Chapter 1: Chemical Approaches to Understanding *O*-GlcNAc Glycosylation in the Brain

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Over the past five years, new chemical approaches have been developed to meet the specific challenges associated with studying *O*-GlcNAc glycosylation in the brain. Methods for chemically tagging and identifying *O*-GlcNAc proteins have vastly expanded the number of neuronal proteins known to be *O*-GlcNAc modified. New pharmacological inhibitors of the *O*-GlcNAc enzymes have been discovered that may overcome some of the limitations of genetic approaches. Quantitative proteomics strategies designed to accommodate post-mitotic neurons and brain tissue have been used to monitor changes in glycosylation levels in response to neuronal stimuli. Combined with recent advances in mass spectrometry, these powerful tools have provided an unprecedented opportunity to explore the *O*-GlcNAc proteome, manipulate glycosylation levels, and study the dynamics of this modification *in vivo*.

O-GlcNAc glycosylation is the covalent attachment of the monosaccharide β-*N*-acetyl-D-glucosamine to the hydroxyl group of serine and threonine residues (**Fig. 1**).

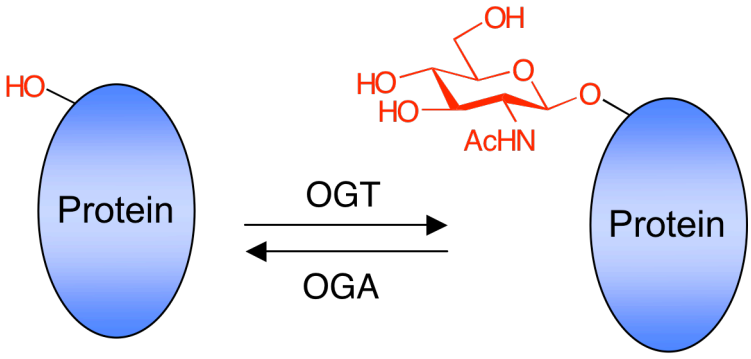

Figure 1: *O*-GlcNAc glycosylation is the addition of β-*N*-acetylglucosamine to serine or threonine residues of proteins.

Recent advances in our understanding of the cellular functions of *O*-GlcNAc have been accelerated by the development of chemical tools for studying the modification. Although *O*-GlcNAc glycosylation was

discovered over twenty years ago[1], its extent and functional significance are only beginning to be fully understood. A major challenge has been the difficulty of detecting and studying the modification *in vivo*. Like other PTMs, *O*-GlcNAc is often dynamic, substoichiometric, targeted to specific subcellular compartments, and prevalent on low-abundance regulatory proteins[2,3]. The sugar is also enzymatically and chemically labile, being subject to reversal by cellular glycosidases and cleavage on a mass spectrometer[2]. As with many protein kinases, the lack of a well-defined consensus sequence for OGT has precluded the determination of *in vivo* modification sites based on primary sequence. Here, we highlight some chemical approaches designed to overcome these challenges and advance a fundamental understanding of *O*-GlcNAc.

Rapid, sensitive detection

Traditional methods for detecting *O*-GlcNAc glycosylation include the use of wheat germ agglutinin (WGA) lectin[4], pan-specific *O*-GlcNAc antibodies[5,6], or radiolabeling using β-1,4-galactosyltransferase (GalT)[7], which transfers [^{3}H]-Gal from UDP-[^{3}H]-galactose to terminal GlcNAc groups. Although these approaches have greatly facilitated studies of the modification, many *O*-GlcNAc proteins still elude their detection. For instance, tritium labeling suffers from low sensitivity, often necessitating exposure times of several days to months. *O*-GlcNAc antibodies and lectins have limited binding affinity and specificity, and thus are best suited for highly glycosylated proteins with multiple modification sites. Recently, chemical approaches have been developed to tag *O*-GlcNAc proteins with reporter groups such as biotin to enable more rapid, sensitive detection. Khidekel and coworkers designed an unnatural substrate for GalT containing a

ketone moiety at the C2 position of UDP-galactose (**Fig. 2a**)[8]. An engineered enzyme with a single Y289L mutation that enlarges the binding pocket[9] was used to transfer the ketogalactose sugar onto *O*-GlcNAc proteins of interest. Once transferred, the ketone functionality was reacted with an aminooxy biotin derivative and detected by chemiluminescence using streptavidin conjugated to horseradish peroxidase. This approach provides a significant improvement in sensitivity, enabling detection of proteins

