Wen Yi *Editor*

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ISBN 978-981-97-4863-1 ISBN 978-981-97-4864-8 (eBook)
https://doi.org/10.1007/978-981-97-4864-8

This Springer imprint is published by the registered company Springer Nature Singapore Pte Ltd.
The registered company address is: 152 Beach Road, #21-01/04 Gateway East, Singapore 189721, Singapore

Preface

Glycosylation is a common and most structurally diverse form of protein posttranslational modification. Among many different types of glycosylation, a single monosaccharide β-*N*-acetylglucosamine (O-GlcNAc) occurring on the serine and/or threonine residues has recently emerged as a unique player in modern glycoscience. Since the discovery by Hart and coworkers in the early 1980s, the number of proteins possessing the O-GlcNAc modification (or O-GlcNAcylation) has now been over 5000. In contrast to the long-held dogma that protein glycosylation is biosynthesized in the endoplasmic reticulum/Golgi apparatus and residing on the cell surface, mounting evidence in the past several decades has clearly demonstrated that O-GlcNAcylation predominantly occurs on intracellular proteins. Another unique property of O-GlcNAcylation is the highly dynamic recycling in response to a variety of stimuli, including but not limited to, nutrient levels, oxygen levels, and intracellular reactive oxygen species levels. Research evidence has now established that O-GlcNAcylation functions as a crucial regulator of nearly all physiological processes in cells. Conversely, perturbation of O-GlcNAcylation homeostasis has been increasingly linked to the onset and progression of various diseases, such as type II diabetes, cancers, and neurodegenerative diseases.

Aided by the recent development of chemical tools, the detailed molecular mechanisms by which O-GlcNAcylation regulates the physio/pathological processes are beginning to be uncovered. The primary aim of this book is to summarize the current understanding of the chemistry and biology of O-GlcNAcylation from a systems perspective with a focus toward the functional role of O-GlcNAcylation in mammalian cells. The combination of chemical and biological studies is in a unique position to reveal the O-GlcNAc code in biology and to develop novel therapeutic strategies to combat diseases. We certainly anticipate more fascinating studies in the years to come.

Hangzhou, China — Wen Yi

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Chapter 1
Chemical Tools for Decoding the Functions of O-GlcNAcylation

Yi Hao, Dangliang Liu, Qi Deng, and Suwei Dong

Abstract O-GlcNAc has been implicated in the modulation of diverse pathophysiological processes, as thoroughly discussed in other chapters of this book. However, due to the dynamic property of O-GlcNAcylation and its coexistence with different posttranslational modifications, it remains a daunting challenge to obtain natural O-GlcNAcylated proteins as homogeneous forms and decipher O-GlcNAc functions in a site-specific and structurally accurate manner. To address this issue, researchers have developed various tools for better understanding the roles of O-GlcNAc, which can be categorized into two main approaches. The first approach takes advantage of the natural biosynthetic pathways and endogenous proteins and utilizes properly designed chemical probes, inhibitors, or affinity-based constructs to label, manipulate, and profile O-GlcNAcylation. The second approach relies on multiple chemical methods to construct the protein of interest in vitro and subsequently study the synthesized sample bearing well-defined O-GlcNAcylation. This chapter will briefly overview these two aspects of developed tools and their applications.

Keywords Glycan labeling · Chemical probes · Native chemical ligation

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W. Yi (ed.), *Chemistry and Biology of O-GlcNAcylation*,
https://doi.org/10.1007/978-981-97-4864-8_1

1 Metabolic Glycan Labeling (MGL)

The MGL strategy [1–4] has emerged as a major tool for monitoring O-GlcNAcylation in living cells over the past two decades. It uses an analogue of *N*-acetylglucosamine (GlcNAc) or *N*-acetylgalactosamine (GalNAc) bearing a bio-orthogonal chemical handle that can be incorporated into cellular O-GlcNAc through the glycan biosynthetic pathway. Then the chemically functionalized glycans are conjugated with fluorophores for imaging or affinity tags for enrichment via bio-orthogonal reactions (Fig. 1.1a). The azide is a principle bio-orthogonal group because it is small in size, inert to cellular components, and totally absent from native biological environments [5–7]. Azido sugars can chemoselectively react with terminal alkynes by copper(I)-catalyzed azide–alkyne cycloaddition (CuAAC, also called click chemistry) [8, 9] (Fig. 1.1b), strained cyclooctynes by strain-promoted azide−alkyne cycloaddition (SPAAC, also called copper-free click chemistry) [10], or triarylphosphines by Staudinger ligation [11]. Due to the popular use of click chemistry, the alkyne functionality can be alternatively installed onto simple sugars for subsequent reactions with azides. These unnatural sugars are generally administered in their per-*O*-acetylated forms to improve cellular uptake (Fig. 1.1c). Upon entering cells, acetyl groups are quickly hydrolyzed by cytosolic esterases, and the deacetylated sugar analogues are further utilized.

