Chemical Glycoproteomics

Krishnan K. Palaniappan[†] and **Carolyn R. Bertozzi**[‡,§,*]

[†]Verily Life Sciences, 269 East Grand Ave., South San Francisco, California 94080, United States

[‡]Department of Chemistry, Stanford University, Stanford, California 94305, United States

[§]Howard Hughes Medical Institute, Stanford University, Stanford, California 94305, United States

Abstract

Chemical tools have accelerated progress in glycoscience, reducing experimental barriers to studying protein glycosylation, the most widespread and complex form of posttranslational modification. For example, chemical glycoproteomics technologies have enabled the identification of specific glycosylation sites and glycan structures that modulate protein function in a number of biological processes. This field is now entering a stage of logarithmic growth, during which chemical innovations combined with mass spectrometry advances could make it possible to fully characterize the human glycoproteome. In this review, we describe the important role that chemical glycoproteomics methods are playing in such efforts. We summarize developments in four key areas: enrichment of glycoproteins and glycopeptides from complex mixtures, emphasizing methods that exploit unique chemical properties of glycans or introduce unnatural functional groups through metabolic labeling and chemoenzymatic tagging; identification of sites of protein glycosylation; targeted glycoproteomics; and functional glycoproteomics, with a focus on probing interactions between glycoproteins and glycan-binding proteins. Our goal with this survey is to provide a foundation on which continued technological advancements can be made to promote further explorations of protein glycosylation.

Graphical Abstract

---

[*]Corresponding Author: bertozzi@stanford.edu.

The authors declare no competing financial interest.
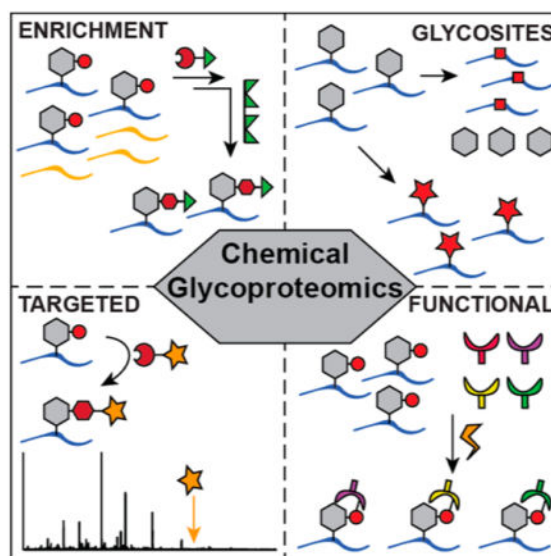
1. INTRODUCTION

Mass spectrometry (MS)-based proteomics is a powerful approach for interrogating biological systems. Over the past decade, the in-depth and quantitative characterization of a number of proteomes has advanced our understanding of basic cellular processes. For example, we now have an initial framework for the protein content of discrete cell types and subcellular structures, and we are beginning to define the connectivity and dynamics among those components.[1–6] Accomplishing this has required the acquisition of information-rich data sets that enumerate and quantitate the components of complex protein mixtures. Advances in both MS (i.e., improved mass resolution and accuracy, increased sensitivity and speed, and new fragmentation methods) and computational methods have been critical in this regard.[4,7–11] However, to align MS-based proteomics with the current and future needs for understanding biological processes, proteins also need to be distinguished by their functions, activities, and interactions with other biomolecules.[12–14]

Posttranslational modifications (PTMs) are a central mechanism by which cells regulate the functional capabilities of their proteome. Identifying and characterizing these PTMs remains a major goal in proteomics.[15] Chemical approaches have emerged as powerful tools for probing PTMs at the proteome scale.[16–19] Of the more than 300 known protein modifications, glycosylation is one of the most abundant and complex PTMs, and it was one of the first to be probed with chemical tools.[20–22] Indeed, the field of glycobiology has long been driven by innovations from chemistry, such as glycan and lectin microarrays, small-molecule inhibitors, and chemically synthesized glycans.[21,23,24]

More recently, chemists have focused on developing tools to help profile protein glycosylation at the level of the proteome. In this review, we discuss key contributions from chemical biology for interrogating the "glycoproteome" by MS. We begin with a discussion of the importance of and challenges in studying protein glycosylation. We then survey chemical approaches for the enrichment of glycoproteins and glycopeptides from complex

mixtures, emphasizing methods that exploit the unique chemical properties of glycans or introduce unnatural functional groups through metabolic labeling and chemoenzymatic tagging. Next, we summarize strategies for identifying sites of protein glycosylation and approaches for targeted glycoproteomics. Finally, we conclude with a discussion of functional glycoproteomics and how chemical tools can be used to detect glycoprotein interactions. The literature covered in this review spans a time period from the 1970s through 2016, with a majority of the work occurring from 2000 onward.

2. GLYCOSYLATION IS AN IMPORTANT PTM THAT INFLUENCES PROTEIN ACTIVITY AND FUNCTION

Protein glycosylation mediates a diversity of cellular processes. It is predicted that 20% of all eukaryotic proteins are glycosylated,[25] but this is likely an underestimation. Unlike DNA transcription and RNA translation, glycosylation is not template-driven; instead, glycoproteins are generated through the enzymatic addition of monosaccharides and complex oligosaccharides onto protein scaffolds, a process that can produce significant diversity and heterogeneity.[26,27] The attached glycans can endow the protein with new properties including cellular trafficking and localization, binding specificity, and thermodynamic stability.[28–30] Glycoproteins on the cell surface facilitate cell recognition, signaling, and adhesion.[31–33] Moreover, changes in protein glycosylation—including in the glycan structure and site occupancy—can modulate cellular phenotypes such as growth, development, and disease.[34–36] Importantly, aberrant glycosylation is well recognized as a hallmark of many diseases and their progression, including microbial pathogenesis, immune deficiencies, neurodegenerative diseases, and many cancers.[37–39] This phenomenon has been exploited through the development of more than 15 glycoproteins as clinical biomarkers.[38,40–43] Consequently, a large effort has been directed toward the accurate characterization of a cell's glycoproteome.

Characterizing the glycoproteome is a challenging endeavor. The nine monosaccharide building blocks used in vertebrates can be assembled in diverse linear and branched patterns to generate a staggeringly complex collection of carbohydrates in an organism, also known as its "glycome." Additional structural diversity results from the attachment of monosaccharides to specific sites on protein scaffolds.[26,44,45] These modifications produce different classes of protein glycosylation, including N-linked, O-linked, and C-linked, with the first two being the most common and the focus of this review.[46–49] In N-glycosylation, glycans are attached to the side-chain nitrogen atoms of asparagine (Asn, N) residues found primarily within the consensus sequence N–X–S/T (where X is any amino acid except proline).[50] N-glycosylation is frequently found on proteins that are translated into the endoplasmic reticulum (ER), where a universal glycan core structure, beginning with a β-linked *N*-acetylglucosamine (GlcNAc), is transferred onto nascent proteins (Figure 1a).[51] The N-glycan precursors are enzymatically edited to yield a plethora of mature glycan structures with compositions that largely depend on cell type, protein localization, and protein structure.[52] However, a majority of N-glycans share a conserved pentasaccharide core structure even after maturation (Figure 1a, red highlight). This feature enables proteome-wide manipulation of N-glycoproteins. As described later, several methods for

identifying N-glycoproteins capitalize on the universality of the conserved core structure for lectin-based enrichment or glycan cleavage using specific enzymes.

In contrast to N-glycosylation, O-glycosylation in vertebrates encompasses considerable structural diversity without a universal core structure. O-glycans are attached to the side-chain oxygen atom of serine (Ser), threonine (Thr), tyrosine, or hydroxylysine residues.[47,53–55] Each type of O-glycosylation is defined by the identity of the peptide-proximal glycan structure. The most common is the mucin-type, which is initiated by an *α*-linked *N*-acetylgalactosamine (GalNAc) residue attached to Ser or Thr residues (Figure 1b).[26] O-glycans can also be initiated with Ser- or Thr-bound glucose, xylose, mannose, or fucose residues.[26] These core monosaccharides are then elaborated by a series of glycosyltransferases to generate high-order structures, such as the tetrasaccharide sialyl Lewis X (Figure 1b, highlighted in blue), and can be found on secreted or cell-surface glycoproteins. One unique subclass of O-glycosylation, found in the cytoplasm and nucleus of most eukaryotic cells, is the addition of a single *β*-linked GlcNAc residue onto either Ser or Thr (Figure 1c) by the enzyme O-GlcNAc transferase (OGT).[56–58] Termed O-GlcNAcylation, this intracellular form of glycosylation is a rapidly reversible, substoichiometric modification, analogous to phosphorylation.[59] The modification is involved in a number of signaling pathways, including cellular metabolism and physiology.[60]

The complexity and heterogeneity inherent to protein glycosylation impart an analytical and computational challenge to the study of this class of PTM. Although a number of biochemical techniques are available to obtain crude structural information regarding a protein-bound glycan, including the use of glycan-binding proteins (i.e., lectins), radioactive labeling, antibodies, and enzymatic deglycosylation, they all suffer from low information content and cumbersome manual analysis. Analytical methods such as NMR spectroscopy, liquid chromatography, and electrophoresis can also be used to characterize protein-associated glycans, but their utility is limited by sample complexity and, in many cases, by the quantity of sample available.[61]

As an alternative to these methods, liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) has emerged as a powerful technology for profiling protein glycosylation. A number of excellent reviews on the subject of MS analysis of glycoproteins have been published.[62–72] The methods primarily employ bottom-up shotgun proteomics, a technique illustrated in Figure 2.[7,9,73,74] In this method, proteins are digested to produce a mixture of peptides, which are then separated by LC and analyzed by MS. Peptide ions in the full-scan mass spectrum are selected for tandem MS analysis, typically on the basis of ion intensity, to generate fragment-ion mass spectra. Comparison to a proteome database allows the assignment of the tandem mass spectrum to its most likely peptide sequence and, ultimately, parent protein of origin.[11,75,76] Despite successes, glycoproteomics remains challenging for a number of reasons. Glycosylation is a labile PTM and requires technical expertise to ensure that the glycan is preserved in its entirety during sample handling or during MS analysis.[77,78] Many interesting glycoproteins are typically present in low abundance, increasing the difficulty of detection in complex biological samples such as human plasma, where protein concentrations can span up to 12 orders of magnitude.[79,80]

Adding to the challenge, glycoproteins usually exist as complex mixtures of glycosylated variants called glycoforms. These structures exhibit both microheterogeneity (i.e., different glycan structures at a specific site of modification) and macroheterogeneity (i.e., differences in the location and number of glycan modifications of the glycoprotein). Additional complexity is introduced through the linkage stereochemistry between monosaccharides and because several monosaccharides are structural isomers of each other with the same molecular weight. These variations complicate the identification process and pose problems for standard bioinformatics software that cannot cope with the enormous variability in glycoprotein structures for a given molecular weight.[81–83]

Considering these formidable obstacles, the successful identification and characterization of glycoproteins by MS will require a combination of new methods, from sample preparation to data analysis.[84] In particular, enrichment through the reduction of sample complexity has played a significant role in the study of glycoproteins in complex mixtures. Following sample preparation, glycoproteins can be characterized using three complementary LC-MS methods: glycoproteomics, proteomics, and glycomics. Although these terms are sometimes used interchangeably and/or inconsistently, glycobiologists have started to adopt specific definitions.[85–87] Here, we define glycoproteomics as the analysis of intact glycopeptides to simultaneously obtain information about the peptide, the site of modification, and the glycan; glycomics as the analysis of free or released glycans from glycoproteins; and proteomics as the analysis of deglycosylated peptides or the nonglycosyated glycoprotein peptides. In this review, we highlight the role of chemical tools in both glycoproteomic and proteomic analyses of glycoproteins; strategies for glycan analysis have been summarized in a number of glycomics reviews.[28,69,88–91]

