Metabolic cross-talk allows labeling of O-linked β-*N*-acetylglucosamine-modified proteins via the *N*-acetylgalactosamine salvage pathway

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Hundreds of mammalian nuclear and cytoplasmic proteins are reversibly glycosylated by O-linked β-*N*-acetylglucosamine (O-GlcNAc) to regulate their function, localization, and stability. Despite its broad functional significance, the dynamic and posttranslational nature of O-GlcNAc signaling makes it challenging to study using traditional molecular and cell biological techniques alone. Here, we report that metabolic cross-talk between the *N*-acetylgalactosamine salvage and O-GlcNAcylation pathways can be exploited for the tagging and identification of O-GlcNAcylated proteins. We found that *N*-azidoacetylgalactosamine (GalNAz) is converted by endogenous mammalian biosynthetic enzymes to UDP-GalNAz and then epimerized to UDP-*N*-azidoacetylglucosamine (GlcNAz). O-GlcNAc transferase accepts UDP-GlcNAz as a nucleotide-sugar donor, appending an azidosugar onto its native substrates, which can then be detected by covalent labeling using azide-reactive chemical probes. In a proof-of-principle proteomics experiment, we used metabolic GalNAz labeling of human cells and a bioorthogonal chemical probe to affinity-purify and identify numerous O-GlcNAcylated proteins. Our work provides a blueprint for a wide variety of future chemical approaches to identify, visualize, and characterize dynamic O-GlcNAc signaling.

Dynamic protein glycosylation has emerged as a critical and ubiquitous intracellular signaling paradigm in animals and plants. Specifically, *N*-acetylglucosamine (GlcNAc) is reversibly attached to serine and threonine side chains of numerous intracellular proteins to regulate their function, localization, and stability (Fig. 1*A*) (1, 2). In mammals, the resulting O-linked β-GlcNAc modification (O-GlcNAc) controls diverse aspects of cell physiology in a range of tissue types, and dysregulation of O-GlcNAc signaling is implicated in such human diseases as type II diabetes and neurodegeneration (3, 4). As a rapidly reversible modification, O-GlcNAcylation is conceptually analogous to phosphorylation in that dedicated enzymes add or remove O-GlcNAc on hundreds of protein substrates in response to diverse biological cues, often on a time scale of minutes (5). Indeed, O-GlcNAc has a complex and dynamic functional interplay with phosphorylation, frequently occurring on the same substrate or even amino acid residues (6). Unlike phosphorylation, however, mammalian O-GlcNAc cycling is performed by only two enzymes: O-GlcNAc transferase (OGT) (7) adds O-GlcNAc and O-GlcNAcase (OGA) (8) hydrolyzes it. O-GlcNAc regulates such processes as nutrient and growth factor sensing (9), cell cycle progression (10), and stress responses (11) and is required for mammalian cell survival (12), underlining its physiological significance. Interestingly, recent studies have demonstrated that rapid, global changes in O-GlcNAc occur in signal-driven processes, such as chemotaxis (5), but the vast majority of the relevant protein substrates remain unidentified. Therefore, systematic and unbiased methods for identifying signal-dependent changes in O-GlcNAcylated proteins are needed to understand these processes.

We have previously developed a general two-step chemical biology method to tag and study glycoproteins in vivo (13–15). First, live cells or organisms are metabolically labeled with synthetic, azide-functionalized monosaccharides ("azidosugars"), which are incorporated into glycans via endogenous biosynthetic pathways. Second, these labeled glycans are identified by covalent chemical tagging with bioorthogonal, azide-reactive probes. We have previously used azidosugars to achieve robust metabolic labeling and chemical detection of glycoproteins in a variety of systems, including cultured cells (13), developing zebrafish embryos (15), and live mice (14). In the particular case of O-GlcNAc, metabolic labeling by azidosugars provides distinct advantages over other techniques for identifying O-GlcNAcylated proteins. For example, azidosugars can be added to cells at defined time points (e.g., along with a stimulus) and can therefore be used to distinguish new and potentially signal-dependent glycosylation from the much more abundant, unchanging "background" glycosylation in the cell. In addition, the well-established, exquisite chemoselectivity of azide-reactive bioorthogonal probes (16) provides greater specificity than do antibodies or lectins, which are prone to cross-reaction with other (glyco)proteins, or chemical modification methods, which can label other O-linked modifications (e.g., phosphorylation).

