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The small molecule 2-Azido-2-deoxy-glucose is a metabolic chemical reporter of O-GlcNAc modifications in mammalian cells, revealing an unexpected promiscuity of O-GlcNAc transferase.

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ABSTRACT: Glycans can be directly labeled using unnatural monosaccharide analogs, termed metabolic chemical reporters (MCRs). These compounds enable the secondary visualization and identification of glycoproteins by taking advantage of bioorthogonal reactions. Most widely-used MCRs have azides or alkynes at the 2-N-acetyl position but are not selective for one class of glycoprotein over others. To address this limitation, we are exploring additional MCRs that have bioorthogonal functionality at other positions. Here, we report the characterization of 2-azido-2-deoxy-glucose (2AzGlc). We find that 2AzGlc selectively labels intracellular O-GlcNAc modifications, which further supports a somewhat unexpected, structural flexibility in this pathway. In contrast to the endogenous modification N-acetyl-glucosamine (GlcNAc), we find that 2AzGlc is not dynamically removed from protein substrates and that treatment with higher concentrations of per-acetylated 2AzGlc is toxic to cells. Finally, we demonstrate that this toxicity is an inherent property of the small-molecule, as removal of the 6-acetyl-group renders the corresponding reporter non-toxic but still results in protein labeling.

Metabolic chemical reporters (MCRs) of glycans are typically monosaccharide analogs that contain bioorthogonal functional groups, such as an alkyne or azide.¹⁻³ Once living cells are treated with one of these analogs, the MCRs can be metabolically transformed into at least one, if not more, high-energy sugar donors that can be subsequently utilized by glycosyltransferases resulting in the incorporation of MCRs into glycans. An ever growing number of bioorthogonal reactions can then be employed for the selective installation of tags for the visualization, characterization, and identification of glycoconjugates, most notably glycoproteins.⁴ In the past five years, we and others have demonstrated that the majority of glycoprotein MCRs are not specific for one type of glycosylation.⁵⁻⁸ For example, the original MCR for intracellular O-GlcNAc modification, *N*-azidoacetyl-glucosamine (GlcNAz),⁹ labels both O-GlcNAcylated proteins and cell-surface glycans (i.e., N-linked and mucin O-linked).^{6,8} Additionally, we have shown that MCRs that have their bioorthogonal functionality at the *N*-acetyl position, which makes up the vast majority of glycan reporters, have the potential to be de-*N*-acetylated, resulting in labeling of the protein acetylation pathway.¹⁰ These issues result in a lack of specificity that can limit the information that can be gained in glycoprotein visualization experiments or the number of a specific type of glycoprotein that can be identified using enrichment and proteomics.

We recently demonstrated that changes to the MCR structure resulted in the first selective reporter of O-GlcNAcylation, 6-azido-6-deoxy-*N*-acetyl-glucosamine (6AzGlcNAc).⁸ O-GlcNAcylation is the addition of the monosaccharide *N*-acetyl-glucosamine to serine and threonine side-chains of proteins located in the cytosol, nucleus, and mitochondria.¹¹⁻¹³ O-GlcNAc transferase (OGT) uses the uridine diphosphate sugar donor UDP-GlcNAc to add O-GlcNAc to protein substrates,

and these modifications can be subsequently removed by O-GlcNAcase (OGA), rendering O-GlcNAcylation dynamic.¹⁴ Genetic knockout of either of these enzymes is lethal in mammals and insects,¹⁵⁻¹⁸ and the modification plays key roles in human disease, including cancer and neurodegeneration.^{19,20} Here, we demonstrate that 2-azido-2-deoxy-glucose (2AzGlc) is also a selective MCR for O-GlcNAc modifications. Treatment of cells with the per-acetylated reporter Ac₂2AzGlc (Figure 1A) resulted in the labeling of a variety of proteins by 2AzGlc in mammalian cells but no detectable incorporation of 2AzGlc into cell-surface glycoproteins. Additionally, bioorthogonal enrichment of proteins that were labeled with Ac₂2AzGlc followed by proteomics identified many known O-GlcNAcylated proteins. In contrast to 6AzGlcNAc, 2AzGlc appears to not be removed from substrate proteins by OGA, consistent with the enzymatic mechanism that requires anchimeric assistance of the *N*-acetyl group.²¹ Additionally, treatment of cells with Ac₂2AzGlc at a high concentration (200 μM) was toxic to cells after overnight exposure. Interestingly, this toxicity is inherent to the structure of the small-molecule, as deprotection of the 6-*O*-acetate to yield Ac₂2AzGlc removed all cell-death but retained similar levels of protein labeling. Importantly, this observation has been made on other MCRs by the Yarema lab, which they attribute to changing the balance between the productive biosynthetic transformation of reporters to their corresponding donor sugars versus toxic side-reactions.²²

RESULTS AND DISCUSSION

Given our interesting previous results that structural changes in the chemical structure of MCRs can change their selectivity, we set out to explore 2AzGlc, as it lacks the acetamido-functionality that is common to the vast majority of monosac-

charide positions found in glycoproteins and that has been a consistent feature of all previous MCRs. Therefore, we rationalized that it may have interesting properties concerning its metabolism and glycoprotein selectivity. Towards this goal, we prepared the per-*O*-acetylated compound, Ac₄2AzGlc, in two steps from glucosamine. These acetates enable diffusion of the reporter through cellular membranes where they are removed by lipases and/or hydrolases. NIH3T3 and H1299 cells were then treated with increasing concentrations of Ac₄2AzGlc for 6 h, and the corresponding cell lysates were subjected to the copper(I)-catalyzed azide-alkyne cycloaddition (CuAAC) with an alkyne-rhodamine tag. In-gel fluorescence scanning showed a range of labeled proteins (Figure 1B). In both cell lines, we observed a large increase in labeling when the concentration of Ac₄2AzGlc was increased from 150 to 200 μ M. Notably, this higher concentration also resulted in toxicity when the cells were treated for longer periods of time (i.e., 16 h). This observation is consistent with previous experiments by the Bertozzi lab, who commented on the toxicity of Ac₄2AzGlc but did not characterize its labeling.²³ To explore this toxicity further, the same cell lines were treated with a range of Ac₄2AzGlc concentrations for 16 h and their NAD(P)H-dependent reductase activity was measured using a commercially available MTS assay (Figure 1C). Interestingly, an increase in activity was observed for lower concentrations of Ac₄2AzGlc, indicating additional metabolic flux into reductive equivalents. However, the cells displayed a dramatic loss of activity between 150 to 200 μ M Ac₄2AzGlc, consistent with our morphological observations.

