*Chapter 1*

# The role of *O*-GlcNAc glycosylation in neurobiology and neurodegeneration

## 1.1 Introduction to glycobiology

The central dogma of biology begins with the DNA code, is followed by transcription to RNA, and ends with translation into proteins.[1] This well choreographed dance allows us to take the template-encoded blueprint inherited from our ancestors and execute complex, robust, and adaptive biological functions. In addition to the aforementioned major macromolecules, carbohydrates and lipids are the understudied brethren whose contributions to biology have been proven time and time again to be every bit as important as DNA, RNA, and proteins. Indeed, both glycans and lipids have been shown to be critical regulators of cellular fate and function and arbiters of disease.[2-4] In particular, despite the prevalence and importance of carbohydrates, their study has been less accessible than the central dogmatic macromolecules largely due to their heterogeneity and their non-template encoded nature. For these reasons, carbohydrates remain neglected and poorly understood in the context of biology.[5] Without the luxury of a blueprint or a "Rosetta Stone," researchers must fundamentally shift the way they approach the study of carbohydrates and develop and exploit novel chemical biological tools in order to decipher the language of the "glycocode" or "sugar code"− the concept that the specific glycan structure conveys biological information to cells.[5-7] Over the course of the next chapter, I will attempt to shed light on the intricacies and importance of a specific glycosylation modification, *O*-GlcNAc glycosylation.

## 1.2 Introduction to *O*-GlcNAc glycosylation

The post-translational modification (PTM) of serine or threonine residues with β-linked *N*-acetylglucosamine (*O*-GlcNAc) is cycled by two proteins in mammalian cells: *O*-GlcNAc transferase (OGT) and *O*-GlcNAcase (OGA), which catalyze the addition and

removal of *O*-GlcNAc respectively (Figure 1.1). *O*-GlcNAc glycosylation is a dynamic and inducible PTM found on over one thousand proteins and has been shown to critically regulate a variety of different cellular processes including transcription, epigenetics, translation, organelle biogenesis, cell cycle, autophagy, metabolism, protein turnover, apoptosis, and stress response.[8-11] Due to the importance of *O*-GlcNAc modification in most cellular processes, dysregulation of *O*-GlcNAcylation is associated with a variety of diseases including diabetes, cancer, and neurodegenerative diseases.[12] *O*-GlcNAc glycosylation has been observed across metazoans, insects, plants, fungi, and several bacteria.[12,13] While plants have two different genes that encode for OGTs, mammals have a single OGT-encoding gene for intracellular *O*-GlcNAc modification.[14,15] In 2011, a separate epidermal growth factor (EGF)-domain specific OGT (EOGT) protein was identified and shown to preferentially *O*-GlcNAcylate EGF-like repeats of extracellular and secreted proteins in the lumen of the endoplasmic reticulum (ER).[16,17] *O*-GlcNAc modification by EOGT has been shown to be important for cell-matrix interactions and notch signaling and its disruption leads to the congenital disorder, Adams-Oliver syndrome.[17-19]

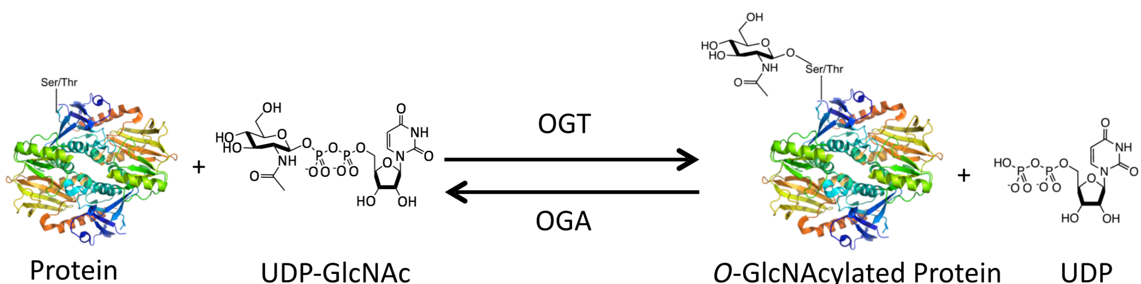



**Figure 1.1 *O*-GlcNAc glycosylation.** *O*-GlcNAc transferase (OGT) catalyzes the transfer of an *N*-acetylglucosamine (GlcNAc) from UDP-GlcNAc to a serine or threonine residue on a protein substrate (shown here is synapsin). *O*-GlcNAcase (OGA) catalyzes the removal of *O*-GlcNAc from protein substrates.

Despite the ubiquitous nature of this modification, *O*-GlcNAcylation was not discovered until the 1980s.[20] The delayed discovery of *O*-GlcNAc can be attributed to challenges inherent to the study of protein *O*-GlcNAc glycosylation; *O*-GlcNAc glycosylation (1) does not have a well-defined consensus sequence; (2) is chemically labile and vulnerable to hydrolysis during cell lysis; (3) adds a small and uncharged moiety to proteins precluding separation using electrophoretic gels; (4) like other PTMs, is substoichiometric on most proteins making MS detection difficult; and finally (5) cannot be separated from unglycosylated forms in reverse phase high performance liquid chromatography (HPLC).[21,22] Due to these difficulties, site detection has remained difficult, and novel techniques have been developed to detect and quantify endogenous *O*-GlcNAcylated sites and proteins, which we will discuss in section 1.5.

**1.3 The enzymes that cycle *O*-GlcNAc: OGT and OGA**

Notwithstanding the difficulties in determining *O*-GlcNAcylated proteins and sites, several properties of *O*-GlcNAc modification have been characterized based on the characterization of the enzymes that cycle *O*-GlcNAc. Human OGT is active as a homodimer or heterotrimer and is alternatively spliced to produce three isoforms: a 116-kDa nuclear/cytoplasmic isoform (ncOGT), a 103-kDa mitochondrial isoform (mOGT), and a 70-kDa short isoform (sOGT).[23,24] Recently, Conrad and colleagues showed alternative splicing further regulates OGT under high *O*-GlcNAc levels through the retention of an intron that leads to decreases in OGT mRNA levels.[25] The crystal structure of a truncated human OGT bound to UDP and a peptide substrate has recently been determined (Figure 1.2).[26] OGT has a C-terminus with a catalytic domain, a phosphatidylinositol (3,4,5)-triphosphate ($PIP_3$)-binding domain, and an N-terminal

protein-protein tetratricopeptide repeat (TPR) domain.[27] While most ncOGT is localized in the cytoplasm and nucleus, ncOGT can be targeted to the plasma membrane through its $PIP_3$-binding domain in response to insulin signaling.[28] OGT's TPR domains form a superhelical structure similar to importin-α, which allows OGT to mediate protein-protein interactions through asparagines along one face.[29] Ablation of these asparagines disrupts protein-protein interactions and decreases *O*-GlcNAcylation globally, demonstrating that the TPR domains are critical for its recruitment to substrates.[30]

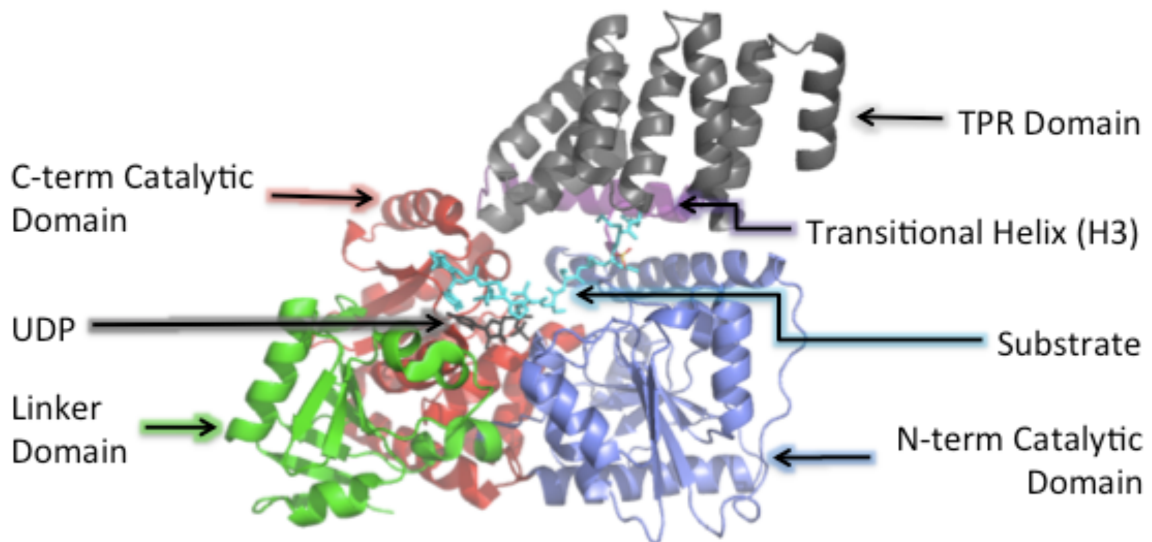



**Figure 1.2. Structure of human OGT with UDP and peptide substrate.** The crystal structure of hOGT displays 4.5 TPR (gray) although the number of TPR motifs differs for each isoform where ncOGT, mOGT, and sOGT, have 11.5, 9.5, and 2.5 TPR motifs respectively. The transitional helix (H3), peptide substrate, N-term catalytic domain, C-term catalytic domain, UDP, and linker domain are shown in purple, cyan, blue, red, black, and green respectively.[26]

A recent study showed that OGT can also *O*-glucosylate proteins with 25-fold less efficiency than its *O*-GlcNAcylation activity, suggesting that OGT may also catalyze the addition of *O*-Glc to proteins. However, more studies are required to definitively demonstrate *in vivo* OGT *O*-glucosylation catalytic activity.[31] In addition to its glycosyltransferase activity, OGT catalyzes the proteolytic cleavage and maturation of host cell factor-1 (HCF-1) in its active site through a unique mechanism whereby an

HCF-1 glutamate is glycosylated followed by pyroglutamate formation and spontaneous backbone cleavage.[32-34] While this is the first demonstration of this cleavage mechanism, glutamate glycosylation-mediated amide hydrolysis may be a general cleavage mechanism employed by OGT or other glycosyltransferases.[33]

OGT's opposite, OGA, is a 103-kDa protein with an N-terminal glycoside hydrolase (GH) catalytic domain (residues 60-366), a nonfunctional C-terminal histone acetyltransferase (pseudoHAT) domain (residues 707-916), and a caspase-3 cleavage site in the stalk domain between the two domains (residues 367-706) (Figure 1.3).[35,36] Cleavage of OGA by caspase-3 at residue 413 occurs during apoptosis, producing two segments that remain tightly associated and maintain the same enzymatic activity.[37] Recently, three research groups independently solved the crystal structure of human OGA with the removal of the flexible linker region in the stalk domain (lacking residues ~400~535).[36,38,39] These structures revealed that OGA acts as a homodimer where the active site is composed of the catalytic domain of one monomer and the C-terminal helical bundle of another monomer.[39] There is also a shorter alternatively-spliced isoform of OGA (sOGA) that only contains the N-terminal *O*-GlcNAcase domain.[40] Full-length ncOGA is localized to the cytoplasm and nucleus while sOGA associates with the ER and lipid droplets.[41,42] Within the nucleus, OGA and OGT are often associated in complexes especially at transcription sites allowing for rapid *O*-GlcNAc cycling.[12] OGT and OGA together form a binary complex (a.k.a. the "*O*-GlcNAczyme"), which has been shown to be important for estrogen-dependent signaling, mammary development, and viral pathogenesis.[43,44] Importantly, OGT and OGA levels are carefully regulated transcriptionally and translationally in order to maintain proper *O*-GlcNAc levels.[25]