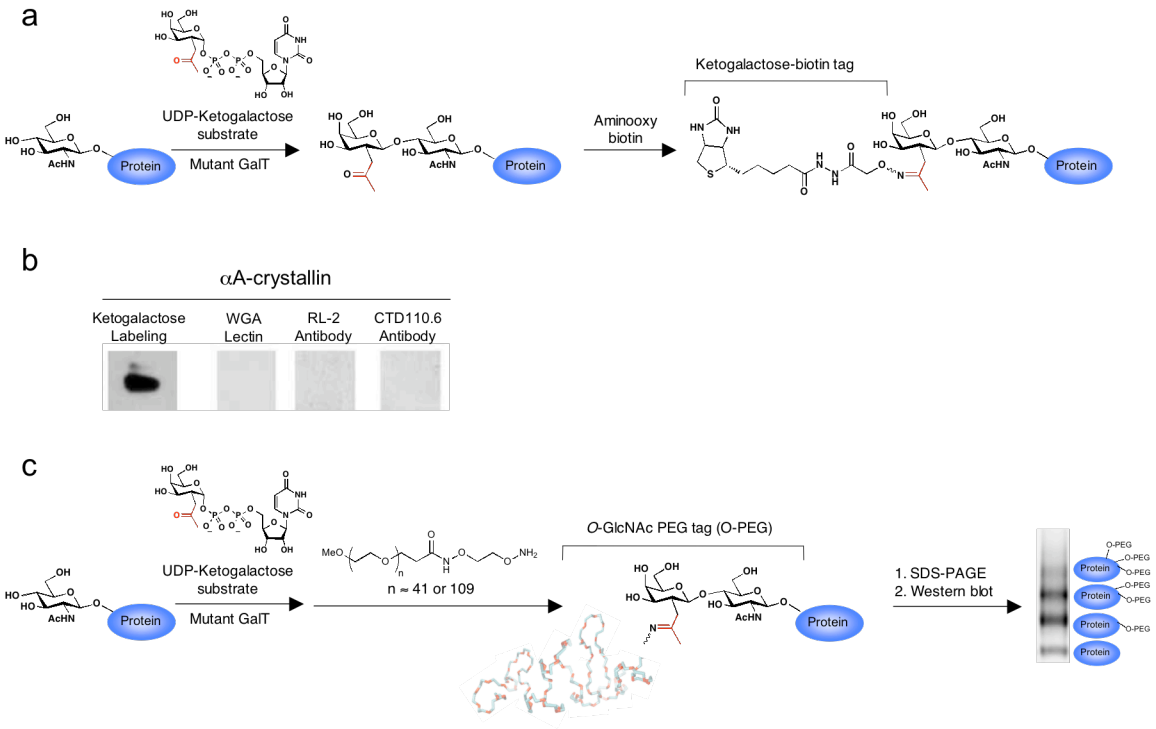

Figure 2: Strategy for chemically tagging *O*-GlcNAc proteins. (a) The UDP-ketogalactose substrate is used with an engineered mutant GalT enzyme to transfer the ketogalactose sugar onto *O*-GlcNAc proteins. Once transferred, the ketone functionality is reacted with an aminooxy biotin derivative. Tagged *O*-GlcNAc glycoproteins are then detected by chemiluminescence or isolated by streptavidin affinity chromatography. (b) Detection of αA-crystallin using the ketogalactose-biotin tagging approach and comparison with other methods. The chemical tagging approach provides significantly improved sensitivity relative to antibodies and lectins. (c) The aminooxy biotin tag can be replaced with an aminooxy PEG tag to afford rapid detection of *O*-GlcNAc stoichiometries and dynamics.