3. ENRICHMENT OF GLYCOPROTEINS AND GLYCOPEPTIDES

3.1. Overview

Reducing interference from more abundant and nonglycosylated species is an important step for the in-depth analysis of glycoproteins in complex mixtures using MS. Typically, enrichment has been achieved by affinity purification using antibodies or lectins that target specific classes of glycoproteins.[38,92,93] Lectins are glycan-binding proteins isolated from various plants, bacteria, and animal sources and have played an important role in the isolation, purification, and characterization of glycoproteins.[94,95] This is most prevalent for glycoproteins bearing N-glycans, where lectins with varying glycan-binding specificities have been used for enrichment.[62] For example, Concanavalin A (ConA), which has been used extensively to enrich glycoproteins, has a strong preference for oligomannose-type N-glycans compared to highly branched or complex-type N-glycans.[62] Lectins that recognize peripheral glycan structures have also been used for enrichment, such as *Sambucus nigra* agglutinin (SNA) and *Aleuria aurantia* lectin (AAL), which have varying affinities to sialic acid and fucose residues, respectively; the lectins' binding promiscuity is based on the monosaccharide's linkage pattern.[96] Nevertheless, these lectins were immobilized on solid supports and used to enrich glycoproteins from human plasma for biomarker detection.[97] In a similar manner, a combination of lectins were placed in an ultrafiltration spin filter [i.e., filter-aided sample preparation (FASP)] for enrichment and used to identify over 2000 N-

glycoproteins from different mouse tissues.[98] This method, termed N-glyco FASP, was also used to profile N-glycosites across seven evolutionarily distant species[99] and was combined with stable isotope labeling by amino acids in culture (SILAC) to differentiate between different patient-derived B-cell lymphoma cell lines.[100]

In contrast to N-glycans, the greater diversity of O-glycan structures restricts the utility of any one lectin as a universal capture agent. In one strategy, serial lectin affinity chromatography, N-glycopeptides were first removed using ConA and then GalNAc-containing peptides were enriched using the lectin Jacalin.[101] For O-GlcNAcylation, the most common lectin enrichment method uses wheat germ agglutinin (WGA),[102] which has also been used in a long-column format, termed lectin weak-affinity chromatography (LWAC), to enrich for O-GlcNAcylated peptides.[103,104] Recently, *Solanum tuberosum* lectin was discovered to recognize O-GlcNAc and then used to enrich glycoproteins from colon cancer tissue.[105] In addition to lectins, several antibodies have also been used to enrich O-GlcNAcylated glycoproteins from complex mixtures.[106–108] Unfortunately, the utility of lectins and antibodies for glycoprotein and glycopeptide enrichment is limited by low substrate binding affinities and/or poor specificities.

Toward addressing these limitations, advancements in chromatographic and solid-phase extraction (SPE) techniques have been applied to glycopeptide enrichment (see these recent reviews for more information).[66,68,84,86,87,109,110] One method is based on hydrophilic interaction liquid chromatography (HILIC), which has enabled a number of glycoproteomics studies by providing a general method for separating and enriching glycopeptides from nonglycosylated peptides.[111,112] Improvements to HILIC for glycopeptides have taken shape in many formats, including the exploration of different stationary phases, introduction of ion-pairing reagents in the mobile phase, and implementation in an SPE format.[112–114] Other strategies include the use of titanium dioxide-functionalized materials to enrich for sialylated glycopeptides[115] or boronic acid-functionalized materials as a more general enrichment method (see section 3.2 for more information).[116]

There is also a growing interest in the use of chemical tools for marking and enriching subproteomes for analysis by MS.[117,118] In early work, chemical probes were developed to target specific amino acids for quantitative proteomics.[117] The progenitors of this approach were the isotope-coded affinity tag (ICAT) and isobaric tags for relative and absolute quantitation (iTRAQ), which were used to label cysteine residues or reactive amines, respectively, for quantitative, comparative proteomics.[119,120] Subsequently, chemical probes that targeted specific classes of enzymes were developed to enrich functionally active species of the proteome. Termed activity-based protein profiling (ABPP), this method employs families of enzymes that are covalently modified with small molecule probes that contain a chemical warhead specific for their active site, allowing for the identification and characterization of many classes of enzymes, including proteases, glycosidases, kinases, and phosphatases.[121–124]

Chemical glycoproteomics methods take advantage of glycans to mark, enrich, and identify glycoproteins or glycopeptides by MS. For example, glycoproteins and glycopeptides can be specifically labeled at their site(s) of glycosylation with a chemical reporter.[125] The reporter

can serve as a handle for either direct analysis or enrichment after covalent modification with a secondary probe. The selections of the chemical reporter and secondary probe are important to ensure that the reaction between the two functional groups is specific and efficient and can take place in a biological environment; such characteristics define a "bioorthogonal" chemical reaction (Figure 3).[126–131] There are three main strategies for introducing a bioorthogonal chemical reporter at sites of glycosylation. The first relies on exploiting chemical reactivity unique to the structure of a monosaccharide (for example, the cis diol in galactose or sialic acid). The second method relies on metabolic labeling, wherein an unnatural monosaccharide is incorporated directly into glycans using the cell's biosynthetic machinery. The third approach relies on using purified enzymes to chemoselectively append a chemical reporter onto a specific monosaccharide in vitro. Each of these three strategies is discussed in turn as it relates to the enrichment of glycoproteins for MS.

3.2. Enrichment by Exploiting Chemical Properties of Carbohydrates

An early approach to enriching glycoproteins from complex mixtures utilized the cis-diol functionality present in many monosaccharides (for example, see Figure 4a, where a cis diol is highlighted in gray). Cis diols can form reversible covalent adducts with boronic acids, or they can be converted to aldehydes after oxidative cleavage, which, in turn, form covalent adducts with hydrazide or aminooxy reagents. These chemistries allow selective conjugation to solid-supported matrixes or affinity probes, enabling enrichment of the glycosylated species.

3.2.1. Boronic Acid Adduct Formation with Cis Diols—Boronic acid-functionalized materials have long been used as chromatography supports for the separation of glycans.[116,132,133] When boronic acids are attached to a solid support, such as agarose particles, magnetic beads, or a mesoporous silica matrix, boronic esters are formed with glycans under basic conditions, enriching both O- and N-glycoproteins.[134,135] Under acidic conditions, glycoproteins are released and can be analyzed and identified by tandem MS. This approach has been employed to detect glycoproteins in human blood samples[136] and N-glycoproteins in human saliva[137] and yeast.[138] To improve the specificity, boronic acid-decorated lectins (BAD-lectins) have been used to enrich specific glycans or glycoproteins from complex mixtures.[139]

3.2.2. Periodate Oxidation to Introduce an Aldehyde—Another enrichment strategy that exploits the chemical properties of glycans is the introduction of aldehyde groups by sodium periodate-mediated oxidative cleavage of cis diols. The aldehyde group serves as a chemical reporter and can react with amine nucleophiles, including hydrazine and aminooxy compounds, to form covalent linkages under physiological conditions (Figure 3a). Aebersold and co-workers were the first to implement this strategy in a glycoprotein profiling experiment: They used hydrazide-functionalized beads to enrich periodate-oxidized glycoproteins from human serum (Figure 4a).[140] With a covalent bond between the glycoproteins and the solid support, nonglycosylated proteins were washed away, and the nonglycosylated portions of the bound glycoproteins were digested away using trypsin. The enzyme peptide:*N*-glycosidase F (PNGase F), which selectively cleaves the amide bond

linking N-glycans to asparagine side chains, was used to release the covalently bound N-glycopeptides from the support, which were then identified by tandem MS. Using this "hydrazide capture technology", the authors identified 145 N-glycosites, mapping to 57 N-glycoproteins, in human serum samples. Subsequently, Zhang and co-workers developed a variant of this method in which glycoproteins are first digested into peptides and then treated with sodium periodate.[141] A number of studies have applied either one of these methods for N-glycosite mapping in human plasma, cerebrospinal fluid, islets, platelets, saliva, the microsomal fraction of an ovarian cancer cell line, and mouse embryonic stem cells.[42,142–150] Most recently, Zhang and co-workers used hydrazide capture technology to perform an integrated proteomic and glycoproteomic analysis of two prostate cancer cell lines, LNCaP and PC3. By analyzing both peptides and intact glycopeptides, the authors were able to compare differences in protein abundance and glycan composition at specific glycosites between the cell lines.[151] Finally, Zou and co-workers developed variants of hydrazide capture technology using hydroxylamine to assist in releasing captured glycopeptides[152] or reductive amination to block N-terminal amines on glycopeptides prior to oxidation of glycans.[153]

There are, however, some limitations to hydrazide capture technology. First, hydrazide-functionalized beads can react with naturally occurring carbonyl-containing biomolecules in cell lysates (including glucose, pyruvate, and pyridoxal-5′-phosphate), which will reduce the selectivity and sensitivity for glycoprotein enrichment. Second, there is a lack of specificity in both the monosaccharides that are oxidized and the class of glycoproteins that are enriched (i.e., both O- and N-glycoproteins can contain oxidized glycans). Whereas the use of PNGase F to release captured glycopeptides will bias the analysis to N-glycosylation sites, a similar strategy is not available for O-glycosite profiling. Finally, side reactions can occur during glycan oxidation with sodium periodate (i.e., oxidation of N-terminal Ser/Thr, cysteine, and methionine residues).[154] Despite these limitations, it should be noted that hydrazide capture technology has broad applicability to a variety of biological samples, including human biofluids.[144]

To improve the sensitivity of hydrazide capture technology for enriching cell-surface glycoproteins, Wollscheid et al. optimized sodium periodate oxidation conditions for live-cell labeling.[155] This strategy enabled treatment of the newly generated aldehydes with a hydrazide-functionalized enrichment probe (biocytin hydrazide) without lysing cells and exposing the intracellular and naturally occurring carbonyl-containing biomolecules. Following proteolytic digestion of labeled-cell lysates, glycopeptides from cell-surface glycoproteins were captured with streptavidin beads. After enzymatic release using PNGase F, the formerly N-glycosylated peptides were identified by tandem MS. This method, termed cell-surface capturing (CSC) technology, was used to identify the differences between the cell-surface N-glycoproteome of T and B cells. In the same work, CSC technology was combined with SILAC to monitor the N-glycoproteome dynamics during T-cell activation and during mouse embryonic-stem-cell differentiation into neurons. CSC technology has also been applied to mouse myoblast cells, mouse β-cells, hepatoma cells, leukemia cells, pluripotent stem cells, *Drosophila* Kc167 cells, and neuronal progenitor cells.[145,156–163] Recently, Wollscheid and co-workers used CSC technology to identify cell-surface proteins

in 41 human and 31 mouse cell lines.[164] This work is summarized in a publically available resource: the Cell Surface Protein Atlas (CSPA).

