Chapter 2
The Functional Implications of *O*-GlcNAc Glycosylation for Gene Expression and Cell Survival

O-GlcNAc glycosylation is a carbohydrate modification found to play a role in the regulation of intracellular processes, such as proteasomal degradation, cytoskeletal dynamics and protein expression. Our interests in elucidating the role of glycosylation in cell signaling and mechanisms that control gene expression directed us to the examination of CREB as a possible *O*-GlcNAc glycosylated protein. To understand the possible role of CREB *O*-GlcNAc glycosylation, I will provide a survey of *O*-GlcNAc glycosylation, its targets and its effects on transcription.

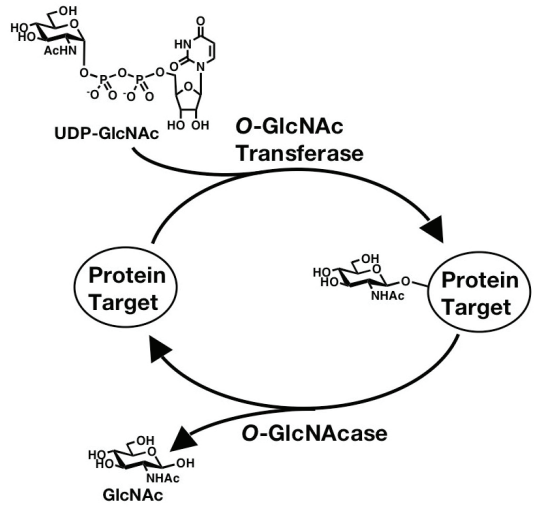


Figure 2-1. *O*-GlcNAc glycosylation is a nuclear/cytosolic post-translational modification that consists of the modification of serine or threonine residues with a single N-acetylglucosamine monosaccharide. A single *O*-GlcNAc transferase and *O*-GlcNAcase responsible for the modification have been identified.

General Description. *O*-linked *N*-acetylglucosamine (*O*-GlcNAc) is a unique post-translational modification [1]. Unlike most forms of glycosylation, which are either displayed on the extracellular domains of transmembrane proteins or exported to the extracellular matrix [2, 3], *O*-GlcNAc glycosylation is a dynamic cytosolic and nuclear modification [4-6] . In this regard *O*-GlcNAc has more in common with protein phosphorylation and, like phosphorylation, *O*-GlcNAc glycosylation can affect a number of protein characteristics from enzyme activity to intracellular localization [7].

O-GlcNAc glycosylation is the addition of a single *N*-acetylglucosamine residue to either serine or threonine residues of the target protein (Fig. 2-1). This modification is mediated by a single *O*-GlcNAc transferase (OGT) that utilizes uridine diphosphate *N*-acetylglucosamine (UDP-GlcNAc) as a substrate. Deletion of this gene is lethal in mice and reflects the critical importance of *O*-GlcNAc glycosylation to cell survival [8]. As with the addition of *O*-GlcNAc, its removal is mediated by a single hexosaminidase (*O*-GlcNAcase). The fact that both OGT and *O*-GlcNAcase are single genes sets *O*-GlcNAc apart from phosphorylation, for which a number of kinases and phosphatases exist, and raises questions as to the mechanism underlying the regulation of *O*-GlcNAc glycosylation and the reasons for its apparent minimalism, despite the wide array of *O*-GlcNAc glycosylated proteins.

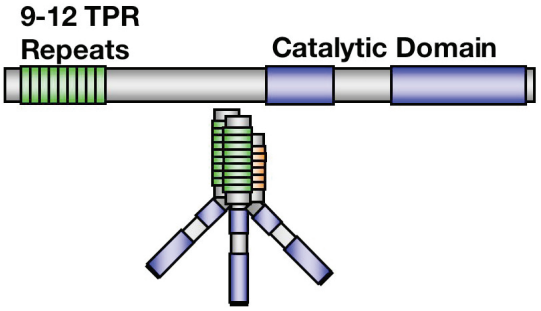


Figure 2-2. *O*-GlcNAc Transferase Domains and Functionality. OGT exists as both 110 and 78 kDa isoforms that interact to form a hetero-trimer of two 110 kDa and one 78 kDa subunits *in vivo*. The *N*-terminal tetratricortico peptide repeats of OGT are organized into a suprahelical structure responsible for protein:protein interactions that help specify the activity of OGT. The C-terminal transferase domain is characterized by a relatively low K_m (500 nM) for UDP-GlcNAc, which allows it to compete with endoplasmic reticulum-based transferases that use UDP-GlcNAc to synthesize glycosaminoglycans.

