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O-GlcNAcomics – Revealing Roles of O-GlcNAcylation in Disease Mechanisms and Development of Potential Diagnostics

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Abstract

O-linked- β -*N*-acetylglucosamine (*O*-GlcNAc) is a dynamic post-translational modification of the 3'-hydroxyl groups of serine or threonine residues of nuclear, cytoplasmic, and mitochondrial proteins. The cycling of this modification is regulated in response to nutrients, stress, and other extracellular stimuli by the catalytic activities of *O*-GlcNAc transferase and *O*-GlcNAcase. *O*-GlcNAc is functionally similar to phosphorylation and has been demonstrated to play critical roles in numerous biological processes, including cell signaling, transcription, and disease etiology. Since its discovery nearly thirty years ago, studies have demonstrated that the *O*-GlcNAc is highly abundant and widespread, like phosphorylation however, the development of methodologies to study *O*-GlcNAc at the site level has been challenging. Recently, a number of studies have overcome these challenges and describe new tagging, enrichment, and mass spectrometric-based approaches to study *O*-GlcNAc in terms of its site identification, stoichiometry, and dynamics on proteins. The development of these methods are key for elucidation of *O*-GlcNAc's functional crosstalk with phosphorylation and other PTMs, and will serve to provide the necessary information for the development of site-specific antibodies, which will aid in the determination of a particular protein's site specific function. In this review, we describe these methods and summarize results obtained from them demonstrating the roles of *O*-GlcNAc in diabetes, cancer, Alzheimer's, and in learning and memory, while also describing how these new strategies have implicated *O*-GlcNAc as a potential diagnostic for the screening of patients for prediabetes.

Keywords

Alzheimer's; Cancer; Diabetes; Glycomics; O-GlcNAc

Introduction

O-GlcNAcylation (termed *O*-GlcNAc) is the addition of a single β -*N*-acetylglucosamine (GlcNAc) moiety to the hydroxyl groups of specific serine and/or threonine amino acids of target proteins. *O*-GlcNAc is a dynamic and inducible post-translational modification occurring in both nuclear and cytoplasmic compartments. However, unlike traditional

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glycosylation, *O*-GlcNAc is not generally elongated into a complex glycan chain. *O*-GlcNAc is similar to phosphorylation in terms of its abundance on proteins and its dynamic nature, and interestingly, on several sites, *O*-GlcNAcylation and phosphorylation are mutually exclusive. This crosstalk with phosphorylation gives *O*-GlcNAc the ability to modulate various phosphorylation-dependent signaling pathways (eg., insulin signaling). *O*-GlcNAc plays an important role in the regulation of many biological processes including, but not limited to, cell cycle progression, cell signaling, transcription, translation, and various aspects of metabolism (for reviews see [1–3]). *O*-GlcNAc plays significant roles in the progression and etiology of various diseases including diabetes, cancer, and Alzheimer's. As a result, understanding how *O*-GlcNAc is regulated has been the focus of numerous studies.

The initial regulation of *O*-GlcNAc occurs via flux through the hexosamine biosynthetic pathway (HBP). Approximately 2–5% of total intracellular glucose is funneled into the HBP [4–7]. After a series of enzymatic steps, glucose is ultimately converted into uridine diphospho-*N*-acetylglucosamine (UDP-GlcNAc), which serves as the substrate donor for protein *O*-GlcNAcylation. Flux through the HBP is comprised of various metabolic inputs including glucose, amino acid, fatty acid, and nucleotide metabolism, which collectively serve to modulate UDP-GlcNAc levels. Substrate availability has an effect on *O*-GlcNAc transferase, as its activity and specificity is extremely sensitive to the levels of UDP-GlcNAc [8]. Therefore, *O*-GlcNAc is a major sensor of the nutrient and metabolic state of the cell.

Briefly, *O*-GlcNAcylation is enzymatically regulated by the concerted activities of two enzymes: namely, uridine diphospho-*N*-acetylglucosamine:polypeptide β -*N*-acetylglucosaminyltransferase (*O*-GlcNAc transferase [OGT]) and β -*N*-acetylglucosaminidase (*O*-GlcNAcase [OGA]). OGT utilizes UDP-GlcNAc as the sugar donor for *O*-GlcNAcylation of target proteins [9, 10], while OGA catalyzes the removal of the modification [11, 12]. To date, more than three thousand proteins have been shown to be *O*-GlcNAcylated. An obvious question is how can only two enzymes regulate the *O*-GlcNAcylation of so many proteins in a highly specific and selective manner? The answer lies in results obtained from studies demonstrating that OGT and OGA have many different binding partners *in vivo*, giving these enzymes the ability to generate transient holoenzyme complexes presumably with different specificities and selectively towards protein substrates [8, 13–15].

