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**Tandem bioorthogonal labeling uncovers endogenous co-translationally O-GlcNAc modified nascent proteins.**

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**ABSTRACT**

Hundreds of nuclear, cytoplasmic, and mitochondrial proteins within multicellular eukaryotes have hydroxyl groups of specific serine and threonine residues modified by the monosaccharide N-acetylglucosamine (GlcNAc). This modification, known as O-GlcNAc, has emerged as a central regulator of both cell physiology and human health. A key emerging function of O-GlcNAc appears to be to regulate cellular protein homeostasis. We previously showed, using overexpressed model proteins, that O-GlcNAc modification can occur co-translationally and that this process prevents premature degradation of such nascent polypeptide chains. Here we use tandem metabolic engineering strategies to label endogenously occurring nascent polypeptide chains within cells using O-propargyl-puromycin (OPP) and target the specific subset of nascent chains that are co-translationally glycosylated with O-GlcNAc by metabolic saccharide engineering using tetra-O-acetyl-2-N-azidoacetyl-2-deoxy-D-galactopyranose (Ac4GalNAz). Using various combinations of sequential chemoselective ligation strategies we go on to tag these analytes with a series of labels, allowing us to define conditions that enable their robust labeling. Two-step enrichment of these glycosylated nascent chains, combined with shotgun proteomics, allows us to identify a set of endogenous co-translationally O-GlcNAc modified proteins. Using alternative targeted methods, we examine three of these identified proteins and further validate their co-translational O-GlcNAcylation. These findings detail strategies to enable isolation and identification of extremely low abundance endogenous analytes present within complex protein mixtures. Moreover, this work opens the way to studies directed at understanding the roles of O-GlcNAc and other co-translational protein modifications and should stimulate an improved understanding of the role of O-GlcNAc in cytoplasmic protein quality control and proteostasis.

**KEYWORDS:** O-GlcNAc, metabolic oligosaccharide engineering, tandem chemoselective ligation, proteostasis, nascent polypeptide

**INTRODUCTION**

Hundreds of nuclear, cytoplasmic, and mitochondrial proteins are modified with N-acetylglucosamine (GlcNAc) units. These GlcNAc monosaccharides are O-linked to the hydroxyl groups of specific serine or threonine residues.1 This modification, known as O-GlcNAc,2 is conserved among multicellular eukaryotes. Levels of O-GlcNAc are regulated by only two enzymes: O-GlcNAc transferase (OGT) installs O-GlcNAc using [uridine 5′-diphosphate-N-acetylglucosamine](about:blank) (UDP-GlcNAc) as a donor substrate3-4 and the hydrolase O-GlcNAcase (OGA) catalyzes the removal of O-GlcNAc.5 This modification plays roles in various fundamental cellular processes including, for example, epigenetic regulation of gene transcription,6-8 stress response9-12 and circadian rhythm.13-14 Moreover, dysregulation of O-GlcNAc is implicated in diverse diseases including cancers, diabetes, and neurodegenerative diseases.1 Despite its importance in both cell physiology and human health, the various mechanisms through which O-GlcNAc participates in these diverse roles remain largely unclear.

Accumulating evidence shows that O-GlcNAc regulates protein stability in various ways including by altering proteolytic susceptibility of proteins14-17 as well as their aggregation propensity.18-20 O-GlcNAc is also emerging as a regulator of key cellular machinery that helps cells cope with varied stresses including, for example, the ubiquitin-proteasome system,21 autophagy22-23 and heat shock response.24 These findings indicate that a major physiological function of O-GlcNAc is to regulate cellular protein homeostasis, or what is generally known as “proteostasis”. Recognizing that OGT associates with actively translating ribosomes25 and binds polypeptide substrates in an extended form within a superhelical groove that guides peptides into the OGT active site,26 we reasoned that O-GlcNAc modification can occur on nascent polypeptide chains as they are being biosynthesized. Using the constitutively O-GlcNAc modified proteins transcription factor Sp1 (Sp1) and nuclear pore glycoprotein p62 (Nup62) as model proteins, we recently demonstrated that this co-translational glycosylation occurs to protect nascent Sp1 and Nup62 polypeptides from premature degradation by the proteasome.27

