A Novel Glycoproteomics Workflow Reveals Dynamic O-GlcNAcylation of COPγ1 as a Candidate Regulator of Protein Trafficking

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O-linked β-N-acetylglucosamine (O-GlcNAc) is an abundant and essential intracellular form of protein glycosylation in animals and plants. In humans, dysregulation of O-GlcNAcylation occurs in a wide range of diseases, including cancer, diabetes, and neurodegeneration. Since its discovery more than 30 years ago, great strides have been made in understanding central aspects of O-GlcNAc signaling, including identifying thousands of its substrates and characterizing the enzymes that govern it. However, while many O-GlcNAcylated proteins have been reported, only a small subset of these change their glycosylation status in response to a typical stimulus or stress. Identifying the functionally important O-GlcNAcylation changes in any given signaling context remains a significant challenge in the field. To address this need, we leveraged chemical biology and quantitative mass spectrometry methods to create a new glycoproteomics workflow for profiling stimulus-dependent changes in O-GlcNAcylated proteins. In proof-of-principle experiments, we used this new workflow to interrogate changes in O-GlcNAc substrates in mammalian protein trafficking pathways. Interestingly, our results revealed dynamic O-GlcNAcylation of COPγ1, an essential component of the coat protein I (COPI) complex that mediates Golgi protein trafficking. Moreover, we detected 11 O-GlcNAc moieties on COPγ1 and found that this modification is reduced by a model secretory stress that halts COPI trafficking. Our results suggest that O-GlcNAcylation may regulate the mammalian COPI system, analogous to its previously reported roles in other protein trafficking pathways. More broadly, our glycoproteomics workflow is applicable to myriad systems and stimuli, empowering future studies of O-GlcNAc in a host of biological contexts.

Keywords: O-GlcNAc, glycoproteomics, SILAC, click chemistry, COPI vesicle trafficking, protein secretion

INTRODUCTION

O-linked β-N-acetylglucosamine (O-GlcNAc) is a major, dynamic post-translational modification (PTM), added by O-GlcNAc transferase (OGT) and removed by O-GlcNAcase (OGA) from serine and threonine residues of intracellular proteins (1–7). O-GlcNAc is broadly conserved among animals, plants and other organisms, and O-GlcNAcylation controls a wide range of cellular
functions, such as nutrient sensing, metabolism and gene expression (1–8). Importantly, aberrant O-GlcNAc cycling is also implicated in numerous human diseases, including cancer (2, 9–12), diabetes (13–16), cardiac dysfunction (17–20), and neurodegeneration (21–24).

Despite this broad pathophysiological significance, major questions about O-GlcNAc signaling remain. For example, O-GlcNAcylation regulates diverse cellular processes and modifies thousands of intracellular proteins, but only a small fraction of substrates change their glycosylation status in response to any given signal or condition (1–4, 25, 26). A central challenge in the field is to identify the most functionally relevant O-GlcNAc changes in response to a stimulus of interest. However, because O-GlcNAc is a transient and sub-stoichiometric PTM, it can be difficult to study with traditional molecular biology or genetics alone.

To address this challenge, we previously reported a two-step chemical biology method to tag and purify O-GlcNAc substrates from live mammalian cells (27–31). Briefly, cells are first metabolically labeled with a peracetylated N-azidocetylgalactosamine (GalNAz), a synthetic, azide-bearing monosaccharide that is non-toxic and cell-permeable (28, 32). GalNAz is accepted by sugar salvage and epimerase enzymes, resulting in the biosynthesis of a nucleotide-azidosugar, “UDP-GalNAz,” which is used by OGT to install an “O-GlcNAz” moiety onto its native substrates (28). O-GlcNAz can then be tagged via chemical ligation to an alkyn-functionalized probe. Azides and alkynes engage in a copper-catalyzed [3+2] cycloaddition, often called “click chemistry,” that proceeds rapidly under biocompatible conditions (33–36). The click reaction between O-GlcNAz moieties and the alknye probe provides exquisitely specific labeling of OGT substrates with useful handles (e.g., biotin, fluorophores) for downstream analysis (27–31). Because GalNAz treatment labels endogenous OGT substrates, it affords time-resolved tagging of O-GlcNAcylated proteins, without the need for a priori knowledge of their identities. We have previously used this strategy to dissect the functional role of O-GlcNAc in a variety of cell biological contexts (28–31).

We envisioned combining GalNAz labeling with quantitative proteomics to discover changes in O-GlcNAcylated proteins in response to physiological stimuli, stresses, or other cues. As a model cellular process for these proof-of-principle experiments, we selected protein trafficking. More than a third of mammalian proteins transit the secretory system to localize to, and recycle from, specific subcellular locations, including the endoplasmic reticulum (ER), Golgi, plasma membrane, endosomes, lysosomes, and the extracellular space (37–39). In most instances, dedicated protein machinery effects the formation and trafficking of vesicles between discrete locations, as in clathrin-mediated endocytosis from the plasma membrane to endosomes (40), coat protein complex II (COPII)-facilitated transport from the ER to Golgi (41–46), and COPI-mediated trafficking among the Golgi cisternae and from the Golgi to the ER (47–49). Properly regulated protein trafficking is critical for cell and tissue physiology, particularly in professional secretory cell types and organs, including the endocrine system.

Indeed, protein trafficking is essential in all eukaryotes and is dysregulated in a wide range of human diseases (44–46).

While the fundamental biochemical steps of vesicle assembly are relatively well understood for some systems (e.g., clathrin, COPII, and COPI), much less is known about how vertebrate cells dynamically adjust trafficking activity in response to developmental cues, fluctuating signals, metabolic demands, or stress (44–46). Interestingly, however, several studies have implicated O-GlcNAc in regulating multiple protein trafficking pathways. For example, key COPII proteins are O-GlcNAcylated (28, 50–53), and we recently demonstrated that specific glycosylation sites on Sec23A, a core COPII protein, are required for its ability to mediate collagen trafficking in both cultured human cells and developing vertebrate embryos (54). Other studies have indicated a role for O-GlcNAc in regulating synaptic vesicle trafficking and clathrin-mediated endocytosis as well (55–63). Taken together, these reports suggest that O-GlcNAc may be a broad regulator of protein trafficking. However, the extent and functional effects of O-GlcNAcylation in mammalian trafficking pathways remain largely uncharacterized.