Identification of *O*-GlcNAc proteins and, more importantly, the determination of their modification sites are of the utmost importance when attempting to delineate how site-specific *O*-GlcNAcylation can regulate a protein's function and play roles in disease progression. Early studies attempting to accomplish this task were met with many difficulties, including lability of the modification, low stoichiometries, ion suppression during mass spectrometry analysis, and poor enrichment methods for *O*-GlcNAc, the main contributing factors in hindering the advancement of the *O*-GlcNAc field when compared to that of the site specific data that can be generated when assessing phosphorylation[16–19]. However, in recent years, new methods have overcome these problems and expanded our knowledge of *O*-GlcNAc by identifying hundreds of novel *O*-GlcNAcylated proteins and *O*-

GlcNAc sites, as well as by revealing insights into its stoichiometries. Interestingly, with the use of these methods, investigators are now able to identify hundreds of *O*-GlcNAc sites in a single study. This review will describe these established glycomic approaches and how the data obtained from these methods have increased our knowledge of *O*-GlcNAc's role in the disease mechanisms of diabetes, cancer, and Alzheimer's disease. We will also review data obtained by one of these methods that has established *O*-GlcNAcylation of specific proteins at specific sites as a potential biomarker for the detection of prediabetes.

Glycomic Approaches

Mass Spectrometry (MS) is now a key tool for site-mapping *O*-GlcNAcylation as well as other post-translational modifications (PTMs). As with most PTMs, enrichment of low abundance, modified peptides greatly enhance MS-based identification. Direct, efficient enrichment of *O*-GlcNAc modified peptides is the ideal approach for mass spectrometry analysis and identification. In one approach, lectin weak affinity chromatography (LWAC) was used to enrich *O*-GlcNAc modified peptides [20]. In "weak affinity" chromatography, *O*-GlcNAc modified peptides migrate slower through a wheat germ agglutinin (WGA, a lectin that recognizes GlcNAc) column and elute later than unmodified peptides, allowing for enrichment. They first demonstrated this approach was feasible by identifying synthetic *O*-GlcNAc modified peptides from a tryptic peptide mixture. Next, they applied this technique to a post-synaptic density preparation and identified 145 unique *O*-GlcNAc modified peptides belonging to proteins with diverse functions in synaptic transmission. Recently, the method was further optimized by re-loading and re-separating a post-synaptic vesicle sample three times [21]. By using this sequential enrichment, 1750 *O*-GlcNAcylation sites from 6621 proteins were identified in synaptosomes. Interestingly, 46 *O*-GlcNAcylated kinases were also identified. This approach using brain synaptosomes has produced, to date, the largest proteomics analysis for protein *O*-GlcNAcylation. However, the affinity of the lectin for *O*-GlcNAc is weak, leading to poor *O*-GlcNAcylated peptide separation on the lectin column. Additionally, as the lectin specificity for *O*-GlcNAc is low, it also has affinity for other type of sugars modifications, such as sialic acids or N-linked GlcNAc.

Even though the "weak affinity" enrichment for *O*-GlcNAcylated peptides has matured, *O*-GlcNAc enrichment methods still lack the robustness and specificity used for enrichment of phosphorylation. A chemoenzymatic approach was developed for enrichment of *O*-GlcNAcylated proteins [22]. This approach combines solid-phase chemical derivatization and isotopic labeling to detect *O*-GlcNAcylation sites and was used to quantify *O*-GlcNAc site-occupancy between normal and diabetic erythrocyte proteins. In this approach, a mutant galactosyltransferase (mGalT1) [23] was used to specifically label *O*-GlcNAcylated peptides with UDP-GalNAz, an azido-modified donor substrate sugar for mGalT1. The modified sugar can be further modified with biotin-PEG-alkyne tags using copper as a catalyst in a Click reaction. The biotin arm allows for highly selective enrichment of *O*-GlcNAcylated peptides using streptavidin chromatography. Release of the glycopeptide from streptavidin beads can be achieved through BEMAD (beta-elimination followed by Michael addition with dithiothreitol (DTT)), which cleaves the peptide from the modified disaccharide leaving a dehydroalanine moiety in place of the *O*-GlcNAcylated serine or threonine. DTT is then reacted with the α,β -unsaturated carbonyl in a Michael addition reaction. However,

BEMAD lacks specificity due to possible side reactions with other PTMs. Hence, a more efficient method to release the peptide from the solid resin is desirable, and cleavable linker strategies have been developed to address this problem.

