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Chemical tools to explore nutrient-driven O-GlcNAc cycling

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Abstract

Posttranslational modifications (PTM) including glycosylation, phosphorylation, acetylation, methylation and ubiquitination dynamically alter the proteome. The evolutionarily conserved enzymes *O*-linked *N*-acetylglucosamine (*O*-GlcNAc) transferase (OGT) and *O*-GlcNAcase are responsible for the addition and removal, respectively, of the nutrient-sensitive PTM of protein serine and threonine residues with *O*-GlcNAc. Indeed, the *O*-GlcNAc modification acts at every step in the “central dogma” of molecular biology and alters signaling pathways leading to amplified or blunted biological responses. The cellular roles of OGT and the dynamic PTM *O*-GlcNAc have been clarified with recently developed chemical tools including high-throughput assays, structural and mechanistic studies and potent enzyme inhibitors. These evolving chemical tools complement genetic and biochemical approaches for exposing the underlying biological information conferred by *O*-GlcNAc cycling.

Keywords

O-GlcNAc; O-GlcNAc transferase; OGT's activity assays; OGT's catalytic mechanism; OGT's inhibitors

Introduction

The modification of serine and threonine residues of nuclear and cytoplasmic proteins by the monosaccharide, 2-acetamideo-2-deoxy-D-glucopyranose (GlcNAc) through a β -glycosidic linkage (*O*-linked *N*-acetylglucosamine; *O*-GlcNAc) is a notable post-translational glycosylation. *O*-GlcNAc is present in all intracellular compartments with a particular abundance on nuclear proteins. Its addition and removal is a dynamic process, turning over more rapidly than the polypeptide backbone of the proteins it modifies. Its rapid, continual cycling poises the modification to influence diverse cellular processes including nutrient signaling (Yang *et al.*, 2008), regulation of gene transcription and translation (Sinclair *et al.*, 2009), neural development (Rex-Mathes *et al.*, 2001), stress response (Zachara *et al.*, 2004) and cell division (Slawson *et al.*, 2005).

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Declaration of interest

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In mammals, only two genes encode the evolutionarily conserved nucleocytoplasmic enzymes responsible for the addition and removal of *O*-GlcNAc. Uridine diphospho-*N*-acetyl-glucosamine:polypeptide β -*N*-acetylglucosaminyl transferase, termed *O*-GlcNAc transferase (OGT), uses the donor substrate uridine diphospho-2-acetamideo-2-deoxy-D-glucopyranose (UDP-GlcNAc) to add the monosaccharide to target protein serine and threonine residues (Haltiwanger *et al.*, 1990; Kreppel *et al.*, 1997; Lubas *et al.*, 1997). A glycoside hydrolase, β -*N*-acetylglucosaminidase, termed *O*-GlcNAcase (OGA), returns glycosylated proteins to their unmodified state by removing *O*-GlcNAc (Gao *et al.*, 2001). Although this review focuses on the protein responsible for the addition of the ubiquitous nuclear and cytoplasmic *O*-GlcNAc, we note a third enzyme that is located in the secretory pathway, eOGT, adds *O*-GlcNAc to a small subset of cell surface proteins (Müller *et al.*, 2013; Sakaidani *et al.*, 2012; Shaheen *et al.*, 2013).

Because the hexosamine biosynthetic pathway (HBP) requires rate-limiting metabolites, nearly every metabolic pathway in the cell affects the production of UDP-GlcNAc (Figure 1). This activated sugar nucleotide product of the hexosamine biosynthetic pathway (HBP) (Marshall *et al.*, 1991) is used by OGT, thereby poising *O*-GlcNAc to respond to cellular stimuli including insulin, nutrients and cellular stress (Hanover, 2001; Hart *et al.*, 2007; Love & Hanover, 2005; Wells *et al.*, 2001) and makes OGT an attractive therapeutic target. Indeed, deregulation of *O*-GlcNAc has been suggested to play a mechanistic role in several diseases processes including diabetes (McClain *et al.*, 2002; Vosseller *et al.*, 2002; Yang *et al.*, 2008), cancers (Caldwell *et al.*, 2010) and neurodegenerative disorders (Lazarus *et al.*, 2009; Liu *et al.*, 2004; Yuzwa *et al.*, 2008) as well as a protective role in acute stress and ischemia/reperfusion injury (Chatham & Marchase, 2010; Laczy *et al.*, 2009). Methods for detecting and modulating *O*-GlcNAc continue to expand with a focus on optimizing the chemical tools that will further our understanding of the biological roles played by OGT and *O*-GlcNAc (Çetinba *et al.*, 2006; Dorfmüller *et al.*, 2011; Gloster *et al.*, 2011; Gross *et al.*, 2005; Jiang *et al.*, 2012; Kim *et al.*, 2006a,b; Macauley *et al.*, 2005). This review highlights assays and molecules that have been applied to elucidate OGT's activity at the molecular level, potent OGT inhibitors, high-throughput OGT activity assays and the structural and mechanistic studies of OGT.

***O*-GlcNAc transferase**

OGT was first characterized from cytosolic fractions of rabbit reticulocytes (Haltiwanger *et al.*, 1990) and was later purified from rat liver cytosol where it was present in two forms: a 110-kDa α -subunit and a 78-kDa β -subunit (Haltiwanger *et al.*, 1992). Subsequently, the OGT genes in rat (Kreppel *et al.*, 1997), *Caenorhabditis elegans* (Lubas *et al.*, 1997), human (Lubas *et al.*, 1997) and plant (Olszewski *et al.*, 2010; Thornton *et al.*, 1999) were cloned. The human OGT gene is composed of 23 exons and 21 introns and resides on the X chromosome near the Xist locus (Xq13.1), which is a region associated with Parkinson's disease (Shafi *et al.*, 2000). Knockout studies have shown that mammalian OGT is essential for the viability of embryonic stem cells and embryonic development (Shafi *et al.*, 2000). Interestingly, OGT null *C. elegans* are viable but exhibit profound metabolic changes, such as insulin resistance and macronutrient storage modifications (Hanover *et al.*, 2005). Although OGT is encoded by a single, highly conserved gene in animals (Kreppel *et al.*,

1997; Lubas & Hanover, 1997), the enzyme has over 1000 protein targets (Hahne *et al.*, 2013; Hart *et al.*, 2007; Love & Hanover, 2005; Wells *et al.*, 2001). The implication of OGT's regulated modification of its numerous protein substrates will be briefly explored at the conclusion of this article.

