Chapter 4

Deciphering the Importance of *O*-GlcNAc Glycosylation Using Chemical Tools

4.1 *O*-GlcNAc Glycosylation of Proteins

Although the human genome only contains between 19,000 and 20,000 genes,[1] cells have developed many mechanisms to increase the chemical diversity of protein structures. Methods of protein diversification can occur both before protein translation through the splicing of messenger RNA molecules and afterwards through chemical changes of amino acid residues, known as post-translational modifications (PTMs). Collectively, these diversifying pathways exponentially increase the number of individual protein chemical structures or “proteoforms.”[2] In turn, structural diversity provides a mechanism to increase functional diversity, allowing cells to adapt to a variety of internal and external stimuli.

One type of PTM known as *O*-GlcNAc glycosylation or *O*-GlcNAcylation is remarkably suited as an environmental response element.[3-7] This modification utilizes uridine 5’-diphospho-*N*-acetylglucosamine (UDP-GlcNAc) to attach a single β-linked GlcNAc (GlcNAc) to Ser or Thr residues of many different proteins (Figure 4-1). Because the biosynthesis of UDP-GlcNAc incorporates precursors from nucleotide, carbohydrate, amino acid, and lipid biosynthesis,[5] it has been hypothesized that *O*-GlcNAcylation functions as an integrating signal for global metabolism,[8-10] allowing it to directly connect environmental conditions like nutrient availability with protein function.

O-GlcNAcylation is uncommon in both its localization, as one of the few known intracellular glycosylation events, and its enzymatic cycling.[11] PTMs

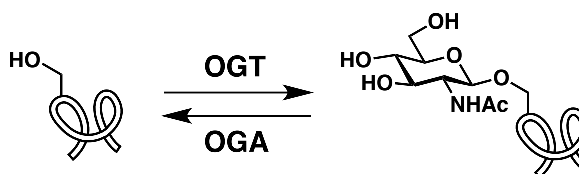

Figure 4-1. *O*-GlcNAc cycling on proteins. *O*-GlcNAc is added to Ser or Thr residues on intracellular proteins by OGT using UDP-GlcNAc and removed by OGA.

generally rely on a highly regulated network of writing and erasing enzymes. For example, protein phosphorylation occurs via a network of hundreds of kinases and phosphatases, each with their own substrate specificities. However, *O*-GlcNAcylation does not occur in such a partitioned fashion. Instead, only two enzymes have been discovered to cycle the *O*-GlcNAc PTM: *O*-GlcNAc transferase (OGT) and *O*-GlcNAcase (OGA). These proteins work in an extremely widespread fashion, with a current tally of over 4,000 putatively *O*-GlcNAcylated proteins.[12] Unsurprisingly, no ubiquitous consensus sequence has been found for OGT recognition,[13] highlighting the promiscuity of OGT and the difficulty in predicting *O*-GlcNAc sites.[14] Interestingly, recent reports have further expanded the substrate scope of OGT to Cys residues as so-called *S*-GlcNAcylation events.[15, 16]

Given the breadth of *O*-GlcNAcylation substrates, this PTM accordingly has far-reaching effects on a variety of biological processes, including transcription, translation, signal transduction, autophagy, protein homeostasis, circadian rhythm, and metabolism.[8, 17-22] The molecular mechanisms by which *O*-GlcNAcylation exerts these different activities are also diverse. For example, *O*-

GlcNAcylation can compete with phosphorylation either directly by modification of the same residue or indirectly by modification of adjacent sites. The former mechanism can be observed on endothelial nitric oxide synthase (eNOS), in which the native Akt phosphorylation site Ser-1177 is blocked by *O*-GlcNAcylation during hyperglycemia to impair its activity.[23] The latter can be found on the tumor suppressor protein p53, on which *O*-GlcNAcylation at Ser-149 prevents phosphorylation at Ser-155 to prevent proteolytic targeting and degradation.[24] *O*-GlcNAcylation can also attenuate or countermand the activity of distal phosphorylation events. This phenomenon was detected on the transcription factor cAMP response element binding (CREB), where *O*-GlcNAcylation at Ser-40 is induced specifically on activated, Ser-133 phosphorylated CREB to lessen neuronal activity-dependent gene expression.[17] *O*-GlcNAcylation can also block protein-small molecule or protein-protein interactions. For example, phosphofructokinase 1 (PFK1) is *O*-GlcNAcylated at Ser-529 in the binding pocket of its allosteric activator fructose-2,6-bisphosphate (FBP), hindering the formation of the active tetrameric form of the enzyme.[8] In stem cells, transcription factor sex determining region Y-box 2 (Sox2) is *O*-GlcNAcylated at Ser-248, disrupting its interaction with poly-ADP ribose polymerase 1 (PARP1) to control self-renewal and differentiation.[25] Conversely, *O*-GlcNAcylation can promote protein-protein interactions; in the case of signal transducer and activator of transcription 5 (Stat5), *O*-GlcNAcylation at Thr-92 facilitates its interaction with CREB-binding protein (CBP) to enable gene