Here, we leverage GalNAz metabolic labeling and quantitative proteomics to create a novel workflow for identifying stimulus-induced changes in O-GlcNAcylated proteins. In a pilot experiment, we used this glycoproteomics workflow to investigate the role of O-GlcNAc in mammalian protein trafficking. Our results indicate that COPγ1, an essential component of the COPII complex, is dynamically O-GlcNAcylated on up to 11 distinct sites under control conditions but deglycosylated upon perturbation of protein secretion. Our study is the first report of COPII protein O-GlcNAcylation and suggests that O-GlcNAc may regulate mammalian intra-Golgi and/or retrograde Golgi-to-ER protein trafficking. More broadly, we expect that our glycoproteomics strategy will be readily extensible to a wide spectrum of experimental stimuli, conditions and systems beyond protein trafficking, permitting the study of O-GlcNAc function in diverse biological contexts.

MATERIALS AND METHODS

Chemical Synthesis

Thiamet-G and Ac₄GalNAz were synthesized as described (28, 64) by the Duke Small Molecule Synthesis Facility. All other chemicals were purchased from Sigma-Aldrich unless otherwise indicated.

Cell Culture

Ramos cells were cultured in Roswell Park Memorial Institute medium (RPMI) containing 10% fetal bovine serum (FBS), 100 units/ml penicillin, and 100 µg/ml streptomycin in 5% CO₂ at 37°C. FL5.12 (parental N6 and XL4.1 lines) were cultured in RPMI containing 10% FBS, 100 units/ml penicillin, 100 µg/ml streptomycin, 55 µM β-mercaptoethanol, 2 mM L-glutamine, 10 mM HEPES and 500 µg/ml recombinant mouse IL-3 (eBioscience) in 5% CO₂ at 37°C.
Cell Viability Assays
Ten thousand Parental FL5.12 (N6) cells in 100 µl RPMI were seeded into clear-bottom 96-well plates and treated with a dose range of brefeldin A (BFA) for 4 or 24 h. Both MTS (Promega, CellTiter 96 AQeueous Proliferation Assay) and ATP (Promega, CellTiter-Glo Luminescent Cell Viability Assay) assays were performed according to the manufacturer’s instructions. Independent replicates were evaluated by a 2 × 2 analysis of variance (ANOVA), with BFA dose and treatment time as the independent factors. Post-hoc tests for differences between BFA doses and treatment times were conducted with Tukey’s honestly significant difference (HSD) test using SAS/IMP software, Version 13.0.0 (SAS Institute Inc.). Significance was defined as p < 0.05 (two-tailed).

Alkyne-Biotin Click Reactions and Affinity Purification
Cells were treated with 100 µM GalNAz up to 24 h prior to harvesting. After harvesting, cells were lysed in click buffer (1% Triton X-100, 1% SDS, 150 mM NaCl, 20 mM Tris pH 7.4) supplemented with protease inhibitors, 5 µM PUGNAc and 50 µM UDP to inhibit hexosaminidases and OGT, respectively. Click reactions were assembled on an ice bucket. The following reaction components were added, in order, to the listed final concentration: protein sample, 5 mM sodium ascorbate, 25 µM alkyne-biotin, 100 µM Tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine (TBTA), 1 mM CuSO4. Reactions were mixed, rotated gently at room temperature for 1 h and then quenched by addition of 10 mM EDTA (final). For immediate analysis, SDS-PAGE sample buffer was added directly to reactions. For further processing and affinity purification, unreacted alkyne-biotin was removed by methanol precipitation as follows. Reactions were mixed with ice-cold methanol (10:1 methanol:sample by volume). After mixing, samples were placed on dry ice or incubated at −80°C for 10 min to increase protein precipitation and then centrifuged at 17,000 g for 10 min at 4°C. Supernatants were removed and pellets were resuspended in methanol and placed on ice. This process was repeated a total of four times. After the final precipitation, the protein pellet was dissolved in 4 M guanidine in phosphate-buffered saline (PBS). Biotinylated proteins were captured from the samples by incubating overnight at 4°C with gentle rotation with NeutrAvidin beads (ThermoFisher, High Capacity NeutrAvidin Agarose). The following day, beads were washed three times with the following buffers, in order: 4 M guanidine in PBS, 5 M NaCl in H2O, 6 M urea in PBS, and 1% SDS in PBS. Captured proteins were eluted by boiling in 2X SDS-PAGE sample buffer. Resistant input samples in 4 M guanidine were bead-exchanged into SDS-PAGE buffer via spin column (BioRad, Bio-Spin 6).

Immunoblotting (IB)
IBs were performed via standard methods as previously described (54). The following primary antibodies were used: mouse monoclonal anti-tubulin (T6074, Sigma-Aldrich; 1:100,000), mouse monoclonal anti-biotin (B7653, Sigma-Aldrich, 1:2,000), mouse monoclonal anti-nucleoporin p62 (610498, BD Biosciences, 1:2,000), mouse monoclonal anti-COPY1 (sc-393977, Santa Cruz Biotechnology, 1:1,000), mouse monoclonal anti-O-GlcNAc antibody 18B10 (MA1-038, ThermoFisher; 1:1,000), mouse monoclonal anti-O-GlcNAc antibody RL2 (SC-59624, Santa Cruz Biotech; 1:500). The following secondary antibody was used: goat anti-mouse IgG (1030-05, horseradish peroxidase (HRP)-conjugated, SouthernBiotech; 1:10,000).