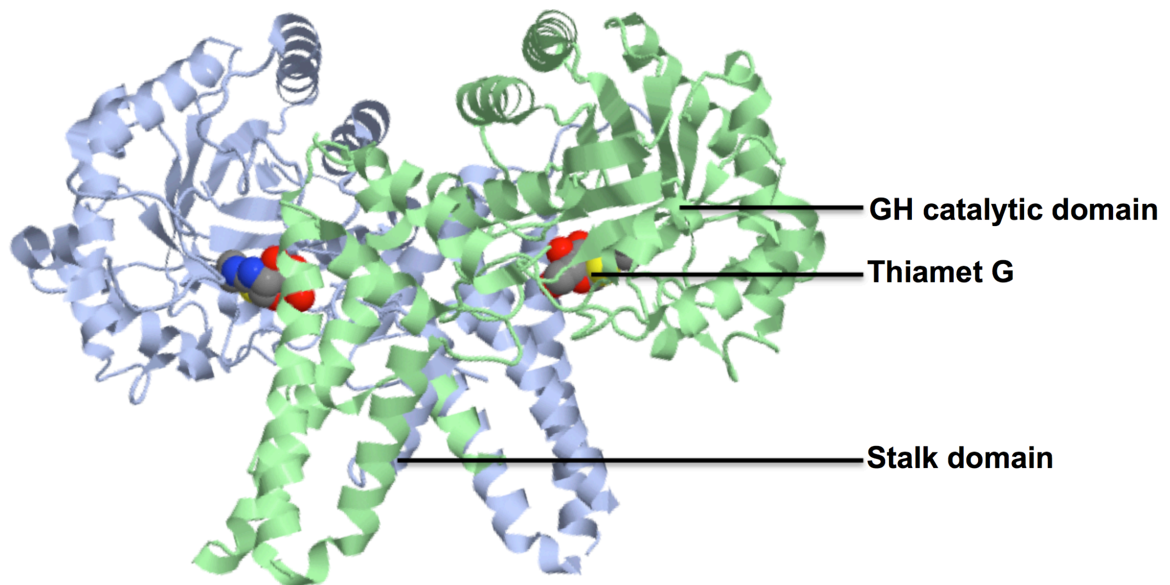



**Figure 1.3 Structure of OGA homodimer.** Shown here is the homodimer crystal structure of OGA including the N-terminal glycoside hydrolase (GH) catalytic domain and the separated stalk domain with the inhibitor Thiamet G (TMG). This figure was made using FirstGlance in Jmol using the crystal structure from Jiang and colleagues (PDB: 5UN9).[36,45]

## 1.4 Methods for the OGT interactome and *O*-GlcNAcome and the OGT substrate specificity hypothesis

The *O*-GlcNAc modification is often compared to phosphorylation due to the fact that it occurs dynamically on serine or threonine residues. However, unlike phosphorylation where there are over 500 of serine/threonine kinases and phosphatases each with their own substrate specificity, only OGT and OGA catalyze the addition and removal of *O*-GlcNAc on intracellular proteins. Several labs have attempted to determine the substrate specificity of OGT, but OGT appears to lack a well-defined consensus sequence although about half of substrates contain a PVS type motif.[12] A more recent study using a peptide library to screen substrates showed that the active site of OGT constrains the OGT-recognition sequence to [TS][PT][VT]**S/T**[RLV][ASY] (probability cutoff of 0.5, in bold is *O*-GlcNAcylated residue).[46] Based on known *O*-GlcNAc sites, several groups have developed computational methods to predict sites, but due to the

ambiguity of the recognition site, these computational methods fail to robustly predict sites, although methods may improve as more sites are identified.[47-50] Aside from the modification site sequence, studies have shown that many substrates have a disordered region C-terminal to the *O*-GlcNAcylation site while other studies have observed that substrates appear to have secondary structure surrounding the site such as coils.[30,51] However, studies have shown that there is no definite three-dimensional structural requirements for the substrate.[51] Additionally, OGT has been shown to *O*-GlcNAcylate proteins co-translationally.[52] Altogether, these studies show that OGT is an inherently promiscuous enzyme that lacks well-defined sequence or structural requirements for its substrates.

Given the high numbers of *O*-GlcNAc substrates and the lack of a consensus sequence or structural constraints, how then does OGT determine which substrates to modify? One prevailing theory in the *O*-GlcNAc field hypothesizes that OGT is recruited or targeted to specific substrates through specific interactors (Figure 1.4). If this hypothesis were true, abolishing the interaction between OGT and a specific interactor would result in reduction in the modification of specific substrates without interfering with other substrates' modification. Indeed, there may exist "hub" interactors, which are critical for recruitment of OGT to a large number of substrates. Several studies have supported this OGT interactor-substrate hypothesis by removing of portions of the OGT protein-binding TPR domains that are responsible for the specific interaction with certain interactors and demonstrating alterations in the *O*-GlcNAcylation of certain substrates. For example, ablation of the OGT TPR domain that interacts with TET2 results in a decrease in the *O*-GlcNAcylation serine 112 of histone 2B (H2B).[53] This TET2-

dependent *O*-GlcNAcylation of the H2B substrate occurs independently of TET2 catalytic activity suggesting that the TET2 interactor presence rather than activity is responsible for recruitment of OGT to this substrate.[53] The chromatin deacetylating mSin3a-HDAC complex interacts with TPR 1-6 region of OGT. Through ablation of OGT TPR 1-6, researchers demonstrated that the protein mSin3a recruits OGT to various substrates including RNA polymerase II and Sp1.[54] Overall, the TPR regions of OGT mediate its protein-protein interactions, which govern downstream substrate *O*-GlcNAcylation.

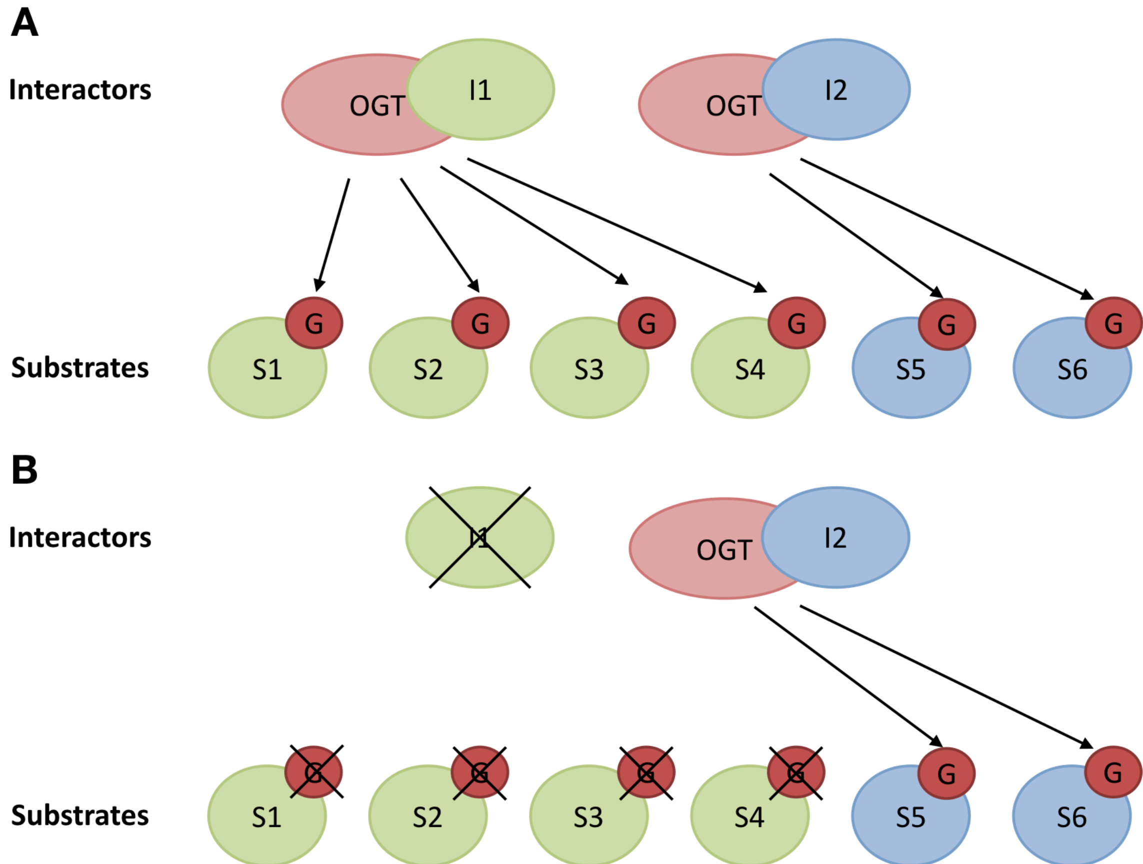



**Figure 1.4 OGT substrate specificity hypothesis.** The major hypothesis for OGT specificity involves OGT interacting with key "hub" interactors through its TPR domains. (A) These interactors then recruit OGT to certain substrates resulting in their *O*-GlcNAcylation. (B) If the interactor-OGT interaction is ablated, the targeted substrates will no longer be *O*-GlcNAcylated. I = interactor, S = substrate, G = *O*-GlcNAc.

Due to the importance of interactor-mediated recruitment of OGT to substrates, several attempts have been made to identify the global OGT interactome. The first such attempt was performed by Gerald Hart's lab, where a yeast two-hybrid study identified 25 different putative OGT interactors. They then verified that several of these putative interactors including MYPT1 and CARM1 interact *in vivo* with OGT and recruit OGT to substrates.[55] This provided a foundation for the next evolution in OGT interactome identification, which was performed by the Yang lab using a tandem affinity purification method. In this study, OGT-FLAG-HA was overexpressed in HEK293T cells, enriched using FLAG and then HA tag pull down, and then identified using MS. Using GFP-FLAG-HA as a control, this study found 853 putative OGT interactors and further verified that OGT interacts with HCF-1, which targets OGT to glycosylate peroxisome proliferator-activated receptor $\gamma$ coactivator 1$\alpha$ (PGC-1$\alpha$).[56] A final approach for the OGT interactome involved the use of a human proteome microarray, which identified 25 putative OGT-binding proteins.[57] These studies help to increase the total number of putative OGT interactors although more studies are required in order to validate these putative interactors and expand the interactome for different species and cell types.

