixed in an aqueous solution of 50% MeOH, 7% acetic acid overnight.

In-Gel Digestion of Captured *O*-GlcNAc Proteins. Individual gel pieces (cytoplasmic, nuclear, and –GalT controls) from the 1D and 2D gels were dehydrated with CH_3CN (2 x 5 min) and then rehydrated with dithiothreitol (1.5 mg/mL in 100 mM NH_4HCO_3, pH 8.0) for 30 min. The excess dithiothreitol was removed and iodoacetamide (10 mg/mL in 100 mM NH_4HCO_3, pH 8.0) was added in the dark for 30 min. Excess iodoacetamide was removed and the gels were washed twice with 100 mM NH_4HCO_3, pH 8.0 and dried with CH_3CN before being dried using a speed vac. Trypsin (20 ng/μL in 50 mM NH_4HCO_3, pH 8.0; 50 μL) was added to each gel piece, and the gel pieces were allowed to swell on ice. After 30 min, excess trypsin was removed, 50 mM NH_4HCO_3, pH 8.0 (15 μL for the 2D gel pieces; 30 μL for the 1D gel pieces) was added, and the digestions were incubated at 37 ºC. After 16 h, the peptides were extracted with H_2O (30 μL for the 2D gel pieces; 60 μL for the 1D gel pieces) for 30 min, and the gel pieces were washed twiced with an aqueous solution of 5% formic acid containing 50% CH_3CN (25 μL for the 2D gel pieces; 40 μL for the 1D gel pieces) for 10 min. The combined extract and washes were concentrated using a speed vac for 1 h to remove the CH_3CN.

LC-MS Analysis of Captured *O*-GlcNAc Proteins. Nano LC-MS of in-gel tryptic digests was performed on a Thermo Fisher Surveyor MS plus HPLC and LTQ XL ion trap mass spectrometer using a modified vented column setup and data-dependent scanning[23]. Samples were loaded onto a 360 x 100 μm precolumn (2 cm, 5 μm Monitor C18) and desalted prior to placing the precolumn in-line with the analytical column.

Peptides were then eluted with a linear gradient of 0% to 40% B in 30 min (A, 0.1M aqueous HOAc; B, 0.1M HOAc in CH_3CN), a flow rate of 250 nL/min and using a 360 x 75 μm self-packed column with integrated electrospray emitter (10 cm of 5 μm Monitor, C18). MS scans were as follows: 1 full scan followed by 5 MS/MS scans of the most intense ions from the full scan using data-dependent analysis with dynamic exclusion. Dynamic exclusion parameters: repeat count — 1; repeat duration — 15s; exclusion duration — 30s.

MS/MS spectra were searched against a human, rat and mouse subset of the European Bioinformatics Institute — International Protein Index (EBI-IPI) database (downloaded 08-01-2007), with an appended reversed database and using Sequest 3.0. A fixed modification of Cys (+57), a variable modification of Met (+16) and trypsin cleavage were specified. Search results were compiled and filtered in Scaffold 2.0 (Proteome Software, Inc, Portland, OR). For analysis of 2D gel bands, a protein identification was accepted if it was established with a 99% probability of a correct identification and a minimum of 2 peptides (90% probability of a correct identification) were matched to the protein. For analysis of 1D gel bands, a protein identification was accepted if a minimum of 3 peptides were matched to the protein and peptide identifications satisfied XCorr versus m/z thresholds of +1/1.8, +2/2.5, and +3/3.5, and a DeltaCn threshold of 0.1. Proteins published as putative *O*-GlcNAc proteins were chosen by taking the list of proteins identified in the experimental eluent lane and subtracting out those proteins found in the corresponding control lane, as well as any extracellular proteins that contaminated the protein fractionations.