OGT Domains and Function. As stated above, only a single OGT gene has been identified and is thought to exist [8]. In mammals, OGT is expressed in all cell types, with the highest level of expression in the pancreas, followed closely by the brain [9-11]. OGT exists in three forms: two nucleocytoplasmic forms and a mitochondrial form [12]. The 110 and 78 kDa nucleocytoplasmic forms are the result of alternative start sites. *In vivo* OGT forms

an heterotrimer with two 110 kDa subunits and a single 78 kDa subunit (Fig. 2-2). Trimerization lowers the K_m of the subunits for UDP-GlcNAc to ~500 nM, which gives OGT the capability to sense fluctuations in intracellular glucose concentrations via the hexosamine biosynthesis pathway (HBP), which produces UDP-GlcNAc, the obligatory substrate of OGT (Scheme 2-1) [13, 14]. In cells, 2-5% of glucose is carried through the hexosamine biosynthesis pathway and converted into uridine 5'-diphospho *N*-acetylhexosamines. This process begins with phosphorylation of glucose to produce glucose-6-phosphate, subsequent converted to fructose-6-phosphate. The bulk of fructose-6-phosphate is used to produce ATP via glycolysis, a smaller amount of fructose-6-phosphate is metabolized by L-glutamine:D-fructose-6-phosphate amidotransferase (GFAT) the rate-limiting step in the HBP [15, 16]. Fluctuations in the HBP caused by over-expression of GFAT, glucosamine treatment and hyperglycemia lead to increases in UDP-GlcNAc production and a parallel rise in the levels of cellular *O*-GlcNAc glycosylation [6, 15-18].

OGT trimerization is mediated by a series of *N*-terminal tetratricortico peptide repeats (TPR). The TPR repeats form a superhelical structure around a hydrophobic core that not only mediates trimerization, but also organizes surfaces required for protein interactions with OGT [19]. The crystal structure of the TPR-based superhelix revealed bowl-shaped surfaces with multiple conserved asparagines residues decorating the surface, similar to the binding motif of the nuclear transport protein importin-α [9, 15, 19]. Through the TPR domains, OGT has been found to interact with a number of proteins involved in the regulation of gene expression and neurite dynamics (Fig. 2-3). Three OGT-associated proteins, mSin3a, N-CoR and SMRT, are constituents of co-

repressor complexes [20, 21]. In mammals, mSin3a does not associate with N-CoR or SMRT, which partner to form large (1.5-2 MDa) complexes. As OGT is found associated with both complexes, it may play a general role in gene silencing [22]. OGT and *O*-GlcNAcase are associated in chromatin-associated repressor complexes [23]. This tight nuclear association is speculated to allow for rapid glycosylation and deglycoslation. Within the brain, *O*-GlcNAc glycosylation is critical to membrane-associated signaling in the soma and at synapses based on interactions between OGT and protein phosphatases 1-β and γ. PP1-β is preferentially localized to the soma and PP1-γ is found strictly within neurites [24, 25]. OGT also associates with a GABA-receptor binding protein and related protein, OIP106, which is capable of binding to RNA polymerase II but has had no function assigned beyond its affinity to OGT [26].