SILAC Labeling
RPMI 1640 medium lacking L-lysine and L-arginine (ThermoFisher) was supplemented with 10% dialyzed and heat-inactivated FBS (Corning), 1% penicillin/streptomycin and amino acids. “Heavy” medium was supplemented with 12.5 mg 13C6N4-arginine, 12.5 mg 13C6N2-lysine, and 5 mg proline per 500 ml. “Light” medium was supplemented with 12.5 mg arginine, 12.5 mg lysine, and 5 mg proline per 500 ml. Proline supplementation prevents conversion of arginine to proline (65). XL4.1 cells were passaged for at least 7 doublings in either heavy or light SILAC medium to achieve >99% isotope incorporation. Isotope incorporation was verified via MS at the Duke Proteomics Facility. Full proteomics data are available as Excel files in the Supplemental Material.

Subcellular Fractionation
Cells were washed once with cold PBS and resuspended in 5 ml of ice-cold Buffer A (1.5 mM MgCl2, 10 mM KCl, 10 mM HEPES, pH 7.9) supplemented with protease inhibitors, 5 µM PUGNAc and 50 µM UDP. Cells were lysed using a pre-chilled Dounce homogenizer and ~30 strokes with a tight pestle. Cell integrity was monitored using a hemocytometer. After Douncing, samples were centrifuged at 228 g for 5 min at 4°C, yielding a crude cytoplasmic fraction (supernatant) and a crude nuclear fraction (pellet). Crude nuclear fractions were resuspended in 3 ml of Buffer S1 (0.35 M sucrose, 0.5 mM MgCl2) supplemented with protease inhibitors, PUGNAc and UDP, layered over a cushion of Buffer S3 (0.88 M sucrose, 0.5 mM MgCl2) and centrifuged at 2,800 g for 10 min at 4°C to obtain a pure nuclear pellet. Crude cytoplasmic fractions were centrifuged at >400,000 g for 1 h at 4°C to obtain a pure cytoplasmic fraction (supernatant). Pure nuclear pellets were lysed in click buffer and pure cytoplasmic fractions were supplemented with appropriate concentrations of click buffer ingredients.

Proteomic Analysis of BFA-Induced Changes in O-GlcNacylated Proteins
XL4.1 cells were seeded at 500,000 cells/ml the day before treatment. Cells were treated with 100 µM GalNAz alone for 2 h, then 500 ng/ml BFA or DMSO (vehicle) was added, and cells were harvested 4 h later. Heavy- and light-labeled cells were pooled 1:1, washed twice with cold PBS and fractionated as above. Protein amounts were quantified by BCA Assay (ThermoFisher) and 2 mg of nuclear or cytoplasmic protein was processed further. Alkyne-agarose beads (Click Chemistry Tools) were washed three times in click buffer. Protein samples were precored with 150 µl bead volume of washed alkyne-agarose beads with gentle rotation for 2 h at room temperature. After
precipitating, supernatants were removed and combined with 50 μl of equilibrated alkyne-agarose beads, 5 mM sodium ascorbate, 100 μM TBTA and 1 mM CuSO₄. Reactions were rotated at room temperature for 2 h and then quenched by addition of 10 mM EDTA. Beads were washed sequentially with three 1 ml washes of each of the following: 1% SDS, 20 mM Tris pH 7.4; 1% SDS, 10 mM dithiothreitol (DTT), 20 mM Tris pH 7.4; 1X PBS; 8 M urea; 1X PBS; 6 M guanidine hydrochloride; 1X PBS; 5 M NaCl; 1X PBS; 10X PBS; 1X PBS; 20% isopropanol; 20% acetonitrile; 50 mM ammonium bicarbonate. Washed beads were stored at 4°C in 100 μl 50 mM ammonium bicarbonate until they were stored for on-bead trypsin digestion, LC-MS/MS analysis and quantification at the Duke Proteomics Facility.

**Sample Preparation and Nano-Flow Liquid Chromatography Electrospray Ionization Tandem Mass Spectrometry (LC-MS/MS) Analysis of SILAC Samples**

Samples immobilized on alkyne-agarose beads were washed with 50 mM ammonium bicarbonate, pH 8.0 and suspended in 30 μl 50 mM ammonium bicarbonate, pH 8.0 supplemented with 0.1% Rapigest SF surfactant (Waters). Samples were reduced with 5 mM DTT for 30 min at 70°C and free sulphydryls were alkylated with 10 mM iodoacetamide for 45 min at room temperature. Proteolytic digestion was accomplished by the addition of 500 ng sequencing grade trypsin (Promega) directly to the beads with incubation at 37°C for 18 h. Supernatants were collected following a 2-min centrifugation at 1,000 rpm, acidified to pH 2.5 with trifluoroacetic acid and incubated at 60°C for 1 h to hydrolyze the remaining Rapigest. Insoluble hydrolyzed surfactant was cleared by centrifugation at 15,000 rpm for 5 min. Samples were dried using vacuum centrifugation and resuspended in 20 μl of 2% acetonitrile/0.1% formic acid. Two microliters of each sample was subjected to chromatographic separation on a Waters NanoAquity UPLC equipped with a 1.7 μm BEH130 C₁₈ 75 μm I.D. X 250 mm reversed-phase column. The mobile phase consisted of (A) 0.1% formic acid in water and (B) 0.1% formic acid in acetonitrile. Following a 5 μl injection, peptides were trapped for 5 min on a 5 μm Symmetry C₁₈ 180 μm I.D. X 20 mm column at 20 μl/minute in 99.9% A. The analytical column was held at 5% B for 5 min, then switched in-line and a linear elution gradient of 5% B to 40% B was performed over 90 min at 300 nl/minute. The analytical column was connected to a fused silica PicoTip emitter (New Objective) with a 10 μm tip orifice and coupled to a QExactive Plus mass spectrometer through an electrospray interface. The instrument was set to acquire a precursor MS scan from m/z 375–1,600 with r = 70,000 at m/z 400 and a target AGC setting of 1e6 ions. In a data-dependent mode of acquisition, MS/MS spectra of the 10 most abundant precursor ions were acquired at r = 17,500 at m/z with a target AGC setting of 5e4 ions. Max fill times were set to 60 ms for full MS scans and 60 ms for MS/MS scans with minimum MS/MS triggering thresholds of 5,000 counts. For all experiments, fragmentation occurred with a higher-energy collisional dissociation setting of 27% and a dynamic exclusion of 60 s were employed for previously fragmented precursor ions.