Although CSC technology reduces cross-reactivity between the enrichment probe and native carbonyl-containing bio-molecules, the use of PNGase F restricts the method to N-glycoprotein identification. Larson and co-workers addressed the limitation by optimizing sodium periodate oxidation conditions to selectively oxidize the cis diol in sialic acid residues, a terminal monosaccharide found on both N- and O-glycoproteins (Figure 4b).[165] After oxidized glycoproteins were captured on hydrazide beads and nonglycosylated peptides were digested off using trypsin, both N- and O-glycopeptides were released using mildly acidic conditions that cleave sialic acid residues from the underlying glycan structure. When applied to human cerebrospinal fluid, the authors identified 36 N-glycopeptides and 44 O-glycopeptides, mapping to a total of 45 glycoproteins. Using a nested fragmentation method (i.e., MS^3) and manual annotation, the authors could distinguish between N- and O-glycopeptides and characterized some of the intact glycopeptide structures. This strategy has also been used to identify glycoproteins in human urine and mouse serum.[166,167]

In a similar fashion, Paulson and co-workers demonstrated that it is possible to selectively introduce aldehydes into cell-surface sialic acid residues using mild sodium periodate oxidation conditions.[168] They improved the efficiency of chemical labeling through analine-catalyzed oxime formation with an aminooxy-biotin enrichment probe. Following enrichment with streptavidin beads, the nonglycosylated peptides were digested off using trypsin and used to identify putative glycoproteins, whereas N-glycopeptides were cleaved off using PNGase F and used to identify N-glycosites.[169]

Recently, hydrazide capture technology was extended to the O-GlcNAc modification. Medzihradszky and co-workers demonstrated that the trans diol of GlcNAc can be oxidatively cleaved into aldehydes using high concentrations of sodium periodate and elevated temperatures.[170] After oxidized glycoproteins were captured on a hydrazide-functionalized resin, nonglycosylated peptides were removed using trypsin, and glycopeptides were obtained by treatment with hydroxylamine under mildly acidic conditions. The authors applied this method to study the *Drosophila melanogaster* proteasome and identified 12 O-GlcNAcylated peptides corresponding to 5 glycoproteins.

In summary, hydrazide capture technology and its derivatives have become valuable chemical enrichment strategies for identifying N-glycoproteins. These technologies have enabled the identification of thousands of new glycoproteins across many organisms. Although some effort has been made to extend the method to O-glycoproteins, the lack of efficient chemical and enzymatic tools for release has limited its utility in this respect.

3.2.3. Enzymatic Oxidation to Introduce an Aldehyde—Hattori and co-workers demonstrated that hydrazide capture technology could be used to enrich two proteins bearing two forms of O-glycosylation unique to collagen: galactosyl hydroxylysine (GHL) and glucosyl galactosyl hydroxylysine (GGHL).[171] Instead of chemically oxidizing the glycans with sodium periodate, which was previously shown to degrade these structures, they used galactose oxidase, an enzyme that oxidizes terminal galactose and GalNAc residues at the

C-6 position to generate an aldehyde (Figure 4c). The authors applied the technique to enrich glycopeptides from bovine type I and type II collagens. After enriching oxidized glycopeptides on hydrazide resin and eluting them under acidic conditions, they identified 37 GHL/GGHL glycopeptides using tandem MS, 13 from type I and 24 from type II collagen. Recently, Paulson and co-workers extended the utility of galactose oxidase to live cells and demonstrated that aldehydes could be selectively introduced into terminal galactose and GalNAc residues on cell surfaces.[169] Toward expanding the substrate scope of galactose oxidase, directed evolution was used to select for enzyme variants that could oxidize mannose and GlcNAc residues.[172] Recently, Kohler and co-workers utilized both enzymatic and chemical oxidation to enrich and identify mammalian glycoprotein substrates for bacterial sialidases.[173]

3.3. Enrichment by Metabolic Labeling with Unnatural Monosaccharide Analogues

Metabolic labeling with chemically functionalized monosaccharide substrates is an emerging strategy for glycoprotein profiling.[16–18,63,70,174–177] The approach can be traced to classic work from the 1970s indicating that glycan biosynthetic enzymes are permissive, in some cases, of unnatural substrates, enabling their metabolic incorporation into glycoproteins. In the 1990s, Reutter and co-workers reported that the N-acyl group of sialic acid can be biosynthetically modulated by feeding cells and animals N-acyl derivatives of its metabolic precursor, *N*-acetylmannosamine (ManNAc).[178,179] These observations suggested a route for incorporating bioorthogonal functional groups into glycoproteins as a means of visualizing them in cells and organisms.[180–184] In the past decade, interest has grown in using metabolic labeling to tag and enrich specific classes of glycoproteins for proteomic analysis. As shown in Figure 5a, cells or organisms are treated with a modified sugar, and the metabolically labeled glycoprotein products are chemically tagged with an enrichment probe. The bioorthogonal chemistries employed for such studies generally involve the azide as a principle reagent. Azides are small in size, easily added to synthetic sugar analogues, stable in vivo, and unreactive with normal cellular components.[126,130] After metabolic incorporation into glycoproteins, azido sugars can be selectively reacted by the Staudinger ligation with triarylphosphines,[180] copper(I)-catalyzed azide–alkyne cyclo-addition (CuAAC) with terminal alkynes,[185,186] or "copper-free click chemistry" with strained cyclooctynes (Figure 3b,c).[187,188] Alternatively, simple sugars can be functionalized with terminal alkynes, which are similar in size to azides, and their metabolic products labeled with azide probes.[126,130]

Metabolic labeling can be used to target specific forms of protein glycosylation for enrichment. For example, sialylated glycoproteins can be selectively labeled using analogues of its biosynthetic precursor, ManNAc. Toward this end, both azido (ManNAz) and alkynyl (ManNAlk) derivatives of ManNAc have been employed (Figure 5b).[180,183,189] Alternatively, sialic acid analogues can be used directly as metabolic labels, circumventing the requirement that upstream enzymes recognize unnatural substrates.[190,191] Likewise, azido and alkynyl variants of GalNAc,[192–194] fucose,[189,195,196] and GlcNAc[197–199] have been used to label and enrich various glycoprotein types. These sugars are predominantly administered in a per-*O*-acetylated form to improve membrane permeability and efficient

cell entry. After the sugars have entered the cells, cytosolic esterases cleave the acetyl groups, and glycan biosynthetic enzymes can utilize the deprotected sugar derivatives.[200]

3.3.1. Labeling Sialylated Glycoproteins with ManNAc Analogues—There has been much interest in the selective profiling of sialylated glycoproteins due to known associations of changes in cellular sialylation states during tumor progression.[201,202] To target this group of glycoproteins, both per-*O*-acetylated *N*-azidoacetylmannosamine (Ac$_4$ManNAz) and per-*O*-acetylated *N*-alkynylacetylmannosamine (Ac$_4$ManNAlk) have been used to metabolically label cellular glycans (Figure 5b).

In an early example, Wong and co-workers fed prostate cancer-derived PC3 cells Ac$_4$ManNAlk and then treated whole-cell lysates with a biotin-azide probe under CuAAC reaction conditions.[203] After enrichment of sialic acid-containing glycoproteins using streptavidin beads, nonglycosylated peptides were removed by on-bead tryptic digestion, and then N-glycopeptides were released using PNGase F. Following tandem MS analysis of the released peptides, the authors identified 219 N-glycosites, corresponding to 108 glycoproteins, of which 69 were previously known glycoproteins.

Semmes and co-workers also combined metabolic labeling and MS to identify sialic acid-containing glycoproteins involved in prostate cancer.[204] The authors treated highly metastatic (ML2 cells) and nonmetastatic (N2 cells) PC3-derived cell lines with Ac$_4$ManNAz and performed the CuAAC reaction on intact cells with a biotin-alkyne tag. After whole-cell lysates had been prepared and biotin-labeled glycoproteins had been captured onto streptavidin beads, glycoproteins were released by boiling and then subjected to in-gel digestion with trypsin. Following tandem MS, the authors identified 324 proteins unique to N2 cells and 372 proteins unique to ML2 cells, of which 36 and 44 proteins, respectively, were predicted to be cell-surface N-glycoproteins.

Another example of metabolic labeling with Ac$_4$ManNAz was performed by Bai and co-workers.[205] The authors fed three different cancer cell lines (A549 lung adenocarcinoma, HeLa cervical carcinoma, and SW1990 pancreatic adenocarcinoma) Ac$_4$ManNAz, performed the CuAAC reaction on intact cells using a thiol-cleavable biotin-alkyne probe, prepared whole-cell extracts, and captured the biotinylated proteins on magnetic streptavidin beads. After chemical release with dithiothreitol, proteins were digested using trypsin and analyzed by tandem MS. Collectively, 310 proteins were identified, 56 of which were predicted to be cell-surface glycoproteins.

In addition to cell-surface glycoproteins, metabolic labeling with Ac$_4$ManNAz was used to identify glycoproteins in the secretome.[206] Drake and co-workers cultured two stromal cell lines (WPMY-1 and HS5) in serum-free or low-serum media containing Ac$_4$ManNAz. The clarified cell culture media were treated with alkyne beads to capture glycoproteins. Using on-bead tryptic digestion and tandem MS, the authors identified over 100 secreted proteins in each stromal cell line's secretome, many of which were previously known glycoproteins. In the same study, the authors also investigated the utility of metabolic labeling with per-*O*-acetylated *N*-azidoacetylgalactosamine (Ac$_4$GalNAz) for identifying glycoproteins in the secretome.

To study glycoprotein dynamics in vivo, Chen and co-workers used $Ac_4ManNAz$ and the CuAAC reaction to identify glycoproteins upregulated during cardiac hypertrophy in live rats.[207] After administration of $Ac_4ManNAz$ to saline- or isoprenaline-treated rats, heart tissue was isolated, lysed, and treated with an alkyne-biotin probe. Following enrichment of biotinylated glycoproteins with streptavidin beads and in-gel digestion, the authors used tandem MS to identify newly synthesized sialylated glycoproteins in hypertrophic hearts. They also demonstrated that $Ac_4GalNAz$ could be used to label heart glycoproteins.

Copper-free click chemistry has also been used to enrich glycoproteins after metabolic labeling with ManNAc analogues. In one example, Lichtenthaler and co-workers used a cyclooctyne probe to enrich for glycoprotein substrates of membrane-bound proteases (i.e., sheddases).[208] The authors enriched glycoproteins from the secretome of primary neurons by culturing cells in $Ac_4ManNAz$, treating the clarified media with a biotin-dibenzocyclooctyne probe, and then incubating with streptavidin beads. Following release and tryptic digestion, they identified 34 substrates for the protease BACE1, an Alzheimer's-relevant sheddase.

In another example, Tackett and co-workers used metabolic labeling to enrich and identify cell-surface glycoproteins from pancreatic cancer cell lines.[209] The authors cultured three pancreatic cell lines (BxPC-3, MIAPaCa-2, and Panc-1) in $Ac_4ManNAz$ and then treated the cells with a biotinylated and thiol-cleavable cyclooctyne probe. Cell lysates were prepared and incubated with streptavidin beads to capture glycoproteins. Following cleavage with a thiol reagent, the authors used tandem MS to identify several hundred proteins between the three cell lines.

In addition to the CuAAC reaction and copper-free click chemistry, the Staudinger ligation has also been combined with metabolic labeling with ManNAc analogues to enrich for glycoproteins. In one study, Autelitano et al. identified biomarkers for malignant glioma targeted therapy.[210] Primary cells isolated from human glioblastoma multiform (GBM) tissues and nontumorous brain tissue were cultured in $Ac_4ManNAz$ and treated with a biotinylated phosphine probe to label cell-surface glycoproteins. Biotinylated glycoproteins were enriched from cell lysates using streptavidin beads and eluted through on-bead digestion with trypsin. Using tandem MS, the authors identified 801 proteins, 606 of which were predicted to be glycoproteins. Additionally, using label-free quantification, the authors identified 35 overexpressed proteins in GBM tumors and validated their expression profiles using gene expression analysis.