beyond the reach of traditional methods (**Fig. 2b**). The biotin handle also facilitates isolation of the glycoproteins from cell or tissue extracts, thus circumventing the need to develop purification procedures for each protein of interest. As such, virtually any protein can be readily interrogated for the modification, and comparisons can be made across specific functional classes. For instance, Tai and coworkers have applied the approach to demonstrate that numerous transcriptional regulatory proteins are *O*-GlcNAc glycosylated *in vivo*, including transcription factors, transcriptional coactivators and corepressors[10]. A variant of this method has recently been developed in which the biotin handle is replaced by a heavy poly-ethylene glycol tag. This tag increase the overall weight of the *O*-GlcNAc-modified fractions on a protein, allowing for rapid detection of *O*-GlcNAc stoichiometry and dynamics (**Fig. 2c**)[11].

A complementary strategy involves tagging *O*-GlcNAc modified proteins through metabolic labeling of living cells with *N*-azidoacetylglucosamine[12]. Vocadlo and coworkers showed that the azido sugar is processed by enzymes in the hexosamine salvage pathway and incorporated into *O*-GlcNAc modified proteins. The bio-orthogonal azide moiety can then be derivatized with a FLAG peptide or biotin tag using the Staudinger ligation. Treatment of Jurkat cells with peracetylated *N*-azidoacetylglucosamine led to labeling of the highly glycosylated nuclear pore protein p62, as detected by immunoprecipitation of p62 and Western blotting. Although the labeling efficiency and sensitivity of this approach could not be evaluated and may depend on cell type, the ability to incorporate bio-orthogonal groups selectively into *O*-GlcNAc proteins represents a powerful strategy for the detection of *O*-GlcNAc proteins in living cells.

Mapping *O*-GlcNAc glycosylation sites

Mapping the sites of *O*-GlcNAc glycosylation within proteins is essential for elucidating the functional roles of *O*-GlcNAc in specific biological contexts. Unfortunately, direct observation of the *O*-GlcNAc moiety by mass spectrometry during collision-induced dissociation (CID) is difficult as the glycosidic linkage is labile and readily cleaved, providing little peptide fragmentation[13]. Moreover, although OGT seems to favor sequences rich in proline, serine, and threonine residues[13], there is no apparent consensus sequence that directs the action of OGT.

The development of chemical tools coupled to mass spectrometry has greatly facilitated the localization of *O*-GlcNAc to short peptide sequences within proteins and can sometimes be used to determine exact glycosylation sites. One approach, which relies on β-elimination followed by Michael addition with dithiothreitol (BEMAD), results in replacement of the labile GlcNAc moiety with a more stable sulfide adduct (**Fig. 3**)[14]. As this adduct is not cleaved upon CID, sites of glycosylation can be more readily determined. However, selectivity controls must be performed to distinguish *O*-GlcNAc from *O*-phosphate and other *O*-linked carbohydrates. Extension of BEMAD to proteomic studies for the high-throughput mapping of *O*-GlcNAc sites is an important future goal.

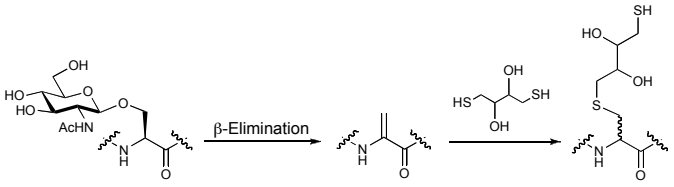

Figure 3: The BEMAD strategy for mapping glycosylation sites. In this approach, the GlcNAc sugar undergoes a β-elimination reaction. Michael addition with dithiothreitol produces a sulfide adduct that is stable to MS/MS analysis.

