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O-Linked β-*N*-Acetylglucosamine (*O*-GlcNAc): Extensive Crosstalk with Phosphorylation to Regulate Signaling and Transcription in Response to Nutrients and Stress

Chutikarn Butkinaree, **Kyoungsook Park**, and **Gerald W. Hart**
Department of Biological Chemistry, Johns Hopkins University School of Medicine, Baltimore, Maryland 21205. http://biolchem.bs.jhmi.edu/

Abstract

Background—Since its discovery in the early 1980s, O-linked-β-*N*-acetylglucosamine (O-GlcNAc), a single sugar modification on the hydroxyl group of serine or threonine residues, has changed our views of protein glycosylation. While other forms of protein glycosylation modify proteins on the cell surface or within luminal compartments of the secretory machinery, O-GlcNAc modifies myriad nucleocytoplasmic proteins. GlcNAcylated proteins are involved in transcription, ubiquitination, cell cycle, and stress responses. GlcNAcylation is similar to protein phosphorylation in terms of stoichiometry, localization and cycling. To date, only two enzymes are known to regulate GlcNAcylation in mammals: O-GlcNAc transferase (OGT), which catalyzes the addition of O-GlcNAc, and β-*N*-acetylglucosaminidase (O-GlcNAcase), a neutral hexosaminidase responsible for O-GlcNAc removal. OGT and O-GlcNAcase are regulated by RNA splicing, by nutrients, and by post-translational modifications. Their specificities are controlled by many transiently associated targeting subunits. As methods for detecting O-GlcNAc have improved our understanding of O-GlcNAc's functions has grown rapidly.

Scope of Review—In this review, the functions of GlcNAcylation in regulating cellular processes, its extensive crosstalk with protein phosphorylation, and regulation of OGT and O-GlcNAcase will be explored.

Major Conclusions—GlcNAcylation rivals phosphorylation in terms of its abundance, protein distribution and its cycling on and off of proteins. GlcNAcylation has extensive crosstalk with phosphorylation to regulate signaling, transcription and the cytoskeleton in response to nutrients and stress.

General Significance—Abnormal crosstalk between GlcNAcylation and phosphorylation underlies dysregulation in diabetes, including glucose toxicity, and defective GlcNAcylation is involved in neurodegenerative disease and cancer and most recently in AIDS.

Keywords

O-GlcNAc; GlcNAcylation; Phosphorylation; OGT; OGA; Stress; glucosamine; Alzheimer's Disease; signaling; diabetes; O-GlcNAcase; O-GlcNAc transferase

Corresponding Author: Gerald W. Hart Professor & Director Department of Biological Chemistry Johns Hopkins University School of Medicine 725 N. Wolfe St. Baltimore, MD 21205-2185 Phone: (410) 614-5993 Fax: (410) 614-8804 gwhart@jhmi.edu.

I. Overview

GlcNAcylation, similar to phosphorylation, is the post-translational cycling of a single O-linked-β-*N*-acetylglucosamine (O-GlcNAc) on the hydroxyl groups of Ser and/or Thr residues of target proteins. O-GlcNAc is a unique type of glycosylation in that it is not elongated to more complex glycan structures and is not restricted to the cell surface and/or luminal face of secreted proteins. In fact, an early study in lymphocytes with exogenous bovine β-1,4-galactosyltransferase demonstrated that a majority of terminal O-GlcNAc modification was found inside the cell [1]. The nucleocytoplasmic localization and structure of O-GlcNAc was confirmed by assessing sensitivity of galactose-labeled O-linked GlcNAc residues to β-elimination and by its resistance to deglycosylation with endo-$\tilde{\beta}$*N*-acetylglucosaminidase F, and the structure was confirmed by high voltage paper electrophoresis or by chromatography on calcium saturated strong cation-exchange columns [2-4]. The addition and removal of *O*-GlcNAc on/off proteins is mediated by the concerted action of O-GlcNAc transferase (OGT) and O-GlcNAcase, respectively. Uridine diphosphate-*N*-acetylglucosamine (UDP-GlcNAc) is the donor substrate of OGT and is the final product of the hexosamine biosynthetic pathway (HBP) (see Fig. 1; see [5] for a more detailed review). Approximately 2 - 5% of intracellular glucose, depending on cell type, enters the HBP, and thus the extent of protein GlcNAcylation is often considered to be sensitive to nutrient (i.e. glucose and/or glucosamine) availability [6-8]. Furthermore, disruption of UDP-GlcNAc synthesis by inhibiting the rate-limiting enzyme of the HBP (glucosamine-fructose-6-phosphate aminotransferase; GFAT) causes vital cellular defects [9], as murine cells lacking GFAT show decreased O-GlcNAc levels, impaired proliferation, and cell adhesion defects. As a result, altering UDP-GlcNAc levels through the HBP either by inhibition of the HBP enzymes or by addition of glucosamine is one of the early approaches used in studies of O-GlcNAc. With increasing knowledge of O-GlcNAc regulatory enzymes, additional biological tools have been applied to O-GlcNAc studies, including over-expression, inhibition, and knockdown of these enzymes. O-GlcNAc can be induced to rapidly cycle on/off proteins in response to activation of numerous intracellular signaling pathways involved in many aspects of life. For example, mitogenic activation of T lymphocytes induces rapid fluctuation of *O*-GlcNAc levels on nuclear and cytosolic proteins within minutes to hours [10,11]. A more detailed review of *O*-GlcNAc's roles in mediating protein activation and/or localization in response to activation of various intracellular signaling pathways is discussed later in this review (see section IV).

Since its discovery, nearly one-thousand O-GlcNAc modified proteins have been identified (for review see [12]). A large number of GlcNAcylated proteins are well-known proteins involved in regulation of intracellular signaling, including transcription regulation, cytoskeletal networks, stress responses, and the ubiquitin-proteasome system (for review see [12,13]). Regulation of some of those GlcNAcylated proteins, such as RNA polymerase II, and transcription factors Sp1 and c-Myc, will be discussed in further detailed. More often, GlcNAcylation modulates functions of these proteins by several mechanisms by influencing protein-protein interactions and by modulating protein localization. Interestingly, all known GlcNAcylated proteins can also be modified by phosphorylation. The two modifications frequently appear to be mutually exclusive, as inhibition of protein kinases, such as protein kinase A and protein kinase C, results in increased global O-GlcNAc levels [14,15]. Also, when global O-GlcNAc levels are elevated by inhibition of O-GlcNAcase in NIH-3T3 cells, a large portion of actively cycling phosphorylation sites from major regulatory proteins show decreased phosphorylation [16]. However, some specific phosphorylation sites actually increase in their extent of phosphorylation upon increased GlcNAcylation [16]. These findings suggest that O-GlcNAc is a cellular regulator that functions, in part, by communicating with protein phosphorylation. However, it is unclear how GlcNAcylation and phosphorylation interplay is regulated, as it may be some combination of the following situations. First, each

modification may influence the other's cycling enzymes. Second, GlcNAcylation and phosphorylation may influence each other by competing through steric hindrance for modification on target proteins at either the same sites or proximal sites, as O-phosphate residues are negatively charged, while O-GlcNAc moieties are neutral, but have a much larger size. The interference between GlcNAcylation and phosphorylation can also arise from proximity of the modification sites in protein tertiary structure. Thus, the dynamic interplay between GlcNAcylation and phosphorylation is surprisingly extensive, and GlcNAcylation has emerged as another important regulator of cellular signaling.

For the past twenty years, O-GlcNAc field has been steadily growing. Roles of GlcNAcylation in various cell-signaling pathways, such as insulin signaling and stress responses, have been explored. While protein phosphorylation employs more than 500 genetically encoded protein kinases and phosphatases, O-GlcNAc cycling is catalyzed by only two highly conserved enzymes (OGT and O-GlcNAcase) that transiently form many distinct holoenzymes in response to various stimuli allowing them to modify many substrates with differing specificities [17-22]. It remains unclear what governs activities and substrate specificities of these two enzymes. In this review, we will discuss possible regulatory mechanisms of O-GlcNAc regulatory enzymes and emerging roles of GlcNAcylation in cellular processes, which are quite often intertwined with those of protein phosphorylation.

II. *O*-GlcNAc Regulatory Enzymes

A. *O*-GlcNAc Transferase (OGT)

i. Structure & Function—OGT (uridine diphospho-*N*-acetylglucosamine:polypeptide β-*N*-acetylglucosaminyltransferase) catalyzes the addition of O-GlcNAc from UDP-GlcNAc onto the hydroxyl group of a serine or a threonine residue on the protein substrates [23]. The gene encoding OGT is evolutionarily conserved from plants to humans [24,25]. In *Arabidopsis*, OGT is encoded by two distinct genes, SPINDLY (SPY) and SECRET AGENT (SEC) [26]. Both genes are required during gametogenesis and embryogenesis, but SPY also functions as a repressor of gibberellin signaling and a positive regulator of the cytokinin response [26,27]. In mammals, *OGT* gene is highly conserved and is present as a single X-linked gene localized near the centromere where recombination rates are low. Using *in situ* hybridization, the mouse OGT gene is mapped to chromosome XD, while the human OGT is mapped to chromosome X q13.1, which is a region associated with Parkinson's disease [28,29].