In another study, Delcourt et al. identified sialylated glycoproteins in hypoxic human umbilical vein endothelial cells.[211] Cells were cultured in $Ac_4ManNAz$ under hypoxic conditions and then treated with a biotinylated phosphine probe to label cell-surface glycoproteins. Biotinylated glycoproteins were enriched from cell lysates using streptavidin beads. Following on-bead digestion and tandem MS, the authors identified 27 proteins upregulated in the hypoxia-treated cells, many of which were previously known sialylated glycoproteins or were validated by Western blot analysis.

Most recently, Zhang and co-workers identified N-glycoproteins as potential biomarkers for pancreatic cancer.[212] The authors cultured SW1990 human pancreatic cells in $Ac_4ManNAz$ and biotinylated azido-glycoproteins using the Staudinger ligation. After biotinylated glycopeptides had been captured using streptavidin beads, the formerly N-glycopeptides were selectively released using PNGase F and analyzed by tandem MS. The authors identified 75 N-glycosites across 55 glycoproteins, 42 of which were linked to cancer. Using immunohistochemistry, they proposed two new pancreatic cancer biomarkers: lysosome-associated membrane protein 1 and hypoxia upregulated protein 1.

3.3.2. Labeling Sialylated Glycoproteins with Sialic Acid Analogues—A major challenge with metabolic labeling in whole organisms is achieving tissue-specific incorporation of unnatural monosaccharide analogues. Chen and co-workers overcame this limitation by encapsulating monosaccharides in ligand-targeted liposomes to direct sugar analogues to tumor cells in vivo.[213,214] The authors encapsulated 9-azido sialic acid into a liposome displaying a cyclic-RGD peptide, injected the construct into a melanoma xenograft mouse model, and observed high expression of azido sialic acid in tumor cells. The authors collected tumor tissue samples at different time points, treated tissue lysates with an alkyne-biotin probe, and enriched for glycosylated proteins using streptavidin beads. Using tandem MS, they identified 204, 180, and 156 glycosylated proteins in day 14, 18, and 21 tumors, respectively; a majority of which were predicted to be membrane or secreted N-glycoproteins. Most recently, Chen and co-workers used this method to enrich glycoproteins in the mouse brain.[215]

3.3.3. Labeling Fucosylated Glycoproteins with Fucose Analogues—
Glycoprotein fucosylation is differentially regulated during normal and pathophysiological processes.[216] Fucose can be a terminal antenna glycan modification, as in the Lewis antigens where it is linked to an underlying GlcNAc or Gal residue, or a modification of the peptide-proximal GlcNAc residue within the N-glycan pentasaccharide core. As well, fucose directly linked to Ser or Thr side chains is the initiating residue of glycans associated with epidermal growth factor (EGF) and thrombospondin repeat (TSR) sequences, as in the EGF repeat-rich Notch protein.[217,218]

To enrich fucosylated glycoproteins, both per-*O*-acetylated *N*-azidoacetylfucose (Ac_4FucAz) and per-*O*-acetylated *N*-alkynylacetylfucose ($Ac_4FucAlk$) have been used to metabolically label cellular glycans (see Figure 5b).[189,195,196] These compounds were designed to access the fucose salvage pathway, where free fucose is converted to fucose-1-phosphate and then to GDP-fucose, the substrate for all fucosyltransferases. However, fucose derivatives can have low labeling efficiencies and can inhibit protein fucosylation.[219] To overcome this limitation, Narimatsu and co-workers generated Namalwa cells that stably overexpressed two important enzymes in the fucose salvage pathway, L-fucokinase/GDP-fucose pyrophosphorylase (FKP) and α-1,3-fucosyltransferase 9 (FUT9).[220] Overexpression of FKP and FUT9 enhanced metabolic labeling with $Ac_4FucAlk$ and enabled the identification of FUT9 substrates.

Recently, FucAlk was used to label proteins bearing core O-fucose residues on their EGF or TSR domains, a modification catalyzed by protein-O-fucosyltransferases 1 and 2 (POFUT1

and POFUT2), respectively.[221] Through metabolic labeling, the O-fucose sites predicted from bioinformatics analysis were verified. Moreover, the unnatural fucose substrates were recognized by glycosyltransferases and elaborated analogously to the native fucose reside. This finding could enable the discovery of unexpected proteins bearing O-fucose residues.

3.3.4. Labeling Glycoproteins with GalNAc Analogues

GalNAc is a versatile monosaccharide that can be used to enrich both mucin-type O-glycoproteins and N-glycoproteins. In the case of mucins, a conserved α-GalNAc residue initiates all possible glycan core structures. GalNAc can also be found in different positions in N-glycan structures. Accordingly, GalNAc analogues that contain either an azide[192] or alkyne[198] (Figure 5b) have been used to enrich a diversity of glycoproteins. Although some studies failed to distinguish the identifications into their respective glycoprotein class, others used lectins and/or PNGase F for N-glycoprotein assignment.

In an effort to identify prostate cancer biomarkers, Bertozzi and co-workers used metabolic labeling to enrich cell-surface glycoproteins from prostate cancer-derived PC3 cells.[222] Cells were fed $Ac_4GalNAz$ and then treated with a membrane-impermeable phosphine-FLAG probe. By performing the bioorthogonal reaction on live cells, rather than cell lysates, the authors were able to select for cell-surface glycoproteins while minimizing potential contamination from intracellular glycoproteins (vide infra). After whole-cell lysates had been prepared, FLAG-labeled glycoproteins were enriched using an anti-FLAG antibody and then subjected to in-gel digestion with trypsin. Using tandem MS, the authors identified 71 proteins, 29 of which were previously known to be sialylated based on metabolic labeling with ManNAlk.[203]

In addition to the Staudinger ligation, Gorfien and coworkers used GalNAz and the CuAAC reaction to enrich glycoproteins from the CHO cell secretome.[223] They cultured two different CHO cell lines, CHO-S and DG44, in $Ac_4GalNAz$ and then treated the clarified cell culture media with an alkyne-functionalized agarose resin. Following on-bead digestion with trypsin, peptides were labeled with iTRAQ reagents, separated by two-dimensional chromatography, and quantified by tandem MS. The authors identified and quantified 325 secreted proteins between the two CHO cell lines.

To complement lectin- and periodate-based enrichment strategies, Smeekens et al. used metabolic labeling with GalNAc derivatives to enrich N-glycoproteins.[224] They cultured HEK293T cells in $Ac_4GalNAz$ and used copper-free click chemistry with a biotin-dibenzocyclooctyne probe to label cell-surface glycoproteins. After whole-cell lysates had been digested with trypsin, glycopeptides were enriched using NeutrAvidin beads and eluted by enzymatic deglycosylation using PNGase F. Following tandem MS, the authors identified 152 N-glycosites, which mapped to 110 glycoproteins. Although metabolic labeling with $Ac_4GalNAz$ is agnostic to N- versus O-glycosylation, the authors restricted their analysis to N-glycoproteins by using PNGase F.

Recently, Xiao et al. evaluated metabolic labeling with three monosaccharide analogues for their ability to enrichment cell-surface N-glycoproteins.[225] The authors demonstrated that GalNAz outperformed both ManNAz and *N*-azidoacetylglucosamine (GlcNAz) when

combined with copper-free click chemistry for glycopeptide enrichment. The authors then used metabolic labeling with GalNAz and SILAC reagents to quantify changes in N-glycosites in statin-treated HepG2 liver cells.

3.3.5. Labeling O-GlcNAcylated Proteins with GalNAc Analogues—In addition to labeling cell-surface glycoproteins, GalNAz can also label intracellular O-GlcNAcylated substrates. Based on the promiscuity in sugar-donor selection by the enzyme UDP-galactose 4-epimerase (GALE), which interconverts UDP-GlcNAc and its C_4 epimer UDP-GalNAc, GalNAz can be converted to GlcNAz. Bertozzi and co-workers demonstrated this metabolic cross-talk by culturing Jurkat cells in Ac_4GalNAz and then treating whole-cell lysates with a phosphine-FLAG-His$_6$ probe.[193] After enrichment of glycoproteins by sequential pull-downs using an anti-FLAG antibody followed by metal affinity chromatography, the authors identified 18 proteins, many of which were previously known to be glycosylated. Depending on the application, the cross-talk between GalNAz and GlcNAz can require careful sample preparation to correctly interpret data from nucleocytosolic proteins and proteins translated in the ER/secretory pathway.[198,223]

Recently, metabolic labeling with Ac_4GalNAz and the CuAAC reaction were combined for differential glycosylation analysis between two biological samples. The method is a modified version of difference gel electrophoresis (DIGE),[226,227] in which two samples are labeled with fluorophores that are distinct (but identical in charge and close in mass) and then combined and analyzed on a two-dimensional gel. After fluorescent gel spots that correspond to incongruity in the two fluorescent signals have been excised, protein abundance changes between the samples (i.e., due to a stimulus) can be identified through in-gel digestion and tandem MS. By integrating DIGE and metabolic labeling, Bertozzi and co-workers were able to rapidly monitor changes in glycoprotein levels in complex biological samples.[228] Termed "glyco-DIGE", this technique was used to examine organelle-specific protein O-GlcNAcylation. After Jurkat cells had been cultured in Ac_4GalNAz, mitochondrial and cytosolic extracts were separately labeled with an alkyne cyanine dye (either Cyanine 3 or Cyanine 5), combined, and then separated on a two-dimensional gel. Following in-gel digestion of fluorescent bands unique to the mitochondria, the authors used tandem MS to identify a mitochondrial outer-membrane protein with crucial roles in glucose metabolism and apoptosis regulation: voltage-dependent anion channel 2.

3.3.6. Labeling O-GlcNAcylated Proteins with GlcNAc Analogues—Intracellular O-GlcNAcylation is highly dynamic and typically substiochiometric, necessitating enrichment methods to identify O-GlcNAcylated proteins. To enrich this class of glycosylation, both per-*O*-acetylated *N*-azidoacetylglucosamine (Ac_4GlcNAz) and per-*O*-acetylated *N*-alkynylacetyl-glucosamine (Ac_4GlcNAlk) have been used for metabolic labeling (see Figure 5b).

In the first example, Zhao and co-workers fed *Drosophila* S2 cells Ac_4GlcNAz, treated cytosolic extracts with a phosphine-biotin probe, and captured labeled proteins on streptavidin beads.[229] After proteins had been digested off the beads with trypsin and analyzed by tandem MS, the authors identified 41 proteins, 10 of which were previously

known to be glycosylated. In a follow-up report, the same group applied an optimized enrichment protocol in HeLa cells and increased the number of identifications.[230]

In addition to the Staudinger ligation, the CuAAC reaction has also been used to enrich GlcNAz-labeled glycoproteins. For example, Kuster and co-workers treated whole-cell lysates from Ac$_4$GlcNAz-fed HEK293 cells with an alkyne-functionalized agarose resin.[231] Following on-bead digestion and tandem MS, they identified approximately 1500 proteins. For a subset of these identifications, the authors were able to resolve 185 O-GlcNAc glycosites using β-elimination (see section 4).

Lemonie and co-workers also used the CuAAC reaction to enrich glycoproteins. They treated nuclear and cytosolic extracts from Ac$_4$GlcNAz-fed MCF-7 cells with a biotin-alkyne probe.[199] After enrichment of glycoproteins with a streptavidin column and in-gel digestion with trypsin, the authors identified 32 proteins using tandem MS, 18 of which were previously known to be glycosylated. In the same work, Lemonie and co-workers tested metabolic labeling with Ac$_4$GlcNAlk, the alkyne-derivatized GlcNAc analogue. However, under their labeling conditions, they were unable to observe metabolic labeling.