Raw LC-MS/MS data files were processed in Mascot distiller (Matrix Science) and then submitted to independent Mascot database searches (Matrix Science) against SwissProt (Mus musculus taxonomy) containing both forward and reverse entries of each protein. Search tolerances were 5 ppm for precursor ions and 0.02 Da for product ions using trypsin specificity with up to two missed cleavages. Carbamidomethylation (+57.0214 Da on Cys) was set as a fixed modification, whereas oxidation (+15.9949 Da on Met) and O-GlcNAcylation (+203 Da on Ser/Thr) were considered variable modifications. All searched spectra were imported into Scaffold (Proteome Software) and protein confidence thresholds were set using a Bayesian statistical algorithm based on the PeptideProphet and ProteinProphet algorithms, which yielded a peptide and protein false discovery rate (FDR) of 1%.

SILAC data were processed using Rosetta Elucidator as previously described (66–68) with the following modifications. Database searching in Mascot used a SwissProt mouse database (downloaded on 4/21/11) with an equal number of reverse entries, 5 ppm precursor and 0.02 Da product ion tolerances and variable modifications on Met (oxidation), Arg (+10), and Lys (+8). Data were annotated at a 1% peptide FDR using the PeptideTeller algorithm. Quantification of labeled pairs required that both members were identified.

**Immunoprecipitation (IP)**

Cells were washed twice with cold PBS and lysed in IP lysis buffer (1% Triton X-100, 150 mM NaCl, 1 mM EDTA, 20 mM Tris-HCl pH 7.4) supplemented with protease inhibitors, 5 μM PUGNAC and 50 μM UDP. Lysates were probe-sonicated, cleared by centrifugation and quantified by BCA protein assay. IPs were performed on 1–5 mg total protein. Cleared lysates were adjusted to a final total protein concentration of ~1 mg/ml using IP lysis buffer. For every 1 mg of protein lysate used, 3 μg of mouse monoclonal anti-COPY1 (sc-393977, Santa Cruz Biotechnology) antibody was added and rotated overnight at 4°C. The following day, 50 μl equilibrated protein A/G UltraLink Resin (ThermoFisher) was added to the lysate and rotated at room temperature for 1 h. Beads were washed three times with 1 ml of IP lysis buffer and then eluted in 2X SDS-PAGE sample buffer with boiling. Eluents were analyzed via IB.

**Cloning**

The COPY1-myc-6xHis construct was generated by amplifying the open reading frame of the human COPY1 cDNA (Harvard PlasmID Repository) by PCR and ligating it into the HinfIII and NotI sites of pcDNA4/myc-6xHis (Invitrogen) using standard methods.

**Transfections**

293T cells plated at ~50% confluence were transfected the following day as previously described (54). In brief, 750 μl of prewarmed OPTI-MEM was placed into 1.5 ml tubes with 45 μl of TransIT-293 transfection reagent (Mirus), vortexed briefly, and incubated for 15 min at room temperature. Next, 15 μg of
human COPγ1-myc-6xHis DNA was added to the tube, vortexed briefly, and incubated for 15 min at room temperature. After the final incubation, the mixture was added dropwise to the cells. Cells were harvested 48 h after transfection.

Tandem Purification of COPγ1-myc-6xHis

293T cells transfected with COPγ1-myc-6xHis were treated 8 h prior to harvest with 50 µM Thiamet-G and 4 mM glucosamine to enhance O-GlcNAcylation. Cells were harvested in cold PBS and lysed in IP lysis buffer supplemented with 0.1% SDS, protease inhibitors, 5 µM PUGNAc and 50 µM UDP. Lysates were probe sonicated, cleared by centrifugation, and quantified by BCA protein assay according to the manufacturer’s instructions. Myc IPs were performed on ~100 mg of total protein for MS analysis. Cleared lysates were adjusted to a final protein concentration of 2 mg/ml using IP lysis buffer supplemented with 0.1% SDS, protease inhibitors, 5 µM PUGNAc, and 50 µM UDP. Three micrograms of mouse monoclonal anti-c-myc (9E10, BioLegend) per mg of total protein was added and rotated overnight at 4 °C. The following day, 50 µl of washed protein A/G UltraLink Resin (53133, ThermoFisher) was added and the mixture was rotated at room temperature for 1 h. Beads were washed three times with 1 ml of IP lysis buffer with 0.1% SDS and eluted twice in 500 µl using Ni-NTA wash buffer (8 M urea, 300 mM NaCl, 1% Triton X-100, and 5 mM imidazole) with rotation at room temperature. The two 500 µl elutions were pooled, 50 µl of washed 6xHisPur Ni-NTA resin (88223, ThermoFisher) was added to the eluate and the mixture rotated for 2 h at room temperature. The Ni-NTA resin was washed three times with 1 ml of Ni-NTA wash buffer and eluted in 8 M urea plus 250 mM imidazole.