As the lack of a conserved consensus sequence makes it difficult to predict OGT's targets *a priori*, MS methods are currently necessary to directly, efficiently, and globally determine sites of glycosylation. However, *O*-GlcNAc modifications are difficult to detect using traditional MS methods due to several properties of *O*-GlcNAc. First, *O*-GlcNAc and glycans generally are extremely labile during harsh mass spectrometric (MS) conditions precluding the use of higher-energy collisional induced dissociation (CID). Furthermore, *O*-GlcNAc is a large, uncharged moiety that leads to ion suppression

with the unglycosylated peptide and an inability to separate from unglycosylated forms in reverse phase high performance liquid chromatography (HPLC); this is exacerbated further by its substoichiometry on many proteins.[22] In order to circumvent these MS limitations, novel *O*-GlcNAc enrichment-LC/tandem MS methods have been developed to identify *O*-GlcNAc modified proteins and their modification sites.[58,59] The first method developed to successfully identify *O*-GlcNAc sites was β-elimination followed by Michael addition with dithiothreitol (DTT) (BEMAD) whereby the *O*-GlcNAc site is eliminated using DTT leaving behind dehydrated serine or threonine residues.[60] With BEMAD, external validation of the sites are required because false positives are possible with this method as any other eliminated serine or threonine modification will produced the same signature. Since the introduction of BEMAD, many other MS-based methods have been developed, including WGA lectin enrichment, mutant OGA enrichment, metabolic labeling, chemoenzymatic labeling, IsoTag, and native methods often coupled with lower energy electron-transfer dissociation (ETD) MS.[61-69] The advantages and disadvantages of many of these methods are discussed in detail in several reviews.[70,71]

In particular, our lab and others have utilized novel chemoenzymatic labeling methods in order to identify the *O*-GlcNAcome.[72] In this method, a mutant enzyme, GalT, specifically recognizes and appends an N-azidoacetylgalactosamine (GalNAz) moiety to *O*-GlcNAcylated proteins or peptides (Figure 1.5). Then, bioorthogonal “click chemistry” (copper (I)-catalyzed azide-alkyne cycloaddition, CuAAC) can be performed to append an alkynyl linker to the *O*-GlcNAc for subsequent *O*-GlcNAcome enrichment (Figure 1.5). Importantly, both the enzymatic and chemical steps are bioorthogonal and occur stoichiometrically.[73] While the enzyme and CuAAC conditions are consistent

across methods, many different linkers have been utilized, which commonly have: (1) a biotin (for streptavidin bead enrichment), (2) alkyne (for CuAAC), and (3) a cleavable linker portion that enables facile elution. Several different cleavable linkers have been used for *O*-GlcNAcome enrichment including a photocleavable linker and an acid-cleavable linker.[22,67] While these approaches have yielded several novel *O*-GlcNAcylation sites, these linkers can suffer from incomplete cleavage upon elution, hydrolysis of *O*-GlcNAc under harsh cleavage conditions, or off-target selectivity or reactivity.[74] Ideally, the linker will overcome these challenges and also confer MS advantages upon cleavage such as (1) a positive charge to enable facile MS identification like with the photocleavable linker, (2) a small size tag to avoid complex fragmentation, (3) isotopic labeling for facilitated MS identification such as in the IsoTag method, and (4) differential labeling (heavy/light) for multiplexing and labeling of multiple different samples.[22,67] In order to fully realize these goals, it is critical to synthesize and/or apply novel linkers toward *O*-GlcNAcome identification. Considerable variety is observed in the *O*-GlcNAcylation of proteins in different species, cell types, and conditions compelling researchers to determine the *O*-GlcNAcome in the specific conditions of interest. In Chapter 3, we will discuss the development of biological and chemical tools to identify the OGT interactome and *O*-GlcNAcome in the murine brain.

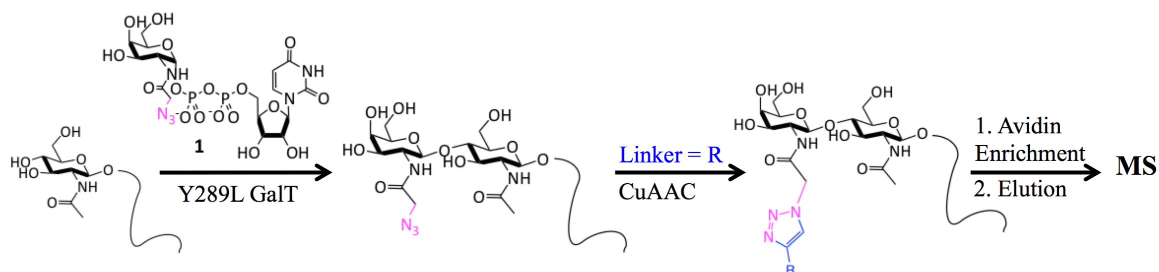



**Figure 1.5 Overview of chemoenzymatic labeling for identification of the *O*-GlcNAcome**. First, *O*-GlcNAcylated proteins or peptides are enzymatically labeled using Y289L GalT and **1** (UDP-GalNAz). After appendage of the azide handle, an alkynyl linker is added using Cu(I) azide-alkyne chemistry

(CuAAC), which appends a biotin. Finally, the *O*-GlcNAcylated peptides or proteins are enriched using streptavidin beads, mildly eluted in the case of a cleavable linker, and then prepared for mass spectrometric analysis.

## 1.5 *O*-GlcNAc crosstalk with other post-translational modifications

As we have touched upon before, the *O*-GlcNAc modification is akin to phosphorylation in that it is highly dynamic, inducible, and occurs on serine or threonine residues. In fact, *O*-GlcNAcylation and phosphorylation are often competing for the same residues where these two processes have a zen-like yin-yang relationship.[12] This relationship is borne out through three general mechanisms: (1) direct competition between phosphorylation and *O*-GlcNAcylation at the same site, (2) OGT association with phosphatases, (3) OGT crosstalk with kinases.

The first demonstration of direct *O*-GlcNAc and phosphate competition was found in the oncogenic transcription factor (TF) c-Myc. Phosphorylation of c-Myc at threonine 58 led to its proteosomal degradation thereby reducing c-Myc activity while *O*-GlcNAcylation at the same site increased its stability and activity.[75] Many more direct competition examples have been discovered since c-Myc with a recent proteomic study identifying a specific yin-yang phosphorylation/*O*-GlcNAcylation interplay motif that is enriched in the human proteome (40% of phosphorylated sites) supporting positive evolutionary selection.[76] Additionally, OGT and phosphatases form complexes that act on the same protein substrates at the same site; *id est*, the phosphatase dephosphorylates the site so that the associated OGT can add an *O*-GlcNAc moiety.[55,77,78] One example of this type of complex is seen in the OGT and PP1β/PP1γ (protein phosphatase 1) complex, which was shown to catalyze the dephosphorylation and subsequent *O*-GlcNAcylation of a peptide.[77] Finally, OGT has been shown to display considerable crosstalk with the

nutrient sensor adenosine-monophosphate-activated protein kinase (AMPK). AMPK-mediated phosphorylation of OGT disrupts OGT's interaction with chromatin, decreases H2B *O*-GlcNAcylation, and alters OGT's overall substrate specificity. In an interesting feedback mechanism, *O*-GlcNAcylation of AMPK increases AMPK's enzymatic activity.[79,80] Furthermore, in response to hyperglycemic conditions, the *O*-GlcNAc glycosylation of CaMKIIδ (calmodulin kinase IIδ) at serine 279 results in elevated CaMKIIδ activity exacerbating arrhythmogenesis in cardiomyocytes.[81] CaMKII is an important $Ca^{2+}$-dependent kinase that regulates cardiac and neuronal function, and its chronic activation leads to pathogenesis.[82,83] *O*-GlcNAcylation of CaMKIIδ links diabetic hyperglycemia to cardiac dysfunction and potentially neurodegenerative diseases. Similar to the case of AMPK, CaMKII phosphorylates and activates OGT, suggesting that this could be a general kinase/OGT feedback loop.[84] Overall, phosphorylation and *O*-GlcNAcylation are inextricably linked post-translational modifications that regulate both homeostatic cellular function and pathology.

Additionally, *O*-GlcNAcylation has been shown to have considerable crosstalk with ubiquitination and its associated ubiquitin-mediated proteosomal degradation.[10,76] First, *O*-GlcNAcylation of H2B at serine 112 facilitates its monoubiquitination at lysine 120 through enhancement of the interaction with the E3 ubiquitin ligase complex, BRE1A/1B. This monoubiquitination event does not affect H2B proteosomal degradation, but rather enhances transcriptional initiation and elongation.[85] Co-translational *O*-GlcNAcylation of the TF Sp1 spares it from ubiquitin-dependent proteosomal degradation, but also impairs its interaction with other transcription factors, thereby disrupting its transcriptional activity.[52,86] Similarly, the glycosylation of the

oncogenic TF p53 spares it from ubiquitin-dependent proteolysis.[87] However, unlike with Sp1, p53 glycosylation leads to enhanced TF activity leading to carcinogenesis.[87-89] The polycomb repressor complex (PRC) members, Bmi-1 (B lymphoma Mo-MLV insertion region 1 homolog) and EZH2 (enhancer of zeste homolog 2), are stabilized by *O*-GlcNAcylation leading to enhanced activity and alterations in oncogenesis.[90,91]

Furthermore, OGT is known to associate with and *O*-GlcNAcylate the deubiquitinase and tumor suppressor, BAP1 (BRCA1 associated protein 1), which regulates DNA replication, dsDNA break repair, and transcription.[92,93] *O*-GlcNAcylation of the TF PGC-1α encourages its association of BAP1, which prevents ubiquitin-mediated degradation of PGC-1α leading to PGC-1α-mediated gluconeogenesis.[56] *O*-GlcNAcylation of BAP1 itself results in BAP1 enhanced activity, which represses retinoic acid receptor (RAR) TF activity and stem cell differentiation.[92] BAP1 deubiquitinates and stabilizes OGT providing another feedback mechanism for OGT activity.[93] Indeed, BAP1 and OGT are often found together in large multiprotein complexes together and have been shown to target each other to different substrates.[94] *O*-GlcNAcylation has been found on dozens of other E1 ubiquitin ligases, E3 ubiquitin ligases, and deubiquitinases although the exact sites and biological consequences of *O*-GlcNAcylation have not yet been determined.[10] Through both specific *O*-GlcNAcylation events and the interaction between OGT and the enzymes that cycle phosphorylation and ubiquitination, *O*-GlcNAcylation displays considerable crosstalk between phosphorylation and ubiquitination.