Although there is only one OGT gene present, mammalian OGT has at least three different isoforms from alternative splicing: 1) the 116-kDa nucleocytoplasmic isoform (ncOGT), 2) the 103-kDa mitochondrial isoform (mOGT), and 3) the short 78-kDa isoform (sOGT) (see Figure 2) [24,29-31]. All OGT isoforms consist of two distinct domains. The N-terminal domain contains tetraticopeptide repeat (TPR) motifs, which are common protein-protein interaction domains, while the C-terminal domain has homology to glycogen phosphorylase [24,32,33]. The main difference among OGT isoforms is the number of TPRs each isoform contains. ncOGT contains ~11.5 TPR motifs and is localized more prominently in the nucleus [24]. sOGT possesses ~2 TPRs. mOGT contains ~9 TPRs and an additional 120-amino acid stretch at the N-terminus identified as the mitochondrial targeting sequence [31]. Deletion of this sequence results in mOGT staying in the cytoplasm and increased O-GlcNAc levels on cytoplasmic proteins [31]. Additionally, expression of each OGT isoform varies among different cell types. All OGT isoforms are highly expressed in blood cell types, including T cells and B cells [34]. Other than that, expression of each isoform is mostly tissue-specific. ncOGT is expressed in all tissues, but at low levels in kidney, and at high levels in brain, pancreas, and uterus [24]. sOGT is found in kidney, liver, muscle, thymus, whole blood, salivary gland, ovary, tonsil, placenta, and pancreatic islet cells [24,34], while tissue distribution of mOGT has not yet been characterized. Tissue-specific expression of OGT

isoforms suggests that different isoforms may have distinct functions and may respond differently to cellular signaling, depending on its tissue distribution [24,34].

ii. Regulation of OGT—OGT is highly regulated by many different mechanisms. The mRNA and protein expression levels of OGT are tissue-specific and are dependent upon specific cellular signaling, such as nutrient availability. For example, in response to glucose starvation, OGT mRNA and protein expression are increased in an AMP-kinase-dependent manner [19]. The activity of OGT, however, does not always correlate with its mRNA and protein expression levels. As illustrated in Figure 3, and discussed below, OGT activity is highly regulated by many factors other than the expression of its catalytic subunit, including donor substrate availability, protein-protein interaction, and post-translational modifications.

Donor Substrate Availability: OGT activity is influenced by the levels of its donor substrate UDP-GlcNAc, as increasing UDP-GlcNAc concentrations can increase OGT affinity toward its peptide substrates [24,30,35]. Additionally, subunit-interaction of OGT is modulated by UDP-GlcNAc levels, as different forms of OGT multimers have different binding affinities for UDP-GlcNAc [35]. Early work suggested that OGT exists in the liver primarily as a heterotrimer, with two 110-kDa and one 78-kDa subunits [30], but a study by Kreppel *et al*. (1999) demonstrates that the 110-kDa subunit of recombinant OGT can function as either homo- or heterotrimers [35]. OGT multimerization, which required at least 6 TPRs, is not necessary for OGT catalytic activity *in vitro*, as both OGT monomer and trimers have similar specific activity and K_m values for the same peptide substrates. However, subunit interaction influences OGT activity by altering binding affinity for UDP-GlcNAc. For example, OGT homotrimers have three distinct K_m values for UDP-GlcNAc of 6, 35 and 217 μM when using CKII peptide (PGGSTPVSSANMM) as substrate, while OGT monomer (OGT mutant with 6 TPR deletions) has two binding affinities for UDP-GlcNAc of 6 and 60 μM.

Protein-Protein Interactions: OGT activity can be regulated by protein-protein interactions. Regulation of OGT interaction with its substrates is proposed to lie mainly in the TPR domain. Jinek *et al.* (2004) reported a partial structure of recombinant OGT, containing 11.5 TPRs and the binding sites of a nuclear pore protein Nup62 and of a transcription repressor mSin3A [36]. In contrast to previous reports, this OGT structure shows the enzyme as a dimer with a large superhelix-inner surface that is similar to the array of conserved asparagines of importin-α and β-catenin. These conserved regions on importin-α and β-catenin are known as their target peptide binding sites, thus, suggesting that OGT may also use its TPR motifs for protein-protein interaction. Recently, another crystal structure of an OGT analog from the bacteria *Xanthomonas campestris (*XcOGT) has been reported [37,38]. XcOGT has 36% identity to the human OGT sequence, but it does not exactly function like OGT, as it catalyzes the transfer of GlcNAc from UDP-GlcNAc to a non-peptide substrate (which, in this study, is water) *in vitro* [37,38]. The catalytic domain of XcOGT forms the classic GT-B (glycosyltransferase-B) fold with a highly conserved active site groove and the UDP-GlcNAc binding site, which is located at the interface between two α/β-folds at the C-terminus. The three TPRs of XcOGT are at the N-terminus, forming a superhelix that is followed by two pairs of anti-parallel α-helices of extra TPR-like repeats. Interestingly, the central axis of these TPRs point toward the active site [40], suggesting that the binding orientation of an acceptor substrate is also critical to OGT activity.

Crystallization of the full mammalian OGT has not yet been successful. So, although the overall structure of XcOGT may not be precisely the same as that of mammalian OGT, it has proven to be a useful model. The catalytic residues are highly conserved in both XcOGT and mammalian OGT. XcOGT structure reveals that the TPRs close to the active site groove are required for OGT activity, as mutations in the putative peptide-binding groove near the active site completely abolish OGT activity [40]. On the other hand, the first few TPRs near the N-

terminus are not completely required for OGT activity. The sequences of those regions are not well conserved and may be responsible for target specificity. Several studies have supported the notion that the TPR domain is responsible for OGT's binding to specific target proteins. Different OGT isoforms have different peptide and protein preferences *in vitro* [34,41]. Additionally, deletion mutants of TPRs also show different substrate specificities [35,42]. Deletion of the first three TPRs of mOGT results in decreased OGT activity toward a nuclear pore protein Nup62, and deletion of six TPRs completely abolishes activity toward Nup62 while increasing OGT auto-GlcNAcylation. OGT mutants with 2.5 and 5.5 TPRs deletion have activity toward CKII peptide, but not a trafficking kinesin-binding 1 protein (OIP106 or TRAK1) [17]. These results suggest that OGT uses different TPR motifs to recognize specific protein substrates.

In support to these findings, studies on OGT purified from rabbit reticulocytes and rat liver cytosol show that OGT has a preferred peptide sequence for GlcNAcylation [23,30]. By comparing amino acid sequences from GlcNAcylated proteins Nup62, band 4.1, and a 65-kDa protein from human erythrocyte, OGT favors a peptide with acidic amino acids followed by serine and proline and subsequent series of serine and threonine residues. This preferred sequence may be due to interaction with those conserved regions in the TPR domains. Recent site mapping studies have now mapped a few hundred O-GlcNAc sites ([43,44], Wang *et al*., submitted), demonstrating that about one-half of the sites have a Pro-Val-Ser/Thr-Ser/Thr (PVS/TS/T) motif. Additionally, a novel TTA motif (two hydroxyl containing amino acids next to an alanine) for O-GlcNAc modification site is also identified in a glycomic study of mouse brain postsynaptic density [43]. Curiously, it is not known whether the TTA motif binds the same regions of OGT as those of the PVS/TS/T motif.