A second approach capitalizes on the ability to selectively biotinylate *O*-GlcNAc proteins using ketone- or azido-containing UDP-galactose sugars as described above (**Fig. 2**). In addition to isolating intact proteins, the biotin handle can be used to enrich *O*-GlcNAc peptides following proteolytic digestion[13]. This enrichment step is essential for mass spectrometric detection as the *O*-GlcNAc-modified species represents only a small fraction of the total peptides. Unlike BEMAD, the approach enables direct detection of the *O*-GlcNAc moiety by mass spectrometry, with the GlcNAc-ketogalactose-biotin tag providing a unique fragmentation pattern for unambiguous identification of *O*-GlcNAc peptides[13]. Notably, the strategy has been applied to both individual proteins and complex mixtures to localize the modification to short sequences within over 50 different proteins. However, mapping exact glycosylation sites remains challenging in most cases due to the lability of the *O*-glycosidic linkage. As a potential solution to this problem, Khidekel and coworkers demonstrated that this ketogalactose-biotin tagging approach could be combined with BEMAD to identify specific glycosylation sites on the HIV-1 Rev binding protein[13]. Similarly, Wang and coworkers recently used azidogalactose-biotin tagging in conjunction with BEMAD to map glycosylation sites on vimentin[15].

Newer mass spectrometry approaches such as electron transfer dissociation (ETD) and electron capture dissociation (ECD) promise to greatly accelerate our ability to identify *O*-GlcNAc modification sites. ETD and ECD produce sequence-specific fragmentation of peptides without the loss of labile PTMs such as phosphorylation and glycosylation[16]. As such, these approaches are ideal for mapping *O*-GlcNAc glycosylation sites. Vosseller and coworkers demonstrated that ECD could be employed

to identify modification sites on several neuronal proteins following enrichment of the glycopeptides by lectin weak affinity chromatography (LWAC)[17]. Khidekel and coworkers applied the ketogalactose-biotin tagging approach in conjunction with ETD to map glycosylation sites on proteins from rat brain lysates (discussed in detail next chapter). More recently, Wang and coworkers have combined ETD with a photocleavable biotin tag to map glycosylation sites from a tau-enriched sample from rat brain[18] and HeLa cells[19], and Chalkley and coworkers have combined ETD with LWAC to map glycosylation sites from the post-synaptic density of mouse brain[20]. With further methodological refinements and advances in database search algorithms for fragment ions, we anticipate that ETD and ECD will become an increasingly powerful tool for the study of *O*-GlcNAc glycosylation.

Proteome-wide analyses

As described earlier, the analysis of *O*-GlcNAc on a proteome level has begun to reveal exciting functional roles for *O*-GlcNAc in the brain. Chemical strategies to tag, enrich, and detect *O*-GlcNAc peptides and proteins have been instrumental in this regard, enabling the first proteome-wide studies of *O*-GlcNAc. Specifically, the ketogalactose-biotin tagging approach has been applied in combination with high-throughput LC-MS/MS analysis to identify over 25 *O*-GlcNAc glycosylated proteins from the mammalian brain[13]. Direct detection of the modified species was observed in each case, which permitted mapping of the glycosylation sites to short peptide sequences. Notably, many of the proteins identified participate in the regulation of gene expression (e.g., CCR4-NOT, Sox2, HCF, TLE-4), neuronal signaling (WNK-1, bassoon, PDZ-GEF), and

synaptic plasticity (synaptopodin), suggesting that *O*-GlcNAc may contribute to neuronal communication processes. A similar approach employing a photo-cleavable biotin tag was used to identify 8 and 141 *O*-GlcNAc glycosylation sites from a tau-enriched sample from rat brain[18]and HeLa cells[19], respectively. In another approach, Nandi and co-workers metabolically labeled HeLa cells with *N*-(2-azidoacetyl)glucosamine[21]. Tryptic digestion of the captured proteins led to the identification of 199 putative *O*-GlcNAc-modified proteins. As the presence of the GlcNAc moiety was inferred rather than detected directly, independent confirmation of the modification by immunoprecipitation was required and demonstrated for 23 of the proteins.