Subsequently, a novel photocleavable linker-based enrichment and site-mapping approach was developed [22]. In this method, first *O*-GlcNAc modified peptides are modified using Click chemistry with a novel biotinylation reagent which is sensitive to cleavage at a specific UV wavelength (see figure 1). *O*-GlcNAcylated peptides are enriched by streptavidin chromatography, released from the solid support by photochemical cleavage, and analyzed by electron transfer dissociation (ETD) mass spectrometry[24]. Specifically, the *O*-GlcNAcylated peptides were labeled with UDP-GalNAz, then tagged with the photo-cleavable linker (PC-biotin-alkyne) by Cu catalyzed Click reaction (CuAAC, containing Cu^{2+} , Tris[(1-benzyl-1H-1,2,3-triazol-4-yl) methyl] amine (TBTA) and sodium ascorbate). This cleavable linker efficiently captures *O*-GlcNAcylated peptides that can then be released by cleavage with UV light for LC-MS/MS analysis. The remaining fragment of the enrichment tag on the peptide, which introduces a positive charge, facilitates *O*-GlcNAc site mapping when used in conjunction with ETD based mass spectrometry. This approach was used to identify *O*-GlcNAc sites on proteins involved in spindle assembly and cytokinesis. This study employed SILAC (Stable Isotope Labeling of Amino Acids in Cell Culture) in order to quantify changes in *O*-GlcNAcylation as well as phosphorylation. These data demonstrated that *O*-GlcNAcylation is a crucial regulator of cyclin dependent kinase 1(CDK1) phosphorylation [25]. Yang and coworkers modified this chemoenzymatic photochemical cleavage approach for enriching *O*-GlcNAcylated peptides [26]. They improved the wash method, increasing the number of *O*-GlcNAc protein identified by 16% while reducing the large amount of hydrophobic TBTA that remained from the CuAAC reaction. With this modified approach, a total of 274 *O*-GlcNAcylated proteins and 458 *O*-GlcNAc sites were identified from peptides from mouse cerebrocortical brain tissue. This approach can work very well with a large amount of sample; however, there are an excessive number of steps, decreasing sample recovery. Therefore, less labor intensive methods need to be developed.

Mass spectrometry-based proteomics research has generated a large amount of tandem MS data. The role of bioinformatics is fundamental for the evaluation of mass spectrometry data due to the amount of data that this technique can produce. Normally, researchers probe large-scale proteomic data for targeted information, which, unfortunately, leaves an enormous amount of other valuable sample information undiscovered within the existing data. Hannes and coworkers have recently developed a bioinformatics tool, termed Oscore, that automatically assesses tandem MS spectra for *O*-GlcNAc diagnostic fragment ions and, in turn, they discovered almost 100 new *O*-GlcNAc modified peptides from existing large-scale proteome data sets not initially used for mapping *O*-GlcNAc sites. These data suggest that *O*-GlcNAcylation is more highly abundant than initially thought. Still, low abundance *O*-GlcNAc proteins will need specific enrichment for discovering *O*-GlcNAcylation sites.

High-throughput approaches to investigate *O*-GlcNAcylation are still needed. Recently, an *in vitro* OGT assay was performed on a commercial kinase microarray containing 152 myc-tagged kinases. The microarray was incubated with recombinant human OGT and UDP-

[³H]-GlcNAc. After washing, protein levels were normalized using an anti myc-Cy3 antibody and then exposed to a ³H-sensitive phosphor screen to measure *O*-GlcNAcylation levels. Forty-two of the 152 printed kinases were *O*-GlcNAcylated *in vitro*, representing 39% of the kinases on the array. This result suggests that *O*-GlcNAcylation may directly regulate a substantial number of kinases and illustrates the increasingly complex relationship between *O*-GlcNAcylation and phosphorylation in cellular signaling. Thus far, all the proteins that were *in vitro* OGT substrates were also found to be *in vivo* substrates; however, the approach still requires *in vivo* *O*-GlcNAcylation verification. Besides the MS and microarray experiments, antibodies have been developed to detect protein *O*-GlcNAcylation[27, 28]. CTD 110.6 [27] and RL2 [28, 29] are commercially available antibodies used to detect *O*-GlcNAc.

Knowledge of relative *O*-GlcNAc stoichiometry is essential in understanding the function and regulation of *O*-GlcNAc. However, it is hard to assess whether proteins exist in singly, doubly or multiply glycosylated states *in vivo*. Elizabeth Rexach and coworkers from Linda Hsieh-Wilson's laboratory [30] have developed a facile approach to measure *O*-GlcNAc stoichiometry using resolvable polyethylene glycol mass tags, which provides information regarding overall *O*-GlcNAc site occupancy that cannot be obtained using mass spectrometry. Basically, they chemoenzymatically labeled *O*-GlcNAc modified proteins using a UDP-ketogalactose analog and GalT1. The keto group on the labeled *O*-GlcNAc protein reacts with an aminooxy-functionalized PEG (polyethylene glycol) mass tag. The different *O*-GlcNAcylated species can be visualized upon SDS-PAGE and immunoblotting with antibodies against the protein of interest. They further applied this approach to investigate the stoichiometry, complex regulation, and cellular dynamics of *O*-GlcNAcylation of cyclic AMP-response element binding protein (CREB)[30, 31], methyl CpG binding protein-2 (MeCP2)[30] and phosphofructokinase-1 (PFK1)[32]. These non-MS methods provide another way to study protein *O*-GlcNAcylation. However, in order to elucidate the site-specific function of *O*-GlcNAcylation, this approach still needs to be combined with site mapping methods to obtain the *O*-GlcNAc site information allowing for further functional studies.