OGT isoforms

Three predominant human OGT isoforms are generated by alternative splicing (Figure 2): the longest isoform produces a protein encoded by exons 1–4 spliced directly to exon 6 yielding a ~ 116 kDa protein, which localizes to both the nucleus and cytoplasm (designated ncOGT) (Hanover *et al.*, 2003). ncOGT has 12 tetratricopeptide repeat (TPR) motifs and is known to be associated with transcriptional repression (Comer & Hart, 2001), proteasomal inhibition (Zhang *et al.*, 2003, 2007) and stress tolerance (Zachara *et al.*, 2004). One striking feature of human OGT is that the fourth intron can also encode an exon (exon 5), which contains an unique ATG. Use of exons 5–23 encodes the second longest isoform producing a ~ 103 kDa protein. This isoform produces an N-terminal domain that contains a unique mitochondrial targeting sequence, followed by a membrane-spanning α -helix that (termed mOGT) (Love *et al.*, 2003). The mOGT variant has nine TPRs and may be associated with the activation of transcription factor Sp1 by mitochondrial superoxide overproduction (Du *et al.*, 2001). The shortest OGT isoform has a truncated amino terminus encoded by exons 10–23, producing a protein of ~ 75 kDa, and it has been termed sOGT for its short length (Hanover *et al.*, 2003). The sOGT variant contains two TPR motifs, is localized to both the nucleus and the cytoplasm (Lazarus *et al.*, 2006) and is proposed to have an anti-apoptotic function (Fletcher *et al.*, 2002).

All OGT isoforms consist of two distinct N-terminal and C-terminal domains separated by an intermediate spacer region (Figure 2). The N-terminal domain contains a variable number of TPR motifs. The TPR motif itself consists of 34 amino acids, of which eight residues are highly conserved (Blatch & Lässle, 1999; D'Andrea & Regan, 2003) and is found as a domain in various proteins throughout many organisms (Blatch & Lässle, 1999). TPR motifs are often found in serial arrays and are arranged as two α -helices packed together to form a superhelix with a large groove responsible for binding protein substrates or accessory proteins (Blatch & Lässle, 1999; D'Andrea & Regan, 2003; Kreppel & Hart, 1999; Lubas & Hanover, 2000). The OGT C-terminal catalytic domain (CD) is identical in all three isoforms and has homology to glycogen phosphorylase (Wrabl & Grishin, 2001). The CD consists of three regions: an N-terminal catalytic region (CD-1), C-terminal catalytic region (CD-2) and the intervening region between CD-1 and CD-2. Expression of all three OGT isoforms vary among cell and tissue types suggesting that each may have distinct functions responding to cellular cues (Hanover *et al.*, 2003; Lazarus *et al.*, 2006). In addition, the OGT isoforms have been shown to exhibit *in vitro* peptide and protein preferences (Lazarus *et al.*, 2006).

With a varying number of TPR motifs, OGT serves as a binding scaffold for numerous partners (Andrali *et al.*, 2005; Cheung *et al.*, 2008; Housley *et al.*, 2009; Iyer & Hart, 2003; Wells *et al.*, 2004; Yang *et al.*, 2002). By the sequential removal of TPR segments, the role of TPR domain length in OGT substrate recognition has been explored. It has been

suggested that OGT uses different TPR motifs to recognize specific protein substrates (Kreppel & Hart, 1999; Lubas & Hanover, 2000) and that the TPR domains affect OGT's catalytic activity (Iyer & Hart, 2003). For example, Iyer *et al.* demonstrated that removal of the first 2.5 TPR domains of rat OGT impaired its catalytic activity but the construct was still able to effectively bind its interacting partner OIP106 (OGT-interacting protein, 106 kDa) (Iyer & Hart, 2003). Regulation of OGT activity has been proposed to occur *via* its interaction with other protein partners such as peroxisome proliferator-activated receptor- γ -co-activator 1 α (PGC1- α) (Housley *et al.*, 2009), myosin phosphatase-targeting subunit 1 (Cheung *et al.*, 2008) and p38 MAP kinase (Cheung & Hart, 2008) as well as the formation of its complex with OGA (Whisenhunt *et al.*, 2006). Recent *in vitro* data, however, does not clearly support the formation of a functionally significant complex between OGT and OGA affecting OGT activity (David *et al.*, 2012). It has been proposed that OGT activity can be regulated *via* post-translational modifications including phosphorylation (Song *et al.*, 2008; Whelan *et al.*, 2008), cysteine nitrosylation (Ryu & Do, 2011) and O-GlcNAc modification (Kreppel *et al.*, 1997). Finally, recent kinetic studies showed that both OGT and OGA give different *in vitro* second-order rate constants toward different protein substrates, and these values can differ in response to the post-translational modification of the target proteins (David *et al.*, 2012).

O-GlcNAc level modulation through small molecule inhibition

Manipulation of intracellular O-GlcNAc levels has been widely used to elucidate the biological functions of the O-GlcNAc modification. Global O-GlcNAc levels can be increased and decreased by both genetic and pharmacological methods, the latter of which is discussed herein. Since an overview of OGA inhibitors and an increase in O-GlcNAc levels has been well described in recent literature (Macauley & Vocadlo, 2010), we discuss several approaches of decreasing O-GlcNAc levels *in vitro* and *in vivo* with a special emphasis on small molecule inhibition of OGT.

Decreasing levels of UDP-GlcNAc

Diminishing the upstream UDP-GlcNAc concentration is one method used to globally decrease O-GlcNAc addition to proteins. To decrease flux through the HBP, inhibitors of GFAT (the first and rate-limiting enzyme of the HBP that converts fructose-6-phosphate to glucosamine-6-phosphate) have been used (Gao *et al.*, 2003; Majumdar *et al.*, 2003). Successful GFAT inhibitors are glutamine analogs, 6-diazo-5-oxo-L-norleucine (**1**, Figure 3) (Dion *et al.*, 1956) and O-(2-diazoacetyl)-L-serine (azaserine, **2**, Figure 3) (Kolm-Litty *et al.*, 1998). Not only do **1** and **2** impact O-GlcNAc levels but they also inhibit amidotransferases in a number of metabolic pathways (Staiano *et al.*, 1980; Wu *et al.*, 1999) suggesting that results obtained using these compounds need to be interpreted with great caution. Indeed, OGT is not the only enzyme that uses UDP-GlcNAc as a substrate: many other N-acetylglucosaminyl transferases use UDP-GlcNAc to construct cell surface glycoconjugates including N-glycans, mucin-type O-linked glycans and glycosphingolipids. Finally, UDP-GlcNAc is a versatile metabolic precursor that is converted into other nucleotide sugar substrates such as cytidine-5'-monophospho-sialic acid (**3**, Figure 3) and UDP-N-acetylgalactosamine (GalNAc, **4**, Figure 3) (Du *et al.*, 2009). Given the central role UDP-

GlcNAc plays in various metabolic pathways, the global decrease of UDP-GlcNAc to study *O*-GlcNAc's biological role is not an ideal research strategy.

OGT inhibition by small-molecule inhibitors

Selective inhibition of OGT's catalytic function is the ideal way to profile the specific roles of the *O*-GlcNAc modification. Small-molecule inhibitors have proven invaluable for elucidating the biological functions of many classes of enzymes (Knight & Shokat, 2007; Mayer, 2003). Cell permeable small-molecule inhibitors provide several advantages over genetic methods: (1) there is no need for transfection reagents or viral infection and (2) cells can be monitored in a dose- and time-dependent manner. Small molecules are especially useful in cells that are not easily transfected and to monitor whether inhibition yields lasting cellular effects upon inhibitor removal.