Despite this toxicity, we next set out to characterize the selectivity of Ac₄2AzGlc as an MCR. To determine if 2AzGlc is incorporated into cell-surface glycans, H1299 cells were treated with Ac₄2AzGlc (200 μ M) for 6 h, which is before the onset of obvious cellular toxicity. The intact cells were then subjected to a strain-promoted azide-alkyne cycloaddition (SPAAC) with DBCO-biotin, which can only label extracellular azides, followed by incubation with FITC-avidin and analysis by flow-cytometry (Figure 2A). As controls, we also simultaneously treated cells with either Ac₄GalNAz (200 μ M), which does label cell-surface glycoproteins,^{8,24} or Ac₆AzGlcNAc (200 μ M), which does not.⁸ We observed essentially no fluorescence signal from cells treated with either Ac₄2AzGlc or Ac₆AzGlcNAc, despite strong signal from the Ac₄GalNAz-treated cells, indicating that Ac₄2AzGlc does not label cell-surface glycans. We next used Ac₄2AzGlc in an unbiased proteomics experiment in NIH3T3 cells, as we have utilized these cells in the past for the identification of labeled proteins with other MCRs. Specifically, NIH3T3 cells were treated in triplicate with Ac₄2AzGlc or Ac₄GlcNAc as a control (both at 200 μ M) for 6 h, again before any signs of toxicity. The cells were then lysed under denaturing conditions (4% SDS) and the labeled proteins underwent CuAAC with an alkyne biotin-tag. The labeled proteomes were then reduced and alkylated with iodoacetamide, followed by incubation with streptavidin-conjugated beads. After extensive washing, the enriched proteins were subjected to on-bead trypsinolysis, and the resulting peptides were analyzed using LC-MS/MS and identified using Proteome Discoverer and Mascot. Spectral counting was used to quantify the proteins enriched in either sample.

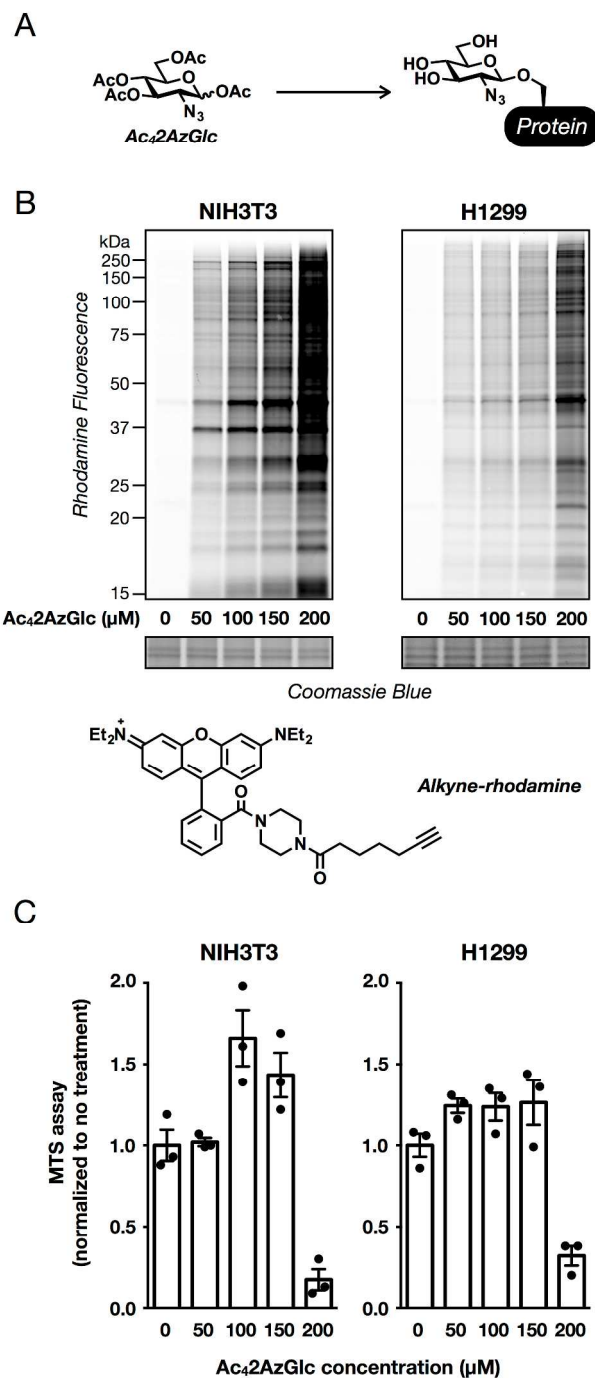


Figure 1. Evaluation of per-acetylated-2-azido-glucose (Ac₄2AzGlc) as a metabolic chemical reporter (MCR). (A) Treatment of living cells with MCRs, like Ac₄2AzGlc, results in modification of proteins that can then be visualized using bioorthogonal reactions. (B) Ac₄2AzGlc-treatment results in labeling of a wide range of proteins. NIH3T3 or H1299 cells were treated with the indicated concentrations of Ac₄2AzGlc for 6 h, followed by CuAAC with alkyne-rhodamine and visualization of labeled proteins by in-gel fluorescence scanning. (C) High concentrations of Ac₄2AzGlc are toxic to mammalian cells. NIH3T3 or H1299 cells were treated with the indicated concentrations of Ac₄2AzGlc for 16 h and their viability was then measured using an MTS assay. Quantitated data is the average of three separate biological experiments, and error bars represent \pm s.e.m. from the mean of biological replicates ($n = 3$).