Recently, biochemical tools such as antibodies and lectins have been exploited for proteomic analyses of *O*-GlcNAc. Vosseller and co-workers used a uniquely packed, 39-foot lectin affinity column to enrich *O*-GlcNAc peptides and identified 18 proteins from the postsynaptic density fraction of murine brain[17]. Antibody affinity chromatography using traditional *O*-GlcNAc antibodies successfully identified 45 putative *O*-GlcNAc proteins from COS7 cells[15]. Additionally, Wells and co-workers developed new *O*-GlcNAc antibodies and identified over 200 *O*-GlcNAc proteins from HEK293T cells and rat liver[22]. The challenge with both approaches is that the weak binding affinity of antibodies and lectins necessitates gentle washing conditions and can lead to false positives, such as interacting proteins or non-specific binding proteins. In many cases, further confirmation of the presence of the modification can be provided by evaluating individually immunoprecipitated proteins or by directly detecting *O*-GlcNAc peptides by mass spectrometry analysis.

Together, proteome-wide studies have revealed that *O*-GlcNAc is a ubiquitous and abundant modification, with considerable functional significance and breadth. In less than 5 years, the number of known *O*-GlcNAc proteins in the brain has expanded from tens to hundreds, with many more proteins yet to be discovered.

Small molecule inhibitors of OGT and OGA

Historically, genetic manipulation of OGT and OGA activity *in vivo* has proven difficult. Whole animal and conditional deletions of the OGT gene have revealed that OGT is essential for cell survival and mouse embryogenesis[23,24]. Modulation of OGT using siRNA is complicated by the long half-life of the protein and often produces only partial knockdown of OGT[25]. Although deletion of OGA in *C. elegans* leads to metabolic changes and increased dauer formation[26], no mammalian knockout of OGA has yet been reported. These features have challenged efforts to study *O*-GlcNAc glycosylation and have limited the use of conventional genetic tools to elucidate its role in cellular processes.

A complementary approach involves the generation of pharmacological agents to inhibit OGT and OGA. A well-established inhibitor of OGT, alloxan, exhibits multiple non-specific effects, such as inhibition of OGA[27], glucokinase inhibition[28], and formation of superoxide radicals[29]. By screening a 64,416-member library of compounds using a novel fluorescence-based assay, Gross and coworkers identified several promising new compounds that inhibit OGT activity *in vitro* (**Fig. 4a**, Compounds **1–3**)[30]. These compounds were selective in inhibiting OGT but not another related enzyme MurG, which also uses UDP-GlcNAc as a substrate. Recently, a benzoxazolinone compound

(**Fig. 4a**, Compound **1**) was shown to inhibit OGT activity in oocytes where it prevents meiotic progression[31]. In the future, these numerous, chemically-distinct OGT inhibitors might be used in parallel to distinguish the potential non-specific effects of individual inhibitors *in vivo*.