An ideal OGT inhibitor has high affinity to OGT, is cell membrane-permeable and exhibits a high specificity to OGT inhibition. The inhibitors described below were discovered by either library screening or rational design. For an in-depth look at several small molecule inhibitors of OGT and OGA, please refer to a recent review and the references therein (Ostrowski & van Aalten, 2013).

UDP—UDP (**5**, Figure 3) is a by-product of the OGT glycosyl transfer reaction using UDP-GlcNAc and is a potent feedback inhibitor of OGT. A reported half-maximal inhibition (IC_{50}) value of UDP is 1.8 μ M for recombinant human ncOGT with the use of a small biotinylated peptide substrate (called biotinylated DEBtide) (Dorfmueller *et al.*, 2011). UDP was reported to have a 50% inhibition value of 0.2 μ M when a multimeric OGT enzyme purified from rat liver cytosol and a synthetic peptide (YSDSPSTST) were used (Haltiwanger *et al.*, 1992). UDP occupies a pocket in the carboxy-terminal domain of the catalytic region of OGT and acts as a competitive UDP-GlcNAc inhibitor (Lazarus *et al.*, 2011). Although UDP is a potent inhibitor of OGT, it also inhibits many other glycosyltransferases that utilize UDP-containing sugar nucleotides.

Alloxan—The diabetogenic compound alloxan (**6**, Figure 3), a uracil analog, is a chemically unstable compound with a half-life of 1.5 min at 37 °C in phosphate buffer at pH 7.4 (Lenzen & Munday, 1991). Nonetheless, alloxan was demonstrated to be an OGT inhibitor (Dorfmueller *et al.*, 2011; Konrad *et al.*, 2002). Konrad *et al.* measured complete inhibition of OGT activity at 1 mM and IC_{50} at 0.1 mM alloxan by a traditional radiometric assay using a recombinant protein substrate, nucleoporin 62 (Nup62) and a radioisotope-labeled sugar donor substrate, UDP- $[^3H]$ -GlcNAc. Other experiments suggest alloxan exhibits more effective OGT inhibition ($IC_{50} \sim 18 \mu$ M) with a small synthetic peptide acceptor substrate (Dorfmueller *et al.*, 2011). The modification of cysteine residues (Konrad *et al.*, 2002) or binding to the uracil binding pocket are two proposed methods of alloxan inhibition (Dorfmueller *et al.*, 2011). Alloxan has been used to investigate OGT's cellular function and has been shown to decrease *O*-GlcNAc levels in several studies (Dehennaut *et al.*, 2007; Kang *et al.*, 2008; Liu *et al.*, 2005, 2006, 2007; Noach *et al.*, 2007). For example, in the study of *O*-GlcNAc's role in cell cycle regulation (Dehennaut *et al.*, 2007), treatment with alloxan decreased *O*-GlcNAc levels without a decrease in the cellular UDP-GlcNAc

pool and blocked the G₂/M transition in an OGT-inhibition dependent manner in *Xenopus laevis* oocyte. One concern for the use of alloxan is its toxicity through reactive oxygen species generation (Zhang *et al.*, 1992), but the study by Dehennaut *et al.* (2007) suggests that the mechanism of G₂/M transition inhibition is not caused by its toxic effect as two detoxifying enzymes were used in conjunction with the experiments. The lack of target specificity is a second concern for alloxan as it may affect many cellular processes beyond OGT catalysis (Lenzen & Panten, 1988). Indeed, alloxan was also shown to act as a weak OGA inhibitor (5mM alloxan was required for 62% inhibition of recombinant OGA activity), although its inhibition mechanism was not fully established (Lee *et al.*, 2006).

UDP-C-GlcNAc and UDP-S-GlcNAc—Replacing the UDP-GlcNAc glycosidic oxygen with nonhydrolysable isosteric linkages was hypothesized to confer resistance toward enzymatic and chemical hydrolysis. To explore this idea, two OGT inhibitors were synthesized and evaluated: UDP-*C*-GlcNAc (**7**, Figure 3) and UDP-*S*-GlcNAc (**8**, Figure 3), in which the glycosidic oxygen is replaced by a carbon or sulfur atom, respectively (Dorfmueller *et al.*, 2011; Hajduch *et al.*, 2008). Despite its promise, UDP-*C*-GlcNAc proved to be a poor inhibitor with an IC₅₀ of more than 5 mM, suggesting that the presence of the glycosidic oxygen in UDP-GlcNAc is important for substrate binding (Hajduch *et al.*, 2008). It is possible that the lack of hydrogen bonds between the isosteric methylene group of the C1-phosphonate analog of UDP-GlcNAc and the active site residues of the enzyme may severely weaken the substrate binding affinity. UDP-*S*-GlcNAc and UDP-*C*-GlcNAc were found to occupy different positions in OGT's active site. Although UDPS-GlcNAc positions the thioglycosidic bond within hydrogen bonding distance of OGT's active site residues, its inhibitory property was reported to be even worse than UDP-*C*-GlcNAc (2.3 times) (Dorfmueller *et al.*, 2011). Moreover, UDP-*C*- and UDP-*S*-GlcNAc are likely to inhibit other glycosyltransferases that utilize UDP-GlcNAc as a donor and neither analog is readily cell-permeable precluding their use for cellular studies.

UDP-5SGlcNAc—Inhibitors for cell-based studies require minimal polarity and charge to ensure cell permeability. Taking advantage of the promiscuity of cellular enzymes that tolerate unnatural sugar analogs (Agard & Bertozzi, 2009; Jones *et al.*, 2004; Keppler *et al.*, 2001; Vocadlo *et al.*, 2003), Gloster *et al.* (2011) introduced cell membrane permeable OGT inhibitor precursors. In their strategy, 2-acetamido-1,3,4,6-tetra-*O*-acetyl-2-deoxy-5-thio- α -D-glucopyranose [(Ac)₄-5SGlcNAc] (**9**, Figure 3) is converted to UDP-5SGlcNAc (**10**, Figure 3) through the HBP and the GlcNAc salvage pathway in cells (Figure 1). UDP-5SGlcNAc **10** is a chemically modified analog of UDP-GlcNAc, with the endocyclic oxygen atom replaced by sulfur. This molecule was found to be a much poorer donor substrate than natural UDP-GlcNAc (~ 0.03% glycosyl transfer compared with that of the natural sugar donor) (Lazarus *et al.*, 2012) and served as a potent OGT inhibitor both *in vitro* and in cells (Gloster *et al.*, 2011). Consistent with previous findings (Offen *et al.*, 2006; Tsuruta *et al.*, 1997, 2003), results demonstrated that no measurable amount of 5SGlcNAc-modified proteins were detected in cell-based assays. In addition, Gloster *et al.* (2011) defined that this is not due to the faster hydrolysis of 5SGlcNAc-modified proteins by OGA but due to the much less efficient transfer of 5SGlcNAc by OGT (UDP-5SGlcNAc was measured to be approximately three times worse substrate for OGT compared to the

corresponding sugar substrate for OGA). Structural and modeling studies have shown that the sulfur substitution in the ring does not cause alteration of the sugar conformation (Lazarus *et al.*, 2012). The proposed dissociative S_N2 mechanism of OGT involves the development and movement of an oxocarbenium ion-like species away from the leaving group toward the acceptor (Lazarus *et al.*, 2012). The substitution of the endocyclic oxygen with sulfur is thought to reduce the stabilizing effect on the anomeric carbon making it resist the formation and movement of an electrophilic anomeric center relative to its oxygen counterpart.