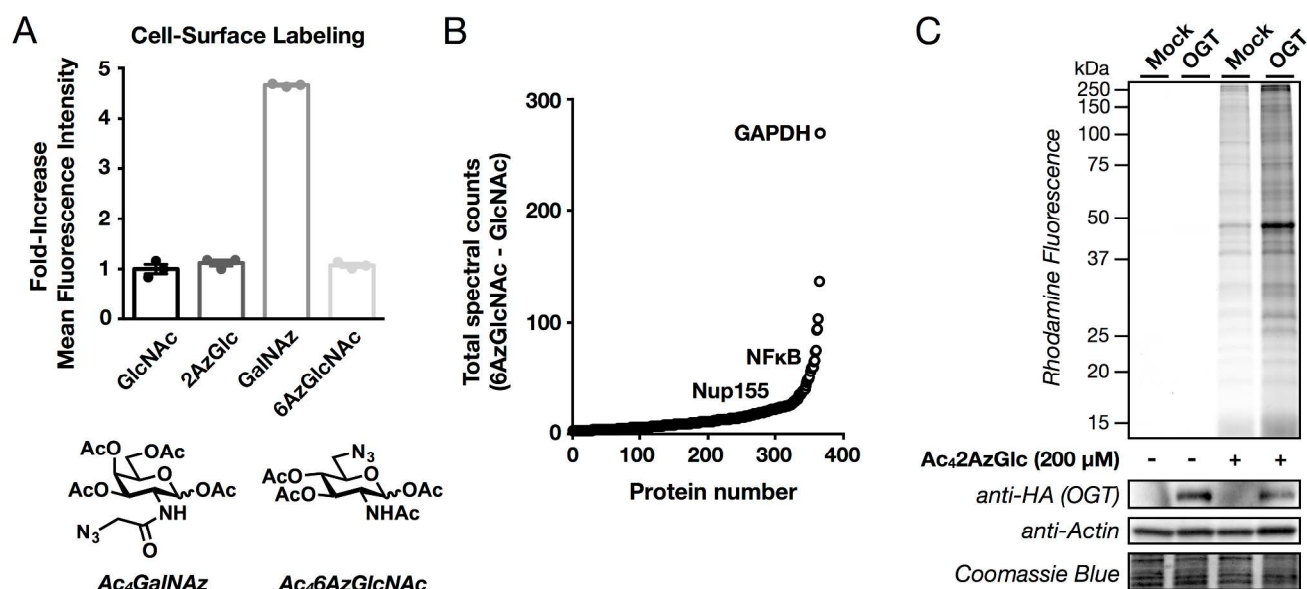


Figure 2. Ac₄2AzGlc is an O-GlcNAc metabolic chemical reporter. (A) Cell surface glycoproteins are not labeled after treatment with Ac₄2AzGlc. H1299 cells were treated with the indicated metabolic chemical reporters (200 μM) for 6 h at which time the cells were collected and subjected to copper-free click chemistry with DBCO-biotin (Click Chemistry Tools). After incubation with FITC-avidin, surface glycoprotein labeling was analyzed by flow cytometry. (B) Unbiased identification of O-GlcNAcylated proteins using 2AzGlc. NIH3T3 cells were treated in triplicate with Ac₄2AzGlc (200 μM) or Ac₄GlcNAc (200 μM) vehicle for 6 h, followed by CuAAC with alkyne-biotin, enrichment with streptavidin-coated beads, and on-bead trypsinolysis. Proteins that were identified using LC-MS/MS are represented as the total number of positive spectral counts minus the total number of control spectral counts. Representative known O-GlcNAcylated proteins are shown. (C) O-GlcNAc transferase (OGT) overexpression increases 2AzGlc labeling. NIH3T3 cells that stably express either HA-tagged OGT or an empty plasmid were treated with Ac₄2AzGlc (200 μM) or DMSO vehicle for 6 h. At that time, the cell lysates were subjected to CuAAC with alkyne-rhodamine and labeled proteins were visualized using in-gel fluorescence.

Proteins were considered to have been labeled by 2AzGlc if they met certain criteria. First, the proteins must have been identified by at least 1 peptide in each of the three Ac₄2AzGlc-treated samples. Second, the sum of the spectral counts from the Ac₄2AzGlc-treated samples must be three-times greater than the sum of the spectral counts for the same protein in the Ac₄GlcNAc-treated sample. Finally, the spectral counts in the MCR-treated samples must be statistically significantly different from the control samples ($P < 0.05$, t-test). With these requirements, we identified 361 proteins (Figure 2B and Table S1). Importantly, in this list were 265 proteins that had been previously identified in other proteomics experiments as being potentially O-GlcNAcylated. We then annotated these proteins based on their subcellular localization, since O-GlcNAcylated proteins will be intracellular while cell-surface glycoproteins will be extra-cellular/luminal or have a transmembrane domain. Using the Uniprot database, we found that Ac₄2AzGlc labeled essentially exclusively intracellular or transmembrane proteins and only resulted in the enrichment of one exclusively extracellular protein, EGF-containing fibulin-like extracellular matrix protein, that could not bear O-GlcNAcylation.

Next, we used retroviral transformation to generate NIH3T3 cells that stably overexpress an HA-tagged OGT or were transduced with an empty plasmid as a negative control. These cells were treated with Ac₄2AzGlc (200 μM) for 6 h, followed by CuAAC with alkyne-rhodamine and analysis using in-gel fluorescence (Figure 2C). Notably, we observed significantly more labeling in the OGT overexpressing cells, further indicating that 2AzGlc is an MCR for O-GlcNAcylation. To ex-

plore the generality of 2AzGlc labeling, we next treated a variety of mammalian cell-lines with Ac₄2AzGlc (200 μM) for 6 h. In-gel fluorescence scanning, after CuAAC with alkyne-rhodamine, showed strong labeling in all of the cell-lines tested (Figure 3), indicating that 2AzGlc is a general MCR for the visualization and identification of O-GlcNAcylated proteins. We next explored the reversibility of 2AzGlc labeling. As stated in the introduction, the enzymatic mechanism of OGA involves anchimeric assistance of the *N*-acetyl group to generate an oxazoline intermediate. A wealth of carbohydrate chemistry has demonstrated that a 2-azido group is incapable of participating in this fashion,²⁵ raising the likely possibility that once 2AzGlc is installed by OGT it cannot be removed by OGA. To directly test this hypothesis, we performed a pulse-chase experiment. Briefly, HeLa cells were treated with Ac₄2AzGlc (200 μM) for 6 h. At this time the media was exchanged for fresh media containing Ac₄GlcNAc (200 μM) and either the OGA inhibitor Thiamet-G (10 μM)²⁶ or DMSO vehicle. After an additional 12 h, the corresponding cell-lysates were subjected to CuAAC with alkyne-rhodamine and analysis by in-gel fluorescence (Figure 4A). In contrast to 6AzGlcNAc labeling that we previously demonstrated is maintained by Thiamet-G treatment,⁸ we observed no difference in the reduction of 2AzGlc labeling, indicating that it is not a substrate for OGA. Interestingly, we did, however, observe a relatively rapid loss in 2AzGlc labeling over 12 h, suggesting that the labeled proteins were being turned over by another mechanism. Proteasomal degradation is one of the major pathways by which proteins are targeted for turnover. To determine if 2AzGlc-labeled proteins are being destroyed by