Recently, several studies have shed new light on regulation of OGT activity. ncOGT activity toward specific target proteins can be modulated through interaction between ncOGT and its binding partners, depending on specific signaling events (See Fig.3 and Fig.4). During glucose deprivation, OGT targeting to substrates is altered, partly, through its interaction with the stress kinase, p38 [19]. In this case, activated p38 interacts with the C-terminus of OGT, not the TPR domain, and p38 kinase does not phosphorylate OGT *in vitro*. Instead, p38 recruits OGT to specific targets, including neurofilament-H (NF-H). Inhibition of p38 reduces O-GlcNAc levels on NF-H and its solubility but not the interaction between OGT and NF-H, suggesting that interaction with p38 influences OGT activity on specific proteins. Similarly, *in vitro* substrate specificity of ncOGT is altered in the presence of a myosin phosphatase targeting protein, MYPT1, or a coactivator-associated arginine methyltransferease1 (CARM1) [45]. Increased GlcNAcylation on specific proteins is also observed when MYPT1 is present *in vivo* [45], suggesting that MYPT1 may serve to target OGT to unique substrates. Another example of OGT activity regulation by protein targeting is O-GlcNAc modification of FoxO1 [7,20]. A mutation at an O-GlcNAc site, Thr 317, on FoxO1 reduces its transcriptional activity stimulated by high glucose and leads to decreased expression of genes involved in gluconeogenesis and in expression of detoxification enzymes, such as manganese superoxide dismutase and catalase [20]. Under hyperglycemic conditions, the transcription coactivator PGC-1α interacts with OGT and facilitates OGT activity on the transcription factor FoxO1 [7,20]. In the presence of PGC-1α, OGT activity toward FoxO1 increases by three-fold *in vitro*, and over-expression of PGC1-α enhances GlcNAcylation on FoxO1 *in vivo*. Additionally, ncOGT can be translocated to another subcellular compartment by its binding partners for targeting. In response to insulin stimulation, ncOGT is recruited from the nucleus to the plasma membrane by phosphatidylinositol 3,4,5-triphosphate (PIP_3) through PI_3K activation [46]. At the plasma membrane, OGT catalyzes O-GlcNAc modification of several proteins involved in the insulin signaling pathway, including insulin receptor substrate-1 (IRS1) and Akt.

OGT activity and localization can also be regulated by transient complex formation with other regulatory proteins. During mitosis, OGT localizes to the mitotic spindle but later to the midbody during cytokinesis, forming a complex with O-GlcNAcase, mitotic aurora B kinase, and protein phosphatase 1c (PP1c) [47]. Inhibition of aurora B kinase activity alters O-GlcNAc levels and disrupts translocation of OGT to the midbody during cytokinesis. Additionally, a cytoskeletal protein vimentin, one of the aurora B kinase substrates during cytokinesis, is also modified by GlcNAcylation at Ser 55 [15,47]. Disturbing O-GlcNAc levels or aurora B kinase activity results in altered mitotic GlcNAcylation and phosphorylation of vimentin [47], potentially causing improper segregation of proteins into daughter cells due to improper filament disassembly. The findings that OGT uses its binding partners for OGT targeting may explain why there are no canonical OGT substrate sequence motifs, as OGT interacts with specific sets of proteins in response to specific signals.

Post-Translational Modifications: OGT activity is also regulated by post-translational modifications. OGT shows auto-GlcNAcylation between amino acids 1037-1046 in the catalytic domain and between amino acids 390-406 in the ninth TPR, and it is phosphorylated at Tyr 979 [24,42,48]. How GlcNAcylation or phosphorylation at these sites affects OGT activity is not yet known, but it has been recently shown that OGT is activated by calcium/calmodulin-dependent proline kinase type IV (CaMKIV) during potassium chloride-induced depolarization in neuronal cells [49]. Similarly, increased OGT activity is observed upon increased tyrosine phosphorylation of OGT following insulin stimulation in 3T3-L1 adipocytes [49,50]. However, it remains unclear where the phosphorylation sites on OGT are and how phosphorylation regulates OGT activity in these specific processes.

B. *O*-GlcNAcase

i. Structure & Function—β-*N*-acetylglucosaminidase (O-GlcNAcase) is a neutral hexosaminidase with a catalytic site similar to that of the family 84 glycoside hydrolase that specifically catalyzes the removal of β-linked GlcNAc on its substrate [51-53]. The gene encoding O-GlcNAcase is identified as meninginoma expressed antigen 5 (MGEA5) [54,55]. By somatic cell hybrid mapping, MGEA5 is mapped to chromosome 10q24.1-q24.3, which is a region associated with Alzheimer's disease. Additionally, O-GlcNAcase gene is also associated with development of diabetes as single nucleotide polymorphism in MGEA5 gene has been linked to the occurrence of type II diabetes in a Mexican population [56]. O-GlcNAcase sequence is highly conserved in higher eukaryotic species, especially in mammals, but absent in prokaryotes and yeast [53]. It does not share significant homology with any proteins, but has a putative acetyl transferase domain at the C-terminus (amino acids 772–898) that is, according to protein sequence analysis, linked to the catalytic center in the N-terminal domain by a highly disordered region of 150 amino acids [52,53,55,57-59]. Although the C-terminal domain of mouse O-GlcNAcase has been shown to possess histone acetyltransferase activity *in vitro*, these findings have not been confirmed by others [60,61].

Two splice variants of O-GlcNAcase were identified (see Fig. 2). The 130-kDa isoform (916 amino acids) localizes predominantly in the cytoplasm [53], while the 75-kDa isoform of O-GlcNAcase (677 amino acids) lacks one third of the protein at the C-terminus and resides in the nucleus [53,57,62]. The two isoforms differ in enzymatic activity. The full length O-GlcNAcase is active on *para*nitrophenol-β-GlcNAc (*p*NP-β-GlcNAc), an *in vitro* O-GlcNAcase substrate commonly used in O-GlcNAcase activity assay. The 75-kDa isoform has little or no activity toward pNP-β-GlcNAc, but its activity is detectable on a more sensitive substrate fluoresecin di(N-acetyl-β-D-glucosaminide (FDGlcNAc) [62,63]. However, this does not mean that the 75-kDa isoform of O-GlcNAcase is more or less active than the full-length isoform *in vivo*, as there may be other factors, such as post-translational modifications or specific cell signaling events that may modulate its activity. The *in vitro* data merely suggests

that the C-terminus of O-GlcNAcase is important for full enzymatic activity, even though the catalytic site of O-GlcNAcase lies in the N-terminus. Noticeably, the O-GlcNAcase activity toward FDGlcNAc of the short O-GlcNAcase isoform is significantly lower than that of the full-length O-GlcNAcase (K_m of 85 μM for the full-length O-GlcNAcase and K_m of 2.1 mM for the short O-GlcNAcase isoform). In addition, the recombinant human O-GlcNAcase mutant containing only the first 350 amino acids the of O-GlcNAcase sequence shows activity toward pNP-β-GlcNAc that is 1,000 fold less than that of the full-length enzyme [52]. In order to better understand how the two domains interact with each other, more information on the three-dimensional structure of O-GlcNAcase is needed.

To-date, attempts to solve the structure of mammalian O-GlcNAcase have not yet been successful. However, the structure of an enzyme similar to human O-GlcNAcase, isolated from the human gut symbiont *Bacteroides thetaiotaomicron* (BtGH4) has been crystallized [58]. BtGH4 is active toward O-GlcNAc modified proteins *in vitro*, and is inhibited by O-GlcNAcase inhibitors, O-(2-acetamido-2-deoxy-D-glucopyranosyldene-amino-N-phenylcarbamate (PUGNAc) and GlcNAc-thiazoline, with similar affinities [58]. While most parts of BtGH4 structure seems to be specific to those of bacterial enzyme, BtGH84 and mammalian O-GlcNAcase share a conserved catalytic mechanism and key catalytic residues [58]. O-GlcNAcase operates via a substrate-assisted catalytic mechanism using the 2-aceto amido group of the substrate [64]. Mutations at the catalytic residues Asp 174 and Asp 175 decreases its catalytic efficiency [52]. This catalytic doublet motif is conserved among predicted hexosaminidases, as mutations at Asp 175 and Asp 177 in mouse O-GlcNAcase and Asp 242 and Asp 243 in BtGH4 result in significantly decreased the enzyme's catalytic activity [58, 59].