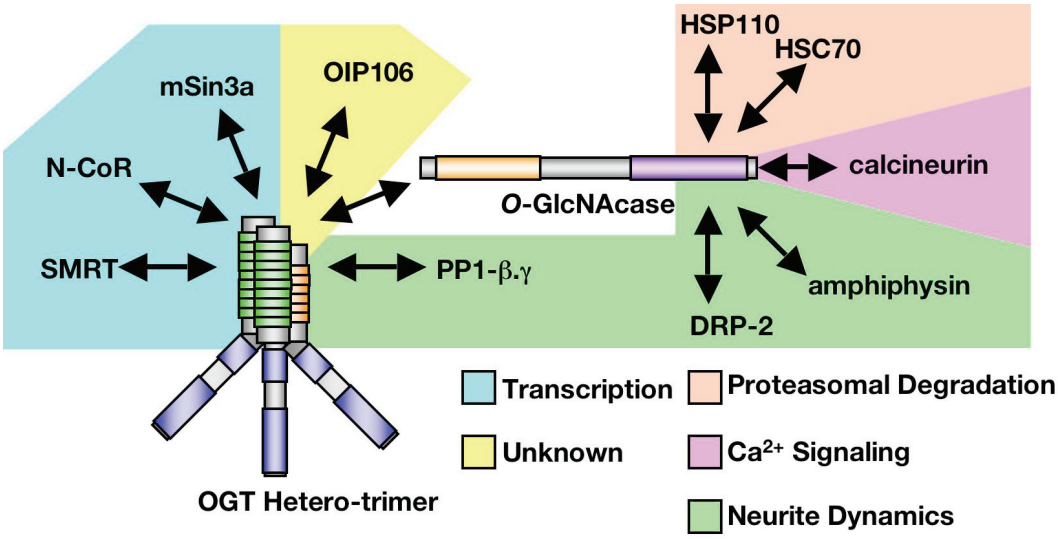
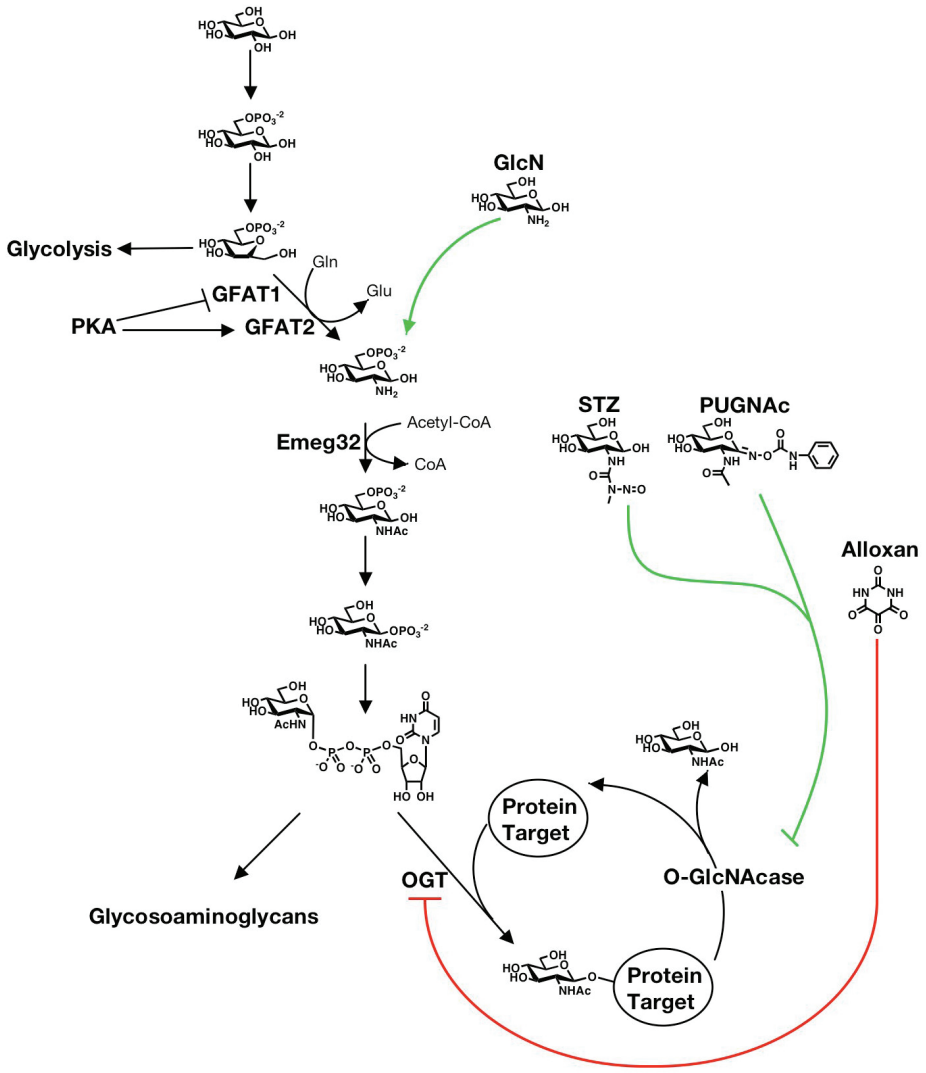