Like several OGT inhibitors described above, UDP-5SGlcNAc is speculated to inhibit other glycosyltransferases that use UDP-GlcNAc as the donor substrate. Interestingly, no effect on cell surface glycosylation is detected by the treatment of cells with (Ac)₄-5SGlcNAc (Gloster *et al.*, 2011). However, testing did not indicate whether UDP-5SGlcNAc inhibits only OGT. There are numerous possible explanations for the absence of cell surface glycosylation changes, including: (1) the *O*-GlcNAc modification turns over at a faster rate than cell surface glycans and is, therefore, more quickly affected in the time frames tested and (2) 5SGlcNAc is not sufficiently distributed in the secretory pathway due to differences in transport rates. The second interesting, but surprising feature of 5SGlcNAc, is that it has no apparent toxicity in mammalian cells yielding no changes in cell morphology or cellular growth rate, despite the fact that its treatment dramatically decreases cellular *O*-GlcNAc levels (Gloster *et al.*, 2011). This result contrasts genetic studies that show that selective OGT knockout, and consequent loss of *O*-GlcNAc leads to neuronal apoptosis (O'Donnell *et al.*, 2004) and loss of embryonic stem cell viability (Shafi *et al.*, 2000). These conflicting results imply that diminishing *O*-GlcNAc levels may not be the direct cause for these crippled cellular functions. Continued efforts toward the development of cell permeable and highly specific OGT inhibitors are essential to clarify the precise basis for the differences observed between the genetic and chemical tools.

Bisubstrate UDP-peptide conjugates—In order to improve the specificity of target enzyme inhibition, molecules that combine elements of both donor and acceptor substrates have been constructed and evaluated as selective inhibitors (Izumi *et al.*, 2009; Lavogina *et al.*, 2010; Palcic *et al.*, 1989). In line with this concept, bisubstrate UDP-peptide conjugates (**11** and **12**, Figure 3) were synthesized and evaluated as OGT inhibitors. These compounds feature a short linker that replaces the GlcNAc moiety of the donor substrate, covalently connect the UDP to the serine of an acceptor peptide (VTPVSTA), and were found to be *in vitro* OGT inhibitors with IC₅₀ values in the micromolar range (Borodkin *et al.*, 2013). Although selectivity was not thoroughly examined, they have some OGT specificity in that bisubstrate UDP-peptide **11** inhibits OGT (IC₅₀ of 18 μM when a synthetic peptide was used) but not the *N*-acetylglucosamine transferase *SmNodC* (Borodkin *et al.*, 2013). However, these bisubstrate UDP-peptide conjugates failed to inhibit OGT in cells, most likely due to the lack of cell permeability (Borodkin *et al.*, 2013).

Neutral diphosphate mimics: dicarbamate core-containing compounds—In 2005, the Walker laboratory elected to utilize a high-throughput library screening strategy and identified several *in vitro* micromolar inhibitors of human OGT (**13–15**) (~ 10–100 μM)

(Gross *et al.*, 2005), some of which were later shown to have utility in cells (Caldwell *et al.*, 2010; Hart *et al.*, 2011; Ngoh *et al.*, 2008). Of the compounds identified, one containing a benzo[d]oxazol-2-one moiety and a phenyl carbamate moiety featuring dicarbamate (**15**, Figure 3) was further studied (Jiang *et al.*, 2012). To deduce the mechanism of OGT inhibition, several dicarbamate derivatives were synthesized (Jiang *et al.*, 2012), and the Walker laboratory identified that in the OGT active site, the dicarbamate moiety resides in the same location as UDPGlcNAc's diphosphate group. Specifically, lysine 842 (K842) points to the dicarbamate carbonyls and OGT inhibition has been proposed to occur *via* a double displacement mechanism (Figure 4). K842 in the enzyme's active site is thought to act as the primary nucleophile attacking the acyclic dicarbamate carbonyl thus forming a covalent bond. Subsequently, a nearby second nucleophile cysteine 917 (C917) attacks the K842 dicarbamate carbonyl adduct to form a C=O crosslink between two active site nucleophiles, resulting in an irreversible loss of enzyme activity. It is interesting to note that the compound **16** (Figure 3) with an electron-donating substituent at the phenyl carbamate moiety gave the most potent inhibition among several derivatives with different substituents on the dicarbamate scaffold tested. This may be explained by the electron-donating substitution of the phenyl carbamate, which decreases the reactivity of the acyclic carbonyl carbon of the dicarbamate toward the nucleophilic substitution by an enzyme's nucleophile. The decreased reactivity of the acyclic carbonyl carbon, in turn, improves its selectivity for the attacking nucleophile of the enzyme.

Even at a few hundred micromolar concentration, the molecules functioning as the neutral diphosphate mimics yielded a significant decrease in the cellular *O*-GlcNAc levels (Dehennaut *et al.*, 2007; Jiang *et al.*, 2012). For example, the benzoxazolinone derivative (**15**, 500 μ M) used in the study of the relationship between *O*-GlcNAc and the cell cycle has been shown to inhibit the increase of progesterone-induced *O*-GlcNAc levels and block G₂/M transition in *Xenopus* immature oocyte *via* the inhibition of OGT (Dehennaut *et al.*, 2007). However, related dicarbamate scaffolds may inhibit other enzymes that bind diphosphate-containing substrates.

OGT activity assay methods

Availability of a useful enzymatic assay to measure OGT activity is a prerequisite for developing potent and selective OGT inhibitors. Indeed, aided by several useful, high-throughput, enzymatic assays and mechanistic and structural insights for OGA, the design of potent and OGA selective inhibitors has flourished in recent years (for reviews, see Gloster & Vocadlo, 2010; Kim, 2011; Macauley & Vocadlo, 2010 and references therein). The use of small-molecule OGA inhibitors has greatly improved our understanding of *O*-GlcNAc's physiological roles. In contrast, efficient discovery of potent OGT inhibitors has been impeded by the lack of a rapid and easy method to continuously monitor OGT activity. OGT enzymatic assay methods adaptable to high-throughput detection technologies have been introduced and are discussed below along with the conventional OGT activity assay.

Direct measurement of OGT enzymatic activity

Conventional *in vitro* OGT activity assay—The conventional *in vitro* OGT activity assay uses a radiolabeled sugar donor substrate such as UDP-[¹⁴C]-GlcNAc or UDP-[³H]-GlcNAc and a sugar acceptor protein substrate such as unmodified Nup62 (Lubas & Hanover, 2000). OGT activity is quantified by measuring radiolabeled GlcNAc incorporation into the protein substrate during the enzymatic reaction. Two detection methods, autoradiography and liquid scintillation counting, are predominately used for assaying incorporated radioactivity. Although the radiometric method is extremely useful in life science studies, it involves a tedious procedure, high cost and inevitable radiochemical waste, which render it unsuitable for rapid high-throughput analysis.