With knowledge of O-GlcNAcase mechanism and structure, several O-GlcNAcase inhibitors were synthesized to study the role of O-GlcNAcase in cellular signaling both *in vitro* and *in vivo*. These inhibitors include PUGNAc, GlcNAcstatin, NbutGT, thiamet-G, GlcNAc-thiazoline, and its derivatives [51,65-72]. However, PUGNAc is not as selective toward O-GlcNAcase as are the newer inhibitors, GlcNAcstatin and GlcNAc-thiazoline [64,67,68]. Streptozotocin (STZ), a common drug used to induce diabetic states in mice and rats is also able to inhibit O-GlcNAcase at very high concentrations [59]. However, STZ is a DNA-alkylating agent that is toxic to cells. When using STZ, researchers need to be able to distinguish whether what they see is caused by O-GlcNAcase inhibition or STZ's toxicity. For example, pancreatic β-cell death was observed when STZ was used to increase O-GlcNAc levels, and it was suggested that pancreatic β-cell death was due to increasing O-GlcNAc levels by STZ [73]. In contrast, other studies show that pancreatic β-cell death is a result from decreased insulin secretion and protein synthesis induced by STZ, which may contribute to energy depletion [59,66,74,75].

ii. Regulation of O-GlcNAcase—Even though the catalytic mechanism of O-GlcNAcase and its roles on cellular function have been intensively studied, the regulation of O-GlcNAcase activity is still a mystery. O-GlcNAcase can be cleaved by activated caspase-3 at Asp 413 *in vitro* and during apoptosis *in vivo* [61,62]. This proteolytic modification, however, does not affect O-GlcNAcase activity, as O-GlcNAcase maintains full enzymatic activity after caspase-3 cleavage. In fact, the two cleavage fragments remain associated, and each fragment alone exhibits no activity *in vitro* [61]. This observation may not be surprising since the caspase-3 cleavage site on O-GlcNAcase lies in the disordered region that links the N-terminal and C-terminal domains. It is known that many disordered regions are more susceptible to proteolysis [76]. However, as disordered regions are known to be involved in molecular recognition and to provide flexibility in substrate binding between the N- and C-terminal domains [76], it is possible that recombining of the two cleavage fragments may contribute to changes in substrate recognition of O-GlcNAcase. As seen during apoptosis, global O-GlcNAc

profiles are altered, suggesting that O-GlcNAcase may act on different sets of substrates upon cleavage by caspase-3. Potentially, O-GlcNAcase activity may also be regulated by post-translational modifications. Using mass spectrometry, O-GlcNAcase has been shown to be modified by phosphorylation at Ser 364 in HeLa cells and GlcNAcylation at Ser 405 in brain. However, how these modifications affects O-GlcNAcase activity or its protein targeting remains unknown [77,78].

O-GlcNAcase activity may also be regulated by transient complex formation. One particular case concerns complex formation between O-GlcNAcase and OGT. OGT and O-GlcNAcase were identified as interacting partners in an OGT yeast-two- hybrid screening [45], and both enzymes are sometimes found in the same complex *in vivo*, although the composition of the other components in the complex is dependent on specific cellular processes. For example, O-GlcNAcase and OGT are found in the same complex with aurora B kinase and PP1c during cytokinesis in HeLa cells [47], but are found with mSin3A, and histone deacetylase-1 (HDAC1) upon stimulation of the estrogen and progesterone signaling in CHO cells [79]. However, since OGT and O-GlcNAcase catalyze opposite reactions, how a complex of the two enzymes is regulated without setting up a futile cycle is not well understood (see Figure 4). In a study of estrogen and progesterone signaling, an OGT and O-GlcNAcase complex is located to the repressed promoter where there is hyper-O-GlcNAcylation, suggesting very low activity of O-GlcNAcase [79,80]. Whisenhunt *et al.* (2006) showed that O-GlcNAcase activity is inhibited when complexed with OGT *in vitro*. However, it is unlikely for O-GlcNAcase to be inactive *in vivo* since O-GlcNAcase activity is important for proper estrogen and progesterone signaling and mammary development, as abnormal elevation of O-GlcNAc levels impair estrogen receptor activation [81]. These observations suggest that O-GlcNAcase activity or OGT activity fluctuates and may influence each other's activity in response to specific cellular conditions.

III. Extensive Crosstalk Between GlcNAcylation and Phosphorylation

O-GlcNAcylation is in many ways similar to protein phosphorylation. Both modifications cycle on their protein substrates with a variable rate, that depends upon the protein, the site of modification and cellular state. They are also highly responsive to nutrients, as their donor substrates are high-energy products from cellular metabolism. All O-GlcNAc modified proteins identified to-date, can also be modified by phosphorylation, suggesting possible interplay between the two modifications.

Several studies have shown that such a crosstalk between GlcNAcylation and phosphorylation exists, as disturbing phosphorylation events affects GlcNAcylation levels and vice versa. In cultured cerebellar neurons, GlcNAcylation of many cytoskeletal proteins is reduced upon activation of protein kinase C (PKC) and protein kinase A (PKA), while inhibition of PKC causes increased O-GlcNAc levels in cytoskeletal proteins [14]. This phenomenon is not specific to one phosphorylation signaling pathway, as elevated levels of GlcNAcylation on many proteins are also observed when glycogen synthase kinase-3 (GSK-3) activity is inhibited in COS-7 cells [15]. This suggests that GlcNAcylation and phosphorylation may influence each other through regulatory enzymes in the signaling pathway. GlcNAcylation may regulate kinases or phosphatases involved in the specific signaling, or alternatively, phosphorylation may affect functions of OGT and O-GlcNAcase. Dias *et al*. (2009) showed that GlcNAcylation of CAMKIV directly inhibits the enzyme since a major O-GlcNAc site is located within the ATP binding region of the enzyme [82]. A recent study by Wang *et al*. (2008) demonstrates that the relationship between GlcNAcylation and phosphorylation is more complicated and much more extensive than previously thought. In NIH-3T3 cells, a modest increase in GlcNAcylation levels by PUGNAc or GlcNAc-thiazoline treatment results in decreased phosphorylation on a large number of actively cycling phosphopeptides [16], and

concomitantly results in increased phosphorylation at many other sites. These findings suggest that GlcNAcylation and phosphorylation not only communicate at the protein substrate level but also by regulating each others cycling enzymes.

Several proteins illustrating the interplay between GlcNAcylation and phosphorylation have been identified. Tau is a microtubule-associated protein important in neuronal cytoskeleton, whose aggregation is involved with neurofibrillary tangles in Alzheimer's disease (AD) (for review see [83]). Tau purified from bovine brain is highly GlcNAcylated in the microtubule-binding domain with high stoichiometry of 4 moles GlcNAc/mole tau [84]. GlcNAcylation of tau has a profound effect on its phosphorylation state. A brain targeted knockout of OGT results in neuronal death and hyper-phosphorylation of tau [85], while inhibition of O-GlcNAcase by thiamet-G treatment in rats results in a reduction in phosphorylation of tau at critical sites for Alzheimer's disease pathology (Thr 231, Ser 396, and Ser 422) in rat cortex and hippocampus [72,85]. Indeed, tau's aggregation state and its involvement in AD is regulated by GlcNAcylation as well as phosphorylation [86]. In the brains of AD patients, GlcNAcylated tau is present more in the non-aggregated pool [87], while hyper-phosphorylated tau is prominently present in the aggregated pool, suggesting that GlcNAcylation of tau is beneficial against AD. Reciprocity between GlcNAcylation and phosphorylation on tau demonstrates that the two modifications can influence each other on a set of proteins, but whether both modifications occurs at the same sites or at proximal sites appear to be protein-specific.

GlcNAcylation and phosphorylation can compete for the same modification site on the protein. For example, a proto-oncogene c-Myc, responsible for transcription of genes regulating cell metabolism and proliferation, can be modified by both phosphorylation and GlcNAcylation at Thr 58, a site most often mutated in Burkitt or AIDS-related lymphomas [88-90]. Inhibition of GSK-3 by lithium leads to increased O-GlcNAc and decreased phosphorylation on Thr 58 [89]. Both GlcNAcylation and phosphorylation on c-Myc are highly responsive to cellular status, as in non-dividing cells Thr 58 is occupied by an O-GlcNAc, but is very rapidly replaced by a phosphate upon serum stimulation (see Fig. 5A) [91]. Alternatively, GlcNAcylation and phosphorylation can compete via steric hindrance when the substrate modification sites are within about 10 amino acids from each other. For example, the CTD (tandem repeats sequence of YSPTSTPS at the C-terminus) of RNA polymerase II can be either GlcNAcylated or phosphorylated [92]. The phosphorylation sites are on Ser 2 and Ser 5 of the CTD sequence, while the O-GlcNAc site is mostly at Thr 4 [93]. *In vitro*, phosphorylation of the CTD prevents GlcNAcylation of CTD, and vice versa [93]. It appears that GlcNAcylation and phosphorylation on the CTD are mutually exclusive because the enzymes responsible for both modifications (OGT and TF IIH CTD kinase) have similar K_m value toward the CTD. Since the CTDs of RNA polymerase are highly phosphorylated after the elongation process starts [94], GlcNAcylation and phosphorylation may play opposite roles in regulation of transcription initiation. Potentially, RNA polymerase II may exist in three states: GlcNAcylated, phosphorylated, or naked, depending on the transcriptional status (see Fig. 5B).

GlcNAcylation and phosphorylation crosstalk can be more complicated than competition between the two modifications on the same protein. Cells sometimes have to find the right balance between these two modifications to decide cell fate. For example, an anti-apoptotic protein Akt needs to be phosphorylated at Thr 308 and Ser 473 for full enzymatic activity [22]. Under hyperglycemia, a condition that can lead to increased flux of UDP-GlcNAc and O-GlcNAc via the HBP, Akt is phosphorylated at Ser 473. On the other hand, upon glucosamine treatment, which can also increase O-GlcNAc levels, Akt shows increased O-GlcNAc and decreased phosphorylation at Ser 473 [8], thus, promoting apoptosis. This suggests that cells need to fine-tune the levels O-GlcNAc and phosphorylation for appropriate survival decisions (see Fig. 5C).