O-GlcNAcylation and Cancer

Cancer can be simply defined as uncontrolled cell growth or proliferation, thus understanding this biological process is important for the development of therapeutics to prevent it. In order for cancer cells to rapidly proliferate, they must consume and utilize nutrients they need to produce metabolites they require. Cancer cells use more glucose, glutamine, acetyl CoA, and nucleotides than normal cells. This is particularly of interest in terms of *O*-GlcNAc as each of these nutrients are known to increase flux through HBP and subsequently increase *O*-GlcNAcylation. As a result, it is not surprising that *O*-GlcNAc is elevated in various cancer cells [33, 34]. However, the simple observation of elevated *O*-GlcNAc is not predictive measure of cancer as OGA activity was higher and *O*-GlcNAc was lower in breast and thyroid cancer tissues compared to paired controls[35]. These and other results demonstrate that regulation of *O*-GlcNAc does have roles in cancer and tumorigenesis, however; they are not well understood. With the advances in glycomic approaches used to study and identify *O*-GlcNAc sites, the field has the means to uncover

how a protein's site specific *O*-GlcNAc modification may regulate function during cell proliferation.

Aneuploidy (abnormal number of chromosomes) is a common phenotype of cancer tumorigenesis[36]. Interestingly, a modest overexpression of OGT, which results in an observable increase in *O*-GlcNAc, promotes aneuploidy with noticeable mitotic defects [25]. During cell proliferation, OGT is localized to the mitotic spindle and goes to the midbody prior to physical separation into two daughter cells. To begin to understand *O*-GlcNAc's role in this process, investigators developed a novel mass spectrometric to identify modified proteins and their sites of modification at the midbody, as described above[24, 25]. Using this approach on purified midbodies, investigators were able to identify over 150 *O*-GlcNAc sites [25]. Interestingly, OGT overexpression was shown to alter the phosphorylation of key proteins involved in cell cycle regulation suggesting that the interplay between *O*-GlcNAc and phosphorylation aids in the proper maintenance of cell proliferation.

Cyclin-dependent protein kinase-1 (CDK1), the master regulatory kinase of M phase progression was among the proteins identified to have its phosphorylation altered upon OGT overexpression. During M phase, CDK1 is dephosphorylated at specific sites resulting in its activation and subsequent phosphorylation of its various downstream targets[37]. Upon OGT overexpression, the increase in global *O*-GlcNAcylation was accompanied with an increase in CDK1 inhibitory phosphorylation cumulating in a decrease in its activity. This reduction in CDK1 activity was explained by the observable decrease in phosphorylation of its upstream kinase MYT1 upon OGT overexpression. These results clearly demonstrate that *O*-GlcNAc has extensive cross talk with phosphorylation and this feature is critical for the proper regulation of M phase progression. Furthermore, the site specific data obtained from this study serves as an initial starting point to begin to uncover how *O*-GlcNAc regulation of these proteins may affect their function during cell cycle progression. Results obtained should provide insight into how misregulation of *O*-GlcNAcylation of proteins can lead to phenotypes associated with cancer.

Cancer cells must consume and utilize elevated levels of nutrients in order to keep up with the cellular demands that are necessary for increased cell proliferation. Cancer cells have the ability to adapt their metabolic pathways to generate molecules necessary for growth. Cancer cells up regulate glycolysis, which is comprised of multiple enzymes that are potential substrates for OGT. Given the observation that *O*-GlcNAc is elevated in some cancers, it is feasible to hypothesize that some glycolytic enzymes could be modified and have their functions regulated by *O*-GlcNAc. Upon overexpression of OGT or inhibition of OGA, investigators discovered that PFK1 is *O*-GlcNAcylated. PFK1 plays a critical role in regulating flux of the glycolytic pathway as it is responsible for catalyzing the irreversible phosphorylation reaction converting fructose-6-phosphate to fructose-1,6-bisphosphate[38], from which after a series of other enzymatically driven reactions results in the production of high energy molecules (ATP and NADH) and pyruvate. Utilizing a method in which a PEG moiety is attached on *O*-GlcNAc residues, it was demonstrated that under hypoxic conditions the population of *O*-GlcNAcylated PFK1 increased. Interestingly, PFK1 was shown to be *O*-GlcNAc modified in many tumors and cancer cell lines, and its modification increased under conditions associated with cancer tumorigenesis.