transcription.[26] In all of these cases, the delineation of the exact site of *O*-GlcNAcylation was instrumental in discovering the functional importance of the PTM. Therefore, it is crucial that robust methods be developed to enable *O*-GlcNAc site identification to better understand the roles of this modification throughout diverse biological processes.

4.2 Methods to Identify Sites of *O*-GlcNAcylation

4.2.1 Complications in the Analysis of *O*-GlcNAcylation

Identifying sites of *O*-GlcNAcylation is complicated by a number of biological and technical factors. Like other PTMs, protein *O*-GlcNAcylation is substoichiometric, making site identification on proteins that may be already poorly abundant even more difficult.[12] During MS analysis, signal intensity from *O*-GlcNAcylated peptides suffers from ion suppression in the presence of unmodified peptides.[27] Furthermore, the *O*-GlcNAc moiety is readily labile for common MS/MS ionization techniques used for PTM analysis like collision-induced dissociation (CID) and higher-energy collisional dissociation (HCD).[28] Efforts on two fronts have helped to overcome these obstacles. First, the newer ionization technique known as electon transfer dissociation (ETD) has been developed as a milder MS/MS method that allows for the sequencing of peptides without loss of the *O*-GlcNAc group.[29] Second, a number of different enrichment techniques have been produced to both concentrate *O*-GlcNAcylated substrates and separate them from unmodified proteins and peptides.[30-32] These techniques

can be broadly separated into three categories: affinity enrichment, group replacement, and chemical functionalization.

4.2.2 Affinity Enrichment of *O*-GlcNAcylated Substrates

One chromatographic method to enrich *O*-GlcNAcylated substrates is known as lectin weak affinity chromatography (LWAC).[30] This method capitalizes on the affinity of natural plant proteins known as lectins for specific carbohydrate structures. In this process, a mixture of modified and unmodified substrates are isocratically passed over a chromatographic column that has wheat germ agglutinin (WGA) covalently attached to the stationary phase. This protein can weakly bind to terminal GlcNAc and sialic acid residues, causing these peptides to be retarded on the column compared to unmodified peptides. The lagging edge of the eluent is kept as the enriched *O*-GlcNAc fraction. As an alternative, succinylated WGA (sWGA) has been developed to no longer bind to sialic acid residues, providing further specificity for *O*-GlcNAcylated proteins.[33] However, this method also enriches for *N*-linked glycans that contain terminal GlcNAc moieties, complicating MS analysis. This can be ameliorated by pre-treatment of samples with PNGase F, which removes all *N*-linked glycans.[34] The natural binding mechanism for WGA requires multiple glycan structures, causing the structure to have a much weaker affinity for single *O*-GlcNAc groups (K_D = ~10 mM for the free monosaccharide).[12] Therefore, longer columns (upwards of 3 m)[31] and/or multiple chromatographic enrichments[32] are often necessary to sufficiently enrich *O*-GlcNAcylated substrates. Nevertheless, this method has

been employed successfully since its development in 2006 in numerous cases to identify sites of *O*-GlcNAcylation.[30-32] The most successful application of this method in combination with downstream high pH reverse phase peptide fractionation identified 1,750 sites from murine synaptosomes, proving its usefulness as a method to identify sites of the *O*-GlcNAc modification.[32] However, the method has only been reported by a single laboratory, so it remains to be seen if LWAC will be more widely embraced by the community.