In a later study, Pratt and co-workers also explored alkyne-functionalized GlcNAc derivatives for metabolic labeling and glycoproteomic profiling.[198] Under low-glucose growth conditions, they demonstrated that Ac$_4$GlcNAlk could be used for metabolic labeling in NIH3T3 cells. After whole-cell lysates were treated with a cleavable enrichment tag (azido-diazo-biotin), glycoproteins were captured onto streptavidin beads and released using sodium dithionite (which reductively cleaves the diazo bond). Using tandem MS, the authors identified 374 proteins, 95 of which were previously known to be glycosylated and three of which were confirmed by immunoprecipitation. The same authors also used this method to study glycoproteins in mice with diabetic retinopathy.[232]

One limitation of both Ac$_4$GlcNAz and Ac$_4$GlcNAlk is that enzymatic pathways can interconvert GlcNAc analogues into GalNAc analogues, necessitating cell fractionation and careful data analysis when assigning the glycosylation state of proteins post enrichment.[193,198] To address this limitation, Pratt and coworkers synthesized 6-azido-6-deoxy-*N*-acetylglucosamine (6AzGlcNAc), an unnatural monosaccharide that they found to be specific for O-GlcNAcylated proteins (Figure 5b).[233] To validate the new GlcNAc analogue, the authors performed a comparative proteomics experiment by culturing NIH3T3 cells with peracetylated 6-azido-6-deoxy-*N*-acetylglucosamine (Ac$_3$6AzGlcNAc), Ac$_4$GlcNAz, or Ac$_4$GalNAz; treating each cell lysate with a biotin-alkyne probe; and enriching glycoproteins with streptavidin-conjugated beads. After eluting peptides by on-bead digestion and analyzing the mixture by tandem MS, they identified 366 proteins using Ac$_3$6AzGlcNAc, 350 of which were annotated as intracellular proteins.

3.3.7. Labeling Bacterial Glycoproteins with GlcNAc Analogues—Metabolic labeling with monosaccharide analogues was recently used to study glycoproteins in bacteria.[234] Dube and co-workers used GlcNAz and the Staudinger ligation to enrich glycoproteins in *Helicobacter pylori*.[235,236] They cultured *H. pylori* in media supplemented with Ac$_4$GlcNAz for 3–5 days, treated whole-cell lysates with a phosphine-FLAG-His6

probe, and enriched glycoproteins by sequential pull-downs using an anti-FLAG antibody and metal affinity chromatography. Using multidimensional chromatography coupled with tandem MS, they identified 125 proteins, including a number of virulence factors. In the future, this strategy could be used to study bacterial glycosylation in different bacterial species.[234]

3.3.8. Advantages and Limitations of Metabolic Labeling—There are two key advantages to using metabolic labeling and the bioorthogonal chemical reporter strategy for glycoprotein enrichment. First, the chemical reporter is directly embedded into the glycan, allowing for glycosylation-specific enrichment using appropriate secondary probes. Unlike lectins or antibodies, which display weak binding and cross-reactivity, bioorthogonal reactions with azide- or alkyne-functionalized monosaccharides allow for stringent enrichment conditions. Second, unnatural monosaccharides can be dosed to cells or animals in a time-dependent manner (along with a stimulus) to reveal the dynamics and signal-dependent changes in glycosylation patterns. This is an appropriate strategy for identifying changes in low-abundance glycoproteins among a background of high-abundance steady-state protein glycosylation.

At the same time, there are limitations to the metabolic labeling strategy. First, some unnatural monosaccharides show low metabolic incorporation efficiency, as discussed with respect to the fucose analogues. This is likely the result of varying enzyme tolerances to substrate structural perturbations. Second, epimerase activity, which can convert one monosaccharide into another, should be characterized for each unnatural monosaccharide. Measurement of this activity would benefit from careful metabolic flux analysis, as was performed by Yarema and co-workers for ManNAc analogues.[237] Third, unnatural monosaccharides could perturb cellular systems and might not be recognized with the same affinity by their wild-type binding partners. Finally, metabolic labeling strategies cannot be used to enrich glycoproteins from human samples.

3.4. Enrichment by Chemoenzymatic Tagging with Unnatural Monosaccharide Analogues

As an alternative to metabolic labeling, glycoproteins can be enriched after chemoenzymatic tagging with bioorthogonal chemical reporters.[174,176,177] In this approach, glycans are enzymatically appended with a functionalized monosaccharide and then covalently ligated to an enrichment probe using bioorthogonal reactions (Figure 6a). The challenge lies in finding or designing appropriate glycosyltransferases that can accept unnatural monosaccharide donors and act on their substrates extracellularly. The motivation for identifying such enzymes is cell-type-independent and high-efficiency labeling of glycoproteins.

3.4.1. Chemoenzymatic Tagging of O-GlcNAc—Chemo-enzymatic tagging of glycans was initially developed to improve the detection of O-GlcNAcylated proteins. It was first demonstrated by Hart and co-workers, who developed a radioassay using ^{3}H-UDP-Gal and the enzyme β-1,4-galactosyl-transferase 1 (GalT1), which transfers Gal from UDP-Gal to any terminal GlcNAc residue.[102,238,239] Subsequently, Qasba and co-workers developed a nonradioactive detection method after they identified the key amino acids that governed GalT1's sugar donor specificity.[240] Using site-directed mutagenesis (i.e., Y289L), they

enlarged the donor-substrate-binding pocket of GalT1, which enabled the enzyme to tolerate UDP-GalNAc and other GalNAc analogues at the 2-position. Hsieh-Wilson and co-workers leveraged the substrate tolerance of the Y289L GalT1 mutant enzyme, installing either GalNAz or the ketone isostere of GalNAc (2-keto-Gal) onto O-GlcNAcylated substrates (Figure 6b).[241–243]

Hsieh-Wilson and co-workers conducted the first glycoproteomics study using the chemoenzymatic tagging enrichment strategy.[244] They prepared nuclear and cytosolic extracts of rat brains, incubated the lysates with UDP-2-keto-Gal and the Y289L GalT1 mutant, and then treated the samples with an aminooxy biotin tag to label and enrich O-GlcNAcylated substrates. Using tandem MS, the authors identified 25 O-GlcNAcylated glycoproteins, two of which were previously known to be O-GlcNAcylated. In a follow-up study, Hsieh-Wilson and co-workers developed a quantitative variant of their chemoenzymatic tagging strategy.[245] In this method, after chemoenzymatic tagging, glycopeptides were generated by trypsin digestion, and free amines were labeled using either isotopically light or heavy versions of formaldehyde and sodium cyanoborohydride. The authors used this protocol to quantify the dynamics of O-GlcNAcylation in the rat brain under different stimulatory conditions. Later, Hart and co-workers used the same enrichment strategy to identify 35 O-GlcNAc sites on 25 glycoproteins in human erythrocytes.[246]

In addition to oxime-forming reactions, the CuAAC reaction has also been used with chemoenzymatic tagging. In the first example, Hsieh-Wilson and co-workers incubated rat forebrain lysates with UDP-GalNAz and the Y289L GalT1 mutant enzyme to install azides onto O-GlcNAcylated substrates.[241] After treatment with a tetramethyl-6-carboxyrhodamine-(TAMRA-) alkyne probe and enrichment by immunoprecipitation, the authors identified 213 proteins using tandem MS, 67 of which were previously known O-GlcNAcylated proteins. Later, Palmisano and co-workers used the same strategy to install azides onto O-GlcNAcylated substrates in mouse brain lysate. The authors then treated the labeled lysate with a phospho-alkyne tag and used titanium dioxide chromatography to simultaneously enrich for phosphopeptides and glycopeptides.[247] Using tandem MS, they identified 12 O-GlcNAcylated peptides and 485 phosphopeptides. Subsequently, after optimizing conditions to favor glycopeptide enrichment, the authors identified 20 O-GlcNAcylated peptides and 23 phosphopeptides. Additionally, Hart and co-workers used chemoenzymatic tagging to determine that histones were O-GlcNAcylated.[248]

To improve the recovery of enriched O-GlcNAcylated substrates, chemoenzymatic tagging was followed by treatment with cleavable enrichment probes. In the first example, Hart and co-workers synthesized a photocleavable biotin-alkyne probe to react with GalNAz-tagged O-GlcNAcylated peptides from rat brain lysates. After affinity enrichment using avidin chromatography, the authors released glycopeptides through exposure to UV light (365 nm) and identified them using tandem MS.[249] The photocleavage reaction reduced the mass of the tagged peptide and generated a basic aminomethyl-triazole group, both favorable features for tandem MS. Hart and co-workers employed this strategy in two large-scale O-GlcNAc profiling studies: One investigated the interplay between O-GlcNAcylation and phosphorylation during mitosis (141 glycosites in 64 glycoproteins),[250] and the other

investigated differences in O-GlcNAcylation in cerebrocortical brain tissue between wild-type mice and an Alzheimer's disease model (458 glycosites in 274 glycoproteins).[251]

More recently, three other cleavable enrichment probes have been combined with chemoenzymatic tagging of O-GlcNAcylated substrates. In one example, Tanaka and co-workers used a 3-ethynylbenzaldehyde probe for reversible hydrazone formation with a hydrazide resin so that enriched glycopeptides could be eluted using hydroxylamine.[252] In another example, Tsumoto et al. used a thiol-alkyne probe for reversible disulfide formation with a thiol-reactive resin.[253] Finally, Hsieh-Wilson and co-workers used an alkyne-functionalized 1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)ethyl biotinylated probe that can be quantitatively cleaved by hydrazine.[254]

3.4.2. Chemoenzymatic Tagging of Sialic Acid

In host–pathogen interactions, sialic acid residues from the pathogen can be transferred to the host's cell surface through a *trans*-sialidase enzyme.[255] In studies of this behavior, it has been difficult to identify cell-surface glycoproteins on host immune cells that act as acceptors for sialic acid residues originating from the pathogen, so Campetella and co-workers developed a chemozymatic tagging strategy to study this interaction.[255,256] They characterized the substrate preference of a *trans*-sialidase from *Trypanosoma cruzi* using an azide-functionalized sialic acid derivative, SiaNAz-α2,3-Lac-β-OMe (Figure 6c).[255] To demonstrate the strategy, they incubated Jurkat cells with the microbial *trans*-sialidase enzyme and the SiaNAz derivative and then performed the Staudinger ligation using a phosphine-FLAG probe on live cells. Cell lysates were prepared, and FLAG-labeled glycoproteins were enriched by immunoprecipitation with an anti-FLAG antibody. Using tandem MS, the authors identified CD45 isoforms as the main glycoprotein acceptor of the SiaNAz derivative.

3.4.3. Chemoenzymatic Tagging of Disaccharides

To extend the technique of chemoenzymatic tagging to other glycans, new enzymes with appropriate target specificity and substrate tolerances are required. This effort has been used to target two disease-relevant disaccharides: *N*-acetyllactosamine (LacNAc, Gal-β-1,4-GlcNAc) and fucose-α-1,2-galactose (Fuc-α-1,2-Gal). In the case of LacNAc, Qasba and co-workers designed a mutant bovine α-1,3-galactosyltransferase that could transfer GalNAz or 2-keto-Gal onto LacNAc residues.[257] In another approach by Wu and co-workers, the authors demonstrated that wild-type α-1,3-fucosyltransferase from *H. pylori* could transfer fucose analogues (e.g., 6-azido-fucose) onto LacNAc residues.[258] Finally, to target Fuc-α-1,2-Gal, Hsieh-Wilson and co-workers demonstrated that the bacterial glycosyltransferase BgtA could transfer GalNAc analogues (e.g., 2-keto-Gal or GalNAz) onto the Gal residue within the Fuc-α-1,2-Gal motif. In the future, we anticipate that these new chemoenzymatic tagging strategies will be coupled with enrichment tags to help identify glycoprotein substrates containing higher-order glycans.