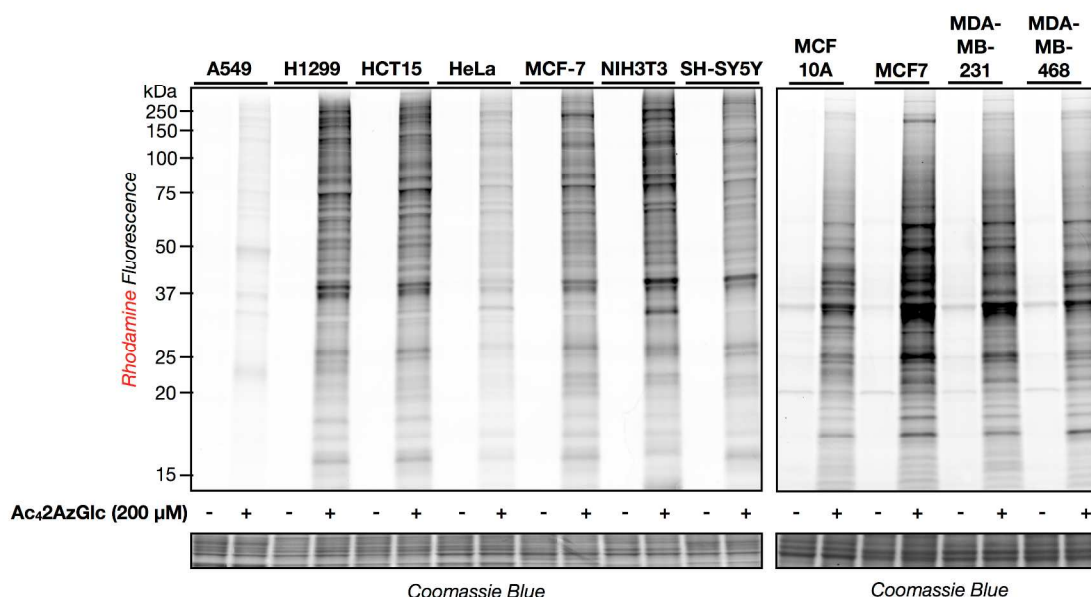


Figure 3. Ac₄2AzGlc is a general chemical reporter in a variety of mammalian cell lines. (A) The indicated cell lines were treated with Ac₄2AzGlc (200 μM) or DMSO vehicle for 6 h. Total cell lysates were then collected and subjected to CuAAC with alkyne-rhodamine, and labeled proteins were visualized using in-gel fluorescence scanning.

this pathway, we performed another pulse-chase experiment. In this case, the pulse was identical to the one above, but in the chase, we added either the proteasome inhibitor MG-132 (10 μM) or DMSO control. In-gel fluorescence scanning following CuAAC with alkyne-rhodamine only showed a slight stabilization of the fluorescent signal (Figure 4B), indicating that the majority of the 2AzGlc-dependent labeling is being removed by an unknown mechanism.

Finally, to explore the potential mechanism underlying the toxicity of Ac₄2AzGlc-treatment, we looked to the elegant experiments performed by the Yarema lab who were exploring the off-target effects of monosaccharides modified by short-chain fatty acids.²² Although the exact biological mechanism behind the toxicity of certain acetylated monosaccharides are not entirely known, the authors demonstrated that the toxicity of these molecules could be eliminated by revealing the 6-hydroxyl functionality, which increases the flux of the carbohydrate into cellular biosynthetic pathways. To test whether this observation could explain the toxicity of Ac₄2AzGlc, we synthesized the 6-OH analog (Ac₄2AzGlc, Figure 5A). First, we examined whether aqueous conditions would result in migration of the 4-*O*-acetate to the 6-hydroxyl group, as can be observed under certain reaction conditions. To accomplish this, we dissolved Ac₃2AzGlc in a 5:1 mixture of D₂O and deuterated DMSO and incubated the mixture for 16 h at room temperature. After this length of time the solution was diluted to 1:1 D₂O and deuterated DMSO and analyzed by proton NMR. Comparison of this spectra to a freshly prepared sample in the same solvent composition showed essentially no differences (Supplemental Information), demonstrating that any acetate migration is minimal and below the detection limit of NMR. Next, treatment with either NIH3T3 or H1299 cells with Ac₃2AzGlc resulted in robust labeling of proteins, as visualized by in-gel fluorescence (Figure 5B). However, unlike Ac₄2AzGlc, the 6-OH analog showed no cellular toxicity

at all of the concentrations tested (Figure 5C). These results support an inherent toxicity mechanism that is largely unrelated to protein labeling by 2AzGlc as an O-GlcNAc reporter.

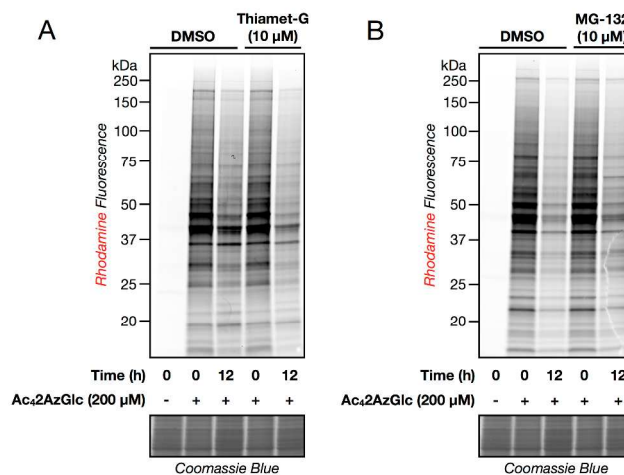


Figure 4. Characterization of the dynamics of 2AzGlc labeling. (A) Protein labeling by 2AzGlc is not enzymatically removed by O-GlcNAcase (OGA). HeLa cells were treated with 200 μM Ac₄2AzGlc for 6 h. At this time, the media was exchanged for fresh media containing 200 μM Ac₄GlcNAc and either the OGA inhibitor Thiamet-G (10 μM) or DMSO. Cells were collected at the indicated times, subjected to CuAAC with alkyne-rhodamine and analyzed by in-gel fluorescence scanning. (B) Labeling by 2AzGlc is not notably stabilized by proteasome inhibition. HeLa cells were treated with 200 μM Ac₄2AzGlc for 6 h. At this time, the media was exchanged for fresh media containing 200 μM Ac₄GlcNAc and either the proteasome inhibitor MG132 (10 μM) or DMSO. Cells were collected at the indicated times, subjected to CuAAC with alkyne-rhodamine and analyzed by in-gel fluorescence scanning.