Azido-enzyme-linked immunosorbent assay—Leavy & Bertozzi (2007) introduced the high-throughput azido-enzyme-linked immunosorbent assay (Figure 5), in order to interrogate OGT's r (avidin and biotin) and an azide-selective chemical reaction (Staudinger ligation). First, a solution-phase enzymatic reaction is performed using unnatural substrates, UDP-*N*-azidoacetyl glucosamine (UDP-GlcNAz) and biotinylated peptide in the presence of OGT. The resulting reaction mixture is then transferred onto avidin-coated microplates to capture the GlcNAz-labeled biotinylated peptide substrates. Subsequently, a chemoselective reaction between the GlcNAz-labeled peptides immobilized on the microplates, and a phosphine-FLAG probe is performed. The resulting FLAG-labeled epitopes bound to the microplate are used for a colorimetric readout of horseradish peroxidase (HRP) activity by using a HRP-conjugated anti-FLAG antibody yielding the relative OGT activity toward each peptide substrate. Transferring the solution-phase enzymatic reaction onto a microplate reduces this method's efficiency and may increase the risk of introducing experimental error. In addition, the method requires the synthesis of the phosphine-FLAG probe as it is not commercially available at the time of this writing. We have recently developed a versatile 96-well plate-based assay where purification of OGT is not required (Kim *et al.*, 2014). This assay is expected to be useful for the identification of new inhibitors, substrates and OGT enzyme variants and all reagents are readily available.

Indirect measurement of OGT enzymatic activity

Protease protection assay—In order to monitor glycosyltransferase activity, the Walker laboratory reported a strategy that measures OGT activity indirectly through protease activity (Figure 6A) (Gross *et al.*, 2008). The “protease protection assay” exploits the fluorescence resonance energy transfer (FRET) phenomenon (Figure 6B) with a FRET pair tethered at each end of a substrate. In FRET, a fluorescent donor absorbs light at a defined wavelength and is excited to the singlet state. Upon relaxation to the lowest excited singlet state, S₁, some of the absorbed energy dissipates. If no acceptor fluorophore is in proximity to the donor, the relaxed singlet state of the donor returns to its ground energy state (S₀) by emitting the remaining light energy at a longer wavelength in the form of specific fluorescence. However, if an acceptor fluorophore is near the donor, the energy released from the donor can be transferred and excite the acceptor fluorophore in a nonradiative process termed “resonance”. The excited acceptor then emits energy at a new wavelength and returns to the ground state. For resonance energy transfer to occur, a FRET pair must simultaneously satisfy compatibility and proximity. In recent years, quencher molecules that

transform the light energy to heat and remain “dark” have emerged to replace fluorescent acceptor molecules. Fluorophore-quencher dual-labeled constructs have no measurable fluorescence as long as the construct remains intact, thus simplifying many fluorescence-based assays (Figure 6).

Walker *et al.* employed substrates containing a fluorescein donor at one terminus and a quencher or fluorescent acceptor molecule at the other terminus (Gross *et al.*, 2008). Their strategy exploits the fact that proteases preferentially cleave less sterically hindered unglycosylated peptides over glycosylated peptides. In this method, FRET-pair labeled peptides are *O*-GlcNAcylated by OGT and then treated with a protease. The degree of peptide *O*-GlcNAcylation is assumed to be inversely related to the amount of proteolysis and is determined by measuring the resulting FRET signal after proteolysis. The indirect nature of the assay presents a potential concern since inhibitors or activators of the protease activity can make negative compounds as appear as positive hits.

Measurement of ligands' binding affinity for OGT: a fluorescent-based substrate displacement method

—A “fluorescent-based substrate displacement” method (Figure 7) was exploited by the Walker group to rapidly screening small-molecule OGT inhibitors (Gross *et al.*, 2005). This strategy involves monitoring the fluorescence polarization of a fluorescent substrate. Fluorescence polarization depends on the mobility of a fluorescent molecule and provides a non-disruptive means of measuring the association of a fluorescent substrate with a larger molecule such as a protein: if the small fluorescent molecule is freely moving, it will emit the light in a different direction from the incident light and tend to “scramble” the polarization of the light. This “scrambling” effect results in a low level of polarization. Conversely, large molecules such as proteins in solution move relatively slowly and will emit polarized light when excited with polarized light. In the fluorescent-based substrate displacement OGT assay, a small unbound fluorescent UDPGlcNAc analog (**17**, Figure 3) rotates rapidly and maintains low levels of polarization after excitation. However, if the fluorescent molecule binds OGT, forming a larger and more stable complex, the bound fluorescent probe rotates more slowly reducing the “scrambling” effect and increasing the amount of polarized light. Therefore, the amount of polarization observed is proportional to the amount of OGT complex formed, which is itself proportional to the concentration of its binding to the OGT partner.

All the assays described above require that the OGT enzyme be added in a highly purified form. OGT activity is sensitive to temperature and loss of the enzyme's activity can occur during the enzyme's purification. In fact, OGT's catalytic activity requires proper storage and preparation conditions and the enzyme's activity is affected by repeated freeze/thaw cycles. Therefore, preparation and purification of catalytically active OGT is not trivial and careful attention needs to be paid to the protocol used (Kim & Hanover, 2013). For a more rapid, facile analysis of OGT activity and screening of OGT inhibitors, a high-throughput assay that does not require the OGT enzyme purification is desired.

Structural and mechanistic studies of OGT

The CDs of OGT have Rossmann-like folds typical of a glycosyltransferase 41 (GT41), type B (GT-B) family in the Carbohydrate-Active EnZymes database (Cantarel *et al.*, 2009). In detail, OGT catalyzes glycosyl transfer from the α -linked sugar on UDP-GlcNAc inverting the stereochemistry to form the β -O-GlcNAc linked protein modification. OGT is exquisitely sensitive to pH with an optimum catalytic activity at pH 6, and its activity is dramatically decreased at a pH lower than 6 or higher than 7 (Haltiwanger *et al.*, 1992). Unlike many other glycosyltransferases, OGT does not require the presence of a divalent metal ion for efficient catalysis (Haltiwanger *et al.*, 1990, 1992). One notable feature of OGT is the intervening domain between the catalytic lobes: together with an adjacent helix of the CD-2 domain, a basic patch is formed and was previously proposed to function as a PIP₃-specific binding region (Yang *et al.*, 2008). Recent mutagenesis studies were not consistent with this proposal, necessitating further investigation to elucidate the exact roles of the intervening domain (Lazarus *et al.*, 2011).