IV. Emerging Roles of GlcNAcylation in Cellular Signaling and Diseases

Phosphorylation has long been known as a major regulatory mechanism in cellular processes. Recently, with its interplay with phosphorylation, GlcNAcylation has emerged as another major regulator of cellular signaling. O-GlcNAc is important for life, as OGT deletion in mice results in loss of embryonic stem cell viability [28]. Therefore, conditional OGT knockout cells were created and used to demonstrate that GlcNAcylation is an absolute requirement for proper cellular regulation. Cells lacking OGT show defects in growth responses, as they fail to respond to serum stimulation, exhibit growth arrest due to improper cell cycle progress, and have decreased expression of transcription factors c-Jun, c-Fos, and c-Myc [85]. In addition, tissue-targeted disruption of OGT in mice also causes apoptosis of T cells and neuronal cells, leading to severe motor defects. Loss of O-GlcNAc in mammalian cells mostly affects cell growth, and viability. However, OGT or O-GlcNAcase knockouts in *Caenorhabditis elegans* yield viable worms [25,28,95]. OGT and O-GlcNAcase knockouts in *C. elegans* show defects in insulin signaling and dauer formation, emphasizing the involvement of GlcNAcylation in diabetes. *C. elegans* lacking OGT are hyper-sensitive to insulin-like signaling and exhibit diminished dauer formation, as they have increased trehalose and glycogen storage and decreased fat storage [25,95]. Opposite effects are seen in O-GlcNAcase knockouts, as those mutants develop augmented dauer formation and insulin resistance, mimicking type II diabetes in human [95]. Interestingly, loss of OGT or O-GlcNAcase also share similar phenotype, as either OGT or O-GlcNAcase knockout results in altered serine/threonine phosphorylation profile and increased in GSK-3 protein and phosphorylation, suggesting that balanced O-GlcNAc cycling is also important [95].

Taken together, these studies demonstrate that inappropriate O-GlcNAc levels in cells can cause serious effects in many cellular processes, partly through miscommunication with phosphorylation signaling. Improper GlcNAcylation response to cellular environment can contribute to the development of diseases, such as cancers, neurodegenerative diseases, and diabetes. Several studies have shown that GlcNAcylation plays critical roles in cellular events, including transcription, cell cycle, cellular stress response, and ubiquitin-proteasome degradation, through a variety of mechanisms, which will be discussed in more detailed below.

A. Transcription

GlcNAcylation is often thought as transcriptional silencer because O-GlcNAc is shown to accumulate more in the transcriptionally inactive areas in *Drosophila* polytene chromosome and in mammalian cells [79,96]. However, after several GlcNAcylated proteins involved in transcription were identified, such as Sp1, C-Myc, c-Jun, c-Fos, CREB-binding protein (CBP), and early growth response-1 (EGR-1, associated with long-term memory formation) [48,85, 92], it appears that GlcNAcylation regulates transcription in more complex ways.

GlcNAcylation regulates gene expression by altering transcriptional activity of a transcription factor through its localization, activity, and binding partners. For example, a transcription factor β-catenin is modified by O-GlcNAc, and its GlcNAcylation state affects its localization [97, 98]. Elevation of O-GlcNAc levels by PUGNAc results in increased GlcNAcylation and cytosolic localization of β-catenin, leading to decreased transcriptional activity of β-catenin. The involvement of GlcNAcylation on β-catenin's activity is further shown, as both prostate cancer and breast cancer cell lines have lower O-GlcNAc on β-catenin in the nucleus and higher β-catenin transcriptional activity than that in normal cells [98,99]. Similarly, localization of Sp1, a transcription factor involved in insulin signaling and in the regulation of cellular metabolism, is regulated by its GlcNAcylation status [100,101]. In response to insulin stimulation in a rat hepatoma cell line, GlcNAcylated Sp1 is translocated to the nucleus, resulting in increased transcription of Sp1 target genes, such as calmodulin (CaM) [101]. Increased nuclear localization of Sp1 by GlcNAcylation was further confirmed, when the HBP

is inhibited by a GFAT inhibitor, 6-diazo-5-oxo-norleucine (DON), non-GlcNAcylated Sp1 is sequestered in the cytoplasm, leading to reduced Sp1 activity and increased Sp1 degradation. However, increased GlcNAcylation on Sp1 does not always result in increased Sp1's activity as shown by Jochman *et al*. (2009). Sp1 is required at the HIV1-LTR promoter for the viral LTR promoter activity, but glucosamine treatment on HIV-infected lymphocyte raises O-GlcNAc levels and represses HIV transcription in a dose-dependent manner [102]. It is shown that inhibition of viral promoter by glucosamine is partly due to the GlcNAcylation status of Sp1, as over-expression of OGT results in GlcNAcylation of Sp1 and inhibition of the HIV-1 LTR promoter activity in T cell lines and primary $CD4^+$ T cell lymphocytes. The mechanism of how GlcNAcylation regulates Sp1 binding to the LTR promoter remains unclear since over-expression of OGT does not inhibit Sp1 DNA binding activity or expression. It is possible that other factors are involved. GlcNAcylation may influence the activity of Sp1 or its interaction with other required binding partners differently, depending on specific cellular signaling. For example, when co-expressed with mSin3A in HepG2 cells, OGT can be recruited to promoters by mSin3A and synergically represses Sp-1-activated transcription [80]. These results, nevertheless, open up a new view on viral replication, which may be dependent on the glucose metabolic state of the host cell.

Alternatively, O-GlcNAc regulates transcription factors by altering their interaction with their binding partners. Under hyperglycemia, GlcNAcylation is increased on the p65 subunit of NFκB at Thr 322 and Thr 352 [103], resulting in decreased interaction between NFκB with IκBα. As a result, IκBα can no longer mask the nuclear localization signals of NFκB and maintain NFκB in the cytoplasm in an inactive state, thus, promoting nuclear translocation and increased activity of NFκB. Similarly, GlcNAcylation inhibits transcription of the spliceosomal U2 snRNA by interfering with the interaction between Sp1 and a POU-domain-containing transcription factor Oct1 [104], which is also an O-GlcNAc modified protein [105].

B. Stress and Cell Death

Several studies have shown that GlcNAcylation plays a protective role against cellular stress and cell death. OGT and O-GlcNAc levels are increased in response to several forms of stress, including UV light, hydrogen peroxide, sodium chloride, ethanol, arsenite, or heat shock [106]. Specifically, upon heat shock treatment, O-GlcNAc is rapidly increased on cellular proteins followed by elevation of heat shock protein 70 (Hsp70). Reducing O-GlcNAc levels results in cells that are more sensitive to stress. Interestingly, some Hsp70 family proteins, including p70, p65, and p55, have specific lectin activity toward O-GlcNAc, and the activity is increased after stress [106-108], suggesting that these proteins may act as chaperones for stabilizing GlcNAcylated proteins during stress. This is actually in agreement with the finding that major components of the stress granules, which regulate the translation and decay of messenger RNA, are modified by O-GlcNAc in response to stress [109]. These proteins include glyceraldehyde-3-phosphate dehydrogenase, receptor for activated C kinase 1 (RACK1), prohibitin-2, and a large number of ribosomal proteins, suggesting that O-GlcNAc is required for stress granule assembly upon stress.

Evidence of protective roles of O-GlcNAc is also found in animal models. Glucosamine treatment raises O-GlcNAc levels and protects cardiacmyocytes after ischemia/reperfusion injury by increasing levels of anti-apoptotic protein Bcl_2, and infarct size of damaged heart can also be reduced by O-GlcNAc elevation upon PUGNAc treatment [110,111]. Furthermore, glucosamine treatment can improve cardiac functions in a rat model of trauma-hemorrhage during resuscitation [112] and reduces circulating inflammatory cytokines by inhibiting the NFκB pathway. Over-expression of OGT gives similar results to those of glucosamine treatment, as it causes a reduction in nuclear NFκB and its DNA binding activity, IκB

phosphorylation and mRNA expression of TNF-α and IL6, suggesting that glucosamine may protect cells from stress through GlcNAcylation.