1.1 Development of Metabolic Chemical Reporters (MCRs) for O-GlcNAc

Per-*O*-acetylated *N*-azidoacetylglucosamine (Ac_4GlcNAz) developed by the Bertozzi group [12] is the first reported MCR for O-GlcNAc. GlcNAz can be accepted by the GlcNAc salvage pathway to generate uridine diphosphate GlcNAz (UDP-GlcNAz). Then UDP-GlcNAz is recognized by O-GlcNAc transferase (OGT) for transfer of the GlcNAz moiety to serine (Ser) and/or threonine (Thr) residues on intracellular proteins. However, Ac_4GlcNAz shows weak labeling efficiency because of an inefficient pyrophosphorylase step in the GlcNAc salvage pathway [13]. Moreover, Ac_4GlcNAz can also label mucin-type O-linked glycans and core pentasaccharides of N-linked glycans [14, 15]. Nevertheless, it was employed for O-GlcNAc glycoproteomic studies [12, 16]. Per-*O*-acetylated *N*-azidoacetylgalactosamine (Ac_4GalNAz) [17] initially designed for mucin-type cell surface glycans was later found to be metabolized to UDP-GalNAz and further interconverted into UDP-GlcNAz by the enzyme UDP-galactose 4′-epimerase (GALE) [13], accounting for O-GlcNAc labeling inside cells. Since GalNAz is well tolerated by the GalNAc salvage pathway, it displays more robust labeling upon O-GlcNAcylation than GlcNAz. Metabolic incorporation of Ac_4GalNAz coupled with click chemistry has been widely used in biological studies. For instance, this strategy enables the discovery of O-GlcNAcylated proteins in mitochondria [18].