3.4.4. Advantages and Limitations of Chemoenzymatic Tagging

The main advantage to using the chemoenzymatic tagging strategy for labeling glycoproteins with bioorthogonal chemical reporters is the high efficiency of the reaction. At the same time, there are limitations to the method. First, chemoenzymatic tagging is best suited to report on steady-state glycosylation rather than on its dynamics. Second, designing enzymes to

tolerate unnatural monosaccharide substrates and synthesizing and purifying sugar derivatives (e.g., UDP-sugars) can be difficult.[259]

4. IDENTIFICATION OF SITES OF GLYCOSYLATION

4.1. Overview

The complete characterization of a proteome includes identifying the locations and the structures of PTMs on proteins. Tandem MS has become an invaluable tool for this task, although considerable challenges exist for the unambiguous assignment of glycosylation sites and glycan structure.[260] This is particularly true in cases involving low protein abundance or low, substoichiometric site occupancy. Glycopeptides also have diverse ionization efficiencies, dependent on both the type of glycan and the type of mass spectrometer, affecting data quality and detectability by MS.[261,262] Adding to the experimental challenges, software tools for analyzing mass spectra of intact glycopeptides are limited, with few solutions for accurately identifying the site of modification and the glycan composition, and for providing confidence metrics for the assignments.[263]

In this section, we discuss how chemical techniques and MS can help identify sites of glycosylation in complex protein mixtures; for more information on characterizing glycan structures, see recent glycomics reviews.[28,69,88–91] In one approach, enzymatic or chemical deglycosylation methods are used to "mark" the glycosite, enabling indirect identification of the glycosite using traditional tandem MS techniques with the deglycosylated peptide. In another approach, advances in tandem MS fragmentation methods [e.g., electron-transfer dissociation (ETD)] are utilized for the direct analysis of intact glycopeptides. In the final approach, homogeneous and well-defined glycans are generated to reduce the computational challenges that typically accompany the analysis of large and heterogeneous glycopeptides.

In parallel with glycosite mapping and glycomics, there are techniques that characterize intact glycopeptides to provide both the site of modification and the glycan composition. These techniques leverage improvements in mass spectrometers and computational tools to simultaneously provide peptide and glycan sequence information. Although we discuss some studies using these techniques in the context of chemical and enzymatic methods, for more information on large-scale intact glycopeptide studies, see recent reviews by Thaysen-Andersen et al.[86,87] and Nilsson,[109] as well as recent work by Medzihradszky and co-workers.[264,265]

4.2. Enzymatic and Chemical Deglycosylation

Deglycosylation methods can improve glycosite mapping in a number of ways. First, after the glycan has been removed, peptides can be identified using standard tandem MS methods with collision-induced dissociation (CID). In the absence of deglycosylation, CID of an intact glycopeptide typically results in glycan cleavage and fragmentation, leaving the peptide intact. Although it is possible to optimize CID settings to improve the peptide backbone fragmentation of intact glycopeptides, a mixture of carbohydrate side-chain and peptide cleavage events typically occur.[78,266–269] With instruments that allow for nested fragmentation (i.e., MS^n), CID fragmentation of the peptide ion resulting after glycan loss

(i.e, MS[3]) can be performed to obtain peptide sequence information.[109,165,270] However, the decrease in fragmention abundance with sequential stages of tandem MS can limit this approach. Second, deglycosylation can improve the proteolytic digestion efficiency by increasing enzymatic access to the amino acid backbone. For these reasons, deglycosylation has become an important step in the identification of both N- and O-glycosites.

4.2.1. Deglycosylation Methods for N-Glycosylation—The most common method for mapping N-glycosites relies on proteomic analysis of enzymatically deglycosylated N-glycopeptides. PNGase F is an amidase that cleaves the entire glycan off an asparagine residue and simultaneously converts the residue into aspartic acid (Asp).[271] This enzymatic deamidation process marks the previously glycosylated Asn residue with a 0.98-Da mass increase, which can be detected using high-resolution tandem MS measurements (Figure 7a).[272] To improve the deglycosylation efficiency of N-terminal Asn residues, Weng et al. succinylated the N-terminus before PNGase F treatment.[273]

To further increase the confidence in N-glycosite assignment, Kaji et al.[274] developed a strategy based on stable isotope labeling.[275] The authors performed PNGase F deglycosylation in the presence of isotopically enriched ^{18}O-water, imparting a 2.98-Da mass increase following the Asn-to-Asp conversion. Termed isotope-coded glycosylation site-specific tagging, the method was used to identify N-glycosites in *Caenorhabditis elegans* and was the first strategy to produce a large-scale map of N-glycosites.[274] Similar strategies have been used to identify N-glycosites in other organisms, including mice[98] and *Trypanosoma cruzi*.[276] One limitation to this method is that the spontaneous deamination of nonglycosylated Asn residues during the course of PNGase F treatment can yield false positive identifications.[277–279]

To reduce the ambiguities in N-glycosite mapping that can accompany complete glycan removal, an alternative approach relies on trimming N-glycans to a single carbohydrate residue. For example, the enzyme endoglycosidase H (Endo H) preserves a single peptide-proximal N-linked GlcNAc residue after treatment (Figure 7a).[280,281] These truncated N-glycopeptides, like their completely deglycosylated counterparts, can be sequenced by tandem MS using standard CID conditions because the N-glycosidic bond is stable to CID fragmentation.[280] Unfortunately, the presence of a single monosaccharide can suppress ionization, potentially biasing data-dependent tandem MS data acquisition against glycopeptide ions.[261,262] Advances in targeted proteomics can be used to overcome this limitation (see section 5).

4.2.2. Deglycosylation Methods for O-Glycosylation—Unlike for N-glycans, there is an absence of universal enzymes that can globally deglycosylate all O-glycan structures. To circumvent this limitation, chemical deglycosylation strategies have been developed. One method relies on periodate oxidation of glycans followed by treatment with a base to sequentially eliminate carbohydrates until just the amino acid backbone remains.[282–284] Another approach, hydrazinolysis, utilizes anhydrous hydrazine to release either all glycans or O-glycans selectively, depending on the specific reaction conditions.[285] Another strategy uses anhydrous trifluoromethanesulfonic acid (TFMSA) for the partial or complete deglycosylation of either N- or O-glycans, respectively.[286] When TFMSA is used at 0 °C,

peripheral carbohydrates are eliminated, whereas the peptide-proximal monosaccharide is preserved (i.e., preservation of O-GalNAc for mucins or N-GlcNAc for N-glycans). However, when it is used at higher temperatures (25 °C or greater), complete deglycosylation of entire O-glycans is possible.[287] Typically, when TFMSA is used, scavengers such as anisole are added to the reaction mixture to minimize degradation of the peptide backbone.[287] Collectively, these chemical deglycosylation methods have primarily been used to identify glycosites on single, purified glycoproteins or a defined glycopeptide mixture; their compatibility with large-scale glycosite mapping studies remains to be demonstrated.

In contrast, β-elimination of O-glycans is one chemical deglycosylation method that has found its way into large-scale glycosite mapping studies. It relies on the base-mediated conversion of glycosylated Ser or Thr residues into dehydroalanine or dehydrobutyric acid, respectively (Figure 7b). The resulting change in the mass of the amino acid can be used to assign O-glycosites in tandem mass spectra.[288,289] Initially, this method was used to identify O-glycosites in simple mixtures of glycopeptides or in purified mucin-type glycoproteins such as MUC1.[290–292] The strategy was later modified by adding amine or thiol nucleophiles to react with the generated α,β-unsaturated carbonyl through a Michael addition. By simultaneously eliminating the glycan and replacing it with a chemical tag that could enable enrichment and was stable to CID fragmentation, this method was quickly applied to a number of glycosite mapping studies. At first, reagents that could act as both a base and a nucleophile were used, including methylamine, ethylamine, and dimethylamine. Later, a two-step variant was developed using sodium hydroxide as the base followed by dithiothreitol (DTT) as the nucleophile. Termed β-elimination followed by Michael addition with dithiothreitol (BEMAD),[293] the method was expanded to include a variety of nucleophiles, including butanethiol, aminoethanethiol, and biotin-pentylamine or biotin-cystamine for enrichment of the deglycosylated peptides (Figure 7b).[244,246,294,295] The BEMAD method has primarily been used to identify sites of O-GlcNAcylation in samples ranging in complexity from purified protein complexes and cellular organelles to mammalian whole-cell lysates.[231,248,262,296] By using isotopically light DTT (DTT-d_0) or heavy DTT (DTT-d_6) during the Michael addition step, differential isotopic labeling for comparative and quantitative analysis of glycosites is possible.[297] Hart and co-workers used this strategy to characterize changes in protein O-GlcNAcylation in cardiac mitochondria isolated from rats treated with or without Thiamet G, an inhibitor of O-GlcNAcylation.[298] Using a similar approach, Hart and co-workers explored the role of O-GlcNAcylation in the mitochondria of diabetic rat hearts.[299]

Despite the potential utility of chemical deglycosylation in glycosite mapping, this method suffers from a number of drawbacks. Most notably, the harsh conditions that are sometimes needed to promote deglycosylation (e.g., high concentrations of acids and bases at elevated temperatures) can affect the peptide backbone, leading to nonspecific cleavage and amino acid modifications. Additionally, because other O-linked PTMs are labile under acidic and basic conditions (e.g., phosphorylation under basic conditions), appropriate controls and careful sample preparation are necessary to correctly interpret the MS data.

4.3. Electron-Transfer Dissociation

Advances in MS fragmentation technology have improved the characterization of labile PTMs. These methods can be used to directly characterize intact glycopeptides, increasing the confidence of glycosite assignments. In particular, electron-capture dissociation (ECD)[300,301] and electron-transfer dissociation (ETD),[77,302,303] which do not use high collisional energies to fragment ions (as does CID), often preserve labile PTMs. Consequently, ECD and ETD preferentially fragment the peptide backbone of glycopeptides, enabling the identification of both the peptide and the site of modification.[78,304–307] Using both ECD and ETD, N- and O-glycosites have been identified in a number of biological samples.[64]

Although both ETD and ECD have been used for glycosite identification, ETD gained popularity after it was integrated with ion-trap mass spectrometers (e.g., LTQ-Orbitrap).[308] These instruments are low-cost alternatives to the Fourier transform ion cyclotron resonance mass spectrometers that are necessary for ECD fragmentation. In particular, ETD has emerged as the fragmentation method of choice for globally mapping sites of O-GlcNAcylation. When implemented, this method has required glycopeptide enrichment through the use of antibodies,[309] LWAC,[103,310–313] or chemoenzymatic methods,[245,249,250,262] followed by ETD-based tandem MS. ETD has also been used for O-GlcNAc glycosite mapping in *Campylobacter jejuni* whole-cell lysates,[314] in the murine synaptosome,[315] in mouse liver and brain tissue,[265] and in *Mycobacterium tuberculosis* culture filtrates.[316] Most recently, ETD was used to quantify the dynamics in O-GlcNAcylation sites after metabolically labeling HeLa cells with $^{13}C_6$-glucose (which was converted to ^{13}C-labeled UDP-GlcNAc).[317]

Compared to CID, precursor-ion fragmentation by ETD (and ECD) is less efficient and best suited for high charge state ions ($Z > 3$) with m/z values less than 1000.[318,319] Accordingly, samples that contain glycopeptides with trimmed glycans and that ionize as high charge state species are ideal for ETD analysis.[64,68] Analysis and interpretation of ETD mass spectra for glycopeptides is also challenging. Darula et al. demonstrated that the number of identified glycopeptides and glycosites could be more than doubled by reprocessing an ETD data set using a new version of Protein Prospector that implemented a modified scoring algorithm.[320,321] The authors also identified side reactions between sialic acid and common enrichment buffers, leading to additional complications during data analysis.[322] In some cases, an iterative data analysis strategy is necessary, where a range of glycan masses are considered as variable modifications on Asn, Ser, or Thr residues. Then, the most likely glycan structure(s) can be estimated based on the measured mass of the modification.[315]

4.4. Well-Defined and Homogeneous Glycans

Mucin-type O-glycosylation has been notoriously difficult to study by MS because of the heterogeneity in O-glycan structures and the high density of adjacent glycosylation sites. To reduce sample complexity, early attempts at O-glycosite mapping required a single, purified glycoprotein; this strategy was employed for MUC1, β-amyloid precursor protein, immunoglobin A1, and lubricin.[54,68,323–326] For complex mixtures, where the interpretation of mucin-type glycopeptide tandem mass spectra is challenged by a large diversity of glycan

structures, two general strategies have been used to address the issue: (1) lectins to enrich for mucin populations that express the same or similar glycans and (2) genetically engineered cell lines to produce homogeneous glycans on all mucins.