A second method of affinity enrichment using *O*-GlcNAc-specific antibodies has also been employed to identify *O*-GlcNAcylation sites.[28] A number of antibodies that recognize *O*-GlcNAc have been generated in the past, with the RL-2[35] and CTD110.6[36] antibodies being the most widely used. However, they generally suffer from either specificity for certain *O*-GlcNAcylated structures like RL-2 for nuclear pore complex proteins or recognition of other terminal GlcNAc moieties as is the case for CTD110.6, precluding their use for enriching all *O*-GlcNAcylated substrates over other GlcNAc-modified structures. Recently, three new general *O*-GlcNAc antibodies have been developed and used originally to enrich *O*-GlcNAcylated proteins.[37] Interestingly, the immunoprecipitated proteins differed for each antibody, highlighting the need to use a combination of these antibodies to enrich *O*-GlcNAcylated proteins in an unbiased fashion. In a subsequent report, enriched proteins from immunoprecipitates of HEK-293T cell lysate using all three antibodies were subjected to MS analysis, where 172 *O*-GlcNAcylated peptides were observed.[28]

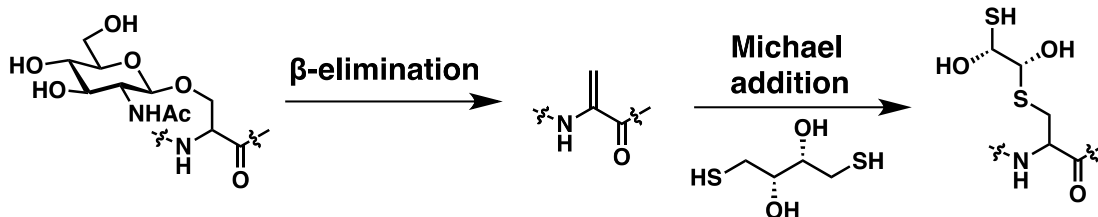

Figure 4-2. Beta-elimination and Michael addition (BEMA). BEMA occurs first by removal of *O*-GlcNAc using basic conditions to produce an α,β-unsaturated carbonyl group, which can then react with nucleophiles like DTT by Michael addition.

4.2.3 *O*-GlcNAc Enrichment by Replacement

The relative lability of the β-glycosidic linkage of *O*-GlcNAc has been exploited in an enrichment protocol termed β-elimination Michael addition (BEMA, Figure 4-2).[38-40] Originally designed to map phosphorylation sites, this method uses mild basic conditions to cause the elimination of *O*-GlcNAc on Ser and Thr residues and form an α,β-unsaturated carbonyl group, which can then be modified using a sulfhydryl nucleophile like DTT or biotinylated cystamine.[40, 41] Also known as BEMAD when using DTT, this method allows for enrichment of previously *O*-GlcNAcylated proteins and peptides via thiol-capture resin. Moreover, DTT isotopically labeled with ^{2}H can be used to differentiate samples for quantification.[42] DTT is stable to CID MS/MS fragmentation, eliminating the need for specialized MS techniques during peptide sequencing. BEMAD can suffer from cross-reactivity, as natural phosphorylated residues as well as alkylated Cys residues produced by reaction with iodoacetamide prior to MS analysis can also undergo β-elimination under stringent conditions.[40] However, treatment of samples with phosphatase prior to analysis and optimization of BEMAD

conditions have improved selectivity.[12] BEMAD has been applied in a variety of biological circumstances to identify *O*-GlcNAcylation sites, including in skeletal muscle, isolated proteasomes, and purified mitochondria.[41-43]

4.2.4 *O*-GlcNAc Enrichment by Chemical Functionalization

The third major method to enrich for *O*-GlcNAcylated substrates is through the chemical functionalization of the GlcNAc moiety. The structure of the GlcNAc residue is readily susceptible to direct modification; therefore, two main avenues have been developed to install reactive groups at the *O*-GlcNAc site: metabolic oligosaccharide engineering (MOE) and chemoenzymatic labeling.