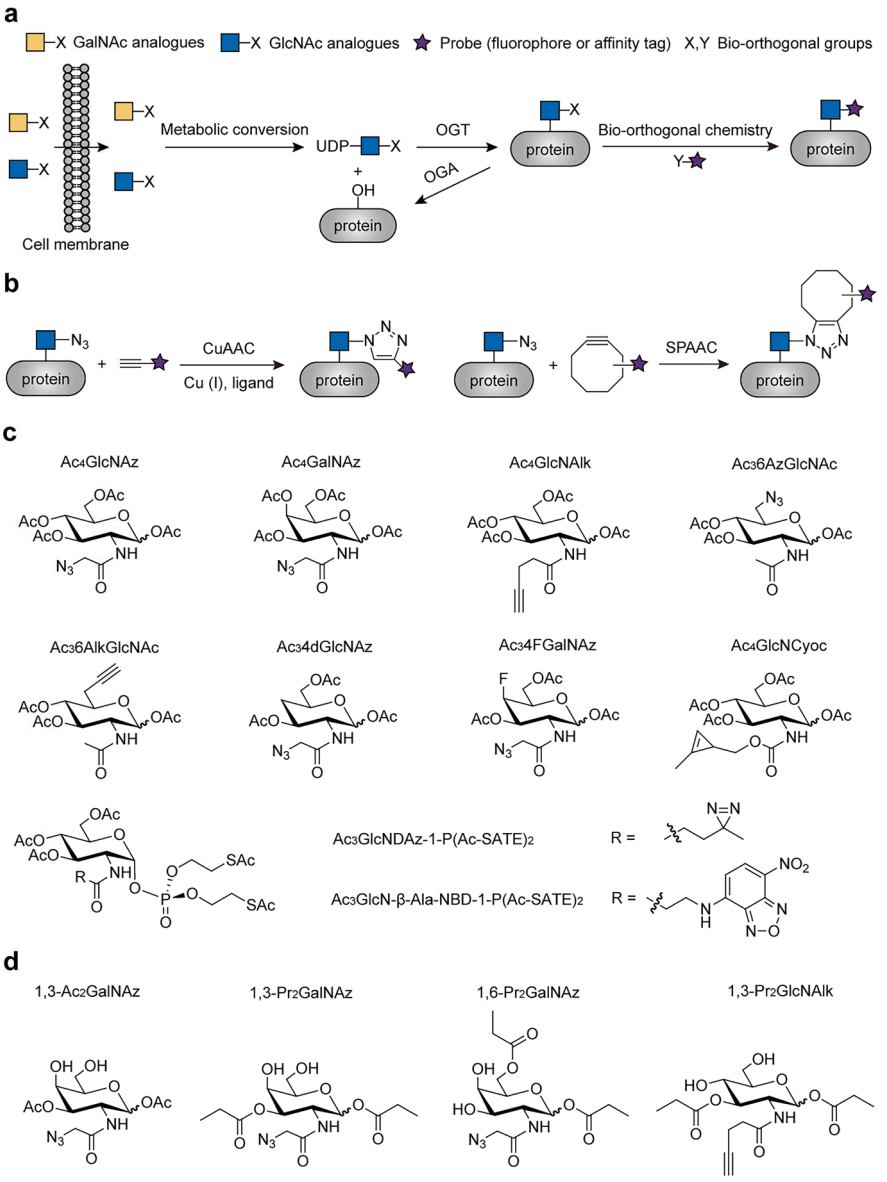

Fig. 1.1 MGL for O-GlcNAc in living cells. (**a**) The MGL procedure for labeling O-GlcNAcylated proteins with fluorophores or affinity tags. (**b**) Schematic of click chemistry. CuAAC and SPAAC are two commonly used bio-orthogonal reactions. (**c**) Per-*O*-acetylated unnatural sugars for MGL. (**d**) Partially protected unnatural sugars designed to avoid artificial S-glyco-modification

Per-*O*-acetylated *N*-pentynylglucosamine (Ac_4GlcNAlk) developed by the Pratt group is a more specific metabolic precursor for O-GlcNAc [19]. GlcNAlk generates minimal levels of alkyne-labeled mucin-type O-glycans because it cannot be efficiently interconverted with GalNAlk. Of note, GlcNAlk is still able to decorate N-linked glycoproteins. To further enhance labeling selectivity, the same group reported per-*O*-acetylated 6-azido-6-deoxy-GlcNAc ($Ac_3$6AzGlcNAc), which exclusively labels O-GlcNAcylation without entering other glycosylation synthetic pathways [20]. The authors showed that 6AzGlcNAc cannot be phosphorylated by *N*-acetylglucosamine kinase (GNK) at the C6 position due to the replacement of the C6-hydroxyl group with an azide. Alternatively, it is directly phosphorylated at its C1-hydroxyl by phosphoacetylglucosamine mutase (AGM1) and incorporated into the salvage pathway to generate UDP-6AzGlcNAc that can be accepted by OGT. Another unnatural monosaccharide specific for O-GlcNAc, per-*O*-acetylated 6-alkynyl-6-deoxy-GlcNAc ($Ac_3$6AlkGlcNAc) [21], was subsequently developed, which labels O-GlcNAcylated proteins with a superior signal-to-noise ratio than $Ac_3$6AzGlcNAc. Applying $Ac_3$6AlkGlcNAc, the authors identified a pivotal regulator of the extrinsic apoptotic pathway, caspase-8, as a novel O-GlcNAc candidate.

Per-*O*-acetylated 2-azidoacetamido-2,4-dideoxy-glucopyranose ($Ac_3$4dGlcNAz) was reported to label O-GlcNAcylated proteins in an OGA-resistant manner [22], which provides a potential tool for dissecting the role of permanent O-GlcNAcylation. It is interesting that 2-azido-2-deoxy-glucose ($Ac_4$2AzGlc) [23, 24] and 6-azido-6-deoxy-glucose ($Ac_4$6AzGlc) [25] exhibit OGT-dependent labeling of intracellular proteins. The authors discovered that both UDP-2AzGlc and UDP-6AzGlc can be accepted by OGT, indicating the large substrate flexibility of this enzyme. Most recently, per-*O*-acetylated 4-deoxy-4-fluoro-GalNAz ($Ac_3$4FGalNAz) [26] was discovered to modify a variety of O-GlcNAcylated proteins, which once again reveals the promiscuity of OGT toward UDP sugar donors.