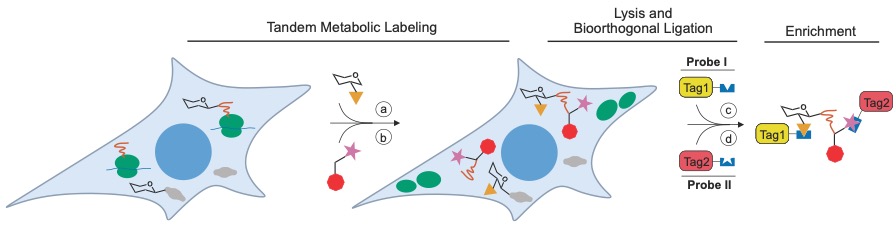
Given that a considerable fraction of nascent polypeptides are degraded to maintain proteostasis28-30, we speculated that co-translational O-GlcNAcylation may occur more widely among the hundreds of proteins that are O-GlcNAcylated within cells.1 Moreover, the previously noted studies focused on two overexpressed target proteins,27 leaving open the question of how widespread co-translational modification might be on endogenous cellular proteins. To address these questions regarding the presence of O-GlcNAc on nascent chains from endogenous proteins and the identities of nascent chains modified in this way, here we use concomitant cellular metabolic labeling of both endogenous nascent polypeptide chains and O-GlcNAc modified polypeptides. We detail methods for the tandem bioorthogonal labeling of these species and, using different sets of reporter probes, we show that co-translational O-GlcNAc occurs on endogenous nascent chains. We then use this tandem bioorthogonal labeling strategy to enrich a subset of doubly labeled proteins and apply high-throughput proteomics to define the first inventory of such nascently O-GlcNAc modified proteins. Finally, we use targeted methods to focus on several identified proteins of interest to further validate that these proteins are co-translationally modified with O-GlcNAc.

**RESULTS**

To assess whether co-translational O-GlcNAc modification widely occurs on endogenous proteins within cells, we set out to develop methods to enrich and identify O-GlcNAc-modified nascent chains. We pursued a tandem metabolic engineering strategy to sequentially tag nascent chains and the O-GlcNAc modified proteome. To this end, mammalian cells were treated with the metabolic reporter Ac4GalNAz (**1**, Figure 1A), an azide-tagged derivative of N-acetylgalactosamine that is transformed by the biosynthetic machinery into UDP-GlcNAz and then used by OGT to install O-GlcNAz in place of endogenous O-GlcNAc within cells.31-32 While this metabolic engineering was underway, we added to the cells O-propargyl-puromycin33 (OPP, **2**, Figure 1A), an analogue of the natural product puromycin that bears a bioorthogonal alkyne functionality. Puromycin and OPP resemble the 3'-end of the aminoacylated tRNA and are incorporated by the ribosome into the nascent polypeptide, thereby terminating translation, and in the case of OPP also tagging nascent chains at their C-terminal ends with an alkyne functionality.33 We reasoned that two sequential rounds of downstream bioorthogonal ligations, including the Staudinger ligation and copper (I)-catalyzed alkyne-azide cycloaddition (CuAAC), could then be used to install various fluorescent reporter groups and affinity ligands to enable either visualization or enrichment of the doubly tagged target proteins (Figure 1B).

A





B

Figure 1. Metabolic labeling and chemoselective ligation strategies used to detect and analyze co-translationally O-GlcNAc modified nascent polypeptide chains. (A) Structures of metabolic labeling agents used in this study to label O-GlcNAc modified proteins and nascent polypeptide chains. Metabolic feeding with Ac4GalNAz (**1**) tags sites of O-GlcNAc with azide-bearing O-GlcNAz, while dosing of cells with OPP (**2**) labels the C-terminus of nascent polypeptides with alkyne functionalities by incorporation of OPP and termination of translation by ribosomes. (B) Generalized design of experiments used for sequential chemoselective labeling of O-GlcNAc modified nascent chains. Tandem metabolic labeling is first done using sequential addition of Ac4GalNAz (Step a) and then OPP (Step b). Cells are next lysed and bioorthogonal labeling is performed in two serial steps. The first step (Step c) uses either the Staudinger ligation or copper (I)-catalyzed alkyne-azide cycloaddition (CuAAC) to ligate Tag 1 (Step c), containing either a fluorophore or biotin, onto either O-GlcNAz or OPP incorporated into nascent polypeptides. The second step (Step d) uses CuAAC to ligate Tag 2, again containing either a fluorophore or biotin, onto the second metabolic chemical reporter. Tandemly tagged polypeptides can be enriched at Step c and/or Step d for downstream analysis by blotting or mass spectrometry.