Structural insights into the TPR domain of OGT

The first structural insights into OGT architecture were provided by the crystal structure of the TPR domain of human ncOGT (Jinek *et al.*, 2004). The crystallized protein lacking the C-terminal domain exists as a homo-dimer [which differs from the trimeric state previously proposed for ncOGT (Kreppel & Hart, 1999)] with each monomer consisting of 23 anti-parallel α -helices that form a right-handed superhelix. This TPR structure showed an extended superhelical architecture with a groove along the lengthwise axis, the center part of which is lined with asparagine residues (Jinek *et al.*, 2004). This asparagine “ladder” in OGT resembles the armadillo repeats found in the structures of importin α (Conti & Kuriyan, 2000; Conti *et al.*, 1998) and β -catenin (Huber & Weis, 2001); in these proteins, the asparagine ladder is important for recognition of their target peptides. The considerable length of the TPR motifs in OGT is suggested to provide a scaffold to accommodate many protein partners in different orientations and binding modes, thereby explaining the absence of an explicit primary sequence motif for the O-GlcNAc modification.

Although there is no obvious consensus sequence that directs for the addition of O-GlcNAc, recent advances in chemical and biological methodologies in conjunction with the development of various mass spectrometry technologies have led to the detailing of the O-GlcNAc proteome. In addition, O-GlcNAc proteomic databases and bioinformatics software tools are available for the *in silico* prediction of potential O-GlcNAc modification sites: namely, YinOYang (Blom *et al.*, 2004) (<http://www.cbs.dtu.dk/services/YinOYang>), OGlcNAcScan (Wang *et al.*, 2011) (<http://cbsb.lombardi.georgetown.edu/hulab/OGAP.html>) and O-GlcNAcPRED (Jia *et al.*, 2013) (website for this tool is not available as of publication). Along with computational analysis (Frequency amino acids analysis with online Protein Sequence Logos), Liu *et al.* (2014) examined key amino acid positions and identities affecting O-GlcNAcylation of peptides experimentally. The authors found that the computational amino acid site analysis was not always consistent with the experimental data suggesting that for improved prediction accuracy, the tools need to be further developed.

Structural insights into the CD of OGT

Structural insights into the C-terminal domain of OGT were first obtained from the structure of a GT41 bacterial homolog of OGT from the plant pathogen *Xanthomonas campestris* (XcGT41) by two independent groups (Clarke *et al.*, 2008; Martinez-Fleites *et al.*, 2008). The XcGT41 structures revealed that the C-terminal CD displays glycosyltransferase GT-B topology and UDP-GlcNAc binding between the two (α/β)-fold domains (Clarke *et al.*, 2008; Martinez-Fleites *et al.*, 2008). Although the bacterial enzyme lacks anintervening region between the two (α/β)-fold domains of the GT-B fold, which appears in all mammalian sequences, these structures enabled the identification of the OGT active site. In addition, this research elucidated how the superhelical groove of the TPR domain extends directly into the CD and active site (Clarke *et al.*, 2008; Martinez-Fleites *et al.*, 2008). The long awaited three-dimensional structure of human OGT, featuring 4.5 TPRs and the CDs was solved by the Walker group in 2011 (Lazarus *et al.*, 2011). The ternary structure of the OGT-UDP-peptide complex revealed that UDP binds in a pocket in the CD-2 domain near the interface with the CD-1 domain. Moreover, the peptide substrate binds in the cleft between the TPR and the catalytic region and extends along the interface between the CD-1 and CD-2 domains, lying over the UDP-GlcNAc binding pocket. The information derived from the human OGT's crystal structure provided numerous clues for deducing an OGT catalytic mechanism although the identification of the catalytic residues and reaction mechanism remains to be defined.

Proposed OGT catalytic mechanisms

A sequential “bi-bi” mechanism—OGT was initially thought to function through a random sequential “bi-bi (two on; two off)” mechanism in which either substrate can bind first (Kreppel & Hart, 1999). However, the crystal structure of human OGT in complex with UDP and peptide revealed that the peptide-binding region lies above the UDP-GlcNAc-binding pocket, thus blocking access to it. This finding supports an ordered, sequential bi-bi mechanism in which UDP-GlcNAc binds first, and then the peptide acceptor binds over the nucleotide sugar (Figure 8) (Lazarus *et al.*, 2011). The structure also revealed that the TPR domain is separated from the CD by a flexible “hinge” region and the conformational motion that occurs around the “hinge” would either allow or restrict protein substrates to enter the active site.

The elusive OGT catalytic base—A big advance in understanding the OGT mechanism through a catalytic base was made in 2012 when UDP-5SGlcNAc was used in complex with human OGT (Lazarus *et al.*, 2012; Schimpl *et al.*, 2012). The enzyme's catalytic base is thought to increase the reactivity of an acceptor protein toward its nucleophilic attack on the anomeric carbon of UDP-GlcNAc by abstracting a proton from a serine or threonine hydroxyl of the acceptor substrate. Histidine residues [human His498 (Lazarus *et al.*, 2011) and human His558 deduced from the His218 in a bacterial OGT structure from the plant pathogen *X. campestris* (Martinez-Fleites *et al.*, 2008)] and Tyr841 (Dorfmueller *et al.*, 2011) were previously proposed as potential bases for OGT's catalysis. However, two recent papers describing crystal structures of the ternary complexes of OGT with UDP-GlcNAc and peptide acceptor analogs rejected the idea of direct interaction of the acceptor with any of those proposed residues as they were too far from the acceptor hydroxyl (Lazarus *et al.*,

2012; Schimpl *et al.*, 2012). In the two independent studies, both groups employed strategies to capture a ternary complex of OGT:UDP-sugar:peptide acceptor since glycosyl transfer tends to occur during the time scale of crystallization when the natural donor substrate (UDP-GlcNAc) is used. In order to reduce the rate of enzymatic turnover or avoid hydrolysis of the donor substrate, the groups used UDP-5SGlcNAc (**10**, Figure 3), an inhibitor of OGT (Gloster *et al.*, 2011) and serine-substituted peptide acceptor analogs.

Lazarus *et al.* chose to use an incompetent peptide acceptor, the CKII peptide analog (CKIIA) wherein the GlcNAc-modification site is removed by the mutation of a serine to alanine and observed that no glycosyl transfer occurred during crystallization when used in conjunction with UDP-5SGlcNAc (Lazarus *et al.*, 2012). In their work, Schimpl *et al.* (2012) used a derivative of the TAB1 acceptor substrate in which the serine hydroxyl was replaced with aminoalanine residue (aaTAB1) and found that glycosyl transfer onto the aminoalanine of the aaTAB1 peptide was very slow when used with UDP-5SGlcNAc. These non-natural substrates were shown to adopt the same binding position and conformation as their natural substrates, providing meaningful insight into the reaction coordinate and catalytic mechanism of OGT. It is interesting to note that the UDP-sugar donor interacts extensively with the enzyme's active site residues and with the peptide acceptor. This observation is consistent with the recently reported kinetic data; apparent K_m values for UDP-GlcNAc vary with regard to the different protein acceptors (David *et al.*, 2012). Overlay of the ternary substrate complex (OGT:UDP-5SGlcNAc:CKIIA) and the ternary product complex (OGT:UDP:GlcNAcylated CKII) revealed that the UDP moiety and the peptide acceptor remain immobile, whereas C5-O5-C1-C2 atoms of the β -linked GlcNAc moiety in the 4C_1 chair conformation vary in position, such that the anomeric carbon (C1), is tilted away from the pyrophosphate group and points toward the nucleophilic serine hydroxyl along the reaction coordinate (Lazarus *et al.*, 2012). The structure of another ternary product complex (OGT:UDP:GlcNAcylated TAB1) solved by Schimpl *et al.* (2012) showed that the *N*-acetyl moiety lies in a relatively spacious pocket between the catalytic core and the TPR repeats, explaining why OGT can tolerate a diverse array of UDP-GlcNAc analogs bearing bulky amido substituents (Gross *et al.*, 2005; Vocadlo *et al.*, 2003). The Walker and van Aalten groups each suggest different residues acting as the catalytic base (Figure 8A and 8B), and their findings are highlighted in a short report from 2012 (Withers & Davies, 2012). General features and implications of the proposed mechanisms are discussed below.