In addition to azide- and alkyne-conjugated sugars, a per-*O*-acetylated GlcNAc derivate equipped with the methylcyclopropene functionality (Ac_4GlcNCyoc) is exploited for labeling O-GlcNAc [27–29]. Methylcyclopropene-tagged O-GlcNAc (O-GlcNCyoc) can react with tetrazine probes bearing dyes or affinity tags via inverse-electron-demand Diels–Alder (DAinv) chemistry [30, 31] for subsequent detection. Recently, the Vocadlo group reported one-step imaging of O-GlcNAcylated proteins in living cells with a fluorescent GlcNAc analogue bearing a small fluorophore 4-nitro-2,1,3-benzoxadiazole (NBD) and a suitable β-alanine (β-Ala) linker at the 2-acetamido position (GlcN-β-Ala-NBD) [32]. To improve cellular uptake, the authors synthesized per-*O*-acetylated 1-α-phospho-GlcN-β-Ala-NBD (Ac_3GlcN-β-Ala-NBD-α-1-P(Ac-SATE)$_2$), in which the phosphate group is masked with two *S*-acetyl-2-thioethyl (SATE) residues. When Ac_3GlcN-β-Ala-NBD-α-1-P(Ac-SATE)$_2$ enters cells, the protecting groups are removed. Under the catalysis of UDP-*N*-acetylglucosamine pyrophosphorylase 1 (AGX1), GlcN-β-Ala-NBD-α-1-P is converted into UDP-GlcN-β-Ala-NBD, which can be recognized by OGT to decorate glycoproteins.

Per-*O*-acetylated unnatural sugars have emerged as powerful tools in the field of MGL for two decades. Chen and coworkers recently discovered that

per-*O*-acetylated monosaccharides nonspecifically react with cysteine (Cys) residues of numerous proteins through an artificial S-glyco-modification [33], which may lead to false positives in O-GlcNAc proteomic profiling. The unacetylated forms of unnatural sugars (e.g., GalNAz) can avoid this issue, but they always need to be administered in high concentrations (1 mM or more) due to the lower cell permeability [33, 34]. Two partially protected unnatural monosaccharides [35], 1,3-di-*O*-acetylated GalNAz (1,3-Ac_2GalNAz) and 1,3-di-*O*-propionyl-*N*-azidoacetylgalactosamine (1,3-Pr_2GalNAz) (Fig. 1.1d), were later reported through a trial and error strategy, which exhibit robust metabolic labeling and have no S-glyco-modification within cells. Notably, the labeling efficiency of 1,3-Pr_2GalNAz is approximately one order of magnitude greater than that of GalNAz. In a follow-up study, Chen and colleagues unveiled the detailed mechanism of the S-glyco-modification [36]. Per-*O*-acetylated monosaccharides in basic microenvironments undergo β-elimination at the C3 position to generate thiol-reactive α,β-unsaturated aldehydes that further react with Cys residues through Michael addition. It should be noted that the S-glyco-modification results in 3-thiolated sugars rather than a typical glycoside in the acetal form. Based on this elimination–addition mechanism, the authors developed 1,6-di-*O*-propionyl-*N*-azidoacetylgalactosamine (1,6-Pr_2GalNAz) as an improved unnatural monosaccharide. In addition, they recently developed a bump-hole approach by integrating 1,3-di-*O*-propionylated *N*-pentynylacetylglucosamine (1,3-Pr_2GlcNAlk) with a UDP-GlcNAc pyrophosphorylase mutant $AGX2^{F383G}$, which achieves in vivo cell-type-specific glycan labeling [37].

1.2 MGL for Monitoring the Turnover Dynamics of O-GlcNAc

Akin to phosphorylation, O-GlcNAcylation rapidly cycles on and off substrate proteins in response to various biological cues [38, 39]. The MGL approach has been applied for determining this dynamic process. For instance, in combination with the stable isotope labeling with amino acids in cell culture (SILAC), Chen and colleagues reported a quantitative time-resolved O-GlcNAc proteomics (qTOP) strategy to measure the turnover rates of O-GlcNAc proteins [40]. In qTOP, the "light" and "heavy" isotope-labeled cells were pulse-treated with $Ac_3$6AzGlcNAc for 36 h to ensure its sufficient incorporation (Fig. 1.2). The heavy (or light) cells were then harvested for use as the time reference, and the light (or heavy) cells were chased with Ac_4GlcNAc for another 12 h in the forward (or reverse) qTOP. After mixing and click labeling of the cell lysates, the 6AzGlcNAc-tagged proteins were captured for subsequent mass spectrometry (MS) detection. The turnover of O-GlcNAc events was determined according to the quantification ratios between reference and chase samples. It is interesting that a subpopulation of quantified proteins, such as core proteins of box C/D small nucleolar ribonucleoprotein complexes, is hyperstable, which exhibited minimal removal of O-GlcNAc within 12 h. Moreover, the authors found that these hyperstable events degrade significantly upon OGT