To test the feasibility of this strategy, we performed a series of different experiments each involving two rounds of ligation chemistry. By using one probe bearing a biotin moiety and the other probe having a pendent cyanine (Cy)-based fluorophore we reasoned we could use immobilized streptavidin to pull-down analytes and then test for the presence of fluorescently labeled proteins within the enriched fractions. Observing such species would reveal the presence of a set of O-GlcNAc-modified nascent chains. For each experiment we also performed two sets of parallel control experiments using cells treated only with either Ac4GalNAz (**1**) or OPP (**2**) alone. We felt these experiments would help assess the feasibility of the approach and address the potential problems arising from side reactions and non-specific enrichment during each step.

In our initial round of experiments, we first tagged the set of O-GlcNAz labeled proteins by Staudinger ligation using triarylphosphine **Probe 3**27 (Figure 2A) bearing biotin and subsequently labeled OPP-modified nascent chains by CuAAC using azide terminated **Probe 4** bearing Cy5.5 (Figure 2A). We then enriched the biotin-tagged O-GlcNAz labeled proteome using streptavidin-agarose resin (Figure 2B). These collective samples were subjected to SDS-PAGE and analyzed after blotting to detect coupling to OPP, by monitoring Cy5.5 fluorescence, and the presence of biotin, by monitoring fluorescence associated with IRDye 800CW-labeled streptavidin. Importantly, analysis of the streptavidin resin-enriched samples (Figure 2C) revealed the presence of Cy5.5 fluorescence arising from OPP-tagged nascent polypeptides only from cells treated with both Ac4GalNAz (**1**) and OPP (**2**). In contrast, polypeptides from cells treated with only Ac4GalNAz (**1**) showed only biotin reactivity. And, finally, no O-GlcNAz-modified polypeptides were enriched and only low background signals of Cy5.5 fluorescence were detected after enrichment step from cells treated with OPP (**2**) alone or vehicle in place of both Ac4GalNAz (**1**) and OPP (**2**) (Figure 2C). Analysis of the input and flow-through samples showed that only polypeptides from cells treated with OPP (**2**) and coupled with **Probe 4** exhibited fluorescence from the Cy5.5 dye. Additionally, only input and flow-through samples treated with Ac4GalNAz (**1**) and coupled to **Probe 3** exhibited biotin reactivity (Figure S1A). Input and flow-through for vehicle-treated lysates exhibited only low levels of background signal (Figure S1A). Together, these data reveal the presence of nascent polypeptide chains within the total set of labeled and enriched O-GlcNAc modified polypeptides and further illustrate that our tandem labeling strategy works effectively to label and enrich such low abundance analytes.

In the second round of experiments, we used a variation of our initial approach to determine if we could confirm these observations by first enriching nascent polypeptides and then looking for fluorescently labeled O-GlcNAz modified proteins within biotin-labeled nascent chains (Figure 2D). Additionally, we wondered if we could replace the relatively slow Staudinger ligation by the faster CuAAC reaction to perform two rounds of CuAAC reactions. However, we recognized that this approach might be complicated by the presence of both azide and alkyne functionalities within the pool of analytes since they could, in theory, couple to one another. To test this approach, we therefore used azide-terminated **Probe 5** (Figure 2A) bearing biotin and alkyne-terminated **Probe 6** (Figure 2A) bearing Cy5.5. We first coupled the OPP-terminated nascent chains with biotin using **Probe** **5** by CuAAC and, after capturing the biotin-labeled nascent proteome on streptavidin-agarose resin, we tagged O-GlcNAz residues with Cy5.5 by on-beadCuAACusing **Probe** **6**. Using this alternative approach, we observed (Figure 2E) results similar to those in the first experiment (Figure 2C). Within the streptavidin-agarose bead-precipitated samples, only nascent polypeptides isolated from cells treated with both Ac4GalNAz (**1**) and OPP (**2**) showed both Cy5.5 and biotin reactivity, whereas polypeptides from cells treated with OPP (**2**) alone showed only biotin reactivity (Figure 2E). As expected, only low background Cy5.5 fluorescence and biotin reactivity were observed in the resin-precipitated sample from cells treated with Ac4GalNAz (**1**) alone indicating that only OPP (**2**)-tagged nascent polypeptides were effectively precipitated (Figure 2E) using this approach. Finally, analysis of the input and flow-through samples (Figure S1B) showed