C. Protein Stability and Ubiquitination-Proteasome Degradation Pathway

Ubiquitination-proteasome degradation is an important mechanism for regulation of protein turnover and steady state concentrations of short-lived proteins in cells. Many O-GlcNAc modified sites have high PEST (Pro-Glu-Ser-Thr) scores, which indicate rapid degradation by ubiquitination [113]. Modification by O-GlcNAc on these sites may inhibit or slow down degradation of these proteins. In fact, GlcNAcylation often protects its substrate against proteasomal degradation. Treating MCF7 cells with STZ increases GlcNAcylation and accumulation of a transcription factor p53 [114]. Although it has not been proven in this study that STZ specifically inhibited O-GlcNAcase activity, it was shown that p53 accumulation was not due to STZ's toxicity, as STZ treatment did not affect mRNA levels of p53 [114]. STZ treatment causes decreased p53 interaction with Mdm2 and decreased p53 ubiquitination, suggesting that increasing O-GlcNAc levels induces p53 accumulation by decreasing p53 protein degradation. Additionally, a competition between GlcNAcylation and phosphorylation for protein substrate at the proximal modification sites is involved in this process. STZ treatment inhibits phosphorylation of p53 on Thr 155, which is induced by COP9 signalosome (CSN) and is required for p53 degradation, and results in increased GlcNAcylation on Ser 149, thus, blocking p53 from being phosphorylated and degraded by ubiquitin-proteasomal mechanism. Similarly, GlcNAcylated forms of murine β-estrogen receptor are more resistant to proteasome degradation than non-GlcNAcylated form, while phosphorylated β-estrogen receptor is more susceptible to proteasomal degradation [115].

GlcNAcylation also regulates the proteasome complex directly. O-GlcNAc protects Sp1 from proteolysis, since increased O-GlcNAc on Sp1 by either high glucose or glucosamine treatment results in more Sp1 stability toward proteasomal degradation [100]. However, later *in vitro* studies argue that the O-GlcNAc state of Sp1 may not have any effects on its degradation [116]. Instead, addition of recombinant OGT partially inhibits the 26S proteasome through its ATPase activity by GlcNAcylating Rpt2 protein on the 19S cap of the proteasome *in vitro*. It should be emphasized that whether GlcNAcylation inhibits the proteasome directly is not known *in vivo*.

Even though GlcNAc modification seems to negatively regulate proteasomal degradation, there may be a common regulation for GlcNAcylation and ubiquitin-proteasome mechanism. Both ubiquitin and O-GlcNAc levels are decreased when blocking the HBP flux, deleting OGT, or during in some case of glucose starvation [117]. Both modifications are also increased upon PUGNAc treatment. Interestingly, E1 family, the enzyme that initiates ubiquitination process, can be GlcNAcylated, and its GlcNAcylation status determines its complex formation with Hsp70. These observations suggest that the ratio of ubiquitin and O-GlcNAc might be a switching point for proteolysis.

D. O-GlcNAc in Neuronal Regulation

O-GlcNAc and OGT levels are abundant in brain and neurons [54,118,119], and GlcNAcylation is directly involved in regulation of neuronal development, synaptic transmission, and synaptic plasticity [43,119,120]. A recent study in primary chicken forebrain neurons shows that O-GlcNAc plays a role in axon branching [119]. Axon branching stimulated by cAMP signaling is inhibited by raising O-GlcNAc levels, while over-expression of O-GlcNAcase increases the number of neurons exhibiting axon branching and axonal filodia number [119]. GlcNAcylation also has important functions in synaptic plasticity and excitatory synapse. In the synaptosome, many O-GlcNAc-modified proteins, such as Bassoon and Piccolo, are mostly involved in synaptic transmission [43]. Elevation of O-GlcNAc levels by

an O-GlcNAcase inhibitor, 9d, up-regulates phosphorylation signaling and synaptic plasticity in hippocampus slice, by activation and phosphorylation events on Erk 1/2 and calmodulin kinase II. Both kinases are required for establishment of long-term potentiation (LTP), which is associated with learning and memory [120]. Additionally, increasing O-GlcNAc levels increases phosphorylation on synapsinI/II at Ser 9 (cAMP-dependent protein kinase activation), Ser 62/67 (activation of Erk1/2 pathway), and Ser 603 (calmodulin kinase II activation), promoting synaptic vesicle in the reserve pool for release during synaptic transmission [120].

E. O-GlcNAc in Diabetes

One of the most important aspects of GlcNAcylation is its involvement in diabetes. GlcNAcylation is highly responsive to glucose levels, [87,121,122]. Transgenic mice over-expressing glucose transporter (GLUT1) in skeletal muscle show increased glucose flux, UDP-GlcNAc concentration, and levels of O-GlcNAc modified proteins, likely, through the HBP [122]. Additionally, GlcNAcylation appears to play important roles in pancreatic β-cells, as high levels of OGT mRNA and O-GlcNAc are found there [83,117]. GlcNAcylation regulates β-cells functions through insulin synthesis through major transcription factors in β-cells. Pancreatic/duodenal homeobox-1 protein (PDX-1) is a transcription factor responsible for pancreas development and insulin gene transcription (for review see [123]). Increased global GlcNAcylation enhances PDX1 DNA binding [124,125]. Although PDX1 is an O-GlcNAc modified protein, it is not known whether increased DNA binding activity of PDX1 is due to elevation of GlcNAcylation on PDX1 or GlcNAcylation effects on the insulin signaling pathway. GlcNAcylation also regulates the localization and activity of the pancreatic β cell-specific transcription factor NeuroD1 in the insulinoma cell line Min6 [126]. Under low glucose conditions, NeuroD1 is localized mainly in the cytosol, while high glucose treatment or inhibition of O-GlcNAcase promotes GlcNAcylation and nuclear transport of NeuroD1. Interestingly, NeuroD1 interacts with OGT in high glucose levels, but interacts with O-GlcNAcase and becomes de-GlcNAcylated under low glucose conditions. Although, the subcellular location where NeuroD1 interacts with OGT or O-GlcNAcase is unclear, it is presumed that interaction with O-GlcNAcase helps NeuroD1 export into cytosol or interaction with OGT facilitates NeuroD1 nuclear import. Taken together, hyperinsulinemia may be due to high glucose influx, causing chronic elevation of GlcNAcylation, followed by increased transcriptional activity of PDX1 and NeuroD1, which leads to improper levels of insulin secretion.

Increased O-GlcNAc levels also appear to be involved with insulin resistance and development of diabetes. High glucose treatment or glucosamine treatment results in GlcNAcylation of glycogen synthase, causing reduced enzyme activity and decreased glucose uptake for glycogen storage [127]. Furthermore, elevation of global O-GlcNAc levels by PUGNAc followed by insulin stimulation leads to reduced glucose uptake and insulin resistance in 3T3-L1 adipocytes [128]. Upon PUGNAc treatment followed by insulin stimulation, insulin-stimulated phosphorylation of Akt at Thr 308 is inhibited, leading to reduced phosphorylation of its substrate GSK-3β at Ser 9. However, recent studies with a more selective O-GlcNAcase inhibitor suggests that other mechanisms may be involved in insulin-resistance [69].

Another major area of O-GlcNAc's role in diabetes lies in the regulation of transcription of genes that are involved in glucose-response signaling. High levels of GlcNAcylation on transcription factors FoxO1, SP1, and the element-binding protein (CREB) 2 (CRTC2) increase expression of gluconeogenic genes in response to high glucose [7,20,129,130]. This over-active transcriptional activity could lead to glucose toxicity. High glucose levels or PUGNAc treatment raise O-GlcNAc levels on Sp1 and results in enhanced transcription of plasminogen activator inhibitor–1 (PAI-1), which is involved in the development of diabetic nephropathy

and atherosclerosis associated with diabetes [131-133]. These effects can be reversed by over-expression of O-GlcNAcase, by siRNA knockdown of OGT, or by over-expressing a dominant negative OGT. Similarly, GlcNAcylation of CRTC2 and FoxO1 induced by high glucose can stimulate hepatic glucogenesis independent of insulin signaling [7,20,130]. Under normal condition, CRTC2 is phosphorylated at Ser 70 and Ser 171, rendering in the cytoplasm by 14-3-3 protein [130]. However, upon high glucose treatment, CRTC2 becomes GlcNAcylated at Ser 70 and Ser 171, allowing nuclear translocation and transcription of glucogenic genes in hepatocyte. These results demonstrate another example of dynamic interplay between GlcNAcylation and phosphorylation.

In addition, in combination with high glucose levels, increased GlcNAcylation levels also contribute to impaired cardiac myocyte functions. Rat myocyte exposed to high glucose exhibits increased GlcNAcylation, causing impaired calcium cycling in the heart by reducing the transcription of a sarcoplasmic reticulum SerCA2a and ATPAse [134]. Furthermore, increased GlcNAcylation by high glucose treatment or by hyperglycemia contributes to cardiac myocyte dysfunction [135]. Several members of the respiratory chain proteins are GlcNAcylated, including subunit NDUFA9 of complex I, and the mitochondrial DNA encoded subunit of complex I (COXI). Increased mitochondrial GlcNAcylation causes decreased mitochondrial calcium and cellular ATP. As increased GlcNAcylation results in impaired activity of complex I, III, and IV, over-expression of O-GlcNAcase can improve the mitochondria functions.