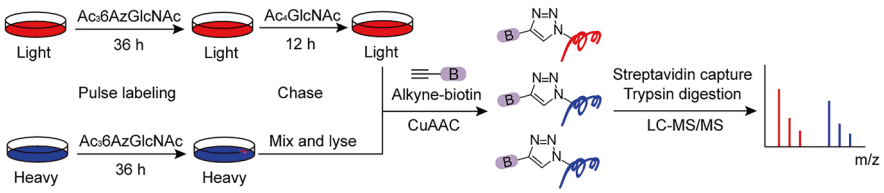

Fig. 1.2 Schematic showing the workflow of qTOP. The O-GlcNAc residues of both light and heavy cells are metabolically labeled with azides. In the forward qTOP, the pulse-labeled heavy cells are immediately collected, and the light cells are chased with $Ac_4GlcNAc$. Then the cells are mixed, lysed, and reacted with alkyne-conjugated biotin tags. After streptavidin bead enrichment and trypsin digestion, the resulting peptides are detected by liquid chromatography tandem MS (LC–MS/MS). In the reverse qTOP, the light cells are collected and the heavy cells are chased

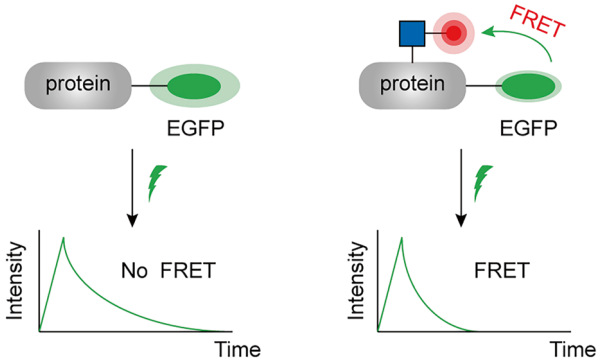

Fig. 1.3 Schematic showing protein-specific imaging of the O-GlcNAc state by a FRET pair

inhibition. These results suggested that the O-GlcNAc with slow turnover kinetics might be important for stabilizing protein substrates.

1.3 FRET-Based Strategy for Imaging O-GlcNAc

Another application of MGL is to visualize O-GlcNAc states on a specific O-GlcNAcylated protein in combination with the intramolecular fluorescence resonance energy transfer (FRET) technique [29, 41]. The protein of interest is genetically tagged with the enhanced green fluorescence protein (EGFP), and the O-GlcNAc moiety labeled by MCRs (e.g., $Ac_4GalNAz$) is tagged with a second fluorophore (e.g., TAMRA) via bio-orthogonal reactions, forming a FRET donor–acceptor pair between EGFP and tagged O-GlcNAc (Fig. 1.3). Upon exciting the donor fluorophore EGFP, the acceptor fluorophore on the same protein is then excited through intramolecular FRET, which results in a shortening of the EGFP fluorescence lifetime for fluorescence lifetime imaging microscopy measurement.

This strategy has been employed to image the O-GlcNAcylation status of several intracellular proteins, including β-catenin, tau, and OGT [29, 41].

1.4 Tandem Metabolic Labeling for the Identification of Cotranslationally Nascent O-GlcNAcylated Proteins

Inspired by recent advances in bio-orthogonal chemistry, the integration of tandem metabolic labeling and specific chemoselective ligation approaches enables probing multiple biomolecules simultaneously [42]. The Vocadlo group exploited this strategy to uncover endogenous cotranslationally nascent O-GlcNAcylated proteins [43], in which O-GlcNAc was labeled with azide by GalNAz, and the nascent polypeptide C-terminus was metabolically tagged with O-propargyl-puromycin (OPP), an analogue of puromycin bearing an alkyne functionality, to terminate its translation. Then the cells were lysed and click-reacted with azide–biotin tags containing an acid labile linker. After streptavidin capture and acid cleavage, the OPP-tagged nascent proteins were released for the second round of click chemistry using alkyne–biotin tags to label O-GlcNAz modified components. Through MS detection, the authors identified a few hundred cotranslationally O-GlcNAcylated nascent proteins (e.g., NUP153), providing insights into the physiological significance of cotranslational O-GlcNAcylation.