MOE, also known as metabolic labeling, uses the endogenous biosynthetic machinery of the cell to incorporate non-natural sugar analogs that contain a chemical handle for further derivatization.[44-46] A number of different functional handles have been used to modify *O*-GlcNAc sites, including azide, alkyne, cyclopropene, and isonitrile groups.[47-50] This method does suffer from some drawbacks, including competition between natural and non-natural substrates leading to substoichiometric incorporation of the functional tag as well as crosstalk between biosynthetic salvage pathways causing the functional tag to be incorporated into a number of different glycan structures. This is epitomized by the discovery that UDP-galactose 4'-epimerase (GALE) interconverts UDP-GlcNAc and UDP-*N*-acetylgalactosamine (GalNAc) as well as the corresponding non-natural sugars.[51] In fact, treatment of cells with peracetylated *N*-azidoacetylgalactosamine (Ac_4GalNAz, Figure 4-3) facilitated labeling of *O*-

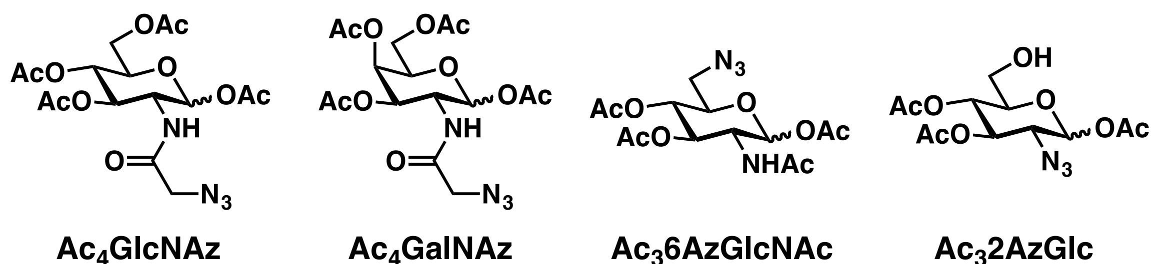

Figure 4-3. Common carbohydrates used in MOE.

GlcNAcylated structures better than treatment with the GlcNAc analog. Moreover, the non-natural sugar can incorporate into any glycan that utilizes GlcNAc, including *N*-linked glycans. Recent advances have been made to provide chemical probes that demonstrate selectivity for *O*-GlcNAc over other structures. For example, installation of the azide group at the C6 position of GlcNAc to produce peracetylated 6-azido-6-deoxy-*N*-acetylglucosamine ($Ac_4$6AzGlcNAc)[52] or at the C2 position of glucose (Glc) to produce acetylated 2-azido-2-deoxy-glucose ($Ac_3$2AzGlc)[53] provided labeling of *O*-GlcNAcylated substrates with minimal surface labeling, suggesting that these probes do not incorporate into *N*-linked glycans like previous probes. However, neither of these specific probes has been used to map sites of *O*-GlcNAcylation. In any case, the installed functional handle can then be elaborated using bioorthogonal chemistry to install an enrichment marker, which is discussed further below.

An alternative way to install a chemical handle for functionalization is through the use of a permissive enzyme via chemoenzymatic labeling (Figure 4-4).[12, 54, 55] In this technique, a mutated form of bovine galactosyltransferase (Y289L GalT)[56]

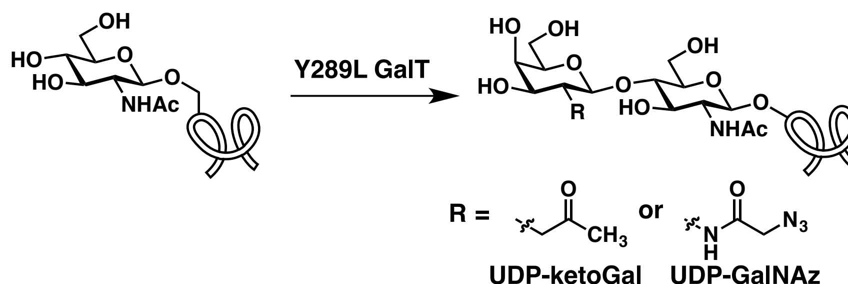

Figure 4-4. Chemoenzymatic labeling. *O*-GlcNAcylated substrates are modified by Y289L GalT with a non-natural UDP-galactose sugar. This approach has been used to append a ketone (UDP-ketoGal) or an azide (UDP-GalNAz) functional handle.

is used to modify terminal GlcNAc moieties with a non-natural UDP-galactose (Gal) or UDP-GalNAc sugar. This method was first used to install a ketone handle with UDP-2-acetonyl-2-deoxygalactose (UDP-ketoGal)[55, 57, 58] and more recently has been employed with the azide-containing UDP-GalNAz.[59] This method has benefits over MOE including the stoichiometric addition of the functional handle, the lack of perturbation of the system by addition of high concentrations of exogenous carbohydrates, and the specificity for GlcNAc structures. As with WGA, Y289L GalT will recognize any terminal GlcNAc moiety,[56] but treatment of samples with PNGase F to remove *N*-linked glycans or subcellular fractionation eliminate this cross-reactivity. As with MOE, this method installs a functional handle that allows for further derivatization and enrichment.