V. Final Remarks

O-GlcNAc modification is a rapidly growing field. Nearly one thousand O-GlcNAc modified proteins have now been identified since its first discovery. Many of these proteins are important regulatory proteins in cellular events, suggesting that GlcNAcylation is one of the major regulators in cellular processes. Inappropriate GlcNAcylation causes serious effects, such as cell cycle arrest and cell death. Importantly, GlcNAcylation has an extensive and dynamic interplay with phosphorylation, and dysregulation of the balance between these two modifications likely underlies chronic diseases. Further studies on functions of GlcNAc-modified proteins and, especially, on the regulation of OGT and O-GlcNAcase will provide a better understanding of the roles of O-GlcNAc modification in cellular signaling.

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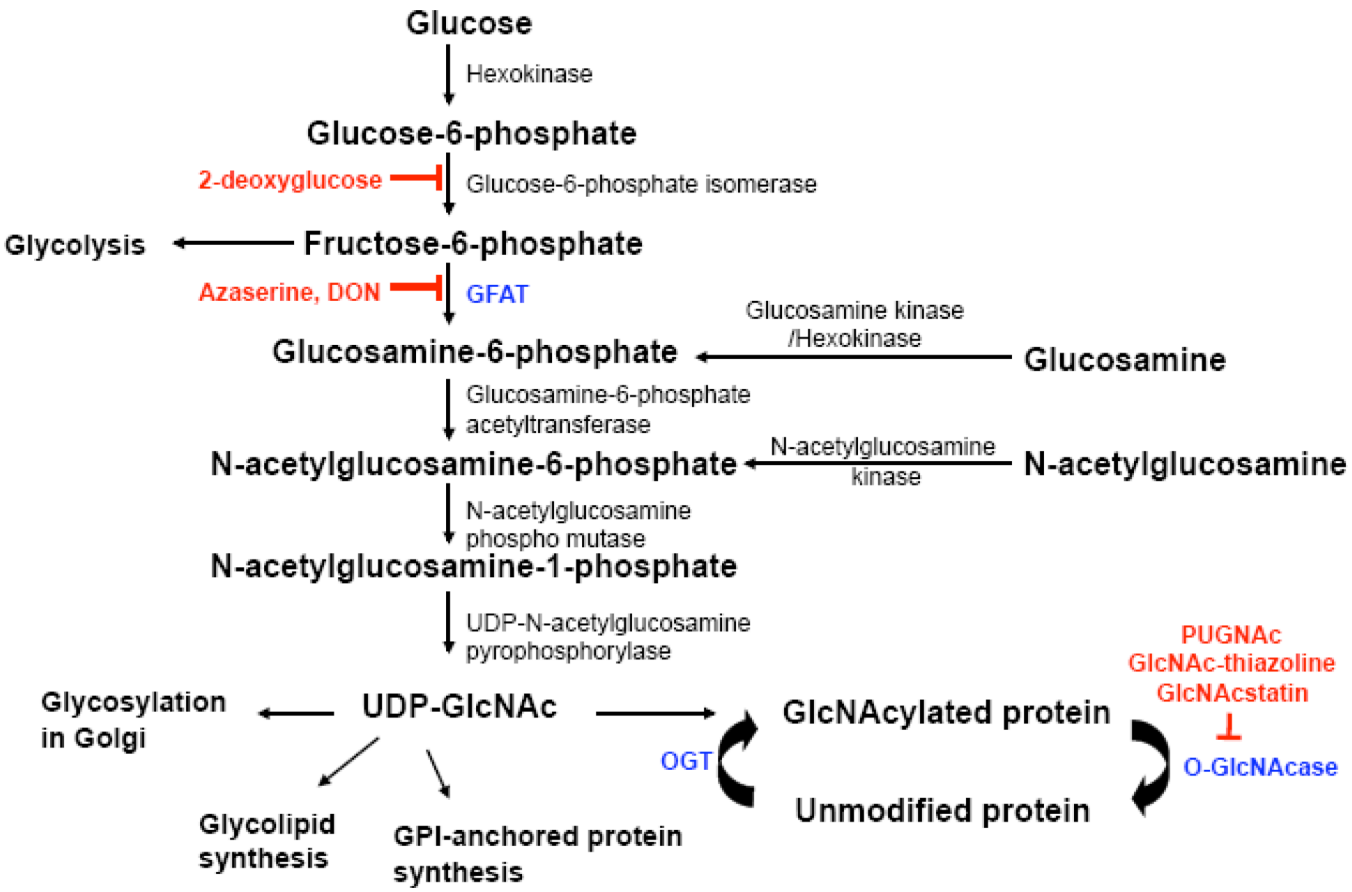

Figure 1.

Hexosamine biosynthetic pathway (HBP). The synthesis of UDP-GlcNAc from glucose and enzymes involved in the process are shown. Commonly used inhibitors of HBP and GlcNAcylation are shown in red.

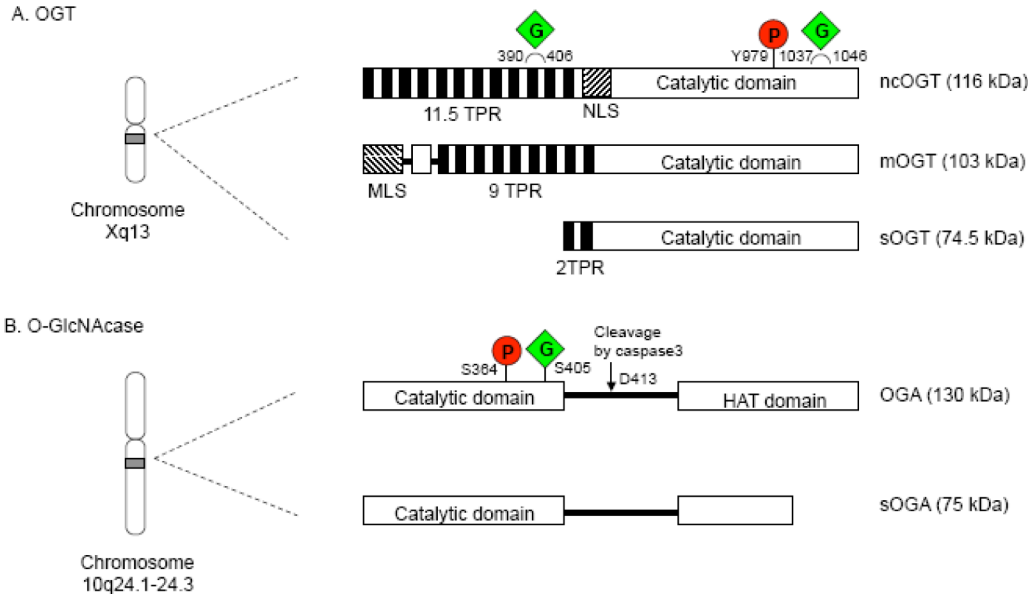

Figure 2.

Schematic structures of OGT and O-GlcNAcase. A. Three different forms of human OGT are produced by alternative splicing from a single gene which resides near the gene of Parkinson's dystonia in chromosome X. Known post-translational modifications are indicated by amino acid positions (if available). B. Two isoforms of O-GlcNAcase are known. The gene is located near a locus of late on-set Alzheimer's disease. Known post-translational modifications are indicated by amino acid positions and a caspase-3 cleavage site is shown. NLS: nuclear localization signal, MLS: mitochondrial localization signal.