4.4.1. Well-Defined Glycans through Lectin Enrichment—Lectins have been used to enrich for proteins or peptides containing specific O-glycan structures. In a workflow developed by Medzihradszky and co-workers, the lectin Jacalin was used to enrich for O-glycopeptides from a bovine serum digest, and 26 O-glycosites were identified using ETD.[321,327] Subsequently, the same group improved the performance of this workflow by coupling additional chromatography steps after lectin enrichment. Using the new method, the authors identified 124 O-glycosites across 51 glycoproteins.[328] A similar lectin enrichment strategy was also employed by Bagdonaite et al. to identify O-glycosites on viral envelope proteins in herpes simplex virus type 1.[329]

4.4.2. Homogeneous Glycans through Genetic Engineering—Clausen and co-workers developed a genetic engineering strategy to identify and study O-glycosites in mucins (see recent review[330]). They generated a cell line, termed "SimpleCells", in which they silenced the chaperone protein cosmic and produced truncated but homogeneous O-glycans (either Tn or STn) on mucin-type glycoproteins.[331,332] Using lectin affinity chromatography and ETD fragmentation, the authors were able to enrich the truncated mucins and identify O-glycosites. Recently, they improved the enrichment protocol through prefractionation of samples with isoelectric focusing, proteolysis with chymotrypsin, and a two-stage LWAC enrichment step.[333]

Using the SimpleCell technology, Clausen and co-workers identified several thousand O-glycosites across a number of cell lines, including CHO cells and 15 human cancer cell lines (HEK293, HeLa, OVCAR-3, MCF-7, MDA-MB-231, T47D, IMR-32, K562, HepG2, HaCaT, COLO-205, Capan-1, AGS, MKN45, and T3M4).[331,334–338] Clausen and co-workers also used the SimpleCell technology to identify substrates for specific glycosyltransferases, focusing on ppGalNAcT isoforms. They performed a comparative glycoproteomics experiment between wild-type and ppGalNAcT2 knockout SimpleCells and identified 73 unique sites of glycosylation for the ppGalNAcT2 isoform.[339]

Beyond mucin-type O-glycosylation, SimpleCells have also been used to identify O-mannosylated glycoproteins. Through the silencing of both cosmic and the glycosyltransferase POMGnT1 in MDA-MB-231 cells, mucin glycans were truncated to a single GalNAc residue, and mannosylated glycans were truncated to a single mannose residue.[340] Using this strategy, Clausen and co-workers identified 235 O-Man glycosites, mapping to 51 glycoproteins. Recently, when this method was extended to study O-mannosylated glycoproteins in yeast, the authors discovered O-Man glycosites on nuclear, cytoplasmic, mitochondrial, and secreted proteins.[341,342]

Although SimpleCells eliminate knowledge about site-specific glycan composition (i.e., microheterogeneity), they provide a path toward large-scale O-glycoproteomics and O-glycosite mapping. The volume of data generated by these studies has already improved

computational models for predicting sites of O-glycosylation in a given protein sequence (e.g., NetOGlyc).[336]

5. TARGETED GLYCOPROTEOMICS

5.1. Overview

In targeted proteomics, the mass spectrometer is instructed to detect specific ions of interest, enabling high sensitivity, reproducibility, and quantitative accuracy.[343,344] Popular implementations include selected reaction monitoring (SRM)/multiple reaction monitoring (MRM) and parallel reaction monitoring (PRM).[345,346] This technology has enabled hypothesis-driven research in systems biology and clinical studies for biomarker discovery, among other applications.[347] Critical to the success of targeted proteomics is the development of repositories containing lists of target proteins, identification of proteotypic peptides, and characterization of a peptide's physical properties to facilitate MS-based measurements.[345] This effort has generated a number of public resources for targeted proteomics, including Skyline, the SRMAtlas, and the CPTAC Assay portal.[348–350] Recently, targeted proteomics was extended to glycoproteins through MRM-based quantitation of glycoforms,[351] PRM assays for N-glycosites,[352] and the introduction of an N-glycosite SRMAt-las.[353] The SRMAtlas was critical in the analysis of human breast tumor samples to identify potential N-glycoprotein biomarkers.[354] A related approach, relying on data-independent acquisition and targeted-data analysis [e.g., sequential window acquisition of all theoretical fragment ion spectra MS (SWATH-MS)], has also been applied to the identification of N-glycosites.[148] Using human tumor tissue samples, Liu et al. employed SWATH-MS to identify N-glycoprotein biomarkers that correlated with the level of aggressiveness of prostate cancer.[355] SWATH-MS has also been used to study N-glycosite micro- and macroheterogeneity in yeast cell wall glycoproteins.[356,357]

Inspired by the benefits of targeted proteomics, Bertozzi and co-workers developed a related technology based on chemically tagging proteins of interest.[358] Once tagged, peptides are detected and sequenced in a manner similar to, but distinct from, that employed in targeted proteomics. This strategy, generally termed chemically directed proteomics, has been used to identify both N- and O-glycosites and glycoproteins in both yeast and human cells.[359,360]

5.2. Chemically Directed Glycoproteomics

A common challenge in proteomics experiments is identifying low-abundance proteins in complex biological samples. This is especially true in studies of glycoproteins, where glycans can suppress ionization. To address this problem, Bertozzi and coworkers developed a chemically directed proteomics strategy for identifying low-abundance species in complex mixtures. The method was quickly extended to study glycoproteins and proved valuable in both resolving the site of glycosylation and sequencing complex, intact glycopeptides. Termed isotopic signature transfer and mass pattern prediction (IsoStamp), the technique exploits the perturbing effects of a dibromide motif on a peptide's isotopic envelope[358] and is an extension of isotopic distribution encoding tagging.[361] As illustrated in Figure 8a, the isotopic envelope of an average peptide changes significantly upon modification with two bromine atoms. Two bromine atoms produce a symmetrical triplet in a mass spectrum, with

major peaks at M, M + 2, and M + 4 at a relative intensity ratio of 1:2:1 due to the abundances of the $^{79}Br_2$, $^{79}Br^{81}Br$, and $^{81}Br_2$ isotopic pairings. The isotopic envelope of the dibromide-tagged peptide then reflects the parent peptide's intrinsic isotopic distribution convoluted with (or "recoded" by) the dibromide distribution.

In the first example of this technology, the dibromide motif was installed onto peptides using a dibrominated tyrosyl iodoacetamide analogue capable of alkylating cysteine residues (Figure 8b).[358] This labeling strategy was used in a proof-of-principle study to develop a computational pattern-searching algorithm capable of identifying isotopically recoded peptides in complex samples with high sensitivity and fidelity. Following pattern identification, the authors used a directed proteomics strategy to identify dibromide-labeled peptides, where inclusion lists containing m/z values and retention times of precursor ions bearing a recoded isotopic envelope were used to trigger tandem MS. Thus, the IsoStamp approach utilizes isotopic pattern rather than ion abundance to drive ion selection for fragmentation. This technique outperforms a traditional data-dependent acquisition strategy, particularly at low target-analyte concentrations.

Subsequently, Bertozzi and co-workers applied the IsoStamp technology to glycoproteins by introducing isotope-targeted glycoproteomics (IsoTaG).[359,362] This method involves metabolic labeling of cells with unnatural monosaccharide analogues, chemical tagging using a dibrominated affinity probe, and chemically directed proteomics to enable fragmentation and identification of glycopeptides. Importantly, IsoTaG is compatible with whole-proteome-scale profiling of intact N-and O-glycopeptides, providing information on both the peptide sequence and glycan structure. The authors used three cancer cell lines (Jurkat, PC-3, and MCF-7) and two unnatural monosaccharide analogues (Ac₄GalNAz and Ac₄ManNAz) to generate a diversity of cell lysate samples. The lysates were treated with a cleavable, dibrominated, biotin-alkyne probe (Figure 8c) that was used to enrich for glycoproteins using streptavidin-agarose beads. After peptides had been eluted by on-bead digestion, glycopeptides were separately enriched by treatment with formic acid to cleave the acid-labile silane linkage in the enrichment probe. Subsequently, peptides were identified using a traditional data-dependent acquisition strategy, and glycopeptides were analyzed using a modified version of the directed proteomics strategy described above. By leveraging the capabilities of an ion-trap mass spectrometer, the authors combined MS^2- and MS^3-level CID information to obtain both glycan and amino acid sequence information. Collectively, the authors identified 32 intact N-glycopeptides and over 500 intact O-glycopeptides from 250 glycoproteins, including multiple glycoforms for some glycopeptides. Recently, IsoTaG was also used to study the role of the glycosyltransferase GALNT3 in an epithelial ovarian cancer cell line.[363]

Although the isotopic signature of a halogenated chemical tag can effectively highlight labeled peptides or glycopeptides within a complex LC-MS data set, some biomolecules are not amenable to chemospecific modification. To address this limitation and expand the utility of the IsoStamp platform, Bertozzi and co-workers demonstrated that it was possible to recode a peptide's isotopic envelope without chemical tagging. Instead, a dibromide-like isotopic signature was embedded into glycans through metabolic labeling.[360] To achieve this effect, a stoichiometrically defined mixture of GlcNAc isotopologues, referred to as a

GlcNAc isomix (Figure 8d), was prepared by combining three GlcNAc isotopologues in a 1:2:1 molar ratio (i.e., to mimic the 1:2:1 dibromide intensity ratio). To evaluate the ability of the GlcNAc isomix to recode the peptide's isotopic envelope, the isomix was fed to a mutant yeast strain with an engineered salvage pathway for GlcNAc.[364] The isomix was metabolically installed into all yeast N-glycans by virtue of the fact that eukaryotic N-glycans contain a conserved GlcNAc-β1,4-GlcNAc disaccharide at their reducing end (see Figure 1a). After metabolic labeling, yeast lysates were prepared and digested with either trypsin or chymotrypsin, and glycopeptides were enriched using a modified version of FASP.[69,168] During the process, N-glycans were truncated to a single GlcNAc residue with Endo H, which has a preference for high-mannose-type oligosaccharides. This strategy preserved the isotopic signature of the GlcNAc isomix and the site of glycosylation. Glycopeptides bearing the isomix signature were subjected to a directed proteomics workflow in which precursor ions were selected for CID using a 4-Da isolation window (IW). A broad IW was used to preserve the isomix signature in fragment ions bearing the GlcNAc isomix modification, further increasing confidence in glycosite assignments. Using this strategy, the authors identified 133 N-glycosites spanning 58 glycoproteins within the yeast proteome, 50% of which were previously biochemically validated as N-glycoproteins.