## 1.6 Role of *O*-GlcNAc in cellular functions: development, survival, stress response, circadian rhythm, longevity, cell cycle, and protein turnover

In order to ascertain the role of *O*-GlcNAc in organismal development and survival, Marth and colleagues first attempted to knockout (KO) the X-linked *Ogt* gene in mice, but found that OGT KO is embryonic lethal.[95] When the Marth lab then created neuron-specific OGT conditional KO (nsOGT cKO) mice, they found that the nsOGT cKO mice were present at only 50% of the expected Mendelian frequencies. The nsOGT cKO led to postnatal death within 10 days due to a failure to develop proper locomotor activity. In addition, OGT knockout in T cells and fibroblasts resulted in apoptosis and growth arrest respectively.[96] Total OGA KO in mice is also embryonic lethal and displays impaired growth and postnatal death within 1 day due to delayed lung development.[97] Brain-specific KO of OGA in mice results in delayed brain differentiation and neurogenesis, obesity, and metabolic perturbations.[98] These OGT and OGA KO studies in mice revealed that *O*-GlcNAc is critical for organismal survival and development.

While most species like mice do not survive knocking out of OGT, an exception to this rule is *C. elegans*, which have led to its use as a model OGT KO organism. An *O*-GlcNAc ChIP-chip study (RL2) in wildtype (WT) *C. elegans* found that the *O*-GlcNAc resided upstream of genes involved in reproductive behavior, aging, axon/neuron differentiation, and glycolysis. When they compared the WT to *ogt-1* and *oga-1* mutants who expressed null/inactive OGT and OGA respectively, they found deregulation in genes involving aging, stress response, innate immunity, and metabolism.[99] A ChIP-chip study (WGA lectin) in *drosophila* showed that *O*-GlcNAc modified transcription factors associate with PREs (polycomb response elements), promoter elements for genes

responsible for homeotic maintenance. As in mice, knocking out the *drosophila* homologue for OGT, super sex combs (KO denoted *sxc*), is required for normal development as *sxc drosophila* die at the pharate adult stage and have highly altered homeotic states.[100] Indeed, many critical regulators of pluripotency and development such as Oct4 and Sox2 are regulated by *O*-GlcNAcylation supporting the demonstrated vital connection between *O*-GlcNAc modification and proper development.[101-105]

In addition to OGT and OGA KO creating developmental abnormalities, *O*-GlcNAc disruption undermines an organism's ability to respond to, recover from, and survive stress stimuli. Several studies have shown that dynamic *O*-GlcNAcylation is important for mounting a defense in cardiomyocytes against oxidative stress.[106-108] In both *ogt-1 C. elegans* (OGT KO) and cardiomyocyte-specific OGT KO mouse (cmOGT cKO) mouse studies, the OGT KO mutants were less tolerant to stress and had lower survival rates in response to stress.[99,109] The essential stress-related TFs, PGC-1α and PGC-1β, as well as their transcription target genes, were downregulated in cmOGT KO mice, which exacerbated heart failure.[99,109] OGT regulates stress through direct *O*-GlcNAcylation of stress-mediating TFs including PGC-1α, HCF-1, HIF-1α (hypoxia-inducible factor 1), and FoxO1/4 (forkhead box O1/4).[110-112]

In *C. elegans*, *daf-2* (IGF-1, insulin-like growth factor 1) mutants display enhanced dauer formation, which is a stress response where worms enter stasis and can survive harsh conditions. Dauer formation was further enhanced in a double *oga-1/daf-2* mutant compared to the *daf-2* single mutants, but reduced in *ogt-1/daf-2* mutants, suggesting that increasing *O*-GlcNAcylation facilitates the stress-induced survival response.[99] *O*-GlcNAcylation confers resistance to heat stress during development across many

different species from worms to zebrafish.[113] Across species, knocking out OGT leads to ER stress, apoptosis, and cell death through reduced $Ca^{2+}$- and Akt-based pro-growth signaling.[114-116] Consistent with a protective role for *O*-GlcNAc, elevation of *O*-GlcNAc levels confers resistance and protection to cardiomyocytes in response to ER stress.[117] In contrast, upregulation of OGT in hepatocellular carcinoma (HCC) increased palmitic acid levels, which in turn, led to elevated ER stress.[114] These studies show that a careful balance of *O*-GlcNAc is necessary for appropriate response to various stress stimuli and survival.

*O*-GlcNAc regulates stress response through many different mechanisms.[118,119] *O*-GlcNAc levels increase in response to physiological and heat stress, which protects cells from various stress stimuli.[115] In a global RNAi screen, OGT was found to be necessary for stress granule formation, a mechanism to conserve energy through decreasing protein synthesis.[120] Formed in response to various stress stimuli, stress granules are composed of P-bodies, mRNA processing enzymes, non-translating mRNAs, and translation initiation proteins.[121] Several key components of stress granules were found to be *O*-GlcNAcylated including RACK1 (receptor for activated C kinase 1), prohibitin-2, GAPDH (glyceraldehyde-3-phosphate dehydrogenase), and ribosomal proteins.[120] Another crucial stress response mechanism is autophagy; a process mediated by the formation of autophagosomes wherein cellular organelles and components are degraded through autophagosomal fusion with lysosomes.[122] *O*-GlcNAcylation has been shown to be necessary for autophagosome maturation through *O*-GlcNAcylation of SNAP-29 (synaptosomal-associated protein 29), a protein that controls the fusion of autophagosomes with lysosomes. Indeed, autophagy is critical for prevention of

aggregation of proteins in proteinopathies, like most neurodegenerative diseases, and is heavily dependent on OGT activity.[9] Across diverse cell types and organisms, *O*-GlcNAc has been shown to be a principle regulator of autophagy.[84,123-125] Thus, *O*-GlcNAcylation acts an important mediator of stress and autophagy, and its level must be carefully tuned to produce appropriate stress survival response.

Longevity and circadian rhythm are also regulated by *O*-GlcNAcylation. A study in *C. elegans* revealed that mutants lacking OGA had effects on overall longevity through its influence on insulin signaling.[99,126] In addition, *O*-GlcNAc modification of the *C. elegans* ortholog of TF Nrf2 (NF-E2-related factor 2) enhanced oxidative stress resistance and anti-aging effects of Nrf2-mediated transcription.[127] As mice age, the levels of *O*-GlcNAcylation gradually decrease along with ncOGT levels while the levels of sOGT increase in the brain.[128] This suggests that *O*-GlcNAc may play a different role in the aging process.

Aside from its role in aging and longevity, *O*-GlcNAc plays a prominent role in circadian rhythm.[129-131] In order to maintain proper circadian rhythm, the TF regulators BMAL1 and CLOCK transcribe the key regulators, period (*Per1/2*) and cryptochrome (*Cry1/2*), which in turn, repress BMAL1 and CLOCK.[132] Additionally, BMAL1 and CLOCK are phosphorylated by GSK3β, a critical regulator of circadian clocks. This leads to their proteosomal degradation and disrupted transcriptional activity.[133-135] These protein turnover and repressive feedback loop mechanisms generate oscillatory activity that gives rise to the 24-hour circadian rhythm within cells.

Altering *O*-GlcNAc levels changes the circadian rhythm in flies and mice; in fact, as the circadian period progresses, global protein *O*-GlcNAcylation changes as well.

OGT regulates circadian rhythm through the *O*-GlcNAcylation of PER2 (period 2), which competes directly with PER2 phosphorylation by casein kinase I (CKIδ) at S662. The *O*-GlcNAcylation of PER2 enhances its repressor activity thereby decreasing CLOCK/BMAL1-mediated transcription.[131] The effects of PER2 *O*-GlcNAcylation mirrors the effects of a S662G-PER2, which leads to reduced PER2 phosphorylation, increases PER2's transcriptional repression, and results in a familial advanced sleep phase disorder.[136,137] As the circadian cycle progresses, GSK3β phosphorylates OGT increasing OGT's activity.[131] Both BMAL1 and CLOCK are *O*-GlcNAcylated in response to high glucose leading to their stabilization likely through the recruitment of BAP1 for deubiquitination.[130] In addition, CLOCK coimmunoprecipitates with OGA at specific circadian times, suggesting that CLOCK is subject to temporally regulated *O*-GlcNAc removal.[131] The stabilization of PER2, BMAL1, and CLOCK through *O*-GlcNAc glycosylation in response to glucose produces a crucial link between the metabolic environment and circadian entrainment.

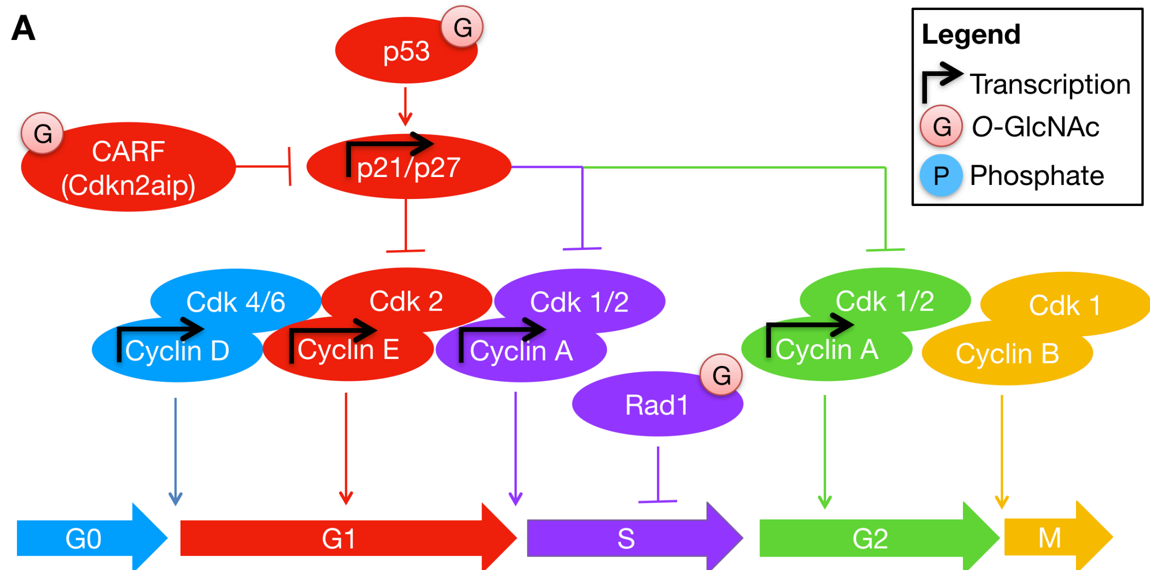
Cell cycle proteins are also subjected to extensive *O*-GlcNAc glycosylation (Figure 1.6).[138-141] Proper cell cycle advancement from G1 phase to S phase (DNA synthesis phase) to G2 phase (post-DNA replication) to M phase (mitosis) and back to G1 phase (or G0 phase) is required for control of appropriate cellular growth or senescence and prevention of the aberrant growth observed in cancer.[142] Manipulation of *O*-GlcNAc levels pharmacologically and genetically has been shown to perturb cytokinesis and mitosis through alterations of the expression of many different cell cycle regulators (Figure 1.6A).[97,138-140] *O*-GlcNAc levels on histones increase in G1 phase, decrease in S phase, and increase again in M phase, suggesting that *O*-GlcNAcylation mediates

chromatin remodeling for cell cycle progression.[143] Increasing OGT levels reduces the activity, mRNA, and protein levels of Cdk1 (cyclin-dependent kinase 1), an important regulator of the G1/S transition through M phase.[144]