Enrichment of *O*-GlcNAc peptides with WGA followed by mass spectrometric analysis using ETD, revealed Ser-529 of PFK1 as an *O*-GlcNAc site. Ser-529 is a highly conserved residue that is important for allosteric regulation of the protein by fructose-2,6-bisphosphosphate (F2,6BP) [39]. Results from structural models suggest that *O*-GlcNAc at Ser-529 could interfere with F2,6BP binding thereby inhibiting its activity. Under either OGT overexpression or hypoxia conditions, cells stably expressing WT PFK1 displayed decreased glycolysis and lactate production; while cells stably expressing PFK1 S529A mutant had no change compared to control cells. This decrease in glycolysis resulted in a redirection of glucose metabolism to the pentose phosphate pathway generating precursors (i.e. NADPH) that are advantageous for cancer cell growth. Taken together results from this study demonstrate that under cancerous conditions (hypoxia) *O*-GlcNAcylation of PFK1 is elevated decreasing its activity while redirecting metabolism to promote cell growth. Strategies targeted at blocking *O*-GlcNAc on PFK1 to increase its activity could be useful to promote proper metabolism and “normal” cell growth thereby serving as a novel avenue to potentially treat cancer.

***O*-GlcNAcylation, Alzheimer’s Disease, and Learning and Memory in the Brain**

Alzheimer’s disease (AD), the most common form of dementia, is a neurodegenerative disease categorized by a decrease of normal brain function including loss of memory, cognitive thinking, and the ability to learn. Currently there is no cure for this disease and the molecular mechanisms that underlie its progression are not completely understood. However, glucose uptake and metabolism in the brain is impaired in AD, suggesting that misregulation of *O*-GlcNAc on specific proteins in the brain may promote AD [40, 41]. A number of proteins that are involved in the disease pathology of AD including tau, neurofilaments, and beta-amyloid precursor protein are known to be *O*-GlcNAcylated [42–44].

One of the characteristic hallmarks of AD is the presence of neurofibrillary tangles resulting from hyperphosphorylation of tau. Interestingly *O*-GlcNAcylation of tau in AD brains is lower than that of control [40]. This observation has led to the notion that preserving tau *O*-GlcNAcylation may provide a therapeutic advantage against the progression of AD. Studies employing mass spectrometric approaches have identified at least 12 *O*-GlcNAc sites on tau [24, 45, 46]. Analysis of the hyperphosphorylated tau revealed that it contains at least 4-fold less *O*-GlcNAc compared to nonphosphorylated tau [40]. This suggested that there exists a reciprocal relationship between *O*-GlcNAc and phosphorylation on tau in AD. This relationship was further observed from studies utilizing tau peptides in which it was shown that phosphorylation at neighboring sites decreased the peptide’s ability to be *O*-GlcNAcylated at Ser-400 and vice versa [45]. Overexpression of OGT leads to an increase in *O*-GlcNAc and a decrease in tau phosphorylation [47], while either knockdown of OGT or inhibition of the HBP leads to a decrease in *O*-GlcNAcylation of tau and an increase in its phosphorylation [40].

Recent studies show that increasing *O*-GlcNAcylation of tau in mouse brain by inhibition of OGA with Thiamet-G leads to an increase in *O*-GlcNAcylation of tau and a decrease in its

site-specific phosphorylation at multiple sites[48]. Similar results were observed when OGA was inhibited in PC-12 cells [49]. Treatment of tau transgenic mice with Thiamet-G also leads to an increase in tau *O*-GlcNAcylation that was accompanied with a decrease in the formation of tau aggregates and neuronal cell loss[41]. Taken together these studies suggest that a possible mechanism by which tau aggregates results in AD is through misregulation of tau *O*-GlcNAcylation.

O-GlcNAc in the brain is similar in abundance to phosphorylation[50, 51]. Numerous proteins in the brain that are involved in proper neuronal function, plasticity, and learning and memory are dependent on phosphorylation. The established crosstalk that exists between *O*-GlcNAc and phosphorylation suggest *O*-GlcNAc also has the potential to play important roles in neuronal function as well. Interestingly, modulation of global *O*-GlcNAc levels affect neuronal signal transmission and neurite growth through its interplay with phosphorylation[52, 53]. Taken together, results suggest *O*-GlcNAc plays a functional role in the brain; however how *O*-GlcNAc accomplishes this mechanistically remains to be established.