LC-MS/MS Analysis of COPγ1 O-GlcNAcylation

Purified COPγ1-myc-6xHis was separated by SDS-PAGE and Coomassie-stained. Stained bands of the correct molecular weight were subjected to standard in-gel trypsin digestion (https://genome.duke.edu/sitesgenome.duke.edu/files/In-gelDigestionProtocolrevised_0.pdf). Extracted peptides were lyophilized to dryness and resuspended in 12 µl of 0.1% formic acid/2% acetonitrile. Each sample was subjected to chromatographic separation on a Waters NanoAquity UPLC equipped with a 1.7 µm BEH130 C18 75 µm I.D. X 250 mm reversed-phase column. The mobile phase consisted of (A) 0.1% formic acid in water and (B) 0.1% formic acid in acetonitrile. Following a 4 µl injection, peptides were trapped for 3 min on a 5 µm Symmetry C18 180 µm I.D. X 20 mm column at 5 µl/minute in 99.9% A. The analytical column was then switched in-line and a linear elution gradient of 5% B to 40% B was performed over 60 min at 400 nl/minute. The analytical column was connected to a fused silica PicoTip emitter (New Objective, Cambridge, MA) with a 10 µm tip orifice and coupled to a QExactive Plus mass spectrometer (Thermo) through an electrospray interface operating in data-dependent acquisition mode. The instrument was set to acquire a precursor MS scan from m/z 350 to 1,800 every 3 s. In data-dependent mode, MS/MS scans of the most abundant precursors were collected following higher-energy collisional dissociation (HCD) fragmentation at an HCD collision energy of 27%. Within the MS/MS spectra, if any diagnostic O-GlcNAc fragment ions (m/z 204.0867, 138.0545, or 366.1396) were observed, a second MS/MS spectrum of the precursor was acquired with electron transfer dissociation (ETD)/HCD fragmentation using charge-dependent ETD reaction times and either 30 or 15% supplemental collision energy for ≥2+ precursor charge states. For all experiments, a 60-s dynamic exclusion was employed for previously fragmented precursor ions.

Raw LC-MS/MS data files were processed in Proteome Discoverer (Thermo Scientific) and then submitted to independent Mascot searches (Matrix Science) against a SwissProt database (human taxonomy) containing both forward and reverse entries of each protein (20,322 forward entries). Search tolerances were 5 ppm for precursor ions and 0.02 Da for product ions using semi-trypsin specificity with up to two missed cleavages. Both y/b-type HCD and c/z-type ETD fragment ions were allowed for interpreting all spectra. Carbamidomethylation (+57.0214 Da on C) was set as a fixed modification, whereas oxidation (+15.9949 Da on M) and O-GlcNAc (+203.0794 Da on S/T) were considered dynamic mass modifications. All searched spectra were imported into Scaffold (v4.3, Proteome Software) and scoring thresholds were set to achieve a peptide FDR of 1% using the PeptideProphet algorithm. When satisfactory ETD fragmentation was not obtained, HCD fragmentation was used to determine O-GlcNAc residue modification, using the number of HexNAcs identified in combination with the number of serines and threonines in the peptide.

RESULTS

We designed a new quantitative glycoproteomics strategy to discover changes in O-GlcNAcylated proteins in response to physiological stimuli, stress, or other cues. In this workflow (Figure 1), cells are first labeled with “light” 12C6-14N2-l-lysine and 12C6-15N4-arginine or “heavy” 13C6-15N2-l-lysine and 13C6-15N4-arginine, in a standard stable isotope labeling of amino acids in cell culture (SILAC) quantitative proteomics protocol (69, 70). Next, all cells are metabolically labeled with a short pulse of GalNAz to prime the biosynthesis of UDP-GlcNAz. Then, one cell population is treated with the stimulus of interest, leaving the other as a control. All cells are then mixed, nuclear and cytoplasmic extracts are prepared by standard biochemical fractionation (to separate O-GlcNAc from secretory pathway glycans) and labeled O-GlcNAc substrates are covalently ligated to alkylene-functionalized agarose beads via a click reaction, permitting extremely stringent washing. Finally, the captured and washed glycoproteins are trypsinized on-bead, and the resulting peptides are analyzed by SILAC mass spectrometry (MS) proteomics, providing an unbiased quantitation of stimulus-dependent changes in O-GlcNAcylated proteins.

For our pilot glycoproteomics studies, we selected the murine pro-B cell line FL5.12, subclone XL4.1 (71–73), because it is a model system for B lymphocyte activation, a process that vastly expands the protein trafficking burden through cell proliferation.
FIGURE 1 | Glycoproteomics workflow. First, cells are stably labeled with either “heavy” (red) or “light” (blue) arginine and lysine to create two distinctly labeled populations, per standard SILAC protocols. Next, cells are treated with GalNAz for a brief period to allow incorporation into endogenous OGT substrates, while limiting the labeling of “housekeeping” proteins. One cell population is treated with a stimulus, inducing changes in O-GlcNAcylation, while the other remains an untreated control. Both cell populations are mixed, fractionated to remove secretory pathway glycans, and covalently ligated to an alkyne-functionalized agarose bead via a click reaction. After extremely stringent washing, the covalently captured glycoproteins are trypsinized on-bead and the resulting peptides are analyzed by quantitative SILAC MS proteomics.

and augmented immunoglobulin secretion (74, 75). Previous work has also indicated that lymphocyte activation induces dramatic changes in global O-GlcNAcylation (76–78). Taken together, these reports suggested that O-GlcNAc might regulate protein trafficking in activated lymphocytes. We incubated XL4.1 cells with GalNAz or vehicle only and captured labeled proteins using our glycoproteomics workflow. Initial MS analysis revealed the strong enrichment of many known O-GlcNAcylated proteins, including numerous nucleoporins (79) and host cell factor 1 (HCF1) (80–82) (Figure 2). We concluded that our method specifically captured O-GlcNAcylated proteins, as intended.