The key cell cycle transcriptional regulators, E2F and Rb (retinoblastoma-like protein), are regulated by *O*-GlcNAc and regulate *O*-GlcNAcylation in turn. The G1/S transition is inhibited by E2F1 association with and sequestration by Rb (Rb1/2).[145] Phosphorylation of Rb by Cdk2/Cyclin E or Cdk4/6/Cyclin D complexes emancipates E2F1 from Rb permitting E2F1's unfettered pursuit of its cell cycle transcriptional programming (Figure 1.6B).[146] For the transition to S phase, E2F carries out its transcription program in complex with HCF-1, which, as we have discussed earlier, requires OGT for its proteolytic activation and is heavily glycosylated.[139,147] In addition, knockout of Rb1 or E2F1 leads to increased OGT and OGA expression.[148] Extensive *O*-GlcNAcylation of Rb1 has been observed in G1 phase, which decreases as cell cycle progresses, suggesting that *O*-GlcNAc may influence Rb1 activity in a cell-cycle dependent manner.[145] Interestingly, ablation of the serine 420 *O*-GlcNAcylation site of Rb2 impairs the activity of OGT supporting reciprocal regulatory roles for *O*-GlcNAc and Rb.[149]

While most cyclins and cyclin-dependent kinases (Cdk) have not yet been found to be *O*-GlcNAcylated, Cyclin K, Cdk12, Cdk13, Cyclin M1, and Cdk5 have been found to be *O*-GlcNAcylated.[150-152] Cyclin K and Cdk12/13 form a complex that phosphorylates RNA polymerase II, leading to enhanced transcriptional elongation (Figure 1.6B).[153] The activity of this cyclin complex is important for the transcription of DNA damage associated genes and therefore has been shown to be critical for DNA repair.[154] *O*-

GlcNAc, OGT, and OGA extensively regulate cell cycle-related transcription, chromatin stability, and DNA damage checkpoints that produce cell cycle arrest, which we will discuss in more detail in Chapter 4. The *O*-GlcNAc sites or peptides have been identified for Cyclin M1, Cdk5, Cdk12, Cdk13, but the sites on Cyclin K have not yet been determined.[150,151] Furthermore, the effects of *O*-GlcNAcylation on their cell cycle functions have not yet been explored. It would be interesting to elucidate the *O*-GlcNAcylation sites on Cyclin K, Cdk12, and Cdk13 and to determine the influence of *O*-GlcNAcylation on the complex's ability to mitigate DNA damage. In summary, *O*-GlcNAcylation is a central regulator of cytokinesis, but more information is needed to determine the role of specific *O*-GlcNAc modifications on cyclin and Cdk cell cycle regulators.



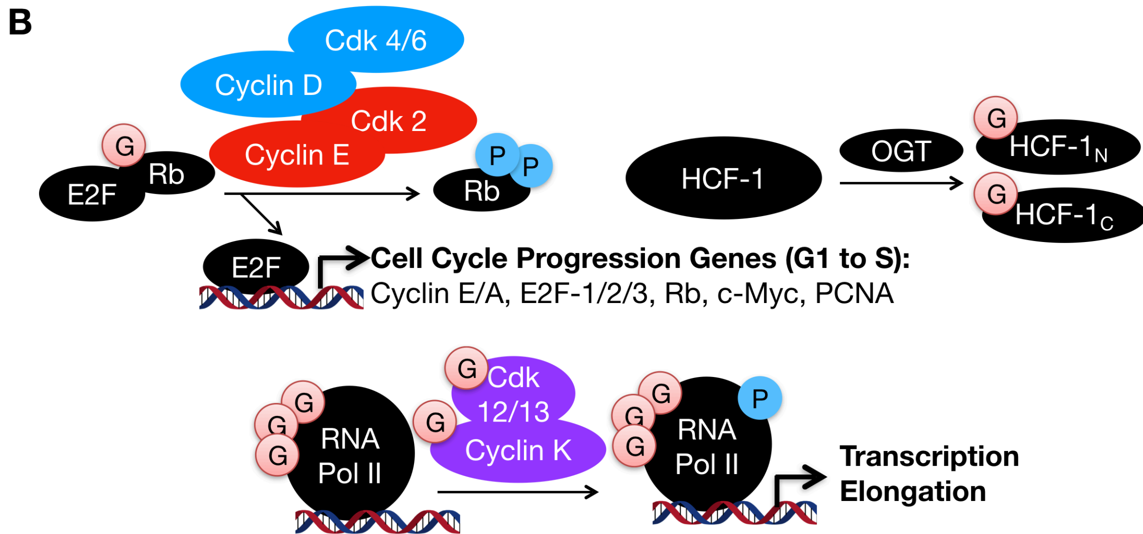



**Figure 1.6 Cell cycle regulators are *O*-GlcNAcylated or regulated by *O*-GlcNAcylation.** (A) Disrupting O-GlcNAc levels can lead to cell growth defects in a variety of organisms through alteration of the expression levels and *O*-GlcNAcylation of different cyclins, and cell cycle-related transcripts. (B) *O*-GlcNAc is involved in the transcriptional regulation of cell cycle. First, Rb is glycosylated in G1 phase, which is associated with association between Rb and E2F leading to inhibition of cell cycle transcription. Upon phosphorylation of Rb by the Cyclin D/Cdk4/6 or Cyclin E/Cdk2 complexes, E2F is freed from Rb sequestration and facilitates transcription of important cell cycle regulators. Additionally, OGT *O*-GlcNAcylates, proteolytically cleaves, and activates the critical cell cycle-regulating TF HCF-1. Finally, the Cdk12/13/Cyclin K complex mediates the phosphorylation of RNA polymerase II, which enhances transcription elongation. Cdk12, Cdk13, and Cyclin K were all found to be glycosylated, but the effect on their activity is not yet known.

*O*-GlcNAc regulates protein synthesis through its mediation of cell cycle, upstream growth-dependent signaling pathways, and the protein synthetic machinery. Protein synthesis is regulated in response to external stimuli such as growth factors and nutrients through intracellular signaling pathways such as the mitogen-activated pathway kinase (MAPK) or phosphatidyl inositide-3 kinase (PI3K) pathway.[155] After extracellular activation, a cascade of phosphorylation and dephosphorylation events are then integrated and converge upon the protein synthetic machinery to prepare for cellular growth.[156] Many of these growth-mediating kinases and phosphatases are *O*-GlcNAcylated (Figure 1.7).[21,157] Protein synthesis occurs in three major steps in eukaryotes: (1) initiation, the most heavily regulated step, which involves a large number of eukaryotic initiation factors (eIFs); (2) elongation, which is regulated by eukaryotic elongation factors (eEFs);

and (3) termination, which is controlled by eukaryotic release factors (eRFs).[158] *O*-GlcNAcylation also occurs on the majority of proteins in the ribosome and many of the proteins involved in translational initiation and elongation (Figure 1.7).[159] While *O*-GlcNAc heavily modifies these proteins, the exact consequences of these *O*-GlcNAc modifications have not yet been delineated.

In addition to regulating the ribosome and protein synthesis, protein turnover is regulated by *O*-GlcNAcylation. OGT cotranslationally modifies proteins such as Sp1 to stabilize them and prevent ubiquitin-mediated proteosomal degradation.[52] Indeed, the proteasome itself and proteosomal regulators are both known to be directly *O*-GlcNAcylated.[160,161] *O*-GlcNAcylation of the 26S and 19S proteasomes reduces proteosomal degradation.[160] The reduction of sOGA impairs proteosomal activity, leading to the accumulation of ubiquitinated proteins. When sOGA is localized to lipid droplets, proteosomal inhibition results in increases in the lipid droplet components, perilipin-2 and perilipin-3, which leads to enhanced lipid droplet formation.[41] Altogether, *O*-GlcNAcylation, OGT, and OGA must act in concert to regulate protein synthesis and degradation.

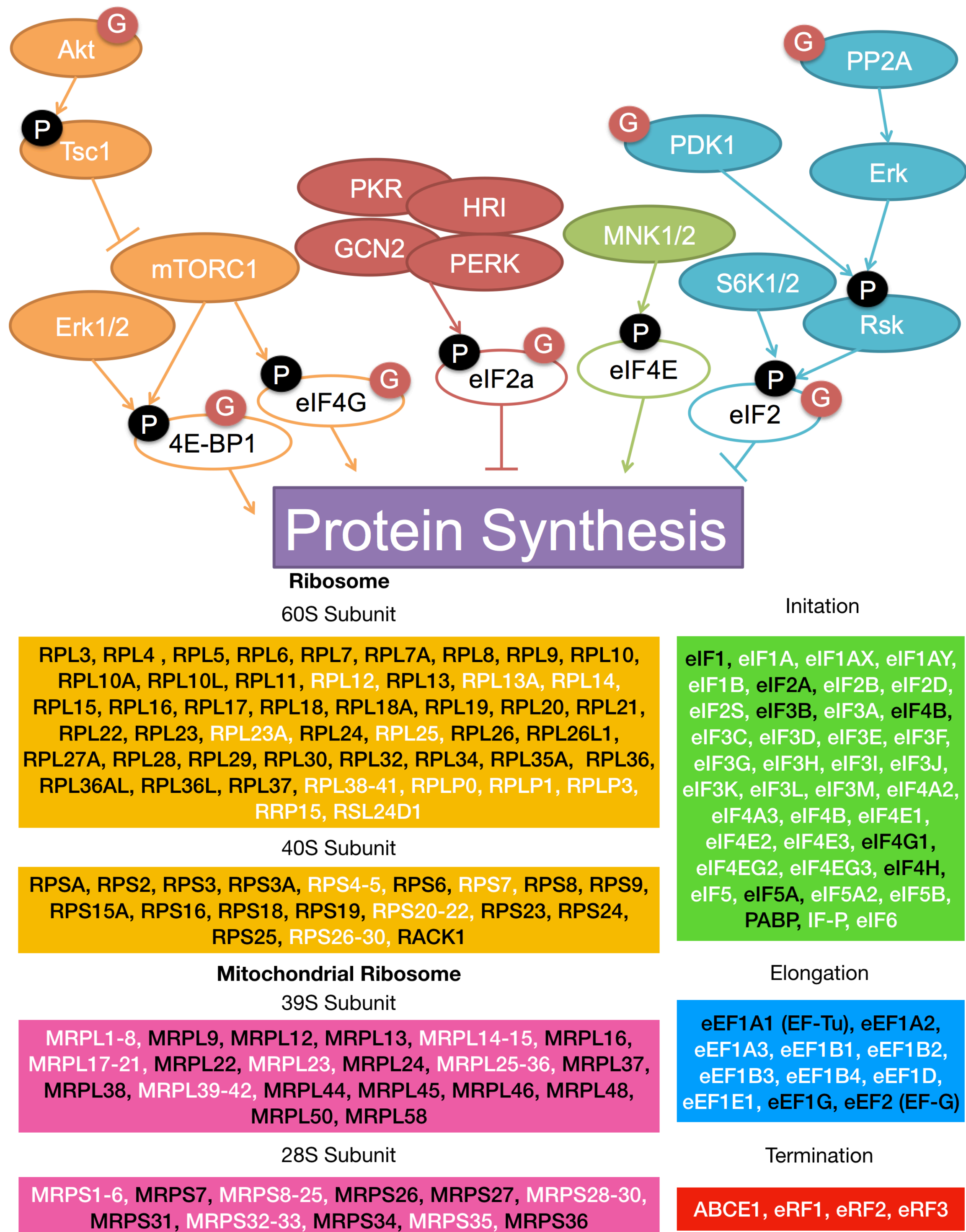



**Figure 1.7 *O*-GlcNAcylation of protein synthesis upstream regulators and machinery.** Shown here are the known glycosylated proteins for the upstream regulators of protein synthesis and the direct protein synthesis regulators including the ribosomal machinery. The critical phosphorylation events that modulate protein synthesis are shown as well as their ultimate effects on protein synthesis. The bottom image shows all the glycosylated proteins that are components of the ribosome and mitochondrial ribosome in addition to proteins involved in initiation, elongation, and termination of protein synthesis. The proteins written in

black are known to be glycosylated, while those written in white are not known to be glycosylated. G = GlcNAc, P = phosphate.