1.5 MGL-Based Photocrosslinking Approach for Dissection of O-GlcNAc-Mediated Protein–Protein Interactions

Despite its pathophysiological significance, O-GlcNAc has been found to regulate essential protein–protein interactions on many substrates [44]. To capture interactive networks of O-GlcNAc proteins, Kohler and coworkers described an MGL-based approach [45] with a GlcNAc analogue bearing a diazirine moiety at the 2-acetamido position (abbreviated GlcNDAz). Given that per-*O*-acetylated GlcNDAz cannot be tolerated by the GlcNAc salvage pathway, 3,4,6-tri-*O*-acetylated 1-α-phospho-GlcNDAz (Ac_3GlcNDAz-1-P(Ac-SATE)$_2$) was synthesized to overcome this metabolic restriction. When Ac_3GlcNDAz-1-P(Ac-SATE)$_2$ diffuses across the cell membrane, it is hydrolyzed by intracellular esterases and phosphatases to readily accumulate GlcNDAz-1-P (Fig. 1.4). GlcNDAz-1-P is a poor substrate for AGX1. Through structure-directed mutagenesis, an AGX1 mutant (AGX1^{F383G}) that leverages the binding pocket of wild-type (WT) AGX1 is designed to accommodate the diazirine substituent, which realizes efficient conversion from GlcNDAz-1-P into UDP-GlcNDAz. Subsequently, UDP-GlcNDAz can be utilized by OGT to decorate intracellular proteins. Upon ultraviolet (UV) irradiation, the diazirine moiety of GlcNDAz-labeled proteins generates a highly reactive carbene,

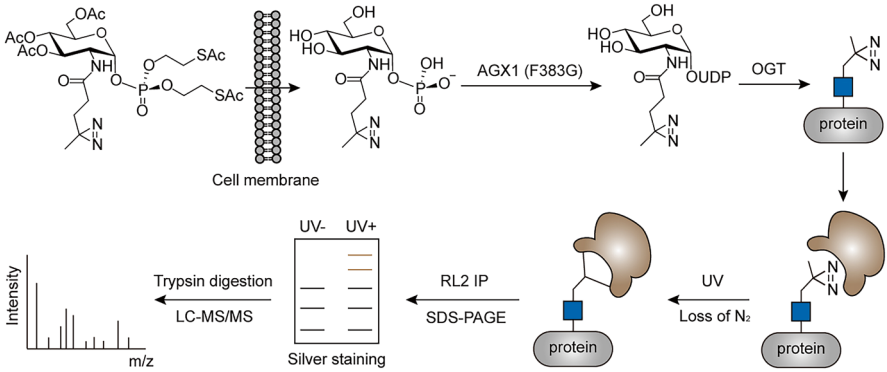

Fig. 1.4 Schematic showing the identification of binding partners of O-GlcNAcylated proteins. The cellular O-GlcNAc is metabolically tagged with a diazirine moiety, which enables covalent crosslinking of neighboring binding partners to the GlcNDAz residue upon short UV treatment. UV irradiation is omitted in the control group. Then the GlcNDAz-labeled proteins are isolated from cell lysates using RL2 immunoprecipitation, followed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and silver staining. After in-gel trypsin digestion, the resulting peptides are subjected to LC–MS/MS analysis. RL2 is an O-GlcNAc-specific antibody (discussed in detail below)

leading to the selective covalent crosslinking of O-GlcNDAz to its direct binding partners within 2–4 Å. Through quantitative MS analysis, the authors revealed protein–protein interactions between O-GlcNAcylated nucleoporins and nuclear transport factors in HeLa cells.

The same group later identified an OGT mutant OGT^{C917A} that prefers to transfer UDP-GlcNDAz rather than natural UDP-GlcNAc [46], which improves the O-GlcNDAz-dependent crosslinking efficiency. Moreover, Boyce and colleagues employed this GlcNDAz-based approach to elucidate how O-GlcNAc regulates the COPII pathway [47, 48]. It was found that O-GlcNAcylation of the essential COPII component Sec23A is required for protein–protein interactions in maintaining COPII activity [48]. Recently, this system was applied to identify 14-3-3 proteins as O-GlcNAc readers in human cells [49].

2 Chemoenzymatic Glycan Labeling

2.1 Advances in Galactosyltransferase (GalT)-Based Chemoenzymatic Approaches for O-GlcNAc Labeling

Chemoenzymatic glycan labeling is another prevailing strategy for O-GlcNAc detection and enrichment. The first chemoenzymatic method to label O-GlcNAcylated proteins utilizes bovine milk β-1,4-galactosyltransferase (GalT) to transfer [^{3}H]-galactose from UDP-[^{3}H]-galactose to terminal GlcNAc moieties [50, 51].

However, this method relies on handling of radioactive materials and requires exposure times of days to months.