CONCLUSIONS

The development of new MCRs for glycans has continued to grow the chemical toolbox for the investigation of glycobiology. In particular, these reporters have been key for the visualization and identification of glycoproteins. We have been focused on understanding the selectivity of different MCRs, including some that would not be predicted to label proteins based on the previously established biosynthetic and salvage pathways. Continuing that theme here, we performed the cellular characterization of 2AzGlc as a chemical reporter of glycosylation. Using bioorthogonal chemistry, we show that 2AzGlc labels a range of proteins in total cell-lysate (Figure 1B and 3), but does not label cell-surface glycans at a detectable level (Figure 2A), suggesting that it might be selective for O-GlcNAc modifications. This selectivity was further supported by an unbiased proteomics experiment that used 2AzGlc to enrich a total of 361 proteins, 73.4% of which had been previously identified in other O-GlcNAcylation proteomics experiments and only 1 exclusively extracellular or luminal glycoprotein (Figure 2C and Table S1). The ability for 2AzGlc to be transferred by OGT from the corresponding UDP-2AzGlc sugar donor in cells is somewhat surprising, given the ternary crystal structures of OGT in complex with its peptide and donor-sugar substrates, as well as the structure with the corresponding products.²⁷ These structures show that the 2-acetamide group of GlcNAc undergoes significant movement during the enzymatic reaction and that the amide nitrogen contributes a hydrogen-bond to the phosphate of the UDP leaving-group. Additionally, the same authors show that *in vitro* OGT will accept UDP-GalNAc but not UDP-Glc or UDP-2-keto-Glc.²⁷ However, another structural and biochemical study found that OGT accepts UDP-GlcNAcF₃ (2-*N*-trifluoroacetamide), which they interpreted as evidence that the electronics of the 2-acetamide are not key for catalysis.²⁸ Given these data, the 2-azido group might either be functionally benign or could participate in weak hydrogen bonds, given its unique electronics.²⁹ We also confirmed a statement from the Bertozzi lab that 2AzGlc is toxic to mammalian cells (Figure 1B), although they did not show any supporting data.²³ Here, we demonstrate that this toxicity can be eliminated by deprotection of the 6-position to reveal the hydroxyl group, which the Yarema lab has shown increased flux of other monosaccharide analogs into the corresponding biosynthetic pathways. Given the mechanism of OGA, we also explored the reversibility of the 2AzGlc modifications. We used a pulse-chase experiment to demonstrate that 2AzGlc is not removed from proteins by OGA (Figure 4A). As stated above, this is not necessarily surprising, given the requirement for anchimeric assistance in the enzymatic mechanism.²¹ Interestingly, although loss of 2AzGlc labeling is not OGA-dependent, the rate of loss is similar to other MCRs that are removed by OGA, such as 6AzGlcNAc,⁸ suggesting to us that the 2AzGlc-labeled proteins might be degraded. However, a pulse-chase experiment with the proteasomal inhibitor MG-132 only showed a modest stabilization of the fluorescent signal (Figure 4B), indicating that another pathway exists to remove these modifications. Further experiments will be needed to directly test this hypothesis. Our proteomics data show that the vast majority of identified proteins have been previously characterized as being potentially O-GlcNAcylated by a variety of chemical and biological techniques (Table S1 - proteins highlighted in blue). This indicates that the specificity for protein substrates between 2AzGlc and other MCRs is similar. However, we can

not rule out that different proteins are more readily modified by OGT with a reporter that lacks the 2-acetamido functionality versus an MCR like 6AzGlcNAc. Therefore, we suggest that 6AzGlcNAc be used for studies aimed at the biology of O-GlcNAcylation and that all potentially-modified proteins be confirmed by either antibodies or the chemoenzymatic modification strategy developed by the Hsieh-Wilson lab.³⁰ In summary, 2AzGlc is the second MCR that has been characterized to be selective for O-GlcNAcylation. It also further highlights the promiscuity of OGT, which now includes 2AzGlc, as well as several N-acetyl-glucosamine monosaccharides,^{5,9,31} and could have interesting implications for the modification of intracellular proteins by natural, non N-acetyl-glucosamine monosaccharides that can be biosynthetically transformed to the corresponding UDP-sugar donors.

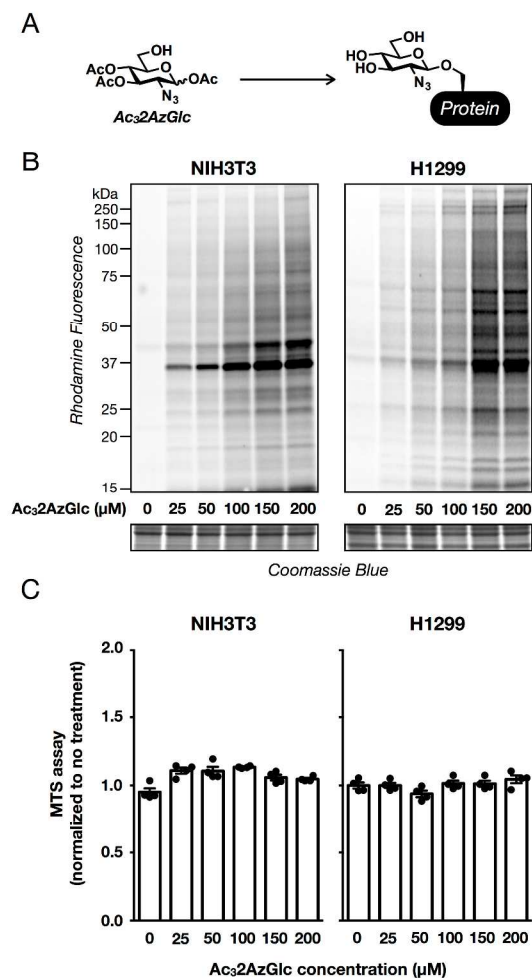


Figure 5. The acetylation pattern and not labeling by 2AzGlc is responsible for cellular toxicity. (A) Proteins can also be labeled by Ac₃2AzGlc, which bears a free hydroxyl at the 6-OH position. (B) Ac₃2AzGlc-treatment results in labeling of a wide range of proteins. NIH3T3 or H1299 cells were treated with the indicated concentrations of Ac₃2AzGlc for 16 h, followed by CuAAC with alkyne-rhodamine and visualization of labeled proteins by in-gel fluorescence scanning. (C) No concentrations of Ac₃2AzGlc are toxic to mammalian cells. NIH3T3 or H1299 cells were treated with the indicated concentrations of Ac₃2AzGlc for 16 h and their viability was then measured using an MTS assay. Quantitated data is the average of four separate biological experiments, and error bars represent \pm s.e.m. from the mean of biological replicates ($n = 4$).

EXPERIMENTAL PROCEDURES

General. All reagents used for chemical synthesis were purchased from Sigma-Aldrich, Alfa Aesar or EMD Millipore unless otherwise specified and used without further purification. All anhydrous reactions were performed under argon or nitrogen atmosphere. Analytical thin-layer chromatography (TLC) was conducted on EMD Silica Gel 60 F₂₅₄ plates with detection by ceric ammonium molybdate (CAM), anisaldehyde or UV. For flash chromatography, 60 Å silica gel (EMD) was utilized. ¹H spectra were obtained at 400, 500, or 600 MHz on a Varian spectrometers Mercury 400, VNMR-500, or -600. Chemical shifts are recorded in ppm (δ) relative to solvent. ¹³C spectra were obtained at 100, 125 or 150 MHz on the same instruments.

Cell lines. NIH3T3 cells stably expressing either HA-tagged O-GlcNAc Transferase (OGT) or an empty vector were generated using Amphopack293 retroviral packaging cell lines according to manufacturer procedure (Clontech).

Synthesis of chemical reporters. Known compounds Thiamet-G, Ac₄GalNAz, Ac₄GlcNAz, Ac₄2AzGlucose and azido-rhodamine were synthesized according to literature procedures.^{8,26,32,33}

Synthesis of 1,3,4,6-Tetra-O-Acetyl-2-Azido-2-Deoxyglucose (Ac₂2AzGlc, 2):

2-Azido-2-Deoxyglucose (1): Commercially available glucosamine hydrochloride was subjected to azide formation using a diazo transfer reaction according to literature procedure.³⁴ ¹H NMR (500 MHz, Deuterium Oxide) of β-anomer δ 4.71 (d, J = 8.1 Hz, 1H), 3.92 – 3.82 (m, 1H), 3.81 – 3.70 (m, 1H), 3.54 – 3.43 (m, 3H), 3.28 (dd, J = 9.6, 8.2 Hz, 1H). The product was used in the subsequent reaction without further characterization.