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Figure 2. A set of nascent mammalian proteins is co-translationally modified with O-GlcNAc. (A) Structures of biotin-tagged triarylphosphine **Probe 3** and azide **Probe 5**, Cy5.5-tagged azide **Probe 4**, and alkyne **Probe 6**. (B) Tandem labeling procedure used for visualizing nascent chains within the enriched O-GlcNAz labeled proteome. HEK293 cells were treated for 14 h with Ac4GalNAz (**1**, 200 M) or vehicle alone. Cells were then treated for 2 h with OPP (**2**, 30 M) or vehicle alone. Following lysis, cell lysates were incubated with triarylphosphine **Probe** **3** to ligate O-GlcNAz-modified proteins to biotin (Step a). After protein precipitation, lysates were reacted with alkyne **Probe 4** to ligate OPP-labeled nascent proteins to the Cy5.5 fluorophore (Step b). Tandem tagged samples were again precipitated and biotinylated analytes were enriched using streptavidin-agarose resin (Step c). (C) Blotting of analytes labeled and enriched as described in (B) using streptavidin-fluorophore conjugates and fluorescence scanning. (D) Tandem labeling procedure used for visualizing O-GlcNAz within the enriched OPP labeled proteome. HEK293 cells were treated as in (B). Cell lysates were first reacted with azide **Probe** **5** to ligate OPP-labeled nascent chains to biotin (Step a). Biotin tagged nascent polypeptides were subsequently captured on streptavidin-agarose resin (Step b) and then reacted on-bead with alkyne **Probe 6** to label O-GlcNAz-modified nascent chains with Cy5.5 (Step c). (E) Blotting of analytes labeled and enriched as described in (D) using streptavidin-fluorophore conjugates and fluorescence scanning.

that only polypeptides from cells treated with OPP (**2**)were tagged with biotin using **Probe 5**, andonly polypeptides from cells treated with Ac4GalNAz (**1**) were labeled with the Cy5.5 of **Probe 6**. These findings from this second round of experiments show that two rounds of CuAAC can be readily applied to samples containing both azide and alkyne labeled analytes. Indeed, while some degree of cross-reactions between the azide- and alkyne-labeled analytes during CuAAC could not be ruled out, these findings suggest that the concentration of probes used here are high enough to avoid significant cross-reactions between the low abundance analytes investigated here. In any event, this approach offers advantages over the first round of experiments by not requiring use of more air sensitive and slower reacting triarylphosphine probes such as **Probe 3** which was used in the first set of experiments.

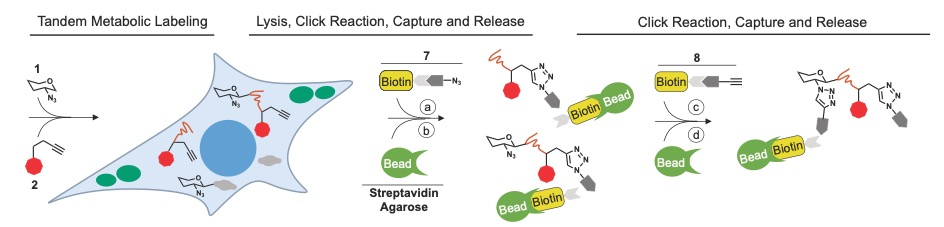
Recognizing the potential benefits of the sequential CuAAC strategy used in the second set of experiments, we set out to perform a third set of experiments to further validate such a tandem CuAAC strategy in a manner similar to previous uses of tandem CuAAC methods.34-35 We reversed the order of the CuAAC reactions, first labeling O-GlcNAz modified proteins with biotin and then fluorescently labeling OPP (**2**)-tagged nascent polypeptide chains. This approach is therefore conceptually similar to the Staudinger-CuAAC approach used in the first set of experiments (Figure 2B). Here we used the alkyne terminated **Probe 10** to install biotin onto O-GlcNAz modified polypeptides using CuAAC. After capturing these species on streptavidin-agarose beads, the second CuAAC reaction was performed on-bead using azide terminated **Probe 4** to label OPP (**2**)-tagged nascent chains with Cy5.5. We observed results that were similar to the experiment as performed in reverse order (Figure 2E), allowing us to detect both Cy5.5 and biotin reactivity only in those samples from cells treated with both Ac4GalNAz (**1**) and OPP (**2**) (Figure S1C). These data from both the second and third set of experiments provide clear support for the utility and robustness of this tandem CuAAC ligation strategy. In addition, the collective results of the three experimental approaches indicate that O-GlcNAz can be found on endogenous nascent polypeptides terminated with OPP (**2**). Moreover, these data support this strategy being a tractable approach to identify proteins that are co-translationally modified with O-GlcNAc.