Figure 2-3. Regulation of OGT and *O*-GlcNAcase. OGT has been shown to interact with a number of proteins including members of transcriptional repressor complexes (mSin3a, N-CoR and SMRT), RNA polymerase II interacting protein OIP106, and signaling enzymes PP1-β/γ and *O*-GlcNAcase. *O*-GlcNAcase was co-purified with proteins involved in the regulation of proteasomal degradation (HSP110 and HSC70), axonal guidance (DRP-2 and amphiphysin) and Ca^{2+} signaling (calcineurin).



Scheme 2-1. The Hexosamine Biosynthesis Pathway (HBP). The HBP produces UDP-*N*-acetylhexosamines from about 2-5% of the intracellular glucose. UDP-GlcNAc is controlled by a combination of GFAT expression and phosphorylation by PKA as well as Emeg32, which is responsible for GlcN acetylation. Increasing the intracellular UDP-GlcNAc concentration by providing GlcN to bypass the rate-limiting step in the HBP will enhance OGT activity and increase *O*-GlcNAc glycosylation (green arrow). The GlcNAc analogs streptozotocin (STZ) and (PUGNAc) will increase *O*-GlcNAc glycosylation by blocking *O*-GlcNAcase activity (green blunt end). The small molecule alloxan will suppress *O*-GlcNAc glycosylation by inhibiting OGT (red blunt end).

The hypothesis that *O*-GlcNAc plays a critical role in a number of processes is supported by the identification of OGT-interacting proteins involved in cell signaling, both at cell-cell contacts and within the nucleus.

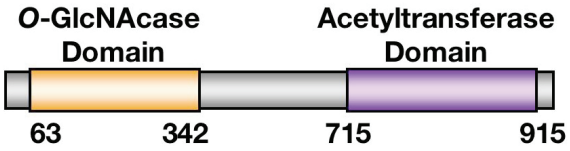


Figure 2-4. *O*-GlcNAcase Domain and Functionality. O-GlcNAcase exists as a nuclear/cytosolic monomer with two functional domains. The O-GlcNAcase activity of the enzyme is localized to a ~280aa N-terminal domain that has homology with the hyaluronidase family of hexosaminidases. The C-terminal region contains an histone acetyltransferase homology domain that exhibits HAT activity *in vitro*.

_O_-GlcNAcase Domains and Function. The protein that catalyzes the removal of *O*-GlcNAc glycosylation, *O*-GlcNAcase, is a unique enzyme. Ubiquitously expressed within mammals, as in the case of OGT, *O*-GlcNAcase expression is highest in the pancreas and the brain [27-29]. Within cells, *O*-GlcNAcase is found preferentially in the cytosol. The observed reciprocal localization of OGT and *O*-GlcNAcase may be a result of regulatory mechanisms meant to control nuclear deglycosylation.

Analysis of the primary sequence of the *O*-GlcNAcase gene found homology not only with hyalouronidases, within the assigned *O*-GlcNAcase domain (aa63-342), but also with histone acetyltransferases (HAT) (aa715-915). The HAT domain is functional *in vitro* [30]. The presence of a HAT domain lends support to the growing body of evidence that *O*-GlcNAc glycosylation generally inhibits gene expression, unlike phosphorylation, which will both activate and repress gene expression (Fig. 2-4).