## 1.7 Role of *O*-GlcNAc in metabolic function and dysfunction

In eukaryotes, *O*-GlcNAcylation acts as a nutrient sensor due to its dependence on the abundance of UDP-GlcNAc, the end product of the hexosamine biosynthetic pathway (HBP) (Figure 1.8). The production of UDP-GlcNAc begins with the uptake of glucose, which can either enter glycolysis, pentose phosphate pathway (PPP), glycogen biosynthesis, or HBP. Specifically, the first three products in the HBP compete with these other pathways for substrates. The first committing and rate-limiting step of the HBP is catalyzed by glutamine:fructose amidotransferase (GFAT), which transfers an amine from a glutamine donor to fructose-6-phosphate in order to generate glucosamine-6-phosphate.[162] Along the HBP, the transformation of glucose to UDP-GlcNAc is reliant on the products of a variety of metabolic pathways, including amino acid, fatty acid, and nucleotide metabolism.[21] Researchers estimate 2-5% of glucose is shunted into the HBP to make UDP-GlcNAc levels the second highest metabolite population after ATP implicating *O*-GlcNAc glycosylation as an important PTM and nutrient sensor.[21] Indeed, *O*-GlcNAc, OGT, and OGA levels are all dependent upon the intracellular UDP-GlcNAc concentrations and therefore HBP flux.[25,162]

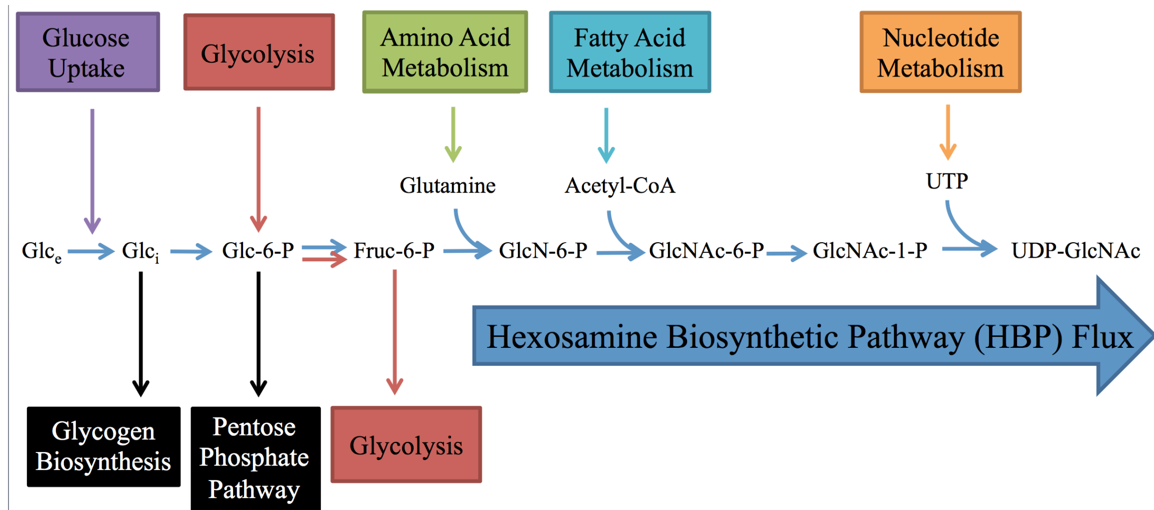



**Figure 1.8 The hexosamine biosynthetic pathway (HBP) requires input from many different metabolic pathways**. The HBP begins with the uptake of extracellular glucose ($Glc_e \rightarrow Glc_i$) followed by two glycolytic steps. The first 3 products are depleted when they enter the competing metabolic processes, glycogen biosynthesis, pentose phosphate pathway, and glycolysis. The first committed and rate-limiting step of HBP occurs when Fruc-6-P is converted to GlcN-6-P. The next HBP steps require metabolites that involved in amino acid, fatty acid, and nucleotide metabolism.

Given the dependence of *O*-GlcNAc on the cell's metabolic pathways, *O*-GlcNAc has been shown to be a key glucose sensor that is exquisitely sensitive to glucose and metabolite levels.[111,163-165] In addition, *O*-GlcNAc regulates glucose uptake, insulin signaling, glycogen synthesis, gluconeogenesis, lipogenesis, and mitochondrial function.[42,166] OGT has been shown to be recruited to the cellular membrane through direct interaction with $PIP_3$ downstream of insulin-induced PI3K signaling. Once localized to the membrane, OGT glycosylates the key insulin signaling regulators, IR-β (insulin receptor β), IRS1 (insulin receptor substrate 1), PDK1 (3-phosphoinositide-dependent protein kinase 1), PI3K, Akt (RAC-α serine/threonine-protein kinase), which changes their phosphorylation states and contributes to insulin resistance.[28] Interestingly, insulin signaling promotes the expression of OGT and the targeting of OGT to lipid rafts.[167]

The connection between O-GlcNAc and insulin signaling has been demonstrated to be present in and contribute to the chronic disease, diabetes.[162,168] Aberrant *O*-GlcNAc glycosylation has been observed in diabetic patients and diabetic mouse models.[81,108] In fact, several studies have proposed and demonstrated that *O*-GlcNAc has utility as a robust diagnostic biomarker for diabetic and, potentially more importantly, pre-diabetic metabolism.[169,170] Reduction of OGA in mice have been shown to cause obesity and insulin resistance implicating OGA in diabetic metabolic and transcriptional changes.[171] *O*-GlcNAcylation of CREB-regulated transcriptional coactivator 2 (CRTC2) reduces its cytoplasmic sequestration, increases its nuclear translocation, and enhances hepatic gluconeogenesis through elevated CRTC2-mediated transcription.[172] In addition to CRTC2, many metabolism-regulating TFs are glycosylated or regulated by *O*-GlcNAc, which affects their transcriptional activity including carbohydrate responsive element binding protein (ChREBP), nuclear receptor liver X receptor (LXR), sterol response element binding protein 1 (SREBP1), CRTC2, PGC-1α, and Sp1. Through these TFs, *O*-GlcNAc directly regulates insulin signaling, glycogen synthesis, gluconeogenesis, and lipogenesis in response to nutrient levels.[173-176]

*O*-GlcNAc has also been shown to regulate nutrient flux and mitochondrial activity. Mitochondria are the powerhouses of the cells, responsible for maximizing the ATP required for cellular activity while minimizing ROS (reactive oxygen species) generation. OGA deficiency modulates insulin and glucose sensitivity through the modulation of mitochondrial homeostasis.[177,178] Interestingly, GlcN (glucosamine), a precursor to UDP-GlcNAc (the product of GFAT), facilitated mitochondrial function, which led to lifespan extension in both *C. elegans* and mice.[179] This result led to the

hypothesis that increasing *O*-GlcNAc levels may be beneficial for mitochondrial function. However, increasing *O*-GlcNAc levels (TMG treatment) led to mitochondrial dysfunction and was associated with lower glycolytic flux and ROS generation while decreasing ncOGT and mOGT levels led to higher glycolysis and ROS generation in mitochondria.[180,181] The conflicting GlcN and TMG results can be explained by the ability for GlcN to affect multiple different metabolic pathways while TMG selectively and specifically inhibits OGA. OGT directly *O*-GlcNAcylates a variety of mitochondrial proteins involved in oxidative phosphorylation.[182] Interestingly, the mitochondrial OGT (mOGT) isoform has been shown to trigger apoptosis and potentially contribute to Alzheimer's disease (AD) pathology in diabetic patients.[23,183] However, the role of *O*-GlcNAc in mitochondrial function has been left unclear by two studies that showed (1) that ncOGT is sufficient for mitochondrial *O*-GlcNAcylation leaving the exact role of mOGT unclear and (2) high glucose can change mitochondrial function independent of *O*-GlcNAc.[184,185] While many studies suggest that *O*-GlcNAc tracks with and may regulate metabolic functions, establishment of either a causative or correlative link between *O*-GlcNAc and these functions require further study.

Cancer exploits perturbations in cell cycle, growth, and metabolism to circumvent constraints on growth and proliferation. Cancer has been shown to display altered metabolism including increased glucose import, aerobic glycolysis (a.k.a. the Warburg effect), glutamine addiction, reduced oxidative phosphorylation, and hyper-*O*-GlcNAcylation.[186] Increased *O*-GlcNAc, OGT, and OGA levels have been observed across many different cancer subtypes from including breast, lung, colon, and prostate cancer, suggesting that *O*-GlcNAc could be a useful malignancy diagnostic biomarker.[187]

Indeed, several studies have shown that *O*-GlcNAc plays a critical role in cancer cell biology, leading to many reviews on the link between *O*-GlcNAc and cancer.[186,188-190] As discussed in the section above, many TFs, transcriptional regulators, and epigenetic regulators that are known to regulate cancer biology are *O*-GlcNAcylated, which alter their oncogenic properties including p53, c-Myc, Bmi-1, EZH2, HIF-1, HCF-1, HDAC, mSin3a, TET, BAP1, Sp1, Rb, and FOXM1.[110,191-193]

*O*-GlcNAc achieves metabolic reprogramming of cancer cells through transcriptional and metabolic changes. The *O*-GlcNAc modification of two glycolytic pathway proteins, PFK1 (phosphofructokinase 1) and G6PD (glucose-6-phosphate dehydrogenase), reduces glycolytic flux, and therefore shunts carbon flux through the PPP.[194,195] Cancer cells rely more heavily on the PPP than glycolysis because the PPP reduces oxidative stress and capacitates rapid growth required for cellular proliferation.[196] Many studies have shown that *O*-GlcNAcylation and HBP flux are perturbed in and important for the cancer epithelial to mesenchymal transition (EMT), the initiative process involved in cancer metastasis.[197-199] Cancer metastasis, invasiveness, and angiogenesis are heavily influenced by *O*-GlcNAcylation.[191,192,200,201] Tumorigenesis, the promotion of tumor growth in cancers, is also enhanced by increasing *O*-GlcNAcylation.[202] Recently, researchers have also shown that cancer stem cell proliferation is also regulated by *O*-GlcNAcylation.[203] Cancer stem cells are the population of cells that with self-renewal and differentiation properties that are believed to be key to tumor initiation, progression, and metastasis.[204] In summary, *O*-GlcNAc has been demonstrated to be a key regulator of cancer biology and could be an important biomarker and therapeutic target.