In prior work, we showed that *N*-azidoacetylglucosamine (GlcNAz) (Fig. 1*B*) is tolerated in vitro by the enzymes of the human GlcNAc salvage pathway (Fig. 1*A*) and that incubation with cell-permeable, peracetylated GlcNAz (Ac_4GlcNAz) labels proteins in cultured human cells (17). However, although O-GlcNAc is highly abundant in mammalian cells, we observed weak metabolic labeling by Ac_4GlcNAz as compared to other azidosugars (see below), prompting us to explore other metabolic routes that would provide access to O-GlcNAcylated proteins by azidosugar reagents. Ac_4GlcNAz exploits the endogenous GlcNAc salvage pathway (Fig. 1*A*), which scavenges GlcNAc to biosynthesize the nucleotide–sugar donor UDP-GlcNAc, but distinct routes of UDP-GlcNAc biosynthesis exist. Indeed, it has long been known that the mammalian enzyme UDP-galactose 4′-epimerase (GALE) interconverts UDP-GlcNAc and its C_4

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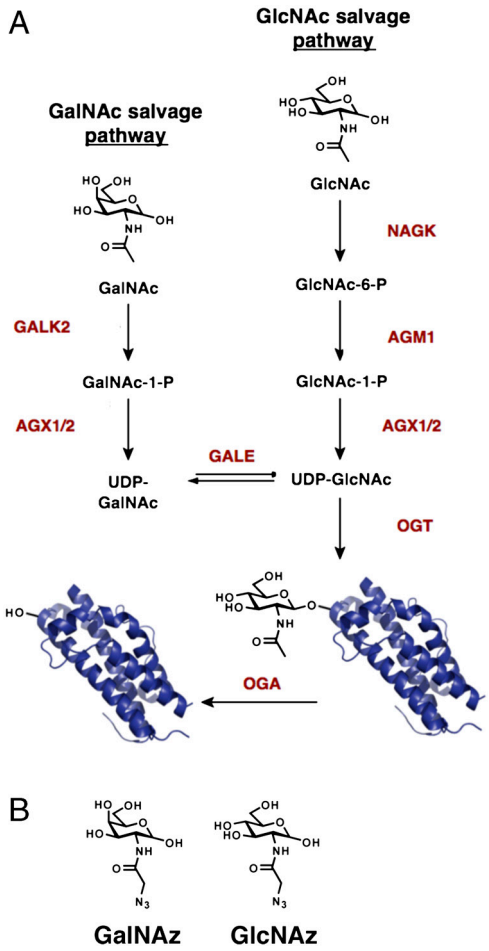

Fig. 1. (*A*) The GlcNAc and GalNAc salvage and O-GlcNAc signaling pathways. Enzyme names shown in red. (*B*) GalNAz and GlcNAz.

epimer, UDP-*N*-acetylgalactosamine (UDP-GalNAc) (Fig. 1*A*) (18), raising the possibility that the GalNAc salvage pathway and GALE epimerase could be exploited by an azido analogue of GalNAc to afford O-GlcNAz inside cells. We have previously synthesized one such azidosugar, *N*-azidoacetylgalactosamine (GalNAz) (Fig. 1*B*), and characterized its ability to label mucin-type cell surface glycoproteins (19). However, the possibility that GalNAz might also label intracellular O-GlcNAcylated proteins via a C_4 epimerization route remained unexplored.

Here, we investigate whether metabolic cross-talk between O-GlcNAcylation and the GalNAc salvage pathway could be harnessed to label O-GlcNAc substrates with azidosugars. Surprisingly, we find that peracetylated GalNAz (Ac_4GalNAz) treatment results in more robust labeling of O-GlcNAcylated proteins than does Ac_4GlcNAz. An analysis of the relevant metabolic pathways suggests that the UDP-GlcNAc pyrophosphorylase step in the GlcNAc salvage pathway is rate-limiting for UDP-GlcNAz biosynthesis, explaining the relatively low labeling of O-GlcNAc by Ac_4GlcNAz. On the other hand, UDP-GalNAz was efficiently converted to UDP-GlcNAz by mammalian GALE, accounting for O-GlcNAc labeling by Ac_4GalNAz. Finally, in a proof-of-principle proteomics experiment, we used Ac_4GalNAz labeling and a tandem affinity purification approach to identify several known O-GlcNAcylated proteins, underlining its utility for studying O-GlcNAc signaling. Our work demonstrates the superior metabolic labeling of native O-GlcNAcylated proteins in mammalian cells using Ac_4GalNAz and provides a road map for using azidosugar to study O-GlcNAc signaling in myriad future applications.

Results

Metabolic Labeling by GalNAz, but not GlcNAz, Robustly and Faithfully Mimics Cellular O-GlcNAc. Although azido GlcNAc analogues are accepted in vitro by the enzymes of the GlcNAc salvage pathway (17), we noticed that the metabolic labeling of cells by Ac_4GlcNAz was consistently weaker than that achieved by other azidosugars (see below), despite the well-known abundance of O-GlcNAc. Because of this, we sought to determine whether Ac_4GlcNAz reliably labeled O-GlcNAcylated proteins. In parallel, we tested the hypothesis that Ac_4GalNAz might be metabolized to UDP-GalNAz and then epimerized to UDP-GlcNAz in cells, thereby providing an alternative, and perhaps preferable, azidosugar tool for interrogating protein O-GlcNAcylation.