O-GlcNAcase associates with the heat shock proteins HSP110 and HSC70, as well as amphiphysin, calcineurin and dihydropyriminidase-related protein-2 (DRP-2) (Fig. 2-3) [28, 29, 31]. HSP110 and HSC70 are involved in the mediation of proteasomal degradation and may help activate the 26S proteasome through complexation with HSP110 and HSC70 *O*-GlcNAcase [32]. Calcineurin is a Ca^{2+}- and calmodulin-dependent protein phosphatase involved in the regulation of a number signaling pathways, including both pro- and anti-apoptotic signaling cascades [33-35]. DRP-2 and amphiphysin are both synaptic proteins. DRP-2, also known as a collapsin response mediator protein-2 (CRMP-2), is involved in axonal guidance, specifically growth cone collapse [36], and amphiphysin is involved in synaptic vesicle recycling [37]. The interaction of *O*-GlcNAcase with these proteins suggests that *O*-GlcNAc is involved in the regulation of synapse dynamics. It is worth noting that while the majority of identified OGT-protein interactions are associated with their role in the regulation of gene expression, the *O*-GlcNAcase-protein interactions reflect the fact that it is predominantly localized to the cytosol and found in association with other cytosolic regulatory proteins.

Targets of *O*-GlcNAc Glycosylation. The development of general proteomic approaches to the identification of *O*-GlcNAc glycosylated proteins has provided us with a more complete picture of the processes and pathways that *O*-GlcNAc glycosylation affects (Table 4) [15, 38-42]. Nearly one third of the *O*-GlcNAc glycosylated proteins identified are involved in the regulation of transcription, emphasizing the importance of *O*-GlcNAc glycosylation in the nucleus. Based on the abundance of proteins involved in synaptic function and axonal guidance isolated in *O*-GlcNAc proteome-wide analysis of

the brain, *O*-GlcNAc glycosylation is likely also critical in neuronal communication [38]. Besides its role in the nucleus, *O*-GlcNAc glycosylation modifies proteins involved in cell growth and survival, the stress response, protein expression and degradation and metabolism (Table 4). The combination of *O*-GlcNAc regulation of these biological functions suggest that *O*-GlcNAc may act as a cell-wide homeostasis sensor. With the ability to affect a number of processes in parallel, *O*-GlcNAc may help orchestrate changes that drive cells towards either apoptosis or survival.

Role of *O*-GlcNAc Glycosylation in the Control of Gene Expression. Several lines of evidence suggest an important role for *O*-GlcNAc in the control of gene expression. First, the modification has been identified on a growing number of transcriptional regulatory proteins, including transcription factors, RNA polymerase II, coactivators and corepressors [38, 39, 43-48]. The subcellular localization of the *O*-GlcNAc modification (nuclear membrane > nuclei > rough microsomes > golgi > cytosol > mitochondria [15]), along with number of *O*-GlcNAc glycosylated proteins involved in the regulation of transcription (Table 4), suggests that the regulation of gene expression is a key function of *O*-GlcNAc glycosylation [1, 12, 14].

O-GlcNAc appears to modulate the activity of certain transcription factors [49, 50] and block phosphorylation of the carboxy-terminal domain (CTD) of RNA polymerase II, a domain required for transcriptional elongation [51, 52]. At least 30 transcription factors have been found to be *O*-GlcNAc glycosylated (Table 4), however, the effect of glycosylation has been characterized in only a fraction those identified. One of the first to be characterized was the transcription factor, Sp1. *O*-GlcNAc reduces

recruitment of $TAF_{II}110$ by Sp1 and as a result reduces its ability to initiate transcription [44, 53]. Interestingly, hyperglycemia-induced *O*-GlcNAc glycosylation of Sp1 was also linked to the activation of *transforming growth factor-β* and *plasminogen activator inhibitor-1* gene expression complicating the function of Sp1 glycosylation. Glycosylation has been characterized in or near the transactivation domains of murine estrogen receptor-β and serum response factor, suggesting a possible role in their regulation [54, 55]. In the case of the transcription factors YY-1 and Stat5a, *O*-GlcNAc glycosylation has been implicated in an enhancement of their activity, by releasing the transcription factor from an inhibitory interaction with retinoblastoma protein in the case of YY-1, or by promoting histone acetyltransferase recruitment with Stat5a [50, 56]. The body of research on the glycosylation of transcription factors shows us that glycosylation, like phosphorylation, may affect gene expression by a number of mechanisms.