In light of the results from these three experiments, coupled with the reported superior efficiency of CuAAC with respect to the Staudinger ligation36, we decided to use the tandem CuAAc strategy in the second set of experiments to perform mass spectrometry (MS)-based proteomic analysis of endogenous O-GlcNAc-modified nascent chains. Toward this goal, we replaced the probes used in the second set of experiments with two cleavable biotin probes that streamlined the strategy by enabling convenient consecutive rounds of enrichment and elution of proteins captured by streptavidin agarose resin (Figure 3B). We first labeled O-GlcNAc-modified nascent chains as above using Ac4GalNAz (**1**) and OPP (**2**). We then tagged labeled nascent chains by CuAAC coupling of the alkyne functionality of OPP (**2**) with

A



B



C

![](data:application/pdf;base64,)

Figure 3. Mass spectrometry (MS)-based identification of a set of nascent mammalian polypeptides that are co-translationally modified with O-GlcNAc. (A) Structures of biotin-tagged cleavable azide **Probe 7** and alkyne **probe 8**. (B) A simplified diagram of the experimental tandem labeling procedure used for the MS-based identification of enriched nascent chains that are O-GlcNAz labeled. HEK293 cells were treated for 14 h with Ac4GalNAz (**1**, 200 M) or vehicle alone. Cells were then treated for 2 h with OPP (**2**, 30 M) or vehicle alone. Following lysis, cell lysates were incubated with cleavable azide-terminated **Probe** **7** to ligate OPP-labeled nascent polypeptides to biotin (Step a). After protein precipitation, polypeptides were resuspended and biotin-tagged nascent polypeptides were captured on streptavidin-agarose resin, washed extensively, and eluted using mild acid (Step b). These samples of enriched nascent chains were incubated with cleavable alkyne-terminated **Probe** **8** to ligate O-GlcNAz-labeled nascent polypeptides to biotin (Step c). Biotin tagged polypeptides were again captured on resin, washed, and eluted using mild acid (Step d). The resulting samples enriched in O-GlcNAz-modified nascent proteins were digested and analyzed by LC-MS/MS. In parallel, control samples from cells treated with either Ac4GalNAz (**1**) or OPP (**2**) alone were subjected to the same experiment process described above and also analyzed by LC-MS/MS as negative controls. (C) Summary of result of the replicates for the MS-based polypeptide identification. High confidence protein hits are defined as those identified in experimental samples but not identified in either negative control sample. The intersection of hits is defined as the list of high confidence hits identified in both replicate experiments and the intersected O-GlcNAcylated hits are those proteins in the intersection of hits that are also known to be O-GlcNAc modified based on previous literature.

the azide terminated cleavable **Probe 7** bearing biotin (Figure 3A). Capture of these nascent polypeptides on streptavidin-agarose beads was followed by multiple rounds of stringent washing and then release of these species using mild acid (10% formic acid) to cleave the acid labile dialkoxydiphenylsilane (DADPS)-based linker.37 After removal of formic acid by evaporation, the resulting enriched OPP-tagged set of nascent polypeptides was submitted to another round of coupling by CuAAC but now using the analogous alkyne terminated cleavable **Probe 8** bearing biotin(Figure 3A) to label O-GlcNAz. The resulting isolated polypeptides obtained through two sequential rounds of CuAAC and enrichment using streptavidin-agarose resin comprise the set of nascent polypeptide chains bearing O-GlcNAz (Figure 3B). We therefore performed tryptic digestion of these nascent polypeptides and analyzed the resulting tryptic peptides by liquid chromatography (LC)-based separation followed by tandem MS (LC-MS/MS) using a Bruker Impact II Q-TOF instrument. Again, for each experiment, we performed two sets of parallel control experiments using cells treated only with either Ac4GalNAz (**1**) or OPP (**2**) alone. After excluding background polypeptides that appeared in the two control samples, we were able to identify, in two independent replicates, 415 and 518 proteins from which these nascent chains were derived (Figure 3C and Supporting Proteomics Dataset). 175 of these proteins were observed in both replicate experiments (Figure 3C and Supporting Proteomics Dataset). We also found that 140 proteins from Replicate 1 and 156 proteins from Replicate 2 had been previously identified as O-GlcNAc modified proteins based on the curated PhosphoSitePlus® database,38 and recent literature.39-41 From these two independently identified subsets of known O-GlcNAc-modified proteins, 75 were observed in both independent biological replicates and we considered these as among the most logical candidates to further validate as proteins that are co-translationally O-GlcNAc modified by OGT (Figure 3C and Supporting Proteomics Dataset). Moreover, among these proteins we also observed nuclear pore protein Nup62, which we previously showed can be co-translationally modified with O-GlcNAc when overexpressed.27 To gain insights on the functional features of these high confidence co-translationally O-GlcNAcylated protein candidates, gene ontology (GO) analysis was performed using DAVID (https://david.ncifcrf.gov/home.jsp). We find significant enrichment of proteins in functional categories including purine nucleoside biosynthesis, nuclear-cytoplasmic transportation, stress response, and regulation of gene expression (Supporting Proteomics Dataset), suggesting physiological roles for co-translational O-GlcNAcylation. To assess which nascent O-GlcNAc modified proteins were most abundant in our dataset, the set of identified proteins were quantified relative to each other using an established label free approach42 using spectral counts of protein hits. In this way, final protein lists observed in both replicates and those that are O-GlcNAcylated were sorted in descending order according to the mean value of their calculated normalized spectral abundance factors, which ranks their abundance within the enriched set of nascent peptides (Supporting Proteomics Dataset). More abundantly co-translationally modified proteins may be more likely to be subject to regulation by this co-translational process, however, further studies would be needed to test this hypothesis.