First, we asked whether metabolic labeling by Ac_4GlcNAz and/or Ac_4GalNAz mimicked the response of natural O-GlcNAc to perturbations in this pathway. We treated 293T cells with Ac_4GlcNAz, Ac_4GalNAz, or vehicle control in the presence or absence of OGT overexpression to increase global protein O-GlcNAcylation (Fig. 2*A*). Then, we made highly purified nuclear and cytoplasmic extracts (Fig. S1) and labeled azidoglycans via the Staudinger ligation, using a phosphine–FLAG peptide probe, which chemoselectively reacts with azides (20). As O-GlcNAc is the only known endogenous protein glycoconjugate in mammalian nuclear and cytoplasmic compartments, this fractionation allows for the specific examination of O-GlcNAcylated proteins. As expected, OGT overexpression strongly increased O-GlcNAc in both the nucleus and cytoplasm (Fig. 2*A*). In contrast, metabolic labeling by Ac_4GlcNAz, as detected by anti-FLAG immunoblot, was weak and not increased by OGT overexpression (Fig. 2*A*). Interestingly, however, labeling of nucleocytoplasmic proteins by Ac_4GalNAz was strong and was potentiated by OGT overexpression, suggesting that Ac_4GalNAz might be metabolized to O-GlcNAz in vivo (Fig. 2*A*).

As a complementary experiment, we treated cells with azidosugars in the presence or absence of OGA overexpression and examined nuclear and cytoplasmic glycoproteins by the same methods (Fig. 2*B*). As expected, OGA overexpression greatly reduced natural O-GlcNAc in both the nuclear and cytoplasmic compartments (Fig. 2*B*). Analogous to the OGT experiment, Ac_4GalNAz but not Ac_4GlcNAz showed a decrease in labeling corresponding to natural O-GlcNAc in response to OGA overexpression (Fig. 2*B*). The relatively low signal from Ac_4GlcNAz in this assay could not explain its lack of responsiveness to OGT and OGA overexpression, because even longer exposures of our immunoblots revealed no clear overexpression-dependent changes (Fig. S2). Furthermore, in some contexts, metabolic labeling of proteins by Ac_4GlcNAz was not removed by in vitro treatment with a recombinant-purified bacterial OGA homologue (21), whereas labeling by Ac_4GalNAz was (Fig. S3). Taken together, these data indicate that metabolic labeling by Ac_4GalNAz, but not Ac_4GlcNAz, faithfully mimics the response of natural O-GlcNAc to such physiologically relevant pathway regulators as OGT and OGA.

GlcNAz Does not Transit the UDP-GlcNAc Pyrophosphorylase Step in the GlcNAc Salvage Pathway. One possible explanation for the failure of OGT overexpression to potentiate metabolic labeling by Ac_4GlcNAz is that treatment of cells with Ac_4GlcNAz does not result in UDP-GlcNAz biosynthesis (Fig. 1). To test this hypothesis, we measured UDP-GlcNAz and UDP-GalNAz levels in cell extracts via high-performance anion exchange chromatography (HPAEC). We found that treatment of two human cell types with Ac_4GalNAz resulted in the production of both UDP-GlcNAz and UDP-GalNAz in an approximate 3 : 1 ratio (Fig. 3*A*). In contrast, however, treatment of cells with Ac_4GlcNAz showed no detectable UDP-GlcNAz or UDP-GalNAz, and these extracts were indistinguishable from those of vehicle-treated controls (Fig. 3*A*).