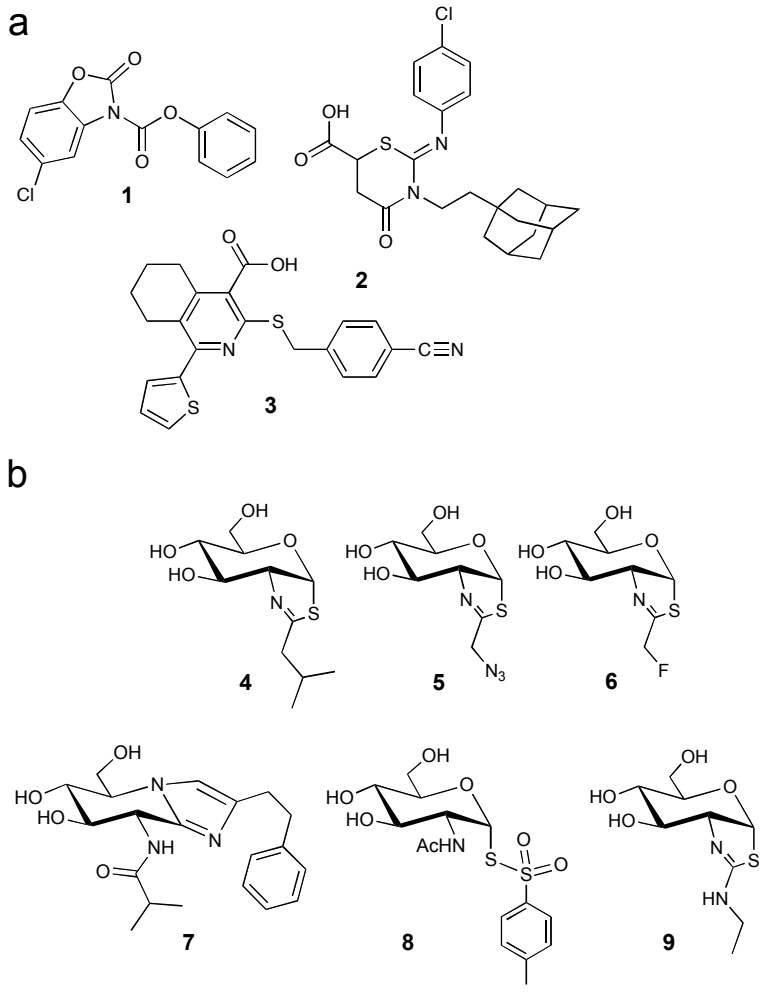

Figure 4: Small molecule inhibitors of OGT and OGA. (a) OGT inhibitors (1–3) identified by screening a 64,416-member library of compounds. The benzoxazolinone compound 1 inhibits OGT activity in oocytes and prevents meiotic progression. (b) Representative OGA inhibitors (4–9) with enhanced selectivity for OGA over β-hexosaminidase

As with OGT, many of the early OGA inhibitors such as PUGNAc exhibit non-specific activity toward β-hexosaminidase[32] and only partially inhibit the short isoform of OGA[33]. Recently, several labs have designed more selective OGA inhibitors that are either structural variants of early hexosaminidase inhibitors and/or have been rationally designed using information about the OGA active site. For instance, Macauley et al.

and Knapp et al. functionalized the non-specific hexosaminidase inhibitor GlcNAc-thiazoline with longer alkyl chains, fluoro, or azido groups to generate new OGA inhibitors that show greater than 3000-fold selective inhibition of OGA over β-hexosaminidase (**Fig. 4b**, Compounds **4–6**)[34,35]. In other studies, extending the *N*-acyl group of PUGNAc led to the creation of new inhibitors with greater than 10-fold selectivity for OGA over β-hexosaminidase[32,36]. A drawback of these compounds is that they inhibit OGA more weakly than PUGNAc. In response to this problem, Dorfmeuller and coworkers designed a nagstatin derivative based on the crystal structure of NagJ, a bacterial homologue of human OGA (**Fig. 4b**, Compound **7**)[37]. The isobutanamido group at the N8 position provides improved selectivity by fitting into a pocket that is larger than the corresponding pockets in other hexosaminidases, while the phenethyl group at the C2 position offered stronger inhibition (K_i = 4.6 ± 0.1 pM) for the bacterial homologue of OGA by interacting with a solvent-exposed tryptophan residue. Nonetheless, a related nagstatin derivative still showed weaker inhibition toward human OGA than PUGNAc[38]. More recently, Kim and coworkers designed a compound that strongly inhibits the short isoform of OGA (**Fig. 4**, Compound **8**)[33], an isoform that is only partially inhibited by 1 mM PUGNAc. However, selectivity may be an issue with this compound as thiosulphonate moieties have been shown to react with exposed cysteine residues of proteins[39]. Notably, compounds **4** and **7** have been tested in cell culture and were shown to increase overall cellular *O*-GlcNAc levels although none of these compounds were shown to have efficacy *in vivo*. Using a mechanism-inspired approach, Yuzwa and coworkers developed thiamet-G a nanomolar inhibitor (K_i = 21 ± 3 nM) of OGA with low or no activity towards human lysosomal β-hexosaminidase and other glycoside

hydrolases and, important, showed that it increases *O*-GlcNAc levels in the brain *in vivo* (**Fig. 4b**, Compound **9**)[40].