Substrate-assisted catalysis: α -phosphate on the UDP-GlcNAc as a general

base.: Schimpl *et al.* (2012). proposed a "substrate-assisted catalysis" where the catalytic base is not provided by the enzyme, but by the α -phosphate on the donor substrate itself. They observed the functional difference between the two chiral α -phosphorothioate derivatives of UDP-GlcNAc in OGT-catalyzed *O*-GlcNAc transfer when aaTAB1 was used as an acceptor (Schimpl *et al.*, 2012): for the one α -phosphorothioate diastereomer, sulfur replaces the α -phosphate oxygen pointing toward the acceptor serine (pro-*R* position, **18**, Figure 3) and is catalytically inactive. The other diastereomer (pro-*S* position, **19**, Figure 3) is a functional donor in an *O*-GlcNAc transfer reaction, although both diastereomers bind to OGT with a similar affinity as UDP-GlcNAc. In the proposal by Schimpl *et al.*, the pro-*R* oxygen of the α -phosphate acts as a base and deprotonates the acceptor, which proceeds as a

nucleophilic attack onto the anomeric carbon, with in-line displacement of the UDP leaving group, resulting in inversion of stereochemistry (Figure 8A). It is widely assumed that the less basic candidate oxygen of α -phosphate in UDP-GlcNAc ($pK_a \sim 2$ in solution for the conjugate acid of this phosphate while the $pK_a \sim 16$ for the conjugate acid of the serine hydroxyl) yields the α -phosphate as a less likely proton acceptor. However, unexpected UDP-GlcNAc ^{31}P -NMR titration data suggested that the pK_a could be much higher than the anticipated value (Jancan & Macnaughtan, 2012).

“Grothuss-type” mechanism: Asp554 acts as a base through a water chain.: In contrast to the proposal for the role of the α -phosphate as a candidate base in the OGT catalysis suggested by Schimpl *et al.*, Lazarus *et al.* (2012) de-emphasized the role of the α -phosphate as the catalytic base. They noted that the oxygen on the α -phosphate in their structure was hydrogen bonded to the amide backbone of the peptide, which is thought to greatly attenuate its basicity. In addition, the modeled serine rotamer, that is properly positioned to interact with the α -phosphate, was argued to be incorrectly aligned for its attack onto the anomeric carbon. Thus, they proposed a “dissociative S_N2 mechanism” involving electrophilic migration of the anomeric center in which a highly dissociative oxocarbenium ion-like species moves away from the leaving group toward the acceptor. In this mechanism, the acceptor serine proton may be shuttled out of the site through a chain of intervening water molecules in a “Grothuss-type” mechanism, possibly to the carboxylate side chain of the conserved residue Asp554 (Figure 8B). However, mutation of Asp554 (D554N) did not inactivate the enzyme (Schimpl *et al.*, 2012), calling into question the possible role of Asp554 in catalysis as a base.

Quantum mechanical considerations: is a catalytic base necessary?: A third independent group performed calculations using quantum mechanical and molecular modeling methods (Tvaroška *et al.*, 2012) that supports the “dissociative S_N2 mechanism” for OGT catalysis, in which the structure of transition state has a significant oxocarbenium ion-like character. However, once again the choice of a base catalyst is problematic. In their model, His498 was assigned as a base catalyst, an assignment rendered highly unlikely by the recent structural and mutational studies (Lazarus *et al.*, 2012; Schimpl *et al.*, 2012). Alternatively, like some inverting glycosidases that seem to lack a base catalyst, a general base catalysis may not be essential for OGT (Koivula *et al.*, 2002). The high affinity binding of UDP-GlcNAc to a deep pocket within the OGT CD is somewhat unique and may serve to increase the reactivity of the nucleotide sugar, thus lowering the energetic barrier presented by the glycosyl transfer step. At this point, the nature of the general base and the identity of the base residue for OGT catalysis are unclear and further studies are, thus, required to answer which, if any, features function in the capacity of a general base.

A role for the GlcNAc acetamido group in the OGT catalysis

A role for the GlcNAc acetamido group of the donor substrate in OGT catalysis has been proposed (Koivula *et al.*, 2002; Lazarus *et al.*, 2012; Schimpl *et al.*, 2012). Both Lazarus *et al.* (2012) and Tvaroška *et al.* (2012) suggested a role for the *N*-acetyl NH moiety (H_{NAc}) in stabilizing the UDP leaving group at the transition state. Lazarus *et al.* (2012) observed a substantial rotation of the acetamido group of the donor substrate upon its conversion to

product and ascribed the stabilizing effect exerted by the H_{NAC} moiety to its hydrogen bonding to the β-phosphate oxygen of UDP. In contrast, Tvaroška *et al.* (2012) ascribed it to its hydrogen bonding to the glycosidic oxygen, but the general base they assigned in their model is unlikely based on known structural information. A product complex obtained by Schimpl *et al.* (2012) also showed that the H_{NAC} moiety coordinates with the β-phosphate oxygen of UDP. Since all three groups demonstrated the importance of the acetamido group of the donor substrate, catalytic activity of OGT is likely to be dependent on the presence of this moiety; OGT is catalytically active with UDP-GalNAc (**4**, Figure 3), but not with UDP-glucose (UDP-Glc **20**, Figure 3) or UDP-2-ketoGlucose (UDP-2-ketoGlc **21**, Figure 3) (Lazarus *et al.*, 2012). The involvement of the GlcNAc acetamido group and possibly the α-phosphate moiety of the nucleotide sugar in the enzymatic reaction is intriguing in light of the proposed role of OGT as a sensor of nutrient-derived UDP-GlcNAc. Although, several catalytic mechanisms of OGT have been proposed to date, none are completely consistent with available data necessitating further research.

Conclusions and perspectives

Cellular processes such as cell cycle regulation, stress response and gene expression are regulated, in part, by the *O*-GlcNAc modification. Many post-translational modifications work together with *O*-GlcNAc in a delicate balance to appropriately maintain cellular homeostasis. With improper regulation of OGT and OGA, cellular processes can go awry yielding pathologies including cancers, diabetes II and neurodegeneration. A number of recent publications also demonstrate that modulation of OGT and *O*-GlcNAc may impart a cytoprotective effect in acute injury responses (Champattanachai *et al.*, 2008; Laczy *et al.*, 2009; Liu *et al.*, 2007; Ngoh *et al.*, 2010; Xing *et al.*, 2008).