1,3,4,6-Tetra-O-Acetyl-2-Azido-2-Deoxyglucose (Ac₂2AzGlc, 2): Compound 1 was resuspended in pyridine and acetic anhydride. A catalytic amount of dimethylaminopyridine was added and reaction stirred at room temperature for 16 h. Upon completion reaction was concentrated, and the crude was resuspended in dichloromethane. The organic layer was washed with equivolume 1 M HCl (1x), saturated Sodium Bicarbonate (x2), Water (x2) and brine (1x). The washed organic layer was then concentrated and subjected to column chromatography to yield the pure product. NMR characterization of this known compound was consistent with previous reports.²³ ¹H NMR (500 MHz, CDCl₃) of β-anomer δ 5.55 (d, J = 8.5 Hz, 1H), 5.15 – 5.00 (m, 2H), 4.34 – 4.25 (m, 1H), 4.11 – 4.05 (m, 1H), 3.84 – 3.77 (m, 1H), 3.71 – 3.62 (m, 1H), 2.19 (s, 3H), 2.09 (s, 3H), 2.07 (d, 3H), 2.02 (s, 3H).

Synthesis of 1,3,4-Tri-O-Acetyl-2-Azido-2-Deoxyglucose (Ac₃2AzGlc, 5):

6-Tert-Butyldimethylsilyl-2-Azido-2-Deoxyglucose (6TBS2AzGlc, 3): Compound 1 (668 mg, 3.26 mmol) was dissolved in dry DMF (8 mL) under nitrogen at room temperature. Imidazole (667 mg, 9.8 mmol) and tert-butyldimethylsilyl chloride (613 mg, 4.07 mmol) were added and the reaction stirred for 16 h. The reaction was diluted in ethyl acetate and washed with sodium bicarbonate (2x), water (2x), and brine (1x). The organic layer was dried with sodium sulfate, filtered, and concentrated by vacuum. The compound was purified by column chromatography (25% ethyl acetate:hexanes) to afford the product as a light brown syrup (327 mg, 31%). ¹H NMR (400 MHz, CDCl₃) of α-, β-anomer mixture

5.18 (d, J = 3.4 Hz, 1H), 4.55 (d, J = 8.0 Hz, 1H), 4.01 (dd, J = 14.0, 6.0 Hz, 1H), 3.95 (t, J = 9.5 Hz, 1H) 3.86-3.65 (m, 6H), 3.53-3.46 (m, 2H), 3.37 (t, J = 9.5 Hz 1H), 3.31-3.25 (m, 1H), 3.20-3.14 (m, 2H), 0.81 (s, 18H), 0.00 (s, 12H). ¹³C NMR (125 MHz, CDCl₃): δ (ppm) 95.89, 91.59, 76.91, 74.47, 72.45, 71.18, 70.08, 66.33, 63.9, 62.99, 25.77, -5.80. ESI-MS calculated for C₁₂H₂₆N₃O₅Si [M + H]⁺: 320.1636, found: 319.5972.

1,3,4-Tri-O-Acetyl-6-Tert-Butyldimethylsilyl-2-Azido-2-Deoxyglucose (Ac₃6TBS2AzGlc, 4):

Compound 3 (150 mg, 0.47 mmol) was dissolved in pyridine (2 mL) and cooled to 0°C. Acetic anhydride (200 μL, 2.11 mmol) was added and the reaction warmed to room temperature over 16 h. The reaction was diluted in DCM and washed with sodium bicarbonate (2x), water (2x), and brine (1x). The organic layer was dried with sodium sulfate, filtered, and concentrated by vacuum before purification by column chromatography (10% ethyl acetate:hexanes) to afford the product as a light brown syrup (170 mg, 81%). ¹H NMR (400 MHz, CDCl₃) of α-, β-anomer mixture: δ (ppm) 6.28 (d, J = 3.6 Hz, 1H), 5.52 (d, J = 8.6 Hz, 1H), 5.46-5.40 (m, 1H), 5.14 (dd, J = 10.2, 9.4 Hz, 1H), 5.08-5.04 (m, 2H), 3.91-3.85 (m, 1H), 3.76-3.70 (m, 1H), 3.69-3.58 (m, 6H), 2.16 (d, J = 1.8 Hz, 6H), 2.09 (d, J = 4.7 Hz, 6H), 2.03-1.99 (m, 6H), 0.86 (s, 18H), 0.04--0.03, (m, 12H). ¹³C NMR (125 MHz, CDCl₃): δ (ppm) 169.7, 169.14, 168.38, 92.23, 89.83, 74.95, 73.01, 72.17, 71.08, 68.01, 62.34, 61.53, 60.19, 25.51, 20.52, -5.72. ESI-MS calculated for C₁₈H₃₁N₃O₈SiNa [M + Na]⁺: 468.1773, found: 468.1766.

1,3,4-Tri-O-Acetyl-2-Azido-2-Deoxyglucose (Ac₃2AzGlc, 5):

Compound 4 (170 mg, 0.38 mmol) was dissolved in THF (2 mL) and cooled to 0°C. Glacial acetic acid (445 μL) was added followed by addition of 1.0 M TBAF in THF (7.8 mL) and the reaction was allowed to warm to room temperature over 16 hours. The solution was diluted with ethyl acetate, washed with sodium bicarb (2x), water (2x), and brine (1x), and dried with sodium sulfate. The resulting mixture was filtered, concentrated by vacuum, and purified by column chromatography (40% ethyl acetate:hexanes) to afford product as a yellow syrup (107 mg, 85%). ¹H NMR (400 MHz, CDCl₃) of α-, β-anomer mixture: δ (ppm) 6.27 (d, J = 3.7 Hz, 1H), 5.54 (d, J = 8.5 Hz, 1H), 5.32 (dd, J = 10.5, 9.3 Hz, 1H), 4.95-4.90 (m, 1H), 4.56-4.47 (m, 2H), 4.21 (dd, J = 12.5, 1.7 Hz, 1H), 4.21 (dd, 12.5, 2.3 Hz, 1H), 3.92-3.87 (m, 1H), 3.65-3.48 (m, 6H), 2.20-2.18 (m, 12H), 2.12 (s, 6H). ¹³C NMR (125 MHz, CDCl₃): δ (ppm) 171.28, 170.95, 168.34, 92.35, 89.91, 74.86, 72.73, 72.03, 68.27, 62.16, 59.92, 20.53. ESI-MS calculated for C₁₂H₁₈N₃O₈ [M + H]⁺: 332.1088, found: 332.1085.