The discovery of potent, selective inhibitors of OGT and OGA provides powerful tools for perturbing *O*-GlcNAc glycosylation in cells and *in vivo*. Application of these inhibitors in specific contexts should reveal new insights into the functional roles of *O*-GlcNAc and cellular mechanisms for the regulation of OGT and OGA.

Monitoring *O*-GlcNAc dynamics

The dynamic nature of *O*-GlcNAc is a unique characteristic that distinguishes it from other forms of glycosylation. As described earlier, this feature has important implications for the regulation of protein structure and function and the interplay with other PTMs. An exciting challenge in the future will be to understand the cellular dynamics of the modification, as well as the signaling pathways and mechanisms by which *O*-GlcNAc is regulated on specific proteins. Toward this end, several groups have developed chemical approaches to monitor changes in *O*-GlcNAc glycosylation levels in response to cellular stimuli.

Carrillo and coworkers designed a FRET-based sensor for the detection of *O*-GlcNAc dynamics in living cells[41]. Their sensor consisted of two fluorophores (enhanced cyan and yellow fluorescent protein) separated by a known peptide substrate for OGT and an *O*-GlcNAc lectin (GafD). Upon *O*-GlcNAc glycosylation, the lectin was expected to bind the glycosylated peptide substrate and bring the two fluorophores in close proximity, producing a stronger FRET signal (**Fig. 5**). As anticipated, an increase in FRET (7–30%) was observed upon stimulation of transfected HeLa cells with

PUGNAc and glucosamine. In the future, it will be interesting to examine changes in OGT activity in response to a variety of cellular stimuli.

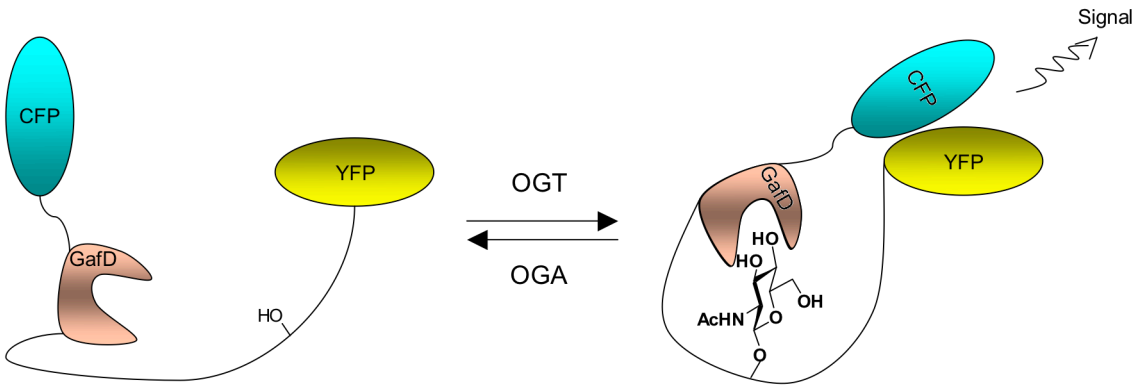

Figure 5: Chemical tools for monitoring O-GlcNAc dynamics. A FRET-based sensor designed to detect the dynamics of *O*-GlcNAc glycosylation in living cells. Upon glycosylation, binding of the GafD lectin to the O-GlcNAc moiety induces a conformational change and produces a stronger FRET signal. CFP, enhanced cyan fluorescent protein; YFP, yellow fluorescent protein

In addition to monitoring OGT activity, identifying the intracellular signaling pathways and dynamics of *O*-GlcNAc glycosylation on specific protein substrates represents an important, challenging goal. General *O*-GlcNAc antibodies (CTD110.6 and RL-2) have been extremely valuable for measuring global changes in glycosylation in response to cellular stimuli[5,6]. However, a limitation of these antibodies is that they detect only a small subset of the *O*-GlcNAc-modified proteins[8,42]. Moreover, it remains difficult to identify the specific proteins that undergo changes in glycosylation. In response to these challenges, Khidekel and coworkers have developed a method to probe the dynamics of *O*-GlcNAc glycosylation *in vivo* using quantitative proteomics (discussed in more detail next chapter). More recently, Wang and coworkers used immunoaffinity chromatography or chemoenzymatic labeling in conjunction with SILAC (stable isotope labeling with amino acids in cell culture), a well-established method for