Hsieh-Wilson and coworkers later developed a mutant GalT (Y289L GalT1)-assisted approach for the rapid detection of O-GlcNAc [52, 53]. This engineered mutant features a larger binding pocket that can tolerate a UDP-galactose (UDP-Gal) derivative bearing a ketone functionality at the C2 position (UDP-2-keto-Gal) and install a keto-Gal moiety onto O-GlcNAc without compromising specificity. The ketone group serves as a versatile handle for the conjugation of aminooxy biotin tags via oxime-forming reactions, thereby enabling the chemoselective enrichment of O-GlcNAc substrates by streptavidin chromatography (Fig. 1.5a). Applying it, the authors performed the first chemoenzymatic labeling-based glycoproteomic study and identified 25 O-GlcNAcylated proteins from rat brains [54]. However, aminooxy tags can react with some functionalities within the cell, resulting in potential nonspecific labeling of proteins. The same group described an advanced chemoenzymatic labeling strategy to reduce this side effect [55]. Using UDP-GalNAz as a sugar donor, O-GlcNAc is first transferred with a GalNAz moiety

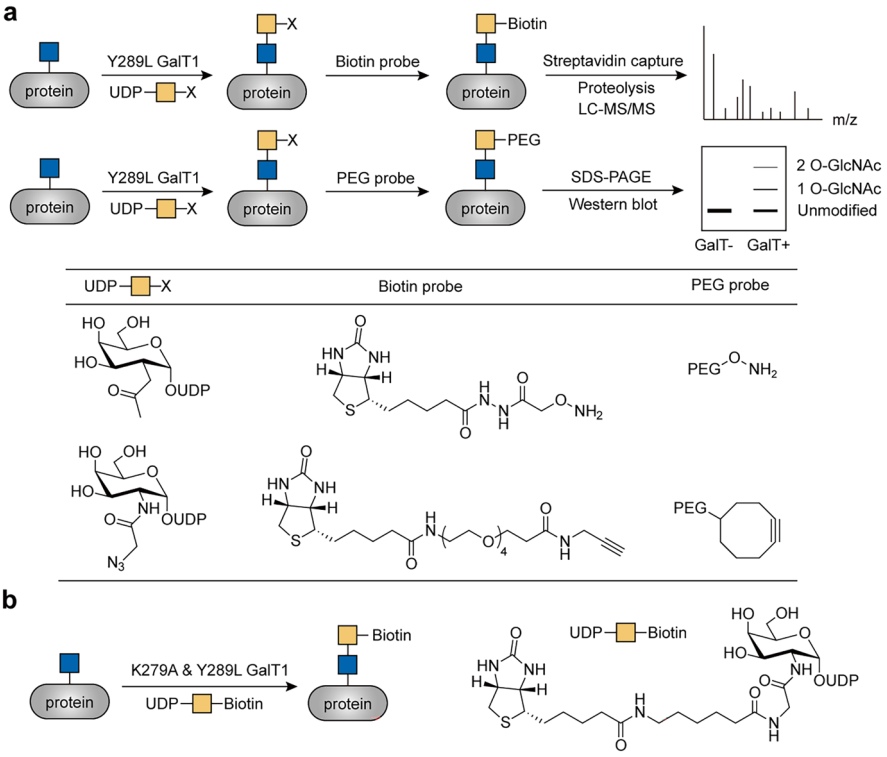

Fig. 1.5 Schematic showing chemoenzymatic labeling of O-GlcNAcylated proteins by GalT mutants and UDP-Gal derivatives. (**a**) The labeled proteins are reacted with biotin or PEG tags via biorthogonal reactions for subsequent detection. (**b**) The principle of one-step chemoenzymatic tagging with no need for bio-orthogonal chemistry

(Fig. 1.5a). The azide-labeled proteins are then conjugated with fluorophore- or biotin-alkyne derivatives for direct in-gel fluorescence, cellular imaging, and proteomic analysis of O-GlcNAcylation. This advanced strategy has been commonly used in various physiological and pathological processes. Another chemoenzymatic histology method relies on UDP-*N*-pentynylgalactosamine (UDP-GalNAlk) [56], an alkyne-bearing GalNAc donor that can be accepted by Y289L GalT1. Compared with UDP-GalNAz, UDP-GalNAlk exhibits more effective chemoenzymatic labeling in human brain specimens. More recently, the Yi group developed one-step enzymatic labeling of O-GlcNAcylation through a double mutant of GalT (K279A/Y289L GalT1) that accepts a biotin-functionalized UDP-GalNAc analogue (UDP-GalNAc-biotin) to directly decorate O-GlcNAc peptides and proteins (Fig. 1.5b) [57]. Although K279A/Y289L GalT1 exploits UDP-GalNAz with much higher efficiency (~65-fold) than UDP-GalNAc-biotin, this method circumvents the need for click chemistry. With this strategy, the authors identified flap endonuclease 1 (FEN1), a necessary enzyme in DNA synthesis, as a novel O-GlcNAc substrate and revealed the functional role of FEN1 O-GlcNAcylation in regulating DNA damage response and cell cycle progression [57].