### 1.8 Role of *O*-GlcNAc in neuronal function

We will now focus on the role of *O*-GlcNAcylation in neurobiology. Studies have shown that OGT and OGA are expressed at their highest levels in the hippocampus, localized to synapses, and modify thousands of proteins in neurons.[205] In fact, an estimated 40% of the neuronal and 19% of the synaptosomal proteomes appear to be *O*-GlcNAcylated with some proteins like bassoon modified with over a dozen *O*-GlcNAcylation sites.[150] In excitatory synapses, OGT is localized to the post-synaptic density where *O*-GlcNAcylation increases upon neuronal stimulation.[206] The addition of the OGA inhibitor, PUGNAc, enhances long term potentiation (LTP), short term potentiation (STP), and axonal branching while an inhibitor for OGT, alloxan, produces the opposite effects (Figure 1.9).[207,208] LTP is a mechanism that underlies learning and memory and becomes increasingly impaired in neurodegenerative diseases.[209] Increasing *in vivo* *O*-GlcNAc levels in synapses enhances the phosphorylation of synapsin I/II, which increases hippocampal synaptic plasticity and activity.[208] OGT KO leads to decreased expression of glutamate AMPA receptors (GluA2/3), reduction of the number of dendritic spines, and an increase in the proportion of immature dendritic spines.[206] On the other hand, another study in excitatory glutamatergic CA3-CA1 hippocampal neurons showed that increasing *O*-GlcNAcylation led to long term depression (LTD), disrupted LTP, and impaired memory formation.[210] Consistent with this observation, increasing *O*-GlcNAc levels was shown to be ameliorate the neuronal hyperexcitability in hippocampal slices and in mice, suggesting a neuroprotective role for *O*-GlcNAc.[211] OGA deficient mice (*Oga*$^{+/-}$) displayed elevated *O*-GlcNAc levels and LTP and LTD impairments through aberrant glutamate receptor phosphorylation.[212] One study showed that

pharmacologically increasing *O*-GlcNAc levels had no effect on basal synaptic transmission while a separate study showed that this facilitated basal synaptic transmission through increased trafficking of AMPA receptors.[208]

The inconsistency of these studies about the role of *O*-GlcNAc on neuronal function can be attributed to pharmacological inhibition vs. genetic ablation methods. In particular, some of the pharmacological agents used to inhibit the *O*-GlcNAc cycling enzymes were found to have off-target effects confounding potential results. Despite their contradictory results, these studies support an essential role of OGT, OGA, and *O*-GlcNAcylation in neuronal function and that the maintenance of homeostatic *O*-GlcNAc levels is necessary for proper neuronal homeostasis.

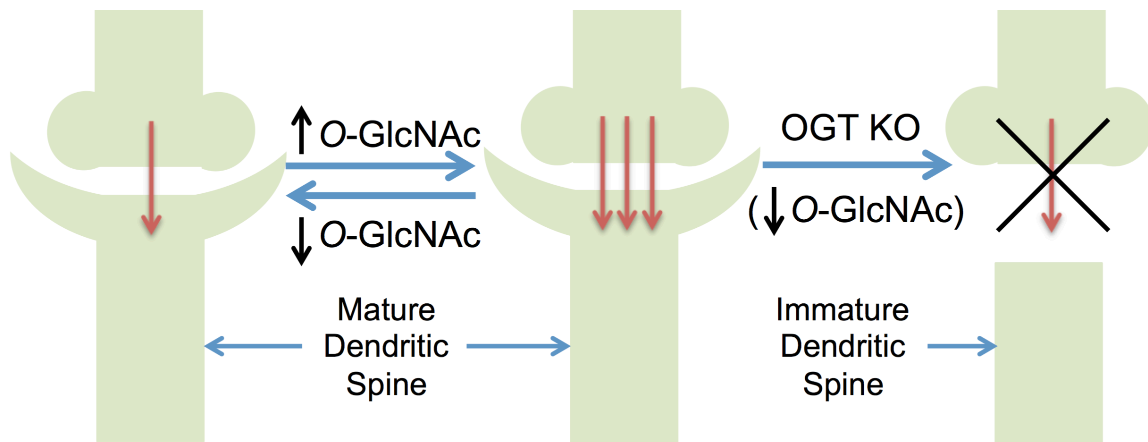



**Figure 1.9 *O*-GlcNAc regulates neuronal activity**. Increasing *O*-GlcNAc levels with an OGA inhibitor enhances LTP, STP, and axonal branching while decreasing *O*-GlcNAc levels with an OGT inhibitor has the reverse effects. Knocking out OGT results in fewer dendritic spines and a higher proportion of mature dendritic spines thereby interfering with neuronal signaling.

Through the examination of the protein- and site-specific effects of *O*-GlcNAcylation on neuronal proteins, the mechanism by which *O*-GlcNAc influences neuronal function has been further elucidated. Ablation of the *O*-GlcNAcylation site of synapsin I at T87 increases synapsin I synaptic localization, the synaptic vesicle localization to axons, as well as the reserve pool of synaptic vesicles, thereby enhancing

synaptic activity.[213] Similarly, *O*-GlcNAcylation of AMPA receptor GluA2 results in LTD and affects GluA2 trafficking to synapses.[209,210] Taken with the studies that globally change *O*-GlcNAc levels, this site-specific data supports a complex and essential role of *O*-GlcNAcylation in mediating neuronal activity and underscores the importance of pursuing and integrating systems level and reductionist approaches in parallel.

*O*-GlcNAc plays a critical role in organismal metabolism through regulation of neuronal activity. In AgRP neurons in the arcuate nucleus of the hypothalamus, *O*-GlcNAc modification of the potassium channel, $K_v$7.3 (*Kcnq3*), increases neuronal excitability, which prevents the conversion of white to brown fat. OGT KO in AgRP neurons resulted in decreased hepatic gluconeogenesis, enhanced thermogenesis, and protection from obesity and insulin resistance.[214] Ablation of OGT in another region of the hypothalamus, the paraventricular nucleus, causes mice to overeat and become obese, demonstrating that specific ablation of OGT in different neurons of the same brain region can result in polar opposite metabolic effects.[215] In these ways, *O*-GlcNAcylation in specific neurons can affect an entire organism's metabolism.

In addition, *O*-GlcNAc plays a vital role in cellular metabolism within neurons. The arborized nature of neurons necessitates proper trafficking and distribution of mitochondria to meet the localized energy demands of dendrites and axons.[216] The mitochondrial motor-adaptor protein, TRAK1 (trafficking kinesin binding protein 1), has been shown to stably interact with OGT, which can alter OGT's substrate specificity.[217] In conditions with high extracellular glucose, *O*-GlcNAcylation of TRAK1/Milton increases, which reduces the motility of migrating mitochondria in axons.[178] Through this mechanism, neurons can rapidly respond to the nutrient milieu. Our lab showed that a

nutrient sensor and important transcriptional regulator of neuronal activity, CREB (cAMP response element binding protein), is *O*-GlcNAc modified at S40 in our lab. This glycosylation event disrupts the association of CREB with the coactivator, CRTC, and thereby suppresses the transcription of neuronal activity genes in response to neuronal depolarization. Ablation of the S40 glycosylation site of CREB was shown to hasten the formation of memory in a fear conditioning paradigm.[218] The role of the *O*-GlcNAcylation of CREB will be explored in significantly greater depth in Chapters 4 and 5. Thus, *O*-GlcNAc regulates neuronal activity through its regulation of the neuronal activity gene transcription and neuronal mitochondrial trafficking in response to neuronal stimulation and glucose increase respectively.

Beyond the importance of *O*-GlcNAc in normal neuronal function, OGT, OGA, and *O*-GlcNAc dysregulation have been implicated in many neurological and psychiatric disorders such as schizophrenia, Adams-Oliver syndrome, X-linked intellectual disability, and stroke.[18,219-221] Given the link between stress and psychiatric and neurological diseases, future studies may reveal more connections between the stress-induced *O*-GlcNAcylation and these diseases. In the next section, we will explore the relationship between *O*-GlcNAcylation and neurodegenerative diseases.

### 1.9 Role of *O*-GlcNAc in neurodegenerative diseases

*O*-GlcNAcylation has also been shown to play a pivotal role in neurodegenerative diseases. Many neurodegenerative diseases are proteinopathies where particular proteins aberrantly aggregate due to hyperphosphorylation and/or differential protease-mediated cleavage. Specifically, these neurodegenerative diseases can be classified into amyloidoses (AD), tauopathies (AD and frontotemporal dementia (FTD)),

synucleinopathies (Parkinson's disease (PD)), TDP-43 (transactivation response-DNA binding protein-43) proteinopathies (amyotrophic lateral sclerosis (ALS) and frontotemporal lobar degeneration with tau-negative and ubiquitin-positive inclusions (FTLD-U)), and polyglutamine (polyQ) proteinopathies (Huntington's disease (HD) and spinocerebellar ataxia (SCA)).[222,223] Through *O*-GlcNAc's fundamental role in regulating autophagy, the major mechanism for removal of protein aggregates, *O*-GlcNAc affects the cellular response to these proteinopathies. In addition, *O*-GlcNAc has been implicated in specific neurodegenerative diseases based on genome-wide association studies (GWAS). The genomic locus for the *Oga* is associated with increased risk for late-onset Alzheimer's disease (AD).[224] In addition, *Oga* is subject to differential alternative splicing in AD.[225] The *Ogt* locus has been associated with X-linked mental retardation and a potential linkage to a rare form of dystonia-parkinsonism although a recent study suggested that the disease might arise from another resident gene on the locus.[226-228]

*O*-GlcNAc has been found on all of these aggregating proteins and has been shown to be important for increasing the solubility of these proteins.[229] Indeed, several studies have established a yin-yang relationship between *O*-GlcNAc and disease-associated hyperphosphorylation for many different proteinopathy-associated proteins including α-synuclein, neurofilaments, and TDP-43.[64] This led to the general hypothesis that hyperphosphorylation leads to protein aggregation while *O*-GlcNAcylation prevents aggregation and confers neuroprotection (Figure 1.9). In addition, *O*-GlcNAc modulates the protease-mediated cleavage of APP (amyloid precursor protein), α-synuclein, and TDP-43, which generates amyloidogenic species.[230] We will now discuss what is known

about the effect of *O*-GlcNAc on the etiology of neurodegenerative diseases and their biological mechanisms.