Next, we sought to use our glycoproteomics workflow to identify O-GlcNAcylation changes that are functionally important in protein trafficking. We reasoned that a short GalNAz pulse followed by a stimulus would afford the preferential labeling of de novo, stimulus-dependent changes in glycoproteins, whereas longer incubations would also label unchanging, background “housekeeping” glycoproteins, as evidenced by the enrichment of nucleoporins after long GalNAz incubation (Figure 2). We first verified that we could label endogenous XL4.1 glycoproteins with brief GalNAz incubations. We treated cells with GalNAz for various times and then reacted lysates with alkyne-biotin to label O-GlcNAc substrates. Anti-biotin immunoblot (IB) revealed that endogenous XL4.1 glycoproteins were labeled as early as 2 h after GalNAz treatment (Figure 3A). We therefore selected 2 h as a pre-stimulus GalNAz incubation time for our subsequent experiment. Next, as a model stimulus to perturb protein secretion, we selected brefeldin A (BFA), a well-characterized fungal metabolite that inhibits COPI and, secondarily, COPII vesicle trafficking (83–85). We hypothesized that secretory pathway disruption by BFA would trigger changes in O-GlcNAcylation events that...
FIGURE 2 | XL4.1 cells were treated with either DMSO vehicle or 100 µM GalNAz for 24 h and processed via the glycoproteomics workflow. To confirm enrichment, proteins with fewer than 25 spectral counts were excluded, and the remaining proteins were rank-ordered by the ratio of spectral counts from the vehicle and GalNAz samples (low to high). Table displays the top 25 proteins ranked this way. Commonly O-GlcNAcylated substrates (e.g., nucleoporins, HCF1) were identified exclusively in the GalNAz-treated samples, confirming the selective enrichment of endogenous OGT substrates by the workflow. Complete proteomics datasets are available as Supplemental Material.

| Protein Name | DMSO (total spectral counts) | GalNAz (total spectral counts) |
|--------------|------------------------------|-------------------------------|
| PRRC1        | 0                            | 48                            |
| SF3A1        | 0                            | 43                            |
| CCAR1        | 0                            | 35                            |
| HYOU1        | 0                            | 32                            |
| RBM26        | 0                            | 31                            |
| RBM14        | 0                            | 31                            |
| P66B         | 0                            | 31                            |
| UBQL2        | 0                            | 29                            |
| PRC2C        | 0                            | 28                            |
| MA2B1        | 0                            | 28                            |
| EP400        | 0                            | 27                            |
| NUP54        | 0                            | 27                            |
| RBM27        | 0                            | 27                            |
| MAVS         | 0                            | 26                            |
| HCFC1        | 1                            | 84                            |
| MILK2        | 1                            | 77                            |
| NU214        | 1                            | 55                            |
| UBP2L        | 1                            | 52                            |
| PICAL        | 1                            | 42                            |
| ARI1A        | 1                            | 40                            |
| PO210        | 1                            | 39                            |
| ERO1A        | 1                            | 36                            |
| CATB         | 1                            | 31                            |
| DIDO1        | 1                            | 27                            |
| TFR1         | 2                            | 50                            |

Frontiers in Endocrinology | www.frontiersin.org 7 October 2018 | Volume 9 | Article 606
To determine the lowest BFA dose that caused strong disruption of the secretory pathway, we treated apoptosis-sensitive parental FL5.12 N6 cells with a range of BFA concentrations for 4 or 24 h and measured cellular ATP and mitochondrial function. In these experiments, 500 ng/ml was the lowest BFA dose that caused significant toxicity after 24 h while having little effect on cell viability after only 4 h. We therefore selected 4 h of 500 ng/ml BFA as a treatment condition to disrupt protein trafficking without inducing the potentially confounding effects of downstream cell death.

We next performed proof-of-principle experiments with BFA and our glycoproteomic workflow. We treated SILAC-labeled XL4.1 cells with GalNAz for 2 h, followed by 500 ng/ml BFA (heavy-labeled cells) or vehicle control (light-labeled cells) for an additional 4 h. Then, we mixed the intact cells, derived nuclear and cytoplasmic subcellular fractions, captured GalNAz-labeled proteins and analyzed BFA-dependent changes in O-GlcNAcylated proteins. We calculated the fold-enrichment of every protein in our control (DMSO) vs. BFA-treated SILAC populations (1,253 nuclear and 792 cytoplasmic IDs). Overall, BFA barely altered the abundance of the vast majority of captured proteins, as expected, with 99% of both nuclear and cytoplasmic IDs changing <4-fold. However, 1 nuclear and 7 cytoplasmic proteins were enriched at least 4-fold in the BFA sample vs. control, and 8 nuclear and 3 cytoplasmic proteins were depleted at least 4-fold in the BFA-treated sample. Similar results were obtained in an independent biological replicate performed with the amino acid and treatment pairings reversed (i.e., heavy/DMSO, light/BFA).

We next applied stringent filters to the data from both biological replicates to identify candidate BFA-dependent changes in O-GlcNAcylated proteins. First, we compared BFA-induced fold-changes across biological replicates and retained only protein IDs with concordant changes (up or down) across replicates. Then, we retained only nuclear proteins with a fold-change magnitude >2, and only cytoplasmic proteins with a fold-change magnitude >1.5. After applying these filters, we identified 80 nuclear and 17 cytoplasmic proteins displaying consistent, BFA-dependent changes across both experiments. Interestingly, several of these proteins participate directly in protein trafficking, including the COPI protein COPγ1 (depleted 2.27- and 2.078-fold, respectively, from the BFA samples in the two biological replicates) and the retromer component...
FIGURE 4 | Glycoproteomics workflow detects global BFA-induced changes in O-GlcNAcylated proteins. Data for nuclear and cytoplasmic proteins are displayed as a log₂ transform of the ratio of detected intensities from the DMSO and BFA samples, as described previously (66–68). (A) In the first biological replicate, 1,252 nuclear and 792 cytoplasmic proteins were identified. (B) In the second biological replicate, 1,703 nuclear and 1,262 cytoplasmic proteins were identified. (C) The nuclear and cytoplasmic proteins exhibiting concordant BFA-dependent changes (up or down) across biological replicates were rank-ordered by the magnitude of the fold-change between DMSO and BFA samples, as described previously (66–68). Tables list the top 15 nuclear and cytoplasmic proteins from each biological replicate by this ranking. Some proteins appear in both tables but at different positions, reflecting rank-order in each case. (D) Representative MS spectra from one SILAC biological replicate, depicting light and heavy versions of the COPγ1 peptide SIATLATILLK. Complete proteomics datasets are available as Supplemental Material.
Vps35 (depleted 3.582- and 2.412-fold from the BFA samples), or are regulators of membrane protein quality control, such as the AAA+ ATPase torsinA (enriched 2.069- and 2.466-fold in the BFA samples) and the ubiquitin E3 ligase NEDD4, which is also a known O-GlcNAc substrate (depleted 3.838- and 3.058-fold from the BFA samples) (Figures 4C,D and Supplemental Material) (86–101). We concluded that our glycoproteomics workflow identified candidate BFA-dependent changes in O-GlcNAc substrates that may impact on protein trafficking.