For further validation, we selected ataxin-2-like protein (Ataxin-2L), nuclear pore complex protein Nup153 (Nup153), and host cell factor 1 (HCF-1) as three proteins of high interest from among these known O-GlcNAc modified candidates. We selected these proteins based on the level of subjective interest in their biological functions and their greater molecular weights. This second criterion was based on the expectation that longer polypeptides would be more likely to yield a series of nascent polypeptides that could be readily detected during analyses. Details on the length and O-GlcNAc sites of the selected candidate proteins are summarized in Table S1.

Having selected these candidate proteins for study, we first transfected HEK293 cells with plasmids encoding each protein bearing on their N-termini a triple-FLAG epitope. Each set of cultured HEK293 cells was then treated with Ac4GalNAz (**1**). After 16 hours, cells were lysed and polysomes were isolated by ultracentrifugation through a sucrose cushion.27 We next released nascent chains from ribosomes by resuspending the polysome-containing fractions in buffer containing 1% SDS, which causes ribosome disassembly.27 The liberated nascent chains bearing O-GlcNAz were then coupled with triarylphosphine **Probe** **9** (Figure 4A) bearing biotin using the Staudinger ligation. From these sets of

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Figure 4. Validation of co-translational O-GlcNAcylation of selected proteins using an alternative method involving genetically-encoded affinity tags and polysome isolation. (A) Structure of biotin-tagged triarylphosphine **Probe 9**. (B) A simplified diagram of the experimental procedure used for visualizing co-translationally O-GlcNAc modified nascent chains on target proteins of interest by isolation of polysomes followed by release and enrichment of nascent chains. HEK293 cells were transfected with plasmids encoding Ataxin-2L, Nup153, and HCF-1 each bearing an N-terminal triple-FLAG tag. Cells were then treated for 16 h with Ac4GalNAz (**1**, 200 M) or vehicle alone. Cells were lysed and nascent chains were enriched by isolation of polysomes by using ultracentrifugation through a sucrose cushion (Step a). Enriched FLAG-tagged nascent chains were released from ribosomes and reacted with triarylphosphine **Probe 9** to tag O-GlcNAz with biotin (Step b). Nascent chains were then immunoprecipitated using anti-FLAG resin (Step c). (C) Blotting of enriched analytes using streptavidin-fluorophore conjugates and anti-FLAG antibody followed by fluorescence scanning. (D) A simplified diagram of the experimental procedure used for visualizing co-translationally O-GlcNAc modified nascent chains on Nup153 by isolation of polysomes followed by release and enrichment of nascent chains. HEK293 cells were transfected with plasmid encoding Nup153 bearing an N-terminal AviTag along with biotin ligase to drive biotinylation of the AviTag within cells. Cells were then treated for 16 h with Ac4GalNAz (**1**, 200 M) or vehicle alone. Cells were lysed and nascent chains were enriched by isolation of polysomes by using ultracentrifugation through a sucrose cushion (Step a). Enriched biotin-tagged nascent chains were released from ribosomes and reacted with alkyne **Probe 6** to tag O-GlcNAz with Cy5.5 (Step b). Nascent chains were then precipitated using streptavidin-agarose resin (Step c). (E) Blotting of enriched analytes using streptavidin-fluorophore conjugates and imaging using fluorescence scanning.