Cell culture. NIH3T3 and MEF cells were cultured in high glucose DMEM media (HyClone, ThermoScientific) enriched with 10% fetal calf serum (FCS, HyClone, ThermoScientific). MDA-MB-231, MDA-MB-468, HeLa and MCF-7 were cultured in high glucose DMEM (HyClone, ThermoScientific) enriched with 10% fetal bovine serum (FBS HyClone, ThermoScientific). H1299 and HCT15 cells were cultured in RPMI 1640 medium (HyClone, ThermoScientific) enriched with 10% FBS. A549 cells were cultured in F-12K medium (HyClone, ThermoScientific) enriched with 10% FBS. MCF10A and SH-SY5Y were cultured in a 1:1 mixture of F-12 and DMEM media (HyClone, ThermoScientific) enriched with 10% FBS. All cell lines were maintained in a humidified incubator at 37 °C and 5.0% CO₂.

Metabolic labeling. To cells at 80-85% confluency, high- or low-glucose media containing Ac₄GlcNAz, Ac₄GalNAz, Ac₂AzGlc (1,000 x stock in DMSO), or DMSO vehicle was added as indicated. For chase experiments, low-glucose media was replaced with high-glucose media supplemented with 200 μ M Ac₄GlcNAc (Sigma) with or without 20 μ M Thiamet-G.

Preparation of NP-40-soluble lysates. The cells were collected by trypsinization and pelleted by centrifugation at for 4 min at 2,000 x g, followed by washing 2x with PBS (1 mL). Cell pellets were then resuspended in 100 μ L of 1% NP-40 lysis buffer [1% NP-40, 150 mM NaCl, 50 mM triethanolamine (TEA) pH 7.4] with Complete, Mini, EDTA-free Protease Inhibitor Cocktail Tablets (Roche) for 20 min and then centrifuged for 10 min at 10,000 x g at 4 °C. The supernatant (soluble cell lysate) was collected and the protein concentration was determined by BCA assay (Pierce, ThermoScientific).

Cu(I)-catalyzed [3 + 2] azide-alkyne cycloaddition (Cu-AAC). Cell lysate (200 μ g) was diluted with 1% NP-40 lysis buffer to obtain a desired concentration of 1 μ g μ L⁻¹. Newly-made click chemistry cocktail (12 μ L) was added to each sample [alkynyl-rhodamine tag (100 μ M, 10 mM stock solution in DMSO); tris(2-carboxyethyl)phosphine hydrochloride (TCEP) (1 mM, 50 mM freshly prepared stock solution in water); tris[(1-benzyl-1-H-1,2,3-triazol-4-yl)methyl]amine (TBTA) (100 μ M, 10 mM stock solution in DMSO); CuSO₄·5H₂O (1 mM, 50 mM freshly prepared stock solution in water)] for a total reaction volume of 200 μ L. The reaction was gently vortexed and allowed to sit at room temperature for 1 h. Upon completion, 1 mL of ice cold methanol was added to the reaction, and it was placed at -20 °C for 2 h to precipitate proteins. The reactions were then centrifuged at 10,000 x g for 10 min at 4 °C. The supernatant was removed, the pellet was allowed to air dry for 15 min, and then 50 μ L 4% SDS buffer (4% SDS, 150 mM NaCl, 50 mM TEA pH 7.4) was added to each sample. The mixture was sonicated in a bath sonicator to ensure complete dissolution, and 50 μ L of 2x SDS-free loading buffer (20% glycerol, 0.2% bromophenol blue, 1.4% β -mercaptoethanol, pH 6.8) was then added. The samples were boiled for 5 min at 97 °C, and 40 μ g of protein was then loaded per lane for SDS-PAGE separation (Any Kd, Criterion Gel, Bio-Rad).

In-gel Fluorescence Scanning. Following SDS-PAGE separation, gels were scanned on a Typhoon 9400 Variable Mode Imager (GE Healthcare) using a 532 nm for excitation and 30 nm bandpass filter centered at 610 nm for detection.

MTS Assay. NIH-3T3 and H1299 cells (1 x 10⁴ cells) were plated per well in a 96-well, white bottom dish 24 hours before treatment with 0-200 μ M Ac₄GlcNAc or Ac₂AzGlucose for 16 hours under low glucose media conditions (1 g/L). CellTiter 96[®] Aqueous Non-Radioactive Cell Proliferation Assay (Promega, Madison, WI) was used according to the provided protocol. Absorbance at 490 nm was read using a BioTek Synergy H4 Multi-Mode Microplate reader.

Flow Cytometry of Cell-Surface Labeling with DBCO-Biotin. H1299 cells grown in 6-well plates at 80-85% confluency were treated with 200 μ M Ac₄GlcNAc, Ac₄GlcNAz, Ac₄GalNAz or Ac₂AzGlc in triplicate for 16 hours at which time media was removed and cells were gently washed with PBS before being detached from the plate with 1 mM EDTA in PBS. Cells were collected by centrifugation (5 min, 300 x g at 4 °C) and were washed three times with PBS (5 min, 300 x

g at 4 °C). Cells were then resuspended in 200 μ L PBS containing DBCO-biotin (Click Chemistry Tools, 60 μ M) for 1 h, after which time they were washed three times with PBS (5 min, 300 x g at 4 °C) before being resuspended in ice-cold PBS containing fluorescein isothiocyanate (FITC) conjugated avidin (Sigma, 5 μ g/mL, 30 min at 4 °C). Cells were then washed three times in PBS (5 min, 300 x g at 4 °C) before being resuspended in 400 μ L PBS for flow-cytometry analysis. A total of 10,000 cells [dead cells were excluded by treatment with propidium iodide (2.5 μ g mL⁻¹ in water, 30 min)] were analyzed on a BD SORP LSRII Flow Cytometer using the 488 nm argon laser.