In-Gel Fluorescence Detection of *O*-GlcNAc Dynamics. HeLa cells were grown to 80–

90% confluence in DMEM containing 10% FBS and penicillin/streptomycin (100 U/mL) and harvested. Cells were incubated in DMEM with PUGNAc (100 μM) or H_2O for 9 h at 37 deg and 5% CO_2. The cells were lysed in boiling 1% SDS, sonicated, and boiled for 5 min. The resulting lysate (200 μg) was chemoenzymatically labeled with **1**, followed by **3**, as described above. A negative control was performed under identical conditions, except that **1** was omitted from the reaction mixture. After TAMRA-labeling, protein (21 μg) was resolved on a 1.0 mm, 12-well NuPAGE 4-12% Bis-Tris Gel. The gel was imaged using a FLA-5100 scanner. Western blotting was done as described for α-crystallin above but using an anti-tubulin antibody (1:10,000).

Total changes in *O*-GlcNAc glycosylation levels with PUGNAc were quantified using Multi Gauge software (Fujifilm). Quantification was determined by taking the ratio of the total fluorescent signal of the PUGNAc lane to the total fluorescent signal of the control lane, corrected to tubulin levels. Quantification represents the mean ± standard deviation for n=2 experiments. The range over which PUGNAc changed *O*-GlcNAc glycosylation levels was determined by taking the ratio of the fluorescent signal of the PUGNAc lane to the fluorescent signal of the control lane for the 18 strongest bands, corrected for tubulin levels.

Chromatin Immunoprecipitation (ChIP). Cortical neuronal cultures were prepared from embryonic day 15 C57BL/6 mice as described[24]. Neurons were cultured for four days in Neurobasal media (Invitrogen), 2 mM Glutamax (Invitrogen), penicillin/streptomycin (Invitrogen, 100 U/ml), 2% B-27 (Invitrogen) for five days, after which the media was removed and the cells were fixed with 1% formaldehyde / PBS

(0.01 M phosphate, 0.138 M NaCl, 0.0027 M KCl, pH 7.4) for 20 min at RT. The formaldehyde was quenched with 50 μl / ml of 2.5 M glycine, the cells were washed three times with 1X PBS, collected in 1X PBS, and then pelleted by centrifugation at 23,500 x g for 5 min at 4 °C. Cells were resuspended in cell lysis buffer (25 mM HEPES pH 7.8, 1.5 mM $MgCl_2$, 10 mM KCl, 0.1% NP-40, 1 mM DTT; 200 μl / 10 cm dish of cells) and incubated on ice for 10 min. The nuclei were pelleted by centrifugation at 10,000 x g for 5 min at 4 °C. The supernatant was removed and the nuclear pellet was resuspended in nuclear lysis buffer (50 mM HEPES pH 7.9, 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% Sodium deoxycholate, 0.1% SDS, 200 μl / 10 cm dish) and incubated on ice for 10 min. The samples were sonicated on ice 5 x 30 sec at 40% amplitude using a Sonics Vibra Cell sonicator, centrifuged for 10 min at 22,000 x g, and the supernatant was retained as the nuclear lysate.

Protein concentration from the nuclear lysate was determined by BCA protein concentration assay. Nuclear lysate (200 μg) was supplemented with nuclear lysis buffer to 165 μl and 4 μl of Complete™ protease inhibitors (50X), 11 μl of $MnCl_2$ (100 mM), 10 μl UDP-GalNAz (0.5 mM), 10 μl GalT. GalT was left out of the control reaction as indicated. The samples were incubated end-over-end overnight at 4°C and then the samples were precipitated by addition of 20 μl NaOAc (5 M, pH 5.2) followed by 1750 μl of ice-cold EtOH (100%). The samples were quickly vortexed, placed at -20°C for 1 hr, and then centrifuged at 23,500xg for 15 minutes. The supernatant was discarded and the pellets were resuspended in 50 μl of 1% SDS, 50 mM Tris pH 8. Labeling with the TAMRA-alkyne dye **3** was performed per the Click-It™ TAMRA Glycoprotein

Detection Kit instructions except for the samples were EtOH precipitated as described above.