nascent proteins, we pulled down our FLAG tagged-target protein of interest by using anti-FLAG immunoprecipitation (IP) resin (Figure 4B). Our analyses revealed a series of nascent polypeptides of different sizes for each candidate protein as detected by probing with anti-FLAG antibody (Figure 4C). Furthermore, the streptavidin reactivity of these nascent chains (Figure 4C) supports these species being O-GlcNAz modified. Notably, these O-GlcNAz-derived signals overlaid well with the immunoreactivity arising from the N-terminal FLAG epitope present on the nascent chains (Figure 4C). To confirm these observations, we selected Nup153 and used an alternative method using an N-terminally fused AviTag as a different genetically-encoded affinity tag and also changing the probe structure to use alkyne-terminated **Probe 6** bearing Cy5.5 (Figure 2A). Biotinylation of the AviTag is driven by biotin ligase which is also encoded in the plasmid harboring the expression construct of Nup153. In this approach we performed a CuAAC reaction using **Probe 6** to install a Cy5.5 fluorescent reporter and then precipitated Nup153 on streptavidin-agarose resin (Figure 4D). On analysis of the samples we observed similar results as seen using the FLAG-tagged Nup153 with a banding pattern that was similar and also showed overlapping biotin reactivity and Cy5.5 fluorescence (Figure 4E). Notably, in both approaches, only weak background signals were seen for samples from cells treated with vehicle in place of Ac4GalNAz (**1**) when monitoring O-GlcNAz labeling using either biotin reactivity, for samples labeled with **Probe 9**, or Cy5.5 fluorescence, for samples labeled with **Probe 6** (Figure 4C and 4E). These data further validate our MS proteomic data providing good support for these candidate proteins being co-translationally O-GlcNAc modified.

Recognizing the complexity of the tandem labeling procedure and subsequent analyses, we considered potential factors that could confound our observations and give rise to false positive identification of protein hits in our high confidence list of co-translationally O-GlcNAc modified proteins. Two potential confounders arise. One stems from the nature of the sample handling, which involves incubation of cells with OPP for 2 hours prior to lysis. The second arises from off target labeling of proteins through the recently identified spontaneous process of cysteine thiol glycosylation by per-O-acetylated monosaccharides.43-44

We first considered the possibility that nascent chains released from ribosomes by OPP within cells may be substrates for OGT, leading to the modification of such released polypeptides with O-GlcNAz during the 2 h incubation time with OPP. This process could lead to undesired false positives during protein identification. One way to assess this potential problem is to liberate nascent chains after cell lysis in the presence of OGT inhibitors. We therefore performed further validation regarding co-translational modification of these three candidate proteins by repeating the same experiments as described above but including OGT inhibitor both in the lysis buffer and during ribosome isolation. Notably, using this modified method we observed similar results as described above (Figure S2A). Furthermore, using these same conditions, we also showed that O-GlcNAz modification could not be detected on nascent chains enriched from cells having never been treated with Ac4GalNAz but having had UDP-GlcNAz added to the lysis buffer. This last experiment confirms effective inhibition of OGT under our experimental conditions (Figure S2A) and rules out OGT acting after cell lysis to give rise to our observations. Collectively, these data indicate that all these three candidate proteins are authentically co-translationally modified with O-GlcNAz within cells and, furthermore, that the high confidence list of candidate co-translationally modified proteins likely has few false positive identifications due to O-GlcNAz modification occurring on nascent chains released by OPP within cells.

The second potential factor that could complicate interpretation of our data is the recently identified non-specific reaction of per-O-acetylated monosaccharides, including Ac4GalNAz used here for protein ID experiments, with the thiol group of cysteine residues of proteins.43-44 This spontaneous process mainly occurs when incubating cell lysates with high concentrations of such per-O-acetylated monosaccharides (Ac4GalNAz > 500 M) and is slower when labeling is performed in live cells, where much lower thiol modification stoichiometry is observed, especially for monosaccharides with high labeling efficiency such as Ac4GalNAz.42 In our experiments we used the “standard labeling conditions” that involves incubating cells with 200 M of Ac4GalNAz for 16 h, which has been shown to enable efficient labeling of O-GlcNAc-modified proteins and negligible cysteine thiol glycosylation.46 Nevertheless, to evaluate the potential of this spontaneous process to confound our experiments, we performed additional validation experiments in which we adopted the recommended approach41 of using the deprotected monosaccharide GalNAz. In these experiments we observed very similar O-GlcNAz labeling patterns on isolated nascent chains as those in previous validation experiments using Ac4GalNAz (Figure 4C and S2A). These data indicate spontaneous cysteine thiol glycosylation is likely not a confounding factor in these experiments giving rise to false positive identification of co-translationally O-GlcNAcylated proteins. Collectively, these validation experiments support the identified proteins being authentically modified with O-GlcNAc in a co-translational manner. Nevertheless, we recommend researchers validate candidate co-translationally O-GlcNAc modified proteins using the pipeline we describe here prior to carrying out any further co-translational O-GlcNAcylation-related studies on proteins identified here.