CREB is a transcription factor that controls the expression of genes important for brain development and memory[54–56], however these studies primarily focused role of CREB phosphorylation during this process. Previously CREB was demonstrated to be *O*-GlcNAcylated[57]; however, knowledge of how *O*-GlcNAcylation of CREB affects its function remain to be discovered. The major site of *O*-GlcNAcylation of CREB is Ser-40. Neuronal stimulation with KCl or N-methyl-D-aspartic acid (NMDA) increases *O*-GlcNAcylation of CREB specifically at Ser-40. Mutation of the Ser-40 site to an alanine (S40A) results in an increases in both luciferase activity and binding to CRTAC compared to wild-type, suggesting that *O*-GlcNAc at Ser-40 of CREB represses its transcriptional activity through impairment of its interaction with the coactivator CRTAC.

Using cortical neurons expressing WT or S40A CREB, the effect of *O*-GlcNAcylation of CREB on dendrite and axon growth was assessed. As expected membrane depolarization simulated the growth of dendrites and axons in WT cells. Interestingly, S40A cells had significantly longer dendrites and axons than WT under non-stimulated conditions with lengths similar to stimulated neurons from WT cells. Using mice either infused with WT or S40A vector in the amygdala, the effect of *O*-GlcNAcylation of CREB on long-term memory was investigated using auditory fear conditioning. Results demonstrated that S40A mice showed enhance memory characteristics after 2 hours and remained higher until 24 hours compared to WT mice, suggesting that CREB *O*-GlcNAcylation at Ser-40 negatively affects long-term memory.

O-GlcNAcylation and Diabetes

Insulin resistance is considered the hallmark of diabetes. One of the noticeable phenotypes of the diabetic condition is elevated glucose or hyperglycemia. Under this condition, it has been demonstrated that *O*-GlcNAcylation of proteins is up regulated. Treating cells with high glucose result in an increase in *O*-GlcNAc and alterations in insulin signaling[58]. By modulating of *O*-GlcNAc levels, studies have linked this post-translational modification to

diabetes. Elevation of *O*-GlcNAc levels by glucosamine treatment and/or inhibition of OGA results in insulin resistance [6, 59]. However, treatment with a more selective inhibitor of OGA, Thiamet-G, does not result in insulin resistance [60]. Targeted overexpression of OGT in muscle and fat tissues resulted in mice displaying an insulin resistance phenotype [61], while overexpression of OGA was shown to be beneficial by relieving insulin resistance [62]. Interestingly, Mexican Americans who have a single nucleotide polymorphism in the gene that encodes OGA (*MGEA5*) have been shown to have a higher risk of developing diabetes [63]. Taken together, these and other studies establish a link between *O*-GlcNAc and diabetes (for review see [64]), but to completely understand this link, identification of *O*-GlcNAc sites on proteins involved in metabolic regulation and delineating their functional significance and/or misregulation is necessary. In this section, we discuss recent studies employing methods described above to uncover *O*-GlcNAc sites on critical proteins that regulate insulin signaling and gluconeogenesis.

Insulin signaling regulates OGT function. Under normal conditions, OGT is localized in the nucleus in most dividing cell types. In the presence of insulin a fraction of OGT translocates to the cytoplasm and is found bound to PIP3 at the membrane. After a series of events and protein modifications, OGT activity is increased and serves to *O*-GlcNAcylate many components in the insulin pathway including insulin receptor substrate-1 (IRS-1), AKT, PI3K, and PDK1. Upon insulin stimulation, IRS-1 *O*-GlcNAcylation is up-regulated[65]. IRS-1 is a major transducer of the insulin signaling pathway, considering its *O*-GlcNAcylation is dynamically regulated in response to insulin, identification of its *O*-GlcNAc sites would be beneficial in understanding how this modification may regulate IRS-1 function at the site level.

Using a mass spectrometric approach coupled with BEMAD, at least four *O*-GlcNAc sites on human IRS-1 have been identified namely Ser-984, Ser-985, Ser-1011, and Ser-1036 [66, 67], with one site found close to the PI3K binding site. As mentioned earlier, elevation of *O*-GlcNAc is linked to insulin resistance. Increasing *O*-GlcNAc in adipocytes by inhibiting OGA results a number of changes to IRS-1; 1) an increase its *O*-GlcNAcylation, 2) a decrease its interaction with PI3K, 3) a decrease in its activating phosphorylation at Tyr-608, and 4) an increase in its phosphorylation at Ser-632 and Ser-635, culminating in an attenuation/impairment of insulin signaling [65, 68]. Now that glycomic methods have identified *O*-GlcNAc sites on IRS-1, investigations regarding *O*-GlcNAc site-specific function on IRS-1 is considered the in the next step.