Currently, the majority of enrichment tags for *O*-GlcNAc site identification are installed onto an azide- or alkyne-tagged version of GlcNAc using copper-catalyzed azide-alkyne cycloaddition (CuAAC). However, a variety of enrichment tags have been used. For example, addition of 4-pentynyl phosphate has been

used in combination with chemoenzymatic labeling to install a phosphorylation mimicking tag that can be purified by titanium dioxide affinity chromatography, revealing 42 sites of *O*-GlcNAcylation from mouse brain lysate.[60] In a second approach using chemoenzymatic labeling, ethynylbenzaldehyde was added via CuAAC and used with hydrazide resin for peptide enrichment; however, this method was validated only with α-crystallin.[61] Finally, a third method utilizing metabolic labeling reacted modified proteins directly to alkyne-containing agarose resin.[62] After on-bead digestion and release of the modified proteins by β-elimination revealed 185 modification sites from HEK-293T lysate.

The most common enrichment method used with MOE and chemoenzymatic labeling is the installation of a biotin group combined with streptavidin affinity purification. One of the first instances of this method to identify *O*-GlcNAc sites was employed in our own lab in combination with chemoenzymatic labeling using UDP-ketoGal.[58] Here, 20 *O*-GlcNAcylated peptides were discovered from rat brain lysate, and incorporation of a peptide isotopic labeling technique known as dimethyl labeling allowed for quantification of modification dynamics on a subset of these sites. Although successful here, the relatively large size of the biotin tag likely hinders peptide ionization and sequencing by MS. Therefore, newer techniques have explored the incorporation of cleavable biotin moieties to reduce the residual size of the appended tag.

The first cleavable biotin tag used to map *O*-GlcNAc sites incorporated a 2-nitrobenzyl group, which can be selectively cleaved by UV irradiation with 365

nm light.[27] This tag leaves only the aminomethyltriazolyl group appended to GalNAz, significantly decreasing the added size from biotinylated compared to non-cleavable variants. Moreover, the amine residue imbues the peptide with an additional positive charge, which can facilitate MS sequencing by ETD. However, cleavage by UV irradiation was shown to be incomplete.[27] Nevertheless, this approach was successfully applied to both purified mitotic spindles and cerebrocortical brain tissue to identify 141 and 458 *O*-GlcNAc sites, respectively.[63, 64]

A second cleavable tag uses the diphenylsilyl diether group, which can be easily removed by mild acid.[65, 66] After reaction with this tag also containing a dibrominated core to provide an isotopic signature in MS1, metabolically labeled proteins were immobilized on streptavidin resin and subjected to on-bead digestion. The remaining modified peptides were cleaved by mild acid treatment and sequenced, providing 357 unique glycopeptides modified by either GalNAz or *N*-azidoacetylmannosamine (ManNAz).[66] This method was recently expanded to use a mixture of ^{1}H- and ^{2}H-labeled probes that also present an isotopic signature in MS.[67] Here, 1765 mono- and di-*O*-GlcNAcylated mass signatures were identified over 15 samples corresponding to 379 uniquely *O*-GlcNAcylation sites. However, 36% of the modified peptides were only identified by the isotopic signature and were unable to be sequenced, underscoring the difficulty in sequencing *O*-GlcNAcylated peptides. Nevertheless, these results collectively demonstrate that the use of a cleavable

biotin tag is a useful approach to enrich for *O*-GlcNAcylated substrates and map modification sites. Newer methods should take advantage of benefits from multiple approaches like minimizing the residual tag left after cleavage while simultaneously improving on shortcomings of current approaches like incomplete cleavage and poor ETD fragmentation.

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