quantitative proteomics[43], to study the dynamic interplay between *O*-GlcNAc and phosphorylation by glycogen synthase kinase-3 (GSK-3)[15] or cytokinesis[19]. In the GSK-3 study, heavy and light isotope-labeled cells were treated with LiCl to inhibit GSK-3, and *O*-GlcNAc proteins of interest were isolated by affinity chromatography using a general *O*-GlcNAc antibody. Forty-five putative *O*-GlcNAc glycosylated proteins were identified by mass spectrometry, ten of which showed enrichment after LiCl treatment, suggesting that these proteins underwent increases in *O*-GlcNAc glycosylation. In four of the cases, the glycosylation increases that were observed indirectly by mass spectrometry were confirmed by immunoprecipitation. Interestingly, other proteins exhibited no change or decreases in glycosylation, suggesting that a complex interplay likely exists between phosphorylation and *O*-GlcNAc glycosylation within signaling networks.

With these new tools, in-depth studies of the dynamics of *O*-GlcNAc within cells are now within reach. Understanding the molecular mechanisms by which this dynamic signaling comes about and regulates specific proteins is a future challenge that promises to propel the field in exciting new directions.

Conclusion and Future Challenges

Many of the new discoveries in the field of *O*-GlcNAc have been accelerated by the development of new chemical tools, such as those for detecting the modification in cells and for inhibiting OGT and OGA. Combined with advances in mass spectrometry, these new technologies have provided an unprecedented opportunity to define the *O*-

GlcNAc proteome, manipulate *O*-GlcNAc enzymes, and explore the dynamics of this modification *in vivo*.

The development of new chemical tools to produce homogeneously glycosylated proteins will represent an important step toward this goal. Unlike phosphorylation, *O*-GlcNAc glycosylation cannot be readily mimicked by any naturally occurring amino acid. The current state-of-the-art involves alanine mutagenesis of specific glycosylation sites within proteins to effect changes in function. Chemical methods such as native chemical ligation[44] may allow for the construction of *O*-GlcNAc glycosylated proteins *in vitro* and in cells. In the future, these methods may provide new insights into whether and how *O*-GlcNAc glycosylation affects protein structure, modulates protein-protein interactions, and influences other post-translational modifications.

One of the central challenges of neuroscience is to understand the unique molecular and cellular heterogeneity of the brain as it relates to systems level phenomena, such as learning and memory. Sensitive methods to detect the modification on small subpopulations of cells or proteins will be required to dissect the role of *O*-GlcNAc in fear, addiction, and other complex learning and memory models. Despite significant progress, faster, higher-throughput methods are still needed to identify *O*-GlcNAc proteins and study *O*-GlcNAc dynamics *in vivo*. For instance, the ability to directly monitor the glycosylation status of specific proteins using chemical tagging approaches or site-specific *O*-GlcNAc antibodies will be essential. To facilitate the production of *O*-GlcNAc antibodies, facile synthetic routes to access *O*-GlcNAc-modified peptides are needed. Moreover, the continued development of methods to precisely map

glycosylation sites, particularly on small quantities of material and on selected proteins of interest, will be critical for any functional studies.

New sensitive and selective OGT and OGA inhibitors will be important tools for finely dissecting the role of each enzyme in neuronal function and dysfunction. Given the diversity of OGT and OGA substrates and the lethality of deleting the OGT gene in mice, creative new genetic or chemical approaches are still needed to more selectively target functional subsets of OGT and OGA by interfering, for instance, with the enzymes in certain subcellular compartments.

From the time of its discovery, the appeal of *O*-GlcNAc has been both the intrigue of understanding its unique biology and the great technical challenges associated with its study. Over the last five years, we have seen a surge of new chemistry designed to meet these obstacles. Strengthened by an arsenal of chemical tools, the future of *O*-GlcNAc is primed for new and exciting discoveries.

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