The samples were resuspended in 20 μl of 0.5% SDS with Complete™ protease inhibitors followed by addition of 173 μl of nuclear lysis buffer and 1 μl of 100% Triton X-100. Protein A Sepharose beads (20 μl), salmon sperm DNA (4 μl, 2 mg/ml), and normal rabbit IgG (5 μl, 0.4 mg/ml) were added to lysate and the samples were rotated end-over-end for 1 h at 4°C. The beads were spun down on a benchtop centrifuge for 30 sec, the supernatant was transferred to a new tube, 10% was saved for input, 2 μg of TAMRA antibody or normal rabbit IgG were added to the remaining sample as indicated, and the samples were rotated end-over-end overnight at 4 °C.

After overnight incubation, 20 μl of Protein A Sepharose beads was added to each sample, and the samples were rotated end-over-end for 1 h at 4 °C. The beads were then spun down on a benchtop centrifuge for 30 sec and wash successively with 1 ml of nuclear lysis buffer, nuclear lysis buffer supplemented with 360 μl NaCl, wash buffer (20 mM Tris pH 8.0, 250 mM LiCl, 1 mM EDTA, 0.5% NP-40, 0.5% Sodium deoxycholate), and TE (20 mM Tris pH 8.0, 1 mM EDTA). The final wash was removed, 100 μl of TE, 1% SDS was added the beads, and the beads were rotated end-over-end for 10 min at 65 °C. The beads were centrifuged, the first eluent was saved, 160 μl TE, 0.67% SDS was added to the sample, and the beads were again rotated end-over-end for 10 min at 65 °C. The beads were centrifuged and the second eluent was combined with the first. TE and SDS was added to the input such that the final input SDS concentration in the inputs was 0.81% and the eluents and inputs were decrosslinked by incubating them at 65 °C for 4

hours. After decrosslinking, 250 μl TE and 10 μl Proteinase K (10 mg/ml) were added and the reactions were incubated for 2 h at 37°C shaking at 235 RPM.

56 μl of 4 M LiCl in TE was added to each sample and the DNA was extracted with 560 μl of Phenol:Chloroform:Isoamyl Alcohol (25:24:1) followed by 560 μl chloroform. 56 μl of NaOAc (5 M, pH 5.2), 1.5 ml ethanol (100%, ice-cold), and 1 μl glycogen (20 mg/mL) was added to each sample, the sample were vortexed and then incubated at -20°C overnight. After overnight incubation, the samples were centrifuged for 30 minutes at 22,000 x g, the supernatant was discarded, the pellets were allowed to air dry, and the pellets were redissolved in 60 μL DNase-free H_2O. These samples were either taken on to PCR or given to Rosemary Tao in the Sun lab (UCLA) for analysis on a promoter microarray.

PCR. 2 μl of DNA from the ChIP experiments was combined with 14.5 μl DNase-free H_2O, 0.5 μl PCR Nucleotide Mix (10 mM), 0.75 μl $MgCl_2$ (50 mM), 2.5 μl 10X Enhancer, 2.5 μl 10X Amplifier, 2 μl primers (10 μM, forward and reverse mix), and 0.25 μl Taq polymerase. The samples were then heated in a thermocycler using the following heating profile: 95 °C, 1 min (1x), 95 °C, 30 sec, annealing temperature, 1 min, 72 °C, 1 min, 30 sec (36x), 72 °C, 7 min (1x), 4 °C, unlimited hold. 5 μl of 6x gel loading dye was added to each sample, and the DNA was resolved on a 2% agarose / TEA gel. The following primers were used for ChIP:

Gene	Forward Primer	Reverse Primer
Pomc	TACCTCCAAATGCCAGGAAG	CGCTGGTGGT TAGGAAGAAC
18S Ribosomal RNA	CGCGGTTCTATTTTGTTGGT	AGTCGGCATCGTTATGGTC