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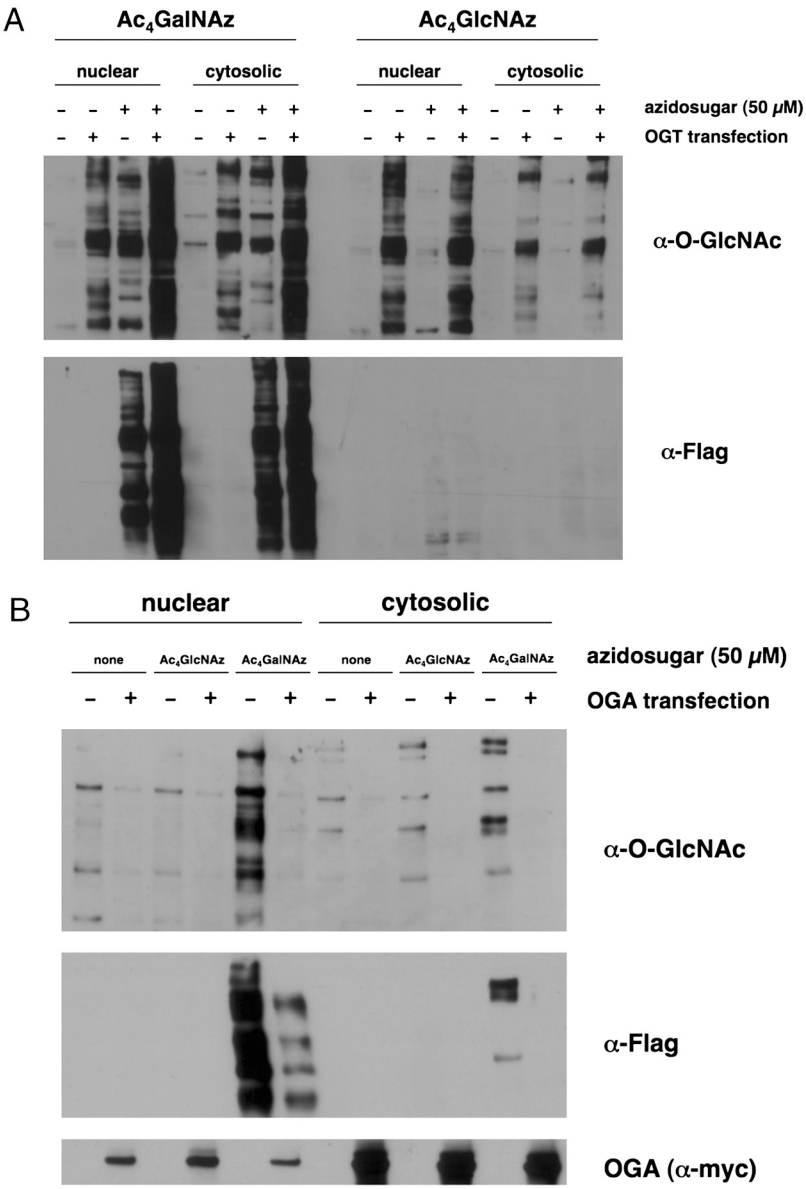

Fig. 2. Metabolic labeling by GalNAz, but not GlcNAz, robustly mimics natural O-GlcNAc. 293T cells were mock-transfected or transfected with a construct expressing (*A*) OGT or (*B*) OGA and treated with vehicle or azidosugar for 24 h. Nuclear and cytoplasmic extracts were prepared, reacted with phosphine-Flag, and analyzed by immunoblot.

These results suggested that the weak metabolic labeling by Ac_4GlcNAz was due to an inefficient step in the GlcNAc salvage pathway upstream of UDP-GlcNAz biosynthesis. The GlcNAc salvage pathway consists of three enzymes: a GlcNAc kinase (NAGK) (22), a GlcNAc-6-phosphate mutase (AGM1) (23), and a UDP-GlcNAc pyrophosphorylase (AGX1 or AGX2, splice-variant isoforms) (24) (Fig. 1*A*). We reasoned that if a rate-limiting step in the GlcNAc salvage pathway prevented the metabolic conversion of Ac_4GlcNAz into O-GlcNAz, we might overcome this block by overexpressing the enzyme controlling that step. We cloned and overexpressed human NAGK, AGM1, and AGX2 individually in the presence or absence of Ac_4GlcNAz and assayed the metabolic labeling as before (Fig. 3*B*). Although NAGK and AGM1 overexpression had no discernible effect on Ac_4GlcNAz metabolic labeling, we observed a strong increase in response to AGX2 overexpression (Fig. 3*B*). This result suggests that Ac_4GlcNAz treatment fails to produce UDP-GlcNAz because it does not efficiently transit the pyrophosphorylase step. Consistent with this model, we detected UDP-GlcNAz formation by HPAEC in AGX2-transfected cells in as little as 3 h after Ac_4GlcNAz treatment, whereas no UDP-GlcNAz was detectable in mock-transfected, Ac_4GlcNAz-treated cells (Fig. 3*C*). Furthermore, AGX2 overexpression restored detectable metabolic labeling of glycoproteins by Ac_4GlcNAz in as little as 6 h of Ac_4GlcNAz treatment (Fig. 3*D*). Together, these experiments suggest that metabolic labeling of mammalian cells by Ac_4GlcNAz is inefficient because of a bottleneck at the pyrophosphorylase step of the GlcNAc salvage pathway, resulting in little or no UDP-GlcNAz biosynthesis.