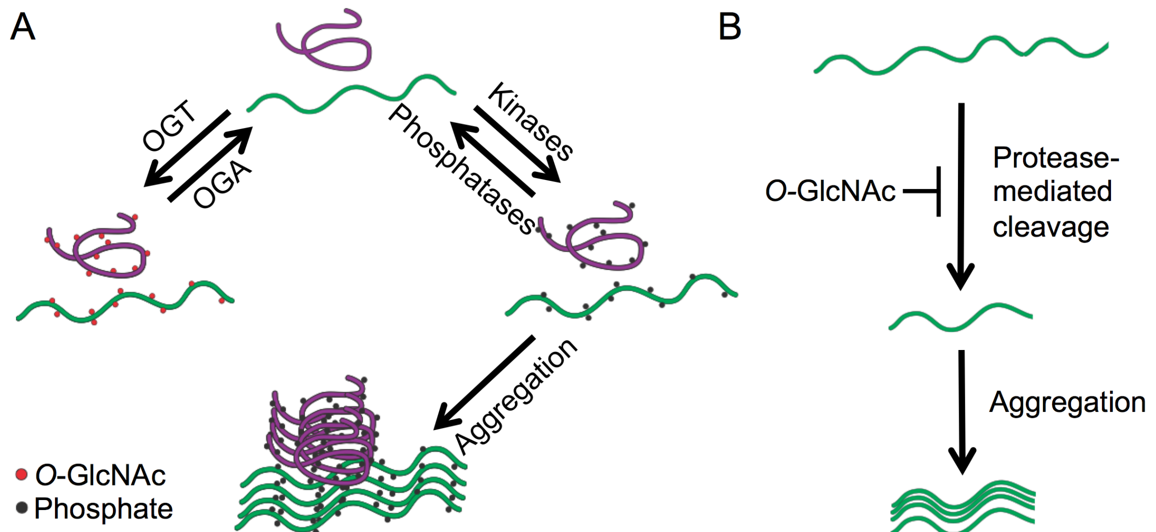



**Figure 1.9 *O*-GlcNAc reduces protein aggregation in proteinopathies**. (A) *O*-GlcNAcylation of proteins increases the solubility of proteins while hyperphosphorylation leads to aggregation and the formation of disease-related plaques, tangles, of inclusions. (B) *O*-GlcNAc also mediates the proteolytic cleavage of proteins in proteinopathies. Proteolytic cleavage of aggregating proteins often enhances their amyloidogenic capabilities and *O*-GlcNAcylation of these proteins can reduce their cleavage.

Alzheimer's disease (AD) is a debilitating disease that is characterized by progressive memory loss and is currently the largest unmet neurological need with no treatment to prevent, slow, or cure the disease.[231] *O*-GlcNAc levels and specific *O*-GlcNAcylation events are perturbed in AD patients.[232,233] The major hallmarks of AD are amyloid plaques and neurofibrillary tangles (NFTs), which are composed of β-amyloid peptide and hyperphosphorylated microtubule-associated protein tau respectively. Both disease-related proteins are known to be *O*-GlcNAcylated *in vivo*, and their *O*-GlcNAcylation is correlated with a decrease in protein aggregation.[234,235] The main familial Alzheimer's disease (FAD) mutations that lead to early-onset AD occur in proteins involved in β-amyloid (Aβ) processing, including mutations in APP, presenilin 1 (PS1), and presenilin 2 (PS2). Amyloid precursor protein is a transmembrane protein that

is subject to proteolytic processing by sheddases. APP can be cleaved either by α-secretase to generate non-aggregating species or β-secretase to generate the βCTF. After cleavage by β-secretase, γ-secretase cleaves βCTF to create Aβ40 and Aβ42, the more amyloidogenic species.[236] The *O*-GlcNAcylation of APP increases α-secretase non-amyloidogenic processing and decreases Aβ40 levels.[237] In addition, *O*-GlcNAc modification of APP at threonine 576 increases the trafficking rate to the membrane, decreases its endocytosis, and decreases Aβ peptide production.[238] Increasing *O*-GlcNAc levels using an OGA inhibitor, NButGT, reduced β-amyloid plaque formation, neuroinflammation, and memory impairment in a mutant mice that expresses FAD mutations in APP and PS1. The underlying mechanism of neuroprotection was via the direct *O*-GlcNAcylation of S708 of nicastrin, a component of γ-secretase, leading to a reduction in γ-secretase activity and Aβ generation.[239] These results led to the theory that *O*-GlcNAc modification plays a neuroprotective role in AD and generally in neurodegenerative diseases.

In addition to AD, FTD, FTD with parkinsonism (FTDP), and progressive supranuclear palsy (PSP) are tauopathies that are characterized by the hyperphosphorylation and then aberrant aggregation of microtubule-associated protein tau to form neurofibrillary tangles (NFTs).[240] OGT is known to modify and regulate glycogen synthase kinase-3b (GSK-3b) and Cdk5, kinases that abnormally hyperphosphorylates APP and tau.[151,241-243] In a mouse model of FTDP expressing amyloidogenic tau$^{P301L}$, the Vocadlo lab evinced that increasing *O*-GlcNAc levels with TMG raised global protein *O*-GlcNAcylation levels and reduced tau aggregation.[244] As the *O*-GlcNAcylation of tau at S400 increased, the solubility of tau increased, although

the hyperphosphorylation of tau was not affected.[245-247] This suggests that *O*-GlcNAcylation alone (without removal of hyperphosphorylation) may be sufficient for reducing the fibrillar tau. Another study showed that *O*-GlcNAc-S400 tau enhanced the solubility of tau relative to WT tau *in vitro*, although this was not compared to the purely phosphorylated species or pseudo-phosphorylated species where phosphorylation sites are replaced with phospho-mimetic residues.[248] Furthermore, increased *O*-GlcNAc levels led to the elevation of *O*-GlcNAc-S400 tau, reduction of tauopathy, decreased cerebrospinal fluid tau, and amelioration of the neurodegenerative phenotype in tau$^{P301L}$ mice.[244,249] In another AD mouse model that expresses tau$^{P301L}$ and APP$^{K670N/M671L}$ (APP$^{Swe}$, Tg2576), treatment with TMG reduces the amount of β-amyloid peptides and plaques and succors cognitive decline.[250] Across mouse models of neurodegeneration and especially of amyloidoses and tauopathies, studies have consistently shown that increasing *O*-GlcNAc levels ameliorates neurodegeneration supporting a neuroprotective role for *O*-GlcNAc.[249,251,252] In fact, several companies, one of which grew out of the Vocadlo group, are developing and exploring the utility and efficacy of *O*-GlcNAcase inhibitors as therapeutics for neurodegenerative diseases. A Swiss company, Asceneuron, now has a drug entering Phase I clinical trials for the treatment of the tauopathy PSP as of this year.[253]

In the synucleinopathy PD, α-synuclein aggregates to form Lewy bodies in dopaminergic neurons in substantia nigra pars compacta (SNpc), which causes neuronal death and progressive motor dysfunction.[254] Elevated *O*-GlcNAc levels were observed in the temporal cortex of PD patients. Enhancing *O*-GlcNAc levels through TMG inhibition of OGA was then shown to reduce autophagy activity, leading to α-synuclein aggregation

in PD, which suggests that globally increasing *O*-GlcNAc may exacerbate PD.[255] Knocking out the second most common causal genetic PD mutation (*LRRK2*) and its homolog *LRRK1* in mice reduced autophagic flux, recapitulated the specific SNpc dopaminergic neurodegeneration observed in PD, and intensified α-synuclein accumulation, suggesting that impaired autophagic flux could be a causal mechanism in PD generally or at least specifically in *LRRK2* mutation-induced PD.[256] *O*-GlcNAcylation of T72 and S87 on α-synuclein prevents α-synuclein hyperphosphorylation and aggregation and associated neurodegeneration.[257-259] In PD, α-synuclein is proteolytically cleaved by calpain to generate fragments found in Lewy bodies. α-synuclein *O*-GlcNAcylation on either T72 or S87 conferred resistance to the calpain-induced cleavage of α-synuclein.[230] Interestingly, increasing *O*-GlcNAc levels through GlcN or PUGNAc diminishes calpain activity and is cardioprotective in rats.[260] Similar to AD, the role of *O*-GlcNAc appears to be neuroprotective for PD through prevention of with aberrant hyperphosphorylation and proteolytic cleavage, which inhibits protein aggregation. In addition, up to half of patients with Parkinson's disease also have dementia with the major hallmarks of Alzheimer's disease, amyloid-β plaques and neurofibrillary tangles, suggesting a link between PD and AD.[261]

Amyotrophic lateral sclerosis (ALS) is another neurodegenerative disease that causes the progressive degeneration of motor neurons leading to paralysis.[262] ALS is characterized by the aggregation of TDP-43, NFs, FUS (FUS RNA-binding protein), and SOD1 (Cu/Zn superoxide dismutase 1) to form inclusion bodies. TDP-43, NF-H, NF-M, NF-L, FUS, and SOD1 are all known to be *O*-GlcNAcylated and at least some of their sites have been mapped.[150,263-266] Like with tau and α-synuclein in AD and PD

respectively, hyperphosphorylation of neurofilaments and TDP-43 occurs with aberrant proteolytic cleavage of TDP-43. TDP-43's *O*-GlcNAc sites overlap with known CK1 phosphorylation sites, suggesting that a yin-yang relationship may exist for *O*-GlcNAcylation and phosphorylation on TDP-43.[263] *O*-GlcNAc levels are decreased in the spinal cords of G93A-SOD1 mutant mice suggesting that *O*-GlcNAcylation is perturbed in ALS.[267] More research is required to explore the connection between ALS and *O*-GlcNAcylation.

Although *O*-GlcNAc modification has been shown to be important in neurodegenerative diseases, a causal link between *O*-GlcNAcylation and neurodegenerative diseases has not been established. This leads to the outstanding question: does *O*-GlcNAc dysregulation precede and produce neurodegeneration or is aberrant *O*-GlcNAcylation merely a side effect of neuronal dysfunction? What are the initial transcriptional or morphological changes that occur upon *O*-GlcNAc depletion, and does this depletion ultimately lead to a disease phenotype? In humans, these questions remain largely inaccessible, as diagnosis for AD, PD, and other idiopathic neurodegenerative diseases are typically made after neurodegeneration. Studies in different model organisms have been contradictory; studies in *C. elegans* show that increasing *O*-GlcNAc levels aggravates protein aggregation and neurodegeneration while studies in mice show that increasing *O*-GlcNAc produces the neuroprotective effects.[229,268,269] More incompatible evidence can be found in studies exploring the levels of *O*-GlcNAc in human samples; different studies have shown increased and decreased *O*-GlcNAc levels even in the same exact disease and brain region. For example, one study found increased levels of *O*-GlcNAc and decreased OGA protein levels in the

cerebral cortices of AD patients while another study found decreased levels of *O*-GlcNAc.[233,252] In light of these apparent antithetical studies, the question remains: is *O*-GlcNAc neuroprotective or neurodegenerative? Additionally, what is the role of *O*-GlcNAc in neuronal function and homeostasis? In order to answer these major questions, our lab has characterized a forebrain-specific OGT KO mouse, which will be the subject of Chapter 2.

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