**CONCLUSIONS**

The development of chemoselective ligation reactions in combination with metabolic engineering has enabled covalent tagging of a wide range of biomolecules47-50 including nascent polypeptides.33, 51-52 The development of tandem chemoselective ligation strategies has advanced in recent years to enable monitoring multiple analytes simultaneously or specific subsets of abundant analytes.53-55 Here we build on these advances to use tandem metabolic labeling in combination with optimized sequential chemoselective labeling and purification to create a strategy that can be applied to low abundance analytes present in a complex matrix. We validate this approach using various probes and apply the method to address the question of whether endogenously occurring nascent polypeptide chains are substrates of OGT and are co-translationally glycosylated with O-GlcNAc. We further go on to uncover the identities of proteins that are co-translationally O-GlcNAcylated by coupling this tandem chemical labeling strategy with traditional shotgun MS-based proteomics analysis. Subsequent validation experiments on three candidates confirm co-translational O-GlcNAcylation of these proteins, supporting the suitability of the methods developed here for identifying O-GlcNAc modified nascent proteins. Notably, it is only through this combination of metabolic labeling and subsequent tandem chemoselective ligations that these experiments could be pursued. This need stems from the known antibodies to O-GlcNAc having relatively modest affinities and only recognizing subsets of O-GlcNAc modified proteins.56

These data provide a useful resource that will help uncover the roles of O-GlcNAc in cellular proteostasis. Emerging data has pointed to some ways in which O-GlcNAc can influence the biosynthesis of proteins, including for example how O-GlcNAc can regulate initiation of protein translation by modifying and altering the function of core factors governing translational initiation.57 The findings described here should also serve as a valuable point of departure for fundamental studies directed toward understanding the functional roles of co-translational O-GlcNAc modification of nascent polypeptides, the sites of co-translational O-GlcNAc modification, and the molecular features controlling this process. We recommend such studies commence with validation of co-translational O-GlcNAc modification using the validation pipeline we describe above. Notably, co-translational O-GlcNAc modification bears some similarities with the N-glycosylation of asparagine residues within the secretory pathway that also occurs in both a post- and co-translational manner.58-61 In this regard it is interesting to speculate that co-translational O-GlcNAc modification may play fundamental roles in regulating protein quality control in ways that are similar to those already well established for co-translational N-glycosylation.62-63 The recent discovery of O-mannosylation in yeast of a similar set of proteins as seen to be modified by O-GlcNAc in metazoans64 lends support to the possibility that perhaps protein glycosylation is an evolutionarily conserved mechanism that has evolved to contribute to protein quality control within both the secretory pathway and nucleocytoplasm. Finally, antagonists of the O-GlcNAc pathway have shown therapeutic value in preclinical models of different diseases18, 65-73 and even entered the clinic.74 Accordingly, dissecting the functional roles of O-GlcNAc within cells and how they regulate specific subsets of proteins should help guide the development of more targeted strategies to manipulate this pathway for therapeutic benefit.

ASSOCIATED CONTENT

Supporting Information

Supporting Figure S1, S2, Table S1 and experimental methods (PDF)

Supporting Proteomics Dataset (XLSX)

This material is available free of charge via the Internet at http://pubs.acs.org

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ABBREVIATIONS

Ac4GalNAz, tetra-O-acetyl-2-N-azidoacetyl-2-deoxy-D-galactopyranose; Ataxin-2L, ataxin-2-like protein; CuAAC, copper (I)-catalyzed alkyne-azide cycloaddition; Cy, cyanine; DADPS, dialkoxydiphenylsilane; HCF-1, host cell factor 1; IP, immunoprecipitation; LC, liquid chromatography; Nup153, nuclear pore complex protein Nup153; Nup62, nuclear pore glycoprotein p62; MS, mass spectrometry; O-GlcNAc, O-linked N-acetylglucosamine; OGA, O-GlcNAcase; OGT, O-GlcNAc transferase; OPP, O-propagryl-puromycin; Sp1, transcription factor Sp1; UDP, Uridine diphosphate.

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**Table of Contents Figure**

A close up of a map

Description automatically generated