Ac_4GalNAz Treatment Results in GALE-Dependent UDP-GlcNAz Biosynthesis and Labeling of O-GlcNAcylated Proteins. In contrast to Ac_4GlcNAz, Ac_4GalNAz treatment of human cells resulted in both UDP-GlcNAz biosynthesis (Fig. 3*A*) and metabolic labeling of O-GlcNAcylated proteins (Fig. 2). Our model for the biosynthesis of UDP-GlcNAz from Ac_4GalNAz invokes the action of the GALE C_4 epimerase (Fig. 1*A*). To test whether metabolic labeling of intracellular proteins by Ac_4GalNAz requires a C_4 epimerase, we turned to the ldlD mutant CHO cell line, in which C_4 epimerase activity is nearly absent (25). We treated parental CHO cells or the ldlD mutant with vehicle only or Ac_4GalNAz and analyzed both UDP-GlcNAz biosynthesis (Fig. S4*C*) and azidosugar incorporation into nucleocytoplasmic proteins (Fig. 4*A*). Ac_4GalNAz-treated CHO cells biosynthesized both UDP-GalNAz and UDP-GlcNAz, whereas ldlD cells biosynthesized only UDP-GalNAz (Fig. S4*C*), consistent with a requirement

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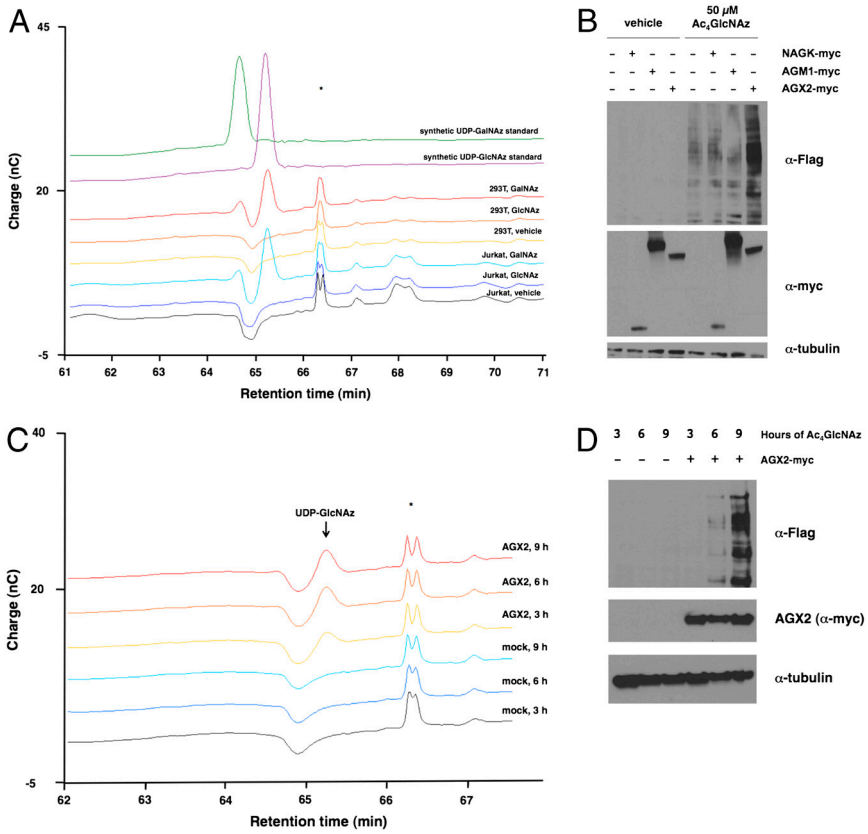

Fig. 3. GlcNAz does not transit the UDP-GlcNAc pyrophosphorylase step in the GlcNAc salvage pathway. (*A*) Jurkat or 293T cells were treated with vehicle or 100 μM azidosugar for 24 h. Ethanol extracts were made and analyzed by HPAEC. Synthetic UDP-GlcNAz and UDP-GalNAz standards were included. Asterisk: an unknown species present in all cell-derived samples. (*B*) 293T cells were mock-transfected or transfected with the expression construct indicated and treated with vehicle or Ac_4GlcNAz for 24 h. Cell lysates were reacted with phosphine-Flag and analyzed by immunoblot. (*C*) 293T cells were mock-transfected or transfected with an AGX2-myc expression construct. After 21 h, all cells were treated with 100 μM Ac_4GlcNAz, samples were harvested by ethanol extraction after 3, 6, or 9 additional hours and analyzed by HPAEC. Asterisk: an unknown species present in all cell-derived samples. (*D*) Ethanol-insoluble protein fractions from the samples in *C* were resuspended in 8 M urea, reacted with phosphine-Flag, and analyzed by immunoblot.