From our filtered glycoproteomics data, we selected COPγ1 for further validation experiments because of its well-established role in protein trafficking. COPγ1 is a core component of the heteroheptameric COPI complex, which is recruited to Golgi membranes by the small GTPase ADP ribosylation factor 1 (Arf1) to mediate vesicle formation and trafficking within the Golgi or to the ER (49, 102, 103). COPγ proteins interact with cargo adaptors in the Golgi membrane, with Arf GTPase-activating proteins and with other COPI components in the coat itself (49, 88, 89, 92). COPγ is highly conserved across eukaryotes and is essential for in vitro COPI vesicle formation and for viability in budding yeast (49, 104, 105). While phosphorylation, arginine methylation, and ubiquitination of COPγ1 have been observed in several studies (106–116), O-GlcNAcylation of COPγ1 has never been reported.

To confirm our MS results with COPγ1, we GalNAz-labeled XL4.1 or Ramos cells (a human B cell line) in the presence or absence of BFA treatment, performed click reactions with alkynelabeled, purified O-GlcNAc substrates by streptavidin affinity chromatography and analyzed the results by IB (Figure 5A). Consistent with our glycoproteomics results, anti-COPγ1 IB indicated that BFA treatment reduced the O-GlcNAcylation of COPγ1 without causing dramatic effects on total COPγ1 levels (Figure 5A). To extend these results to natural O-GlcNAc, we immunoprecipitated (IP-ed) endogenous COPγ1 from XL4.1 or Ramos cells and observed that it was recognized by anti-O-GlcNAc monoclonal antibodies (Figure 5B). This signal was specific, because treatment of cells with Thiamet-G, a small molecule inhibitor of OGA (64), increased both global O-GlcNAc signal and anti-O-GlcNAc immunoreactivity of COPγ1 (Figure 5B). We concluded that endogenous COPγ1 is dynamically O-GlcNAcylated in mammalian cells under homeostatic conditions, and deglycosylated upon disruption of protein trafficking by BFA.

As a first step toward characterizing the function of COPγ1 O-GlcNAcylation, we expressed and purified epitope-tagged human COPγ1 to homogeneity from human cells and used MS to map O-GlcNAc-modified residues. We detected 11 O-GlcNAc moieties across six unique peptides and unambiguously assigned five glycosylation sites: T132, S134, T135, T552, and S554 (Figure 6A and Supplemental Material). COPγ1 is highly conserved between human and mouse (97.3% identical and 99.4% similar), and all candidate O-GlcNAc sites that we identified are identical between the orthologs (Figures 6A,B). The candidate O-GlcNAc sites occur in several regions of the COPγ1 protein, with most lying within the last HEAT repeat of the adaptin N-terminal domain or in the appendage domain (Figure 6B). The appendage domain interacts with ARFGAP2, which binds the α/β/ε COP1 subcomplex and influences vesicle uncoating, suggesting that O-GlcNAcylation in this domain could influence these functions (88). Finally, we modeled the observed O-GlcNAc sites onto crystal structures of COPγ1 in the COPI coat “triad” complex (Figures 6C,D (PDB: 5A1U) (117). Interestingly, the T552 and S554 glycosylation sites of COPγ1 lie close to COPβ1-binding interface and might impact on this
interaction, which is essential for COP1 function (117). Taken together, our BFA and MS results suggest that site-specific COP1 O-GlcNAcylation may promote or license its activity in the COPI pathway.

**DISCUSSION**

O-GlcNAc is a highly dynamic PTM that modifies thousands of nuclear, cytoplasmic and mitochondrial proteins. While the number of identified O-GlcNAc substrates continues to rise, the specific functions of O-GlcNAc on most proteins are elusive and assessing stimulus-triggered changes in O-GlcNAcylated proteins remains a significant challenge in the field. Here, we report a novel glycoproteomics workflow enabling the proteome-wide identification and quantification of changes in O-GlcNAc-modified proteins and use it to discover cycling O-GlcNAcylation of mammalian COP1 as a candidate regulatory event in Golgi protein trafficking.

Several proteomics-compatible approaches for enriching natural O-GlcNAc exist, including lectin weak-affinity chromatography (LWAC), chemical modification of O-GlcNAc moieties (e.g., β-elimination followed by Michael addition) and chemoenzymatic methods that harness an engineered galactosyltransferase (9, 59, 81, 82, 118–134). Each of these is a well-established and powerful tool for elucidating O-GlcNAc signaling. Our glycoproteomics workflow leverages GalNAz metabolic labeling, which complements these methods in important ways. In our approach, a pulse of GalNAz is added to cultured cells only shortly before the stimulus of interest, permitting the preferential enrichment and characterization of relatively new glycosylation changes. Therefore, our workflow provides time resolution and reduces the labeling of long-lived, unchanging O-GlcNAc moieties (e.g., on the nuclear
pore complex) that could otherwise dominate the proteomics results (Figure 2). In addition, our workflow affords the covalent capture of O-GlcNAcylated proteins onto a solid matrix, allowing extremely stringent washing conditions to remove unglycosylated proteins. Although the individual components of our strategy have been reported previously, we have assembled them into a new and optimized workflow in which the proteome-wide profiling of stimulus-dependent O-GlcNAc changes in SILAC-labeled cells can be performed by one worker in as little as 1 week. Moreover, our workflow can be implemented in any cell type or organism that supports azidosugar and SILAC labeling, and can be used to study a wide range of stimuli, stresses or other experimental comparisons. We anticipate that this method will be a useful addition to the quantitative analysis of O-GlcNAc signaling.