2.2 *Chemoenzymatic Mass Tagging for O-GlcNAc Stoichiometry Detection*

Chemoenzymatic labeling and resolvable poly(ethylene glycol) (PEG) polymers of defined molecular mass can be jointly used to analyze endogenous O-GlcNAcylation stoichiometry [58–60]. The installed PEG polymers result in mass-shifted bands between the O-GlcNAcylated and nonglycosylated fraction of labeled proteins during SDS–PAGE, which could be further detected by immunoblotting with an antibody against the protein target (Fig. 1.5a). Moreover, the number of shifted bands monitors the O-GlcNAc site numbers. Recently, Pratt and colleagues optimized this procedure using UDP-GalNAz and SPAAC chemistry for expedited quantification of the O-GlcNAc stoichiometry [61].

2.3 *PLA-Coupled Chemoenzymatic Labeling Approach*

In combination with the proximity ligation assay (PLA) and quantitative polymerase chain reaction (qPCR) [62], Glyco-seek, a chemoenzymatic labeling-based strategy, was developed to detect O-GlcNAc states with ultrahigh sensitivity [63]. In Glyco-seek, the O-GlcNAc moiety is chemoenzymatically tagged with a GalNAz residue and ligated with biotin tags (Fig. 1.6). Next, the biotinylated samples are reacted with two single-stranded DNA (ssDNA)-antibody conjugates, one of which targets a specific protein of interest and the other recognizes biotin. Through

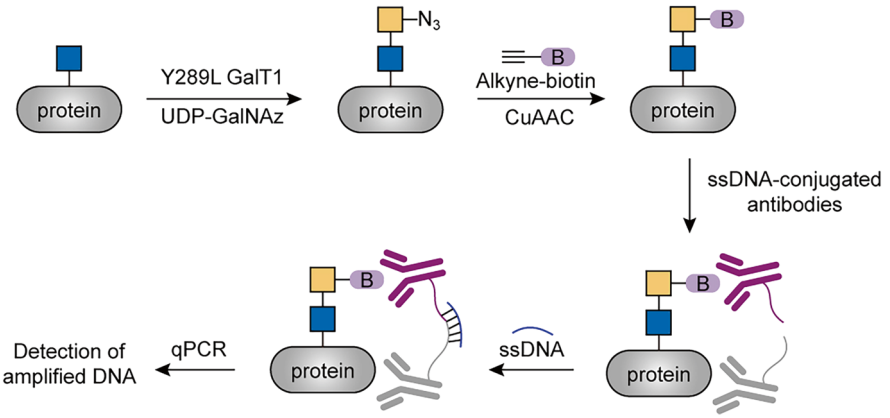

Fig. 1.6 Workflow of Glyco-seek for quantification of the O-GlcNAcylation status

proximity ligation, a short ssDNA complementary to the above two DNA halves and DNA ligase is added to produce a double-stranded segment for qPCR amplification and detection. This method has been applied for directly analyzing the endogenous O-GlcNAcylation of low-abundance transcription factors [63].

2.4 Tandem Chemoenzymatic Labeling Strategy for O-GlcNAc Detection

A combination of two enzymes B3GALNT2 and OGT was designed to determine the O-GlcNAc site state [64]. For a given protein, it may contain various Ser and/or Thr residues that could be potentially modified by O-GlcNAc. The O-GlcNAcylated residues are referred to as closed sites, while the unmodified residues are termed open sites (Fig. 1.7a). B3GALNT2 is a β-1,3-*N*-acetylgalactosaminyltransferase that can incorporate GalNAc or GalNAz into an O-GlcNAc moiety [65, 66], which allows for closed-site labeling. The open sites can be probed with OGT and UDP-GlcNAz. The azide-labeled samples are then reacted with clickable fluorophores or biotin tags for subsequent detection. This approach was exploited to analyze the O-GlcNAc modification degree on purified proteins and cellular extracts. Chen and coworkers reported sequential labeling to image O-GlcNAcylation [67]. O-GlcNAc is first galactosylated by GalT in this method, and then α-2,6-sialyltransferase (ST6Gal1), a sialyltransferase that can accept cytidine monophosphate (CMP)-sialic acid analogues [68], is used to label this disaccharide with a fluorophore-conjugated sialic acid (Fig. 1.7b). Most recently, Ye and coworkers reported a reversible strategy for O-GlcNAcylated peptide capture [69]. The O-GlcNAc moiety is conjugated with a complex-type mimic N-glycan oxazoline (CT-ox) by Endo-M N175Q [70], an Endo-M mutant that possesses glycosynthase-like activity toward N-linked glycans (Fig. 1.7c). Hydrophilic interaction liquid chromatography