for C_4 epimerase activity in metabolizing Ac_4GalNAz to UDP-GlcNAz. Furthermore, CHO cells showed robust labeling of nucleocytoplasmic proteins by Ac_4GalNAz, whereas ldlD cells did not (Fig. 4*A*). As before (Fig. 2), the majority of the Ac_4GalNAz labeling was observed in the nucleus, where natural O-GlcNAc is known to be especially abundant (Fig. 4*A*) (1). In addition, total (Fig. S4*A*) and cell surface (Fig. S4*B*) Ac_4GalNAz labeling was higher in CHO versus ldlD CHO cells, consonant with our results and previous reports of defective secretory pathway glycosylation in the mutant (25, 26). To confirm these results in an independent assay, we treated parental CHO or ldlD mutants with vehicle or Ac_4GalNAz, fixed them, labeled azidoglycans by phosphine–FLAG ligation, and visualized them via anti-FLAG immunofluorescence (IF) (Fig. 4*B*). Consistent with our biochemical fractionation results, IF revealed that metabolic labeling by Ac_4GalNAz was predominantly nuclear and that all labeling was substantially reduced in the ldlD mutant, as compared to parental CHO cells (Fig. 4*B*).

To verify that the human C_4 epimerase can mediate metabolic labeling of intracellular proteins by Ac_4GalNAz, we stably expressed epitope-tagged human GALE in ldlD CHO cells. As expected, GALE expression, but not an empty vector, restored both the biosynthesis of UDP-GlcNAz (Fig. S4*D*) and robust nucleocytoplasmic protein labeling (Fig. S4*E*) in Ac_4GalNAz-treated ldlD CHO cells, with the majority of labeled protein observed in the nucleus. Together, these experiments demonstrate that Ac_4GalNAz treatment results in the biosynthesis of UDP-GlcNAz and subsequent metabolic labeling of nucleocytoplasmic proteins in a C_4 epimerase-dependent manner. Based on these and our earlier results (Figs. 2 and 3*A* and Fig. S3), we concluded that Ac_4GalNAz provides a faithful and robust chemical tool for labeling O-GlcNAcylated proteins in mammalian cells.

A Pilot Proteomics Experiment Demonstrates the Utility of Ac_4GalNAz in Studying Protein O-GlcNAcylation. Our results suggested that Ac_4GalNAz might be a powerful tool for studying protein O-GlcNAcylation in mammalian cells. To test the utility of Ac_4GalNAz as a metabolic reporter, we performed a proof-of-principle proteomics experiment. We treated Jurkat cells with vehicle only or Ac_4GalNAz for 3 d and labeled azidoglyco-

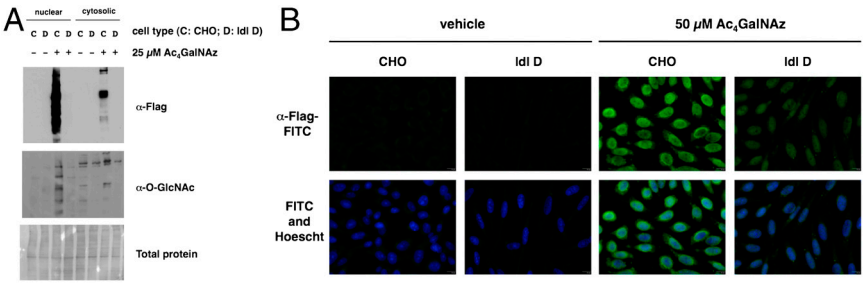

Fig. 4. Ac_4GalNAz treatment results in GALE-dependent UDP-GlcNAz biosynthesis and labeling of O-GlcNAcylated proteins. (*A*) CHO or ldlD CHO cells were treated with vehicle or Ac_4GalNAz for 24 h. Nuclear and cytoplasmic extracts were prepared, reacted with phosphine-Flag, and analyzed by immunoblot. Total protein was visualized by India ink staining. (*B*) CHO or ldlD mutant CHO cells were treated with vehicle or Ac_4GalNAz for 72 h. Cells were fixed and azidoglycans detected via reaction with phosphine-Flag and immunofluorescence microscopy using an anti-Flag-FITC antibody conjugate. A Hoechst 33258 stain was included to visualize nuclei. (*Top*) FITC only. (*Bottom*): FITC/Hoechst merge.

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proteins in cell extracts with a phosphine–FLAG-His$_6$ probe (Fig. S5*A*). Then, we purified the labeled azidoglycoproteins via sequential anti-FLAG and immobilized metal affinity chromatographic steps and identified the captured proteins by two-dimensional liquid chromatography and tandem mass spectrometry. Importantly, numerous reported O-GlcNAcylated proteins were specifically enriched in the Ac$_4$GalNAz-treated cells and absent from samples from vehicle-treated cells, including several components of the nuclear pore complex (known to be heavily glycosylated) (27, 28), a Sec24 family member (29), glyceraldehyde-3-phosphate dehydrogenase (30), host cell factor C1 (31, 32), Elf-1 (33), and OGT itself (34, 35) (Figs. S5*B* and S6).