Impairment or misregulation of gluconeogenic gene expression (gluconeogenesis) in response to glucose contributes to glucose toxicity under insulin resistance conditions. Gluconeogenesis (the production of glucose by the liver) is regulated by various coactivators and transcription factors including PGC-1 α , Foxo1, and CRTC2, all of which are *O*-GlcNAcylated[58, 62, 69]. Mass spectrometric techniques have mapped Ser-550, Thr-648, Ser-654, and Thr-317/Ser-318 of Foxo1[58] and Ser-70 and Ser-171 of CRTC2[62] as being *O*-GlcNAc sites. Under normal conditions, both Foxo1 and CRTC2 are phosphorylated resulting in their cytoplasmic localizations. Hyperglycemic conditions results in increases in the *O*-GlcNAcylation of Foxo1 and CRTC2 (at Ser-40 and Ser-171), their nuclear translocations, and their transcriptional activations of gluconeogenic genes (i.e.

phosphoenolpyruvate carboxykinase [PEPCK], and glucose 6-phosphate [G6P]). Mutations of Thr-317 of Foxo1 and Ser-70 and Ser-171 of CRTC2 resulted in decreases in their respective transcriptional activations and the nuclear localization of CRTC2, suggesting that *O*-GlcNAc plays a definitive role in the localization and transcriptional activations of these proteins. Taken together, these and other studies illustrates the importance of site specific *O*-GlcNAcylation of Foxo1 and CRTC2 under insulin resistance conditions and how they play a role promoting glucose toxicity.

***O*-GlcNAc as a Biomarker for the detection of Prediabetes**

O-GlcNAc was demonstrated to occur on human erythrocyte proteins over 20 years ago [29], however *O*-GlcNAc's role on erythrocyte proteins remains unclear. *O*-GlcNAc's presence on erythrocytes proteins are of particular interest when considering the possibility that it could be used as a biomarker for the detection of prediabetes specifically, as red blood cells are directly exposed to the chronic levels of glucose under hyperglycemic conditions that result from having diabetes. Since *O*-GlcNAc is rapidly regulated by glucose, it is feasible to suggest that aspects of the regulation of *O*-GlcNAc and/or the site-specific modification of proteins could serve as a potential a "marker" for early detection of diabetic progression (i.e. prediabetes). As currently there is no cure for diabetes, detection of prediabetes is advantageous as lifestyle changes could be implemented to delay and/or prevent the onset of diabetes. Biochemical analysis and the development of a chemoenzymatic/enrichment approach that combined solid phase chemical derivatization and isotopic labeling procedure by Wang *et. al.* (as described above), allowed for the testing of this hypothesis[22, 70].

The biochemical analysis of erythrocyte proteins from patients diagnosed as normal, prediabetic, or diabetic yielded some interesting results[22, 70]. Erythrocyte proteins are indeed modified by *O*-GlcNAc and the levels of both protein *O*-GlcNAcylation and OGA were elevated in patients having diabetes compared to that of normal patients, while OGT levels displayed no significant difference among the samples. Data also showed that protein *O*-GlcNAcylation and OGA levels were significantly elevated in prediabetic samples as well. Interestingly, when comparing the levels of OGA from all samples, OGA levels were significantly higher in diabetic samples compared to prediabetic, which were found to be higher than that of normal samples. This finding was extremely important as assessment of hemoglobin A1c (HbA1c), (universal marker for diabetes), was unable to distinguish between normal and prediabetic samples. Taken together these results suggest that assessment of a patient's OGA levels in erythrocytes could be developed and used to diagnose the prediabetic condition.

Results that demonstrated that global *O*-GlcNAc levels were elevated in prediabetic and diabetic erythrocytes, served as a launch point for further investigations for the identification of these *O*-GlcNAcylated proteins and more importantly the sites of their modifications during the progression of diabetes. The chemoenzymatic tagging and enrichment method as described by Wang *et. al.* [22, 24] allowed for the identification and quantification of site specific *O*-GlcNAc occupancy levels (relative occupancy ratio, ROR) between normal and diabetic samples. Twenty-five *O*-GlcNAcylated protein were identified and among them 35

O-GlcNAc sites were uncovered and their ROR between normal and diabetic samples were calculated. Among the proteins that were identified, the *O*-GlcNAcylation of catalase at serine-114 had a ROR of over three when comparing diabetic to normal.

Results demonstrated that the degree of *O*-GlcNAcylation at particular site(s) within proteins were either increased, decreased, or had no change when comparing diabetic to normal samples. This suggested that the increase in *O*-GlcNAcylation of proteins observed in the erythrocytes of patients with diabetes is not a random event. This further suggested that the development of *O*-GlcNAc site-specific antibodies targeted at these sites might have the potential to detect diabetes in its earliest stages. Based on this, an *O*-GlcNAc site-specific antibody toward serine-114 of catalase was generated. Results using this site specific antibody revealed similar results as with assessment of OGA levels (K. Park, and G.W. Hart, unpublished data). Specifically, *O*-GlcNAcylation at serine-114 of catalase was increased approximately 2-fold in diabetic samples compared to normal samples. Furthermore when comparing the *O*-GlcNAcylation of serine-114 among all samples, it was demonstrated that its levels could be used to distinguish normal from prediabetic and prediabetic from diabetic samples. More studies and analysis of more samples are needed however; these results highlight the potential that lies in the detection of site-specific *O*-GlcNAcylation of specific proteins for the screening of patients for prediabetes and diabetes. Interestingly recent study has demonstrated that *O*-GlcNAc analysis of proteins contained within white blood cells could have the potential for use as a prediabetic diagnostic as well [71].