Interactions between OGT and transcriptional co-repressors has also strengthened arguments that *O*-GlcNAc play an important role in the regulation of gene expression. OGT has been shown to associate with the transcriptional repressors mSin3A, NCoR and SMRT and cooperatively repress transcription [20, 21]. OGT glycosylates the transcription co-activator and histone acetyltransferase CREB-binding protein (CBP) implicating OGT in the possible regulation of CBP activity, which itself is linked to the activity of a number of transcription factors [39]. Finally, the glycosidase responsible for reversing the *O*-GlcNAc modification (*O*-GlcNAcase) possesses histone acetyltransferase activity *in vitro* [30, 57]. These results suggest a generally repressive role for *O*-GlcNAc glycosylation.

Transcription Factors	**Phosphatases and Kinases**	**Reactive Oxygen Species**
Sox2	Nuclear tyrosine phosphatase p65	Thioredoxin peroxidase 1
Sp1	Casein Kinase II	Slow superoxide dismutase
AP-1 (c-Jun and c-Fos)	Insulin Receptor Substrate 1 and 2	Glutathione transferase
CTF	GSK-3	Glutathione-S-transferase 1
HNF-1	PI3-Kinase	Aldehyde reductase
V-erbA	Akt/PKB	
Pancreas-specific transcription factor	WNK-1	**Proteasomal Degradation**
Serum Response Factor	Phosphatase 2A inhibitor	Ubiquitin carboxy hydrolase
Steroid receptor coactivator 1	Rho GDP-dissociation inhibitor I	Ubiquitin Activating Enzyme
c-Myc		Proteasome α 6 subunit
Stat5a	**Cytoskeletal proteins**	Proteasome α 4 subunit
p53	Intermediate keratins 8, 13, 18	Proteasome component C2
Estrogen Receptor	Actin-based B and 41	
h-Catenin	Talin	**Intracellular transport**
Nuclear Factor-κB	Ankyrin	Hrb
ELF-1	Myosin	GRASP55
PAX-6	Cofilin	
Enhancer facotr 2D	Dynein	**Metabolic Enzymes**
Human C1 transcription factor	Spectrin β chain	eNOS
Oct1	Neurofilaments H,M, L	Enolase
Plakoglobin	Tau	GAPDH
ATF-1	MAP 1, 2 and 4	Phosphoglycerate kinase
ATF-2	Synapsin 1	Pyruvate kinase
CREB	Piccolo	UDP-glucose phosphorylase
PDX-1	Bassoon	Glycogen Synthase
RB	Synaptopodin	
CCR-NOT4	α-Tubulin	**Signaling Enzymes**
CCR-NOT subunit 2	Hsp 27	CRMP-2
HCF	HsC70	OGT
P107	HsP90	*O* -GlcNAcase
	HsP70	
Transcriptional Cofactors		**RNA Binding Proteins/Translation**
CREB-Binding Protein	**Transmembrane Proteins**	Ewing-sarcoma RNA-binding protein
mSin3a	p62	Eukaryotic initiation factor 4A1
meCP2	Nup180	Elongation factor 1
Transduction-like enhancer protein 4	Nup153,214,358	40S ribosomal protein s24
	Nup54,155	Translation elongation factor 1
Transcription	Glut-1	Eukaryotic peptide chain initiation factor-2
RNA polymerase II		Zn finger RNA-binding protein

Table 2-1. *O*-GlcNAc Glycosylated Proteins. A list of *O*-GlcNAc glycosylated proteins found 105 glycoproteins spanning a number of functions, with the majority of glycoproteins involved in the regulation of transcription. Compiled from Love and Hanover [15], Tai et al. [39], Khidekel et al. [38], and Wells et al. [42].

Studies of the glycosylated transcription factors and protein interactions of OGT and *O*-GlcNAcase within the nucleus paint a complex picture of the function of *O*-GlcNAc glycosylation, with evidence for a role in the activation and repression of gene expression. The developing role of *O*-GlcNAc glycosylation in the dynamic of gene expression offered an exciting opportunity to contribute to the understanding of a PTM-based system of regulation. With the concentration of *O*-GlcNAc glycosylated proteins

involved in the regulation of gene expression, neuronal communication and cell homeostasis, we concentrated our efforts on the identification of proteins that are involved in all three areas. Based on these criteria I pursued study of the transcription factor CREB as an *O*-GlcNAc glycosylated protein.

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