Biotin Enrichment and On-bead Trypsinolysis. NIH3T3 or H1299 cell-pellets labeled with Ac₃6AzGlcNAc, Ac₃2AzGlc or Ac₄GlcNAc for 16 hours were resuspended in 200 μ L H₂O, 60 μ L PMSF in H₂O (250 mM), and 500 μ L 0.05% SDS buffer (0.05% SDS, 10 mM TEA pH 7.4, 150 mM NaCl) with Complete Mini protease inhibitor cocktail (Roche Biosciences). To this was added 8 μ L Benzonase (Sigma), and the cells were incubated on ice for 30 min. Then, 4% SDS buffer (2000 μ L) was added, and the cells were briefly sonicated in a bath sonicator followed by centrifugation (20,000 x g for 10 min at 15 °C). Soluble protein concentration was normalized by BCA assay (Pierce, ThermoScientific) to 1 mg mL⁻¹, and 10 mg of total protein was subjected to the appropriate amount of click chemistry cocktail containing alkyne-PEG3-biotin (5 mM, Click Chemistry Tools) for 1 h, after which time 10 volumes of ice-cold MeOH were added. Precipitation proceeded 2 hours at -20 °C. Precipitated proteins were centrifuged at 5,200 x g for 30 min at 0 °C and washed 3 times with 40 mL ice-cold MeOH, with resuspension of the pellet each time. The pellet was then air-dried for 1 h. To capture the biotinylated proteins by streptavidin beads, the air-dried protein pellet was resuspended in 2 mL of resuspension buffer (6 M urea, 2 M thiourea, 10 mM HEPES pH 8.0) by bath sonication. To cap cysteine residues, 100 μ L of freshly-made TCEP (200 mM stock solution, Thermo) was then added and the mixture incubated for 30 min, followed by 40 μ L of freshly prepared iodoacetamide (1 M stock solution, Sigma) and incubation for a further 30 min in the dark. Streptavidin beads (250 μ L of a 50% slurry per sample, Thermo) were washed 2 X with 1 mL PBS and 1 X with 1 mL resuspension buffer and resuspended in resuspension buffer (200 μ L). Each sample was combined with streptavidin beads and incubated on a rotator for 2 h. These mixtures were then transferred to Mini Bio-Spin[®] columns (Bio-Rad) and placed on a vacuum manifold. Captured proteins were then washed with agitation 5 X with resuspension buffer (10 mL), 5 X PBS (10 mL), 5x with 1% SDS in PBS (10 mL), 30x with PBS (1 mL per wash, vacuum applied between each wash), and 5x 2M urea in PBS (1 mL per wash, vacuum applied between each wash). Beads were then resuspended in 2 M urea in PBS (1 mL), transferred to screw-top tubes, and pelleted by centrifugation (2000 x g for 2 min). At this time, 800 μ L of the supernatant was removed, leaving a volume of 200 μ L. To this bead-mixture was added 2 μ L of 1 mg mL⁻¹ sequence grade trypsin (Promega) and incubated at 37 °C for 18 hours. The resulting mixtures of tryptic peptides and beads were transferred to Mini Bio-Spin[®] columns (Bio-Rad) and the eluent was collected by centrifugation (1,000 x g for 2 min). Any remaining peptides were eluted by addition of 100 μ L of 2 M urea in PBS followed by centrifugation as immediately above. The tryptic peptides were then applied to

C18 spin columns (Pierce) according to manufacturer's instructions, eluted with 70% acetonitrile in H₂O, and concentrated to dryness on a speedvac.

LC-MS/MS Proteomic Analysis. Peptides were desalted on a trap column following separation on a 12cm/75um reversed phase C18 column (Nikkoy Technos Co., Ltd. Japan). A 3 hour gradient increasing from 10% B to 45% B in 3 hours (A: 0.1% Formic Acid, B: Acetonitrile/0.1% Formic Acid) was delivered at 150 nL min⁻¹. The liquid chromatography setup (Dionex, Boston, MA, USA) was connected to an Orbitrap XL (Thermo, San Jose, CA, USA) operated in top-5-mode. Acquired tandem MS spectra (CID) were extracted using ProteomeDiscoverer v. 1.3 (Thermo, Bremen, Germany) and queried against the human Uniprot protein database using MASCOT 2.3.02 (Matrixscience, London, UK). Peptides fulfilling a Percolator calculated 1% false discovery rate threshold were reported. All LC-MS/MS analysis were carried out at the Proteomics Resource Center at The Rockefeller University, New York, NY, USA. Excel files containing identified proteins will be made available upon request.

ASSOCIATED CONTENT

Supporting figures and proteomic data tables. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Author Contribution

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Notes

The authors have no competing financial interests.

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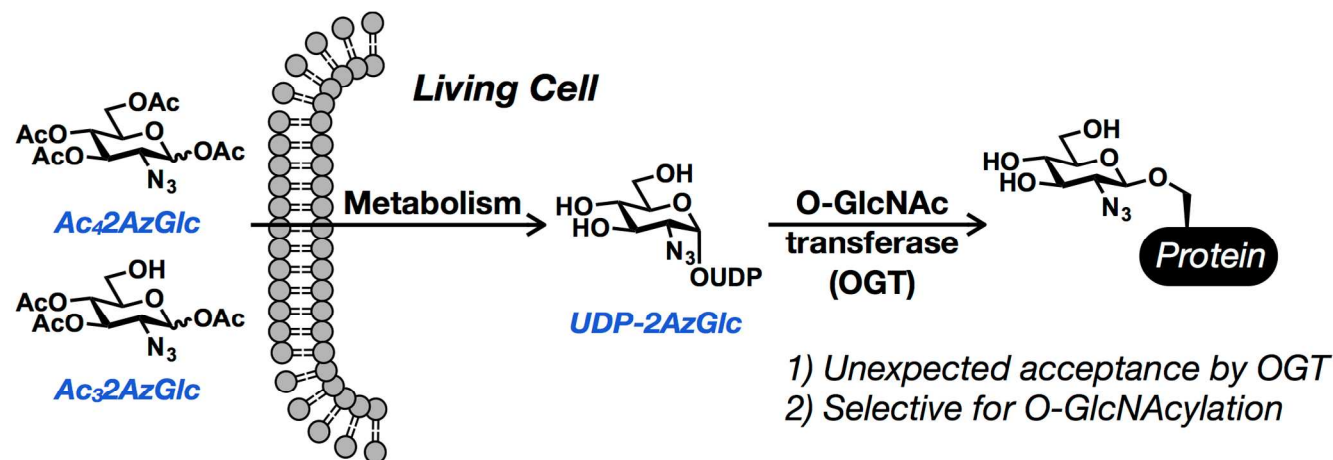
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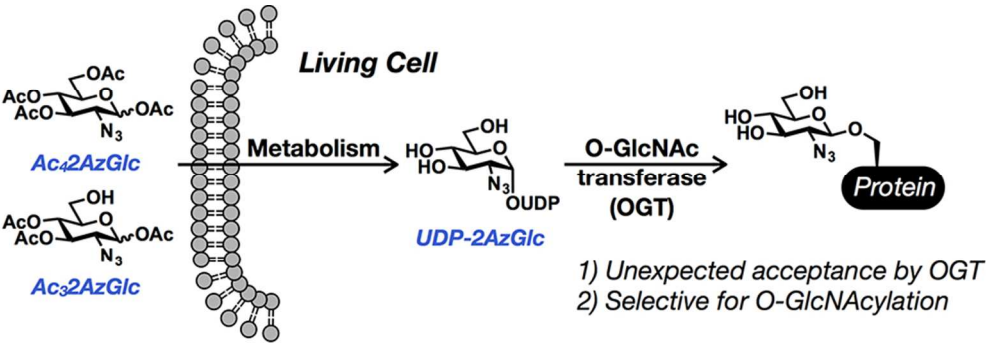
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