Highly active research efforts including the development of high-throughput OGT assay methods and the solution of the structure of OGT comprising both catalytic and TPR domains have yielded a better understanding of the catalytic mechanism in order to generate potent inhibitors. Continued efforts toward developing simpler OGT assay methods in which the enzyme needs not be purified, and the generation of OGT-specific and isoform-specific OGT inhibitors are on the horizon and promise to spur forward our understanding of the *O*-GlcNAc post-translational modification.

There are still challenging questions remaining: (1) how are OGT and OGA regulated in order to modulate *O*-GlcNAc levels? and (2) how does one single enzyme target so many substrates while retaining substrate specificity? The activities of OGT and OGA are likely regulated by their own post-translational modifications. The existence of multiple isoforms of the OGT and OGA arising from alternative splicing (Hanover *et al.*, 2003; Nolte & Müller, 2002; Shafi *et al.*, 2000) may partly explain how these enzymes can have a variety of substrates. Techniques to manipulate both OGT and *O*-GlcNAc levels will provide additional tools to answer these questions. Moreover, the study of gain/loss of function of OGT in either mammalian tissue culture cells or model organisms may prove equally useful.

Several proteins such as myosin phosphatase targeting subunit 1 (Cheung *et al.*, 2008), trafficking kinesin-binding protein 1 (formerly known as OGT-interacting protein 106)

(Beck *et al.*, 2002; Iyer & Hart, 2003; Iyer *et al.*, 2003;), p38 MAPK (Cheung *et al.*, 2008) and PGC 1 α (Housley *et al.*, 2009) have been revealed to interact with and target OGT to unique substrates during a signaling event. Studies on such proteins and their roles demonstrate that the substrate specificities of OGT can be determined, in part, by its transient interactions with many binding partners to form various substrate specific holoenzymes. Many of these proteins are known to be modified by *O*-GlcNAc, which might also affect their interaction and targeting (Iyer & Hart, 2003). Discoveries of such proteins and elucidation of their roles and the nature of their interactions are also good avenues for researchers to explore.

Despite absence of a eukaryotic OGA crystal structure, the domain responsible for OGA's glycoside hydrolase activity, and its detailed catalytic mechanism including general acid catalytic residues are well established (Çetinba *et al.*, 2006; He *et al.*, 2010). Although OGA is presumed to be targeted to *O*-GlcNAc-modified proteins by interacting proteins, this has yet to be fully addressed. Interestingly, the C-terminal domain of OGA which is absent in the bacterial sequences, has a putative GCN-5-related histone acetyltransferase-like domain; however, whether this domain actually has the ability to acetylate histones remains controversial (Butkinaree *et al.*, 2008; Toleman *et al.*, 2004). Most recently, Rao *et al.* demonstrated that the C-terminal domain of human OGA does not possess the key residues required for acetyltransferase activity and is a catalytically incompetent "pseudo"-acetyltransferase based on their crystal structure of a marine bacterial homolog of the human OGA C-terminal acetyltransferase domain (Rao *et al.*, 2013). Ultimately the crystal structure of the human OGA will help to address this question.

Determining the functional roles of the various OGT and OGA isoforms will be an important area for future research. Recently, dynamic *O*-GlcNAcylation during signaling has been demonstrated using an *O*-GlcNAc sensor (Carrillo *et al.*, 2011) and a surprising study that OGT possesses proteolytic activity against HCF1 has also been reported (Capotosti *et al.*, 2011). Continued development of technologies and tools will deepen our understanding of the roles of OGT, OGA and *O*-GlcNAc play in basic biology and, ultimately, human health.

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Figure 1.

The *de novo* hexosamine biosynthetic pathway (HBP) and GlcNAc salvage pathway. In most cell types, 2–5% of cellular glucose (Glc) enters the HBP, resulting in the end product, UDP-GlcNAc. UDP-GlcNAc can be used by OGT to catalyze the addition of GlcNAc to nuclear and cytoplasmic proteins, whereas OGA catalyzes the removal of the O-GlcNAc PTM. GFAT is the rate-limiting enzyme for the HBP. GK, glucokinase; G-6-Pase, glucose-6-phosphate isomerase; GFAT, glutamine:fructose-6-phosphate amidotransferase; GAT, acetyl-CoA:D-glucosamine-6-phosphate N-acetyltransferase; GNK, GlcNAc kinase; AGM, phosphor-N-acetylglucosamine mutase; AGX1, UDP-GlcNAc pyrophosphorylase. (see colour version of this figure at www.informahealthcare.com/bmg).

Figure 2.

Genomic structure of human OGT splice variants (A). OGT protein domain structure (B) and OGT immunoblot (C). The OGT gene is composed of 23 exons and the three isoforms differ at their amino terminus yielding ncOGT, mOGT and sOGT. Alternative use of intron 4 (exon 5) to produce an altered amino terminus is indicated by the light rectangular box. The exons encoding the nucleocytoplasmic, mitochondrial, and short isoforms are numbered and the different start codons are denoted by the arrows. The potential promoters are indicated as P1 and P2. The predicted downstream promoter (P2) could only produce a transcript encoding the shorter isoforms of OGT. Immunoblot analysis shows the presence of three OGT isoforms in HeLa cells (Hanover *et al.*, 2003). MTS, mitochondrial targeting sequence; CD, catalytic domain; TPR, tetra-tricopeptide repeat. (see colour version of this figure at www.informahhealthcare.com/bmg).

Figure 3.

Structures of the molecules described in the article. (see colour version of this figure at www.informahealthcare.com/bmg).

Figure 4.

Proposed double displacement mechanism for the inhibition of OGT by the dicarbamate core-containing compounds (compound 16 pictured here) (Jiang *et al.*, 2012). One dicarbamate acyclic carbonyl reacts with an active site lysine (K842) and the lysine adduct reacts again with a nearby cysteine (C917) to crosslink the OGT active site yielding irreversible loss of enzymatic activity. (see colour version of this figure at www.informahealthcare.com/bmg).

Figure 5.

Schematic representation of the azido-ELISA method for measuring OGT activity. (see colour version of this figure at www.informahhealthcare.com/bmg).

Figure 6. The “protease-protection OGT assay” (A) and a representation of the “FRET” phenomenon (B). (see colour version of this figure at www.informahealthcare.com/bmg).

Figure 7.

A “fluorescent-based substrate displacement” method for high-throughput screening of OGT inhibitors. (see colour version of this figure at www.informahealthcare.com/bmg).

Figure 8. Schematic representation of an ordered “bi-bi” kinetic mechanism of OGT and two proposed mechanisms for O-GlcNAc transfer. (A) A mechanism proposed by Schimpl *et al.* in which the α -phosphate oxygen serves as the base catalyst (Schimpl *et al.*, 2012). (B) A mechanism suggested by Lazarus *et al.* in which the proton of the acceptor hydroxyl is relayed out of the active site through a chain of intervening water molecules to an acceptor (Asp554) in a “Grotthuss-type” mechanism (Lazarus *et al.*, 2012). (see colour version of this figure at www.informahealthcare.com/bmg).