We next sought to validate selected candidates from our proteomics hit list of putative azide-labeled, O-GlcNAcylated proteins using a mass spectrometry-independent method. We reacted nuclear/cytoplasmic extracts from control or Ac$_4$GalNAz-treated Jurkat cells with phosphine–biotin, captured biotinylated proteins by NeutrAvidin affinity chromatography, and analyzed them by immunoblot. As expected, the candidate proteins tested were biotinylated and captured in an Ac$_4$GalNAz-dependent manner (Fig. S5*C*). Importantly, the biotin-mediated capture of candidate proteins was abrogated by in vitro treatment with OGA prior to phosphine-biotin reaction (Fig. S5*D*), indicating that the azide modification on these proteins is O-GlcNAz, as predicted. We concluded that Ac$_4$GalNAz is a useful tool for specifically labeling O-GlcNAcylated proteins in live mammalian cells.

Discussion

As a rapidly reversible posttranslational modification, protein O-GlcNAcylation is challenging to study using traditional molecular and cell biology techniques alone. Here, we report an optimized chemical biology strategy for the metabolic labeling and subsequent characterization of O-GlcNAcylated proteins in mammalian cells using Ac$_4$GalNAz. We have shown that Ac$_4$GalNAz is metabolized by human cells to UDP-GlcNAz in a C$_4$ epimerase-dependent manner and that GalNAz provides a robust and faithful reporter for cellular O-GlcNAc. Furthermore, we demonstrated that Ac$_4$GalNAz could be used to label, purify, and identify O-GlcNAcylated proteins. Importantly, extensive previous work from our lab and others' has demonstrated that multiple azide-reactive functional groups, including terminal alkynes, cyclooctynes, and phosphines, can be used in conjunction with a range of imaging and affinity probes to label and analyze azide-tagged biomolecules in disparate biological systems (20, 36, 37). Given this extensive toolkit of bioorthogonal chemical probes, we anticipate that metabolic labeling of O-GlcNAcylated proteins using Ac$_4$GalNAz, coupled with secondary detection reagents, will provide a powerful strategy for analyzing O-GlcNAc signaling in a variety of experimental contexts. In particular, our metabolic labeling strategy has the advantage of providing time-resolved tagging of newly created O-GlcNAc, a valuable asset for studying this dynamic posttranslational modification.

Our results show that treating cells with Ac$_4$GlcNAz does not afford optimal labeling of O-GlcNAcylated proteins, likely because of a metabolic bottleneck at the UDP-GlcNAc pyrophosphorylase step of the GlcNAc salvage pathway. We previously found that all human GlcNAc salvage pathway enzymes accept azide-functionalized substrate analogues with only modestly reduced in vitro kinetics, though the UDP-GlcNAc pyrophosphorylase showed the greatest loss of catalytic efficiency (17). In many cell types, the expression level and/or posttranslational regulation of AGX1/2 may prevent the conversion of GlcNAz-1-phosphate (GlcNAz-1-P) into UDP-GlcNAz. However, our current cell biological data show that exploiting the GlcNAc salvage pathway to metabolically label O-GlcNAcylated proteins is, in principle, a sound strategy because overexpression of AGX2 overcame the metabolic bottleneck and restored both UDP-GlcNAz biosynthesis (Fig. 3*C*) and glycoprotein labeling (Fig. 3*D*). Indeed, although our results show that Ac$_4$GalNAz is the preferred azidosugar for metabolic labeling of mammalian O-GlcNAc, GlcNAz is the reagent of choice for other applications (38) and may serve as an efficient label for O-GlcNAc in non-mammalian systems, a possibility we have not yet investigated.

In contrast to Ac$_4$GlcNAz, treatment of cells with Ac$_4$GalNAz resulted in UDP-GlcNAz biosynthesis (Fig. 3*A*) and robust labeling of O-GlcNAcylated proteins (Fig. 2). Interestingly, AGX1/2 enzymes also serve as the pyrophosphorylase in the GalNAc salvage pathway (Fig. 1*A*) (24), indicating that the conversion of GalNAz-1-phosphate (GalNAz-1-P) to UDP-GalNAz occurs with relatively greater efficiency in cells than does the corresponding conversion of GlcNAz-1-P. Indeed, in vitro, both human pyrophosphorylase isoforms are more active on GalNAc-1-P than on GlcNAc-1-phosphate (24). Therefore, the kinetics, expression levels, and/or posttranslational regulation of AGX1 and AGX2 may account for the relatively efficient conversion of GalNAz-1-P to UDP-GalNAz, even in cells that fail to convert Ac$_4$GlcNAz to UDP-GlcNAz.