Summary

O-GlcNAc is an abundant and nutrient sensing post-translational modification (PTMs) that plays a major role in many biological functions and in the disease etiologies of cancer, Alzheimer's, and diabetes by regulating specific proteins' functions. As with all PTMs, to critically elucidate their mechanistic function on a protein, identification of modification sites are required. Early studies of *O*-GlcNAc have been met with hardships with respect to this and validated the essential need for the development of new glycomic approaches to map *O*-GlcNAc sites. The recent development of these approaches has greatly advanced the field, as now hundreds of *O*-GlcNAc sites are capable of being identified in a single study. These approaches now allow for the study of *O*-GlcNAc in terms of their site-specific stoichiometries and their relative occupancy changes in response to various stimuli. These approaches have been applied to further our understanding of the roles of how misregulation of *O*-GlcNAc at the site-specific level can have direct impact on diabetes, cancer, learning and memory, and in Alzheimer's disease. Currently, the number of *O*-GlcNAc site-specific antibodies is small. However, with the identification of regulatory *O*-GlcNAc sites on the rise, the generation of more *O*-GlcNAc site-specific antibodies should be forthcoming. Thereby allowing investigators to conduct site-specific studies similar to phosphorylation. The use of these methods has provided data demonstrating the feasibility of using site specific *O*-GlcNAcylation the screen of patients for prediabetes. Future development of these glycomic approaches and the data obtained from their use could lead to novel therapeutic strategies for the treatment and/or diagnosis of various disease states of which *O*-GlcNAc is known to play a role.

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Abbreviations

(O-GlcNAc)	<i>O</i> -GlcNAcylation
(GlcNAc)	β - <i>N</i> -acetylglucosamine
(HBP)	hexosamine biosynthetic pathway
(GFAT)	glutamine:fructose 6-phosphate aminotransferase
(UDP-GlcNAc)	uridine diphospho- <i>N</i> -acetylglucosamine
(OGT)	<i>O</i> -GlcNAc transferase
(OGA or O-GlcNAcase)	β - <i>N</i> -acetylglucosaminidase
(LWAC)	lectin weak affinity chromatography
(WGA)	wheat germ agglutinin
(mGalT1)	mutant galactosyltransferase-1
(BEMAD)	beta elimination followed by Michael addition
(UV)	ultraviolet light
(TBTA)	Tris[(1-benzyl-1H-1,2,3-triazol-4-yl) methyl]
(ETD)	electron dissociation
(SILAC)	stable isotope labeling of amino acids in cell culture
(CDK1)	cyclin dependent kinase 1
(CREB)	cyclic AMP-response element binding protein
(CRTC2)	transducer of CREB2
(MeCP2)	methyl CpG binding protein-2
(PEPCK)	phosphoenolpyruvate carboxykinase
(G6P)	glucose 6-phosphate
(PFK1)	phosphofructokinase-1
(MYPT1)	myosin phosphatase target subunit 1
(F2,6BP)	fructose-2,6-bisphosphosphate
(Sp1)	Specificity protein 1
(Foxo1)	Forkhead box protein 1

(CRTC2)	transducer of regulated cyclic adenosine monophosphate response element binding protein 2
(MGEA5)	meningioma-expressed antigen 5
(ROR)	relative occupancy ratio
(CTD 110.6)	C-terminal domain <i>O</i> -GlcNAc pan-specific antibody
(AD)	Alzheimer's Disease
(WT)	wild type
(IRS1)	insulin receptor substrate 1
(PI3K)	phosphoinositides 3-kinase
(PDK1)	3-phosphoinositide dependent protein kinase 1
(HbA1c)	hemoglobin Alc
(CID)	collision induced dissociation

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Figure 1. O-GlcNAc Site Mapping using a photo-cleavable biotin tagging approach

A) O-GlcNAcylated peptides were labeled with UDP-GalNAz by Gal T1, and further tagged with the photo-cleavable linker (PC-biotin-alkyne) by Cu²⁺ catalyst Click reaction. (B) The cleavable biotin linker allows for efficient capture of the O-GlcNAcylated peptides by streptavidin. (C) After washing off the non-specific binding peptides, the photo-cleavable linker allows to release the tagged-peptide by UV light for further LC-MS/MS analysis. (D) LC-MS/MS analysis of the tagged peptide. The tag of the peptide will generate two diagnostic fragment ions for CID analysis, and the additional positive charge from the tag is also benefit for ETD analysis.