Chemoenzymatic Labeling and Fluorescence Imaging of *O*-GlcNAc Proteins in Cells. HeLa cells were counted, diluted into DMEM containing 10% FBS and penicillin/streptomycin (100 U/mL) and seeded on poly-D-lysine-coated (0.1 mg/mL poly-D-lysine in 50 mM sodium borate, pH 10, 100 μL/coverslip for 30 min at 37 °C) 15 mm glass coverslips (Carolina Biologicals) at a density of 75 cells/mm^2 (100 μL/coverslip). After 30 min, 400 μL of media was added to each coverslip, and the cultures were incubated at 5% CO_2 at 37 °C for 6 h.

Cortical neuronal cultures were prepared from embryonic day 18 Long Evans rats as described[24]. Neurons were counted, diluted into supplemented Basal Media Eagle (BME, Sigma; 450 mL media, 10 mL L-glutamine (200 mM), 5 mL penicillin/streptomycin (10,000 U/mL), 10 mL B-27 serum-free supplement (50X stock), 25 mL FBS) and seeded on poly-DL-ornithine-coated 18-mm glass coverslips (Carolina Biologicals) at a density of 100 cells/mm^2 (150 μL/coverslip). After 30 min, 350 μL of supplemented BME media was added to each coverslip. The cultures were incubated in 5% CO_2 at 37 °C for 7 days.

To image *O*-GlcNAc glycosylated proteins, the media was removed, and the coverslips were rinsed one time with PBS, fixed in 4% paraformaldehyde for 20 min at RT, washed twice with PBS, permeabilized in 0.3% Triton X-100 for 5 min at RT, and washed twice with enzymatic labeling buffer (50 mM HEPES, 125 mM NaCl, pH 7.9). Reaction mixtures and negative controls without UDP-GalNAz **1** were prepared as described in the Click-It™ *O*-GlcNAc Enzymatic Labeling System instructions except that Component C, the enzymatic labeling buffer, was replaced with a buffer containing

125 mM NaCl, 50 mM HEPES, pH 7.9. These mixtures were added to each coverslip (50 μL), and the coverslips were incubated at 4 °C for 14–20 h. For the HeLa cells, PNGase F (2500 U/mL) was added to the enzymatic labeling reaction mixture; no difference in staining was observed in the presence or absence of PNGaseF. Coverslips were washed one time with 125 mM NaCl, 50 mM HEPES, pH 7.9 and twice with 50 mM Tris, pH 8.0. Biotin labeling reaction mixtures were prepared as per the Click-It™ Biotin Glycoprotein Detection Kit instructions using 50 mM Tris, pH 8.0 without SDS, added to each coverslip (50 μL), and the reaction allowed to proceed for 1 h at RT. For TAMRA labeling, TAMRA-alkyne **3** was substituted above for biotin-alkyne **2**. The TAMRA-alkyne **3** produced high background labeling in the absence of GalT, likely due to noncovalently sticking of **3** to hydrophobic regions of membranes and proteins. The background could be reduced by washing the cells with organic solvents (similar to the precipitation steps after TAMRA labeling on lysates), but these solvents also distorted and destroyed the fixed cells.

After the reaction was finished, the coverslips were washed once with PBS, three times with 0.1% Triton-X100 in PBS, and once with PBS. Following the PBS wash, nonspecific binding was blocked by incubating with 3% BSA in PBS for 1 h at RT and then rinsing once with PBS. Cells were then incubated with streptavidin-AlexaFluor 488 (1:800; Molecular Probes) in 3% BSA in PBS for 1 h at 37 °C. Coverslips were rinsed three times with 0.2% Triton-X100 in PBS and once with PBS. The coverslips were mounted onto glass slides using Vectashield mounting medium with DAPI (2 μL; Vector Labs) and sealed with clear nail polish. Cells were imaged using a Nikon Eclipse

TE2000-S inverted microscope, and images were captured with Metamorph software using a 40x plan fluor oil objective.

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