Downstream of AGX1/2, we have shown that the GALE epimerase efficiently converts UDP-GalNAz to UDP-GlcNAz in mammalian cells (Fig. 4). Interestingly, our HPAEC data indicate that this equilibrium substantially favors UDP-GlcNAz over UDP-GalNAz, which were formed in an approximate 3:1 ratio in Ac$_4$GalNAz-treated cells (Fig. 3*A*). This ratio might be explained by a greater thermodynamic stability of UDP-GlcNAz versus UDP-GalNAz. Although we have not directly tested this hypothesis, it is consistent with in vitro studies of a bacterial UDP-GlcNAc C$_4$ epimerase, which produced a ~7:3 ratio of UDP-GlcNAc to UDP-GalNAc at equilibrium (39). Furthermore, our results are in excellent agreement with the observed 3:1 ratio in human cells of UDP-glucose to UDP-galactose (40), two other metabolites that can be interconverted by human GALE and that likely have similar relative thermodynamic stabilities to those of UDP-GlcNAz and UDP-GalNAz, respectively.

We have previously shown (19) that Ac$_4$GalNAz treatment results in cell surface mucin-type glycoprotein labeling, likely via the O-linked α-GalNAc residue that forms the core of all mucin-type glycans. Indeed, here we have reconfirmed this finding using human mucin-18 (Fig. S7), and note that some secretory pathway glycoproteins were identified in our proteomics results (Figs. S5*B* and S6). The heterogeneous labeling of different glycan populations by Ac$_4$GalNAz may be undesirable for some experimental applications, and the possibility that Ac$_4$GalNAz may produce both GalNAz- or GlcNAz-bearing secretory pathway glycans may complicate studies of Ac$_4$GalNAz-labeled cell surface glycoproteins in particular. However, simple subcellular fractionation methods to purify nuclear and cytoplasmic proteins away from membrane-bound mucin-type glycoproteins permit a specific examination of O-GlcNAc itself. Moreover, we show here that most nucleotide-azidosugar arising from Ac$_4$GalNAz treatment is UDP-GlcNAz (Fig. 3*A*), and most glycoprotein labeling by Ac$_4$GalNAz is O-GlcNAz, as judged by its potentiation by OGT overexpression (Fig. 2*A*), sensitivity to human (Fig. 2*B*) or bacterial (Fig. S3) OGA enzymes, and subcellular localization (Fig. 4). Together, these data indicate that Ac$_4$GalNAz is a faithful and robust metabolic reporter for O-GlcNAc.

Finally, our results may have implications for a systems-level understanding of endogenous amino sugar metabolic flux in mammalian cells, and perhaps even human diseases where these pathways are dysregulated. For example, a recent study demonstrated that perturbing the GlcNAc salvage pathway via treatment with high or low GlcNAc concentrations impacts on diverse aspects of cellular physiology, including growth factor signaling, cell cycle, nutrient sensing, and endocytosis (41). Our results show that UDP-GlcNAc pyrophosphorylase is a critical, rate-limiting control point in the GlcNAc salvage pathway for GlcNAz meta-

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bolism. It will be interesting to see whether future studies reveal this to be true for natural GlcNAc as well, in which case the pyrophosphorylase step in the GlcNAc salvage pathway may control the response of these downstream cellular processes to fluctuations in GlcNAc availability. In addition, our results with Ac_4GalNAz demonstrate that human GALE regulates cellular concentrations of UDP-GalNAz and UDP-GlcNAz. If this is also the case for natural UDP-GlcNAc and UDP-GalNAc, it may point to unanticipated roles for the GALE C_4 epimerase in cell physiology and, perhaps, disease. For example, rare forms of human galactosemia are caused by loss-of-function mutations in the GALE gene (42). Although most studies have assumed that the clinical consequences of GALE deficiency are due to galactose toxicity, it is tempting to speculate that loss-of-function mutations in GALE may also significantly alter the physiological concentrations of UDP-GalNAc and UDP-GlcNAc and thereby perhaps impinge on O-GlcNAc signaling and *N*-linked protein glycosylation, which both rely on UDP-GlcNAc. Therefore, the kinetic and cell biological characterization of the GALE C_4 epimerase will prove an interesting topic for future research.

Materials and Methods

High-Performance Anion Exchange Chromatography. Equal numbers of cells were treated as indicated, washed twice in PBS, and lysed by sonication in 75% ethanol. Supernatants were cleared by centrifugation, dried in a speedvac, and resuspended in 40 mM sodium phosphate, pH 7.0. Small molecule metabolites were collected via filtration through a spin concentrator with a 10-kDa molecular weight cutoff (Amicon). HPAEC analysis was performed essentially as described (43).

Enrichment and Identification of Azide-Labeled Glycoproteins. Control and Ac_4GalNAz-treated cells were harvested and azidoglycoproteins were enriched from processed samples essentially as described (15).

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