In pilot experiments, we used our workflow to address the role of O-GlcNAcylation changes in mammalian protein trafficking, using BFA as an established tool compound. These studies identified the dynamic glycosylation of COPγ1 in mammalian cells (Figures 4–6 and Supplemental Material), and validation experiments demonstrated that endogenous COPγ1 is reversibly modified by natural O-GlcNAc at least 11 sites, confirming the utility of our approach in characterizing native signaling pathways (Figures 5, 6). (We note that numerous Golgi proteins partitioned to the "nuclear" sample in our fractionation procedure, likely explaining the presence of COPγ1—see full proteomics datasets in the Supplemental Material).

COPI trafficking relies on the guanine nucleotide exchange factor GDP1 to exchange GDP for GTP on Arf1 (47–49, 102, 103). Arf1 undergoes a conformational change upon GTP binding, inserting an N-terminal amphipathic α-helix into the Golgi membrane (49, 102, 103). Membrane-bound Arf1 then recruits the stable heteroheptameric COPI coat complex, which includes COPγ1 (49, 102, 103, 135). The assembling COPI heteroheptamers also undergo a major conformational change, promoting oligomerization of the coat complex and eventual vesicle formation and scission (117, 135, 136). BFA perturbs protein trafficking by stabilizing an abortive intermediate of the Arf1 complex, disrupting both the COPI and, subsequently, the COPII pathways (83–85).

Our results suggest that COPγ1 glycosylation may regulate protein trafficking within or from the Golgi. Consistent with this hypothesis, a prior proteomics study identified a putative biochemical interaction between OGT and the COPI component COPγ (137). The authors proposed that O-GlcNAc might govern intra-Golgi vesicle transport, although no direct glycosylation of any COPI protein was demonstrated (137). The precise biochemical and functional effects of O-GlcNAcylation on COPγ1 remain to be determined, but our results, combined with prior reports, suggest several possibilities. First, because O-GlcNAc can regulate protein-protein interactions in a variety of contexts (138), glycosylation may affect the interaction of COPγ1 with specific binding partners, such as COPβ1, COPγ, Arf1, or p24 cargo adaptors (49, 105, 139–143). Consistent with this notion, our MS site-mapping revealed O-GlcNAcylation on two sites, T552 and S554, located in close proximity to the interface with COPβ1 in the COPI triad structure (Figures 6C,D) (117). Addition of one or more bulky O-GlcNAc moieties in this region of COPγ1 may alter this interaction, which is essential for COPI function. Second, O-GlcNAcylation of COPγ1 may promote or inhibit one of the significant conformational changes that occur during COPI coat assembly (49, 102, 103, 117, 135, 136, 142, 143). Third, O-GlcNAcylation of COPγ1 may regulate the membrane recruitment of the heteroheptameric complex. We have previously demonstrated an analogous role for O-GlcNAc signaling in the COPII pathway, as OGA inhibition impairs the membrane recruitment of the COPII proteins Sec31A and Sec23A (54). Fourth, O-GlcNAc may regulate COPγ1 through cross-talk with other PTMs. Interestingly, five of the candidate O-GlcNAc sites we identified on COPγ1 (S356, S554, T718, T723, and S725) are also reported phosphorylation sites (144). Therefore, COPγ1 function may be regulated by the well-documented, complex interplay between O-GlcNAcylation and O-phosphorylation (2, 145–149). Our site-mapping data have paved the way for future studies to test these hypotheses, and experiments with single and compound glycosylation site mutants are currently underway to determine the impact of COPγ1 O-GlcNAcylation in live-cell trafficking assays.

While many excellent studies have dissected the structures and functions of the core COPI machinery (49, 142, 143), much less is known about how this critical pathway is regulated by mammalian cells in response to rapidly changing physiological and pathological signals. PTMs likely serve as one important mode of COPI regulation. Indeed, several proteomics studies have reported phosphorylation, ubiquitination and arginine methylation of COPγ in particular (107–116), and one study provided functional evidence that phosphorylation of COPβ and COPγ influences coatomer assembly or membrane recruitment (106). Therefore, COPI trafficking may be governed in part by COPγ PTMs. Our results indicate that O-GlcNAc may be a functionally important PTM in the COPI system as well. Moreover, we detected putative BFA-dependent O-GlcNAc changes on proteins operating in distinct parts of the secretory pathway, including Vps35, torsinA, and NEDD4 (Figure 4), and previous studies have implicated O-GlcNAcylation in other vesicle transport pathways beyond COPI as well (28, 50–63). Based on these observations, we propose that O-GlcNAcylation may be a widespread mode of dynamic regulation in mammalian protein trafficking.

**DATA AVAILABILITY STATEMENT**

All datasets generated in this study are included in the manuscript and supplementary files.

**AUTHOR CONTRIBUTIONS**

NC, ES, and MB contributed conception and design of the study. NC, PL, and TS performed experiments and prepared samples. NC and ES performed and analyzed glycoproteomics
FUNDING

This work was supported by a Scholar Award from the Rita Allen Foundation, a Research Grant from the Mizutani Foundation for Glycoscience and NIH grant R01GM117473 to MB.

ACKNOWLEDGMENTS

We thank Dr. Matthew Foster (Duke University Proteomics and Metabolomics Core Facility) for help with SILAC data analysis and members of the Boyce Lab for helpful discussion and reagents.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2018.00606/full#supplementary-material
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