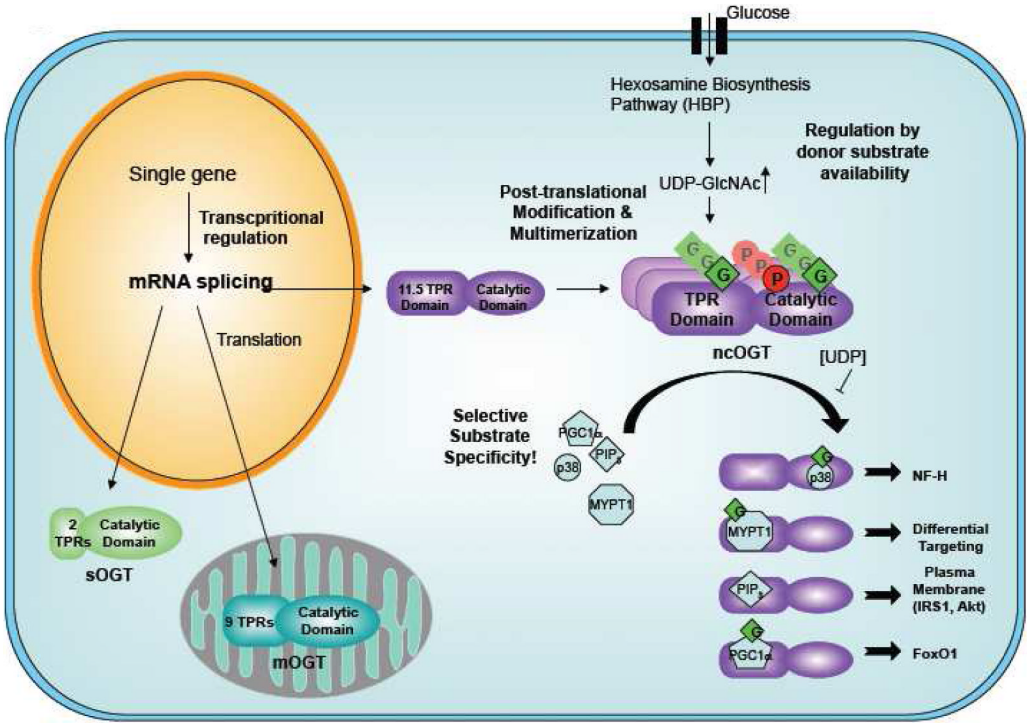

Figure 3.

Mechanism of OGT regulation. OGT is regulated by multiple factors including transcriptional regulation, mRNA splicing, donor substrate availability, post-translational modification and multimerization. Multimerized ncOGTs form dynamic holoenzymes with many different protein partners and regulate differential targeting of proteins as well as their GlcNAcylation.

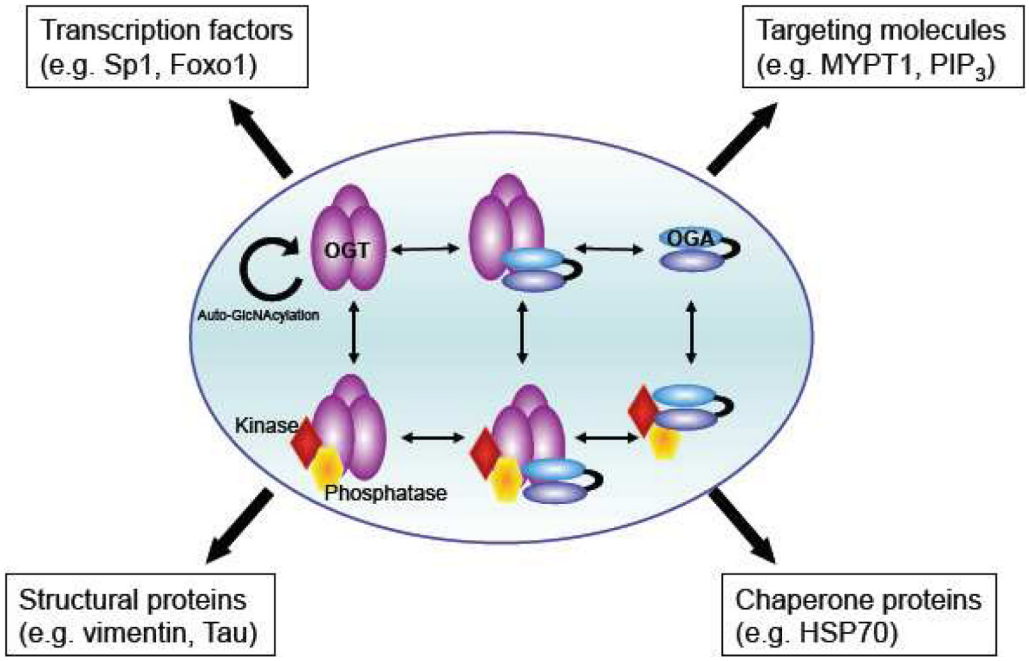

Figure 4.

Complex regulation of OGT and O-GlcNAcase. OGT and O-GlcNAcase can form very dynamic complexes with each other and/or kinases and phosphatases under different cellular signalings. Transient complex formation between O-GlcNAc regulatory enzymes and various binding partners enables a highly sensitive regulation of GlcNAcylation in response to specific cellular conditions. OGT: purple, O-GlcNAcase: blue, Kinase: red, Phosphatase: yellow.

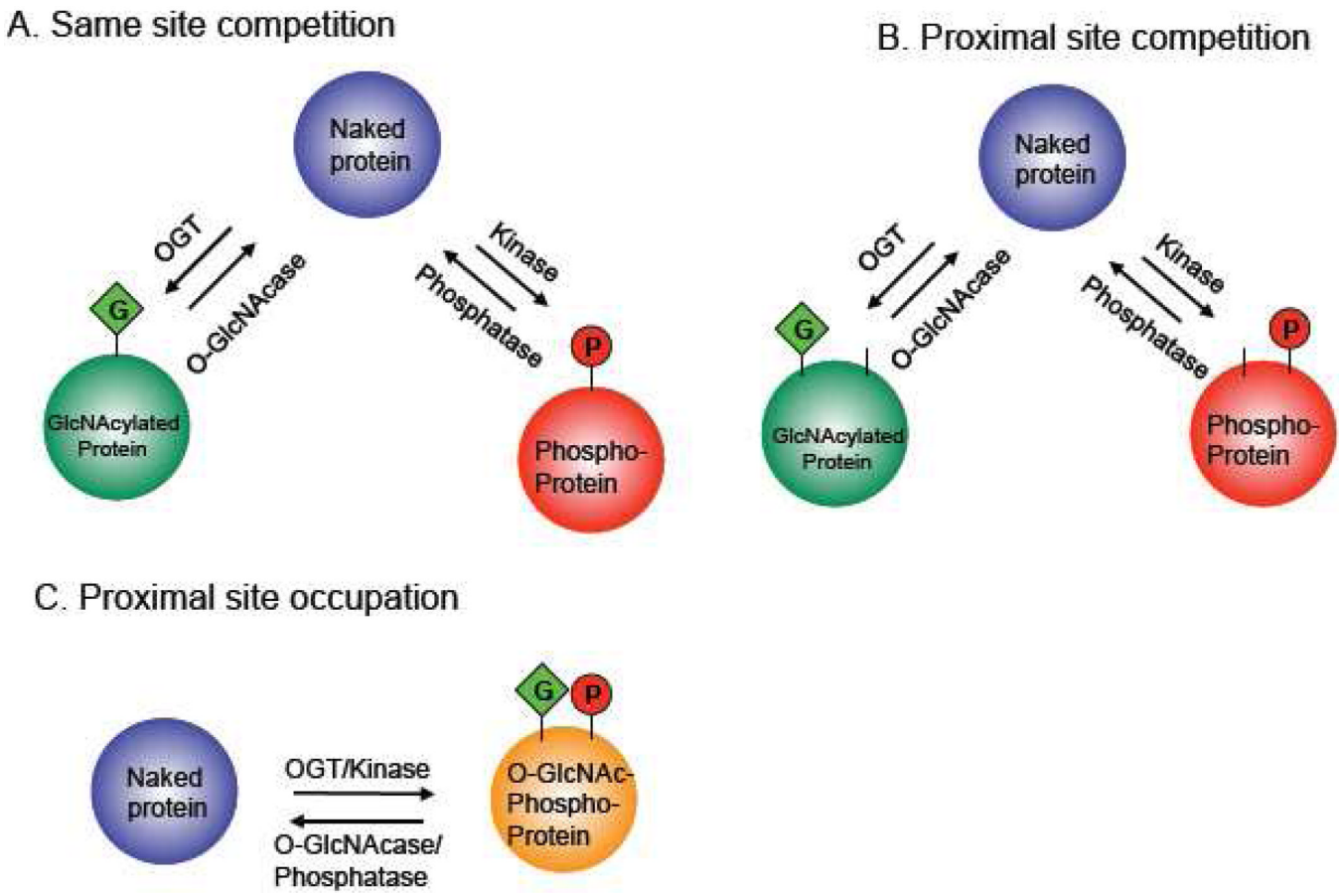

Figure 5.

Dynamic interplay between of GlcNAcylation and phosphorylation. A. O-GlcNAc and O-phosphate can compete for the same site. This competition can change the activity or stability of the proteins (e.g. c-Myc; Thr 58). B. In some cases, O-GlcNAc and O-phostate modification occurs within ~10 amino acids range, regulating the function of the protein substrates (e.g. CTD repeat; Ser 2 and 5 for O-phosphate, Thr 4 for O-GlcNAc). C. GlcNAcylation and phosphorylation can occur on the same protein at proximal sites. The balance between GlcNAcylation and phosphorylation can change the cellular function of the protein (e.g. Akt; Thr 308 and Ser 473 for O-phosphate, Ser 473 for O-GlcNAc).