Chemically directed glycoproteomics is a versatile platform for characterizing glycoproteins in complex biological samples. The IsoStamp technology enables the detection of low-abundance species and offers an enhanced level of confidence for mapping glycosites. With the introduction of IsoTaG, whole-proteome-scale profiling of intact N- and O-glycopeptides is feasible. At the same time, there are limitations to the technology. In particular, the isotopic tagging method dilutes the signal intensity of the parent ion across multiple isotope peaks.[358] Additionally, halogenated glycopeptides can have low ETD fragmentation efficiency, necessitating nested CID fragmentation (MS^2/MS^3) for glycopeptide sequencing.[359,362] However, with continued development, this strategy could be adapted to other chemistries and could be used to profile PTMs beyond glycosylation.

6. FUNCTIONAL GLYCOPROTEOMICS

6.1. Overview

An important aspect of connecting glycoproteins to cellular functions is building interaction networks that can delineate the connectivity between a glycoprotein and its binding partners. One approach to constructing these maps is to stabilize glycoprotein interactions with chemical cross-linking probes and then identify the interacting proteins with MS. Unfortunately, most chemical cross-linking probes target amino acids and ignore glycosylation-dependent interactions.[365] These interactions should also be studied in a physiologically relevant environment to capture biologically relevant proteins.[366] In this section, we discuss methods for studying glycoprotein interactions in a glycosylation-dependent manner and in the context of living cells.

6.2. Capturing Cell-Surface Glycoprotein Receptors

Wollscheid and co-workers developed a method for identifying the cell-surface glycoprotein receptor for a given protein ligand.[367] Termed ligand-based receptor-capture (LRC)

technology, this method builds on cell-surface capturing technology (see section 3.2.2).[155] The LRC technology uses a trifunctional chemoproteomics probe called TRICEPS (Figure 9a) that contains an *N*-hydroxysuccinimide ester for conjugation to a ligand protein, a trifluoroacetylated hydrazine for condensation with aldehydes introduced into glycans through oxidation, and a biotin moiety for affinity enrichment. After the TRICEPS probe was conjugated to a ligand of interest, it was incubated with sodium periodate-treated cells or tissue samples. Given that many cell-surface proteins are glycosylated, ligand–receptor interactions were stabilized through covalent capture between the hydrazine functionality of the TRICEPS probe and aldehydes introduced on oxidized glycans in cell-surface glycoproteins. After labeling, the cells were lysed, digested with trypsin, and the TRICEPS-modified glycopeptides were enriched with streptavidin beads. Following the release of N-glycopeptides by PNGase F treatment and analysis by quantitative MS, the enrichment of a receptor, relative to a negative control sample, indicated a true ligand–receptor interaction. A number of well-characterized ligands, including insulin, transferrin, apelin-17, epidermal growth factor, and the antibody trastuzumab, were conjugated to the TRICEPS probe, and in each case only the known cell-surface receptor was identified. The TRICEPS probe was used to identify the cell-surface receptors targeted by vaccinia virus. Collectively, the LRC technology provides a route to identify specific ligand–glycoprotein receptor interactions.

6.3. Identifying Glycan-Binding Proteins

Glycan-binding proteins (GBPs) are difficult to isolate using traditional enrichment techniques, because they typically have low binding affinities and the complexes they form can be short-lived.[368] One strategy to access these complexes relies on the formation of covalent interactions between the glycan and the binding protein using photo-cross-linking reagents.[369] Early work in this field focused on the synthesis of glycans, glycopeptides, and glycolipids containing both a photo-cross-linking group and an enrichment moiety (see recent reviews by Kohler and co-workers for more information).[24,376] These reagents were used to enrich and identify a variety of GBPs from cell culture media, including the asialoglycoprotein receptor, the adhesion factor BabA on *H. pylori*, and the B-subunit of cholera toxin.[370–372]

Another way to use photo-cross-linking reagents to identify GBPs is through metabolic incorporation of photo-cross-linker-functionalized monosaccharides (Figure 9b,c).[366,369] For example, two sialic acid analogues bearing an aryl azide photo-cross-linking group (Figure 9b), per-*O*-acetylated 5-aryl azide-*N*-acetylneruaminic acid (Ac_5-5-AAz-NeuNAc)[191] and per-*O*-acetylated 9-aryl azide-*N*-acetylneruaminic acid (Ac_4-9-AAz-NeuNAc),[190] were synthesized and metabolically incorporated into cellular glycans. To demonstrate the utility of these probes, Paulson and co-workers used Ac_4-9-AAz-NeuNAc to discover that CD22, a sialic acid-binding glycoprotein, forms homomultimeric complexes on the surface of B cells.[190] Paulson and co-workers also used Ac_4-9-AAz-NeuNAc to identify the trans ligands of CD22.[373] In this study, the authors incubated a CD22 fusion protein with B cells that were metabolically labeled with 9-AAz-NeuNAc and then used UV irradiation to stabilize CD22–glycoprotein complexes. Following immunoprecipitation of CD22 and quantitative proteomics, they identified 27 cell-surface glycoproteins. These results highlight the

potential of using photo-cross-linking reagents to identify glycan-dependent protein interactions.

In the design of unnatural monosaccharides as photo-cross-linking reagents, the size and position of the photo-cross-linking functional group are important considerations. For example, large photo-cross-linkers can reduce the efficiency of metabolic incorporation and impact glycan-epitope recognition.[374,375] To address these issues, Kohler and co-workers synthesized unnatural monosaccharides containing a diazirine, which is a small photoactivatable cross-linking functional group.[376] The authors introduced a diazirine into the *N*-acyl side chains of sialic acid and ManNAc, generating two unnatural monosaccharides (Figure 9c): per-*O*-acetylated 5-diazirine-*N*-acetylneruaminic acid (Ac$_5$-5-DAz-NeuNAc) and per-*O*-acetylated *N*-diazirine-acetylmannosamine (Ac$_4$ManNDAz).[377,378] Both Ac$_5$-5-DAz-NeuNAc and Ac$_4$ManNDAz were incorporated into cell-surface glycoproteins in the form of sialic acid and were used to study ganglioside–protein interactions.[379,380] Kohler and co-workers also used Ac$_4$ManNDAz to identify cell-surface ligands for cholera toxin on colonic epithelial cells, which led to the discovery of fucosylated glycoprotein ligands that might constitute the true ligands involved in cholera in humans.[381] To enable both cross-linking and enrichment, Chen and coworkers synthesized a bifunctional sialic acid derivative that contained an azide at the C9 position and a diazirine on the *N*-acyl side chain (Figure 9c): 5-diazirine-9-azido-*N*-acetylner-uaminic acid (5-DAz-9-Az-NeuNAc).[382] Using Daudi cells as a model system, the authors incorporated 5-DAz-9-Az-NeuNAc into cell-surface glycans, captured GBPs using UV irradiation, and enriched the complexes after conjugation to an affinity probe using the CuAAC reaction. To increase both the metabolic labeling and cross-linking efficiencies with these different sialic acid derivatives, the endogenous sialic acid residues can be enzymatically removed from cell-surface glycans.[383]

Beyond sialic acid and ManNAc derivatives, Kohler and coworkers also synthesized diazirine derivatives of GlcNAc (Figure 9d). The first analogue, per-*O*-acetylated *N*-diazirine-acetylglucosamine (Ac$_4$GlcNDAz), was not a compatible substrate for the GlcNAc salvage pathway.[384] To overcome this barrier, Kohler and co-workers synthesized a peracetylated diazirine-functionalized GlcNAc precursor with an *S*-acetyl-2-thioethyl- (Ac-SATE-) protected phosphate group [Ac$_3$GlcNDAz-1-P(Ac-SATE)]. The authors also mutated the enzyme AGX1, which converts GlcNAc-1-P into UDP-GlcNAc. Using this combination of synthetic chemistry and genetic engineering, they demonstrated that HeLa cells cultured with Ac$_3$GlcNDAz-1-P(Ac-SATE) produce O-GlcNDAz-modified proteins.[384] Subsequently, they employed structure-guided mutagenesis to enhance the efficiency of OGT with UDP-GlcNDAz as a substrate.[383] In the future, it will be exciting to see diazirine derivatives of GlcNAc used to globally profile GBPs.

Although photo-cross-linkable monosaccharides have been used in relatively few studies and in limited-scale proteomics or glycoproteomics experiments, their ability to capture glycan-dependent interactions in a native cellular environment offers a powerful tool for glycobiologists. In the future, a wide range of glycan-dependent interactions could be identified by expanding the types of monosaccharides functionalized with cross-linkers. Additionally, by improving the computational tools required to analyze glycoproteomics

data sets from cross-linked samples, it might become possible to identify the cross-linked sites within the GBP.

7. SUMMARY AND FUTURE OUTLOOK

Chemical tools have enabled tremendous growth in glycobiology, reducing experimental barriers that once restricted access to the study of protein glycosylation. In particular, chemical glycoproteomics has allowed the glycobiology community to enrich and identify glycoproteins and glycan structures associated with a number of biological processes. Continued technological advances are necessary to sustain this growth and exploration of glycoproteins and their biological relevance.

From the standpoint of basic research, it remains imperative that we strive toward a complete parts list for the human glycoproteome. We believe that it is a reasonable target that, within the next decade, the questions of which proteins are glycosylated at what sites with which structures, under specified conditions and with quantitative measurements, should be answerable. However, this state of knowledge will require accelerated advances in instrumentation; informatics; and the availability of reagents for binding, tagging, and enriching the various sectors of the glycoproteome. Thus, tool development remains central to the vision of a complete description of the human glycoproteome. As well, the tools that we now have on hand, powerful as they are, have not yet been democratized so that they can be wielded by nonexperts. For this reason, clinical translation of glycoproteomics is largely unrealized but constitutes an important and aspirational goal. Disease biomarkers are undoubtedly lurking in the glycoproteome, waiting to be discovered and exploited.

Important efforts that are well underway include technologies for the complete structural assignment of intact and complex glycopeptides, simultaneously resolving both the amino acid and glycan sequences at the proteome scale. New tandem MS technologies could allow techniques such as ETD to become more sensitive and routine. Ion-mobility MS offers an additional level of separation based on a molecule's collisional cross section, which affects its rate of migration in the gas phase.[385] This can be used to analyze complex glycopeptide mixtures, resolving identical peptides with different sites of glycosylation as well as isomeric glycan structures.[386–390] MS technologies are improving exponentially, and challenges in glycoproteomics should help direct that growth. Likewise, bioinformatics platforms capable of extracting glycosites and structures from complex MS^n data sets must keep up with new experimental technologies. (For more information, see the recent review by Hu et al.[391]) Commercial analysis tools such as Byonic (Protein Metrics)[392] and SimGlycan (Premier Biosoft);[393] several open source and academic analysis tools;[391,394] and database tools such as GlycoMod, Glyco-SuiteDB,[395] and UniCarbKB[396,397] are already having an impact in this regard. As well, big data studies correlating disease-associated mutations and genetic variations with PTMs are emerging and should help frame studies of the glycoproteome.[398,399]

Another exciting goal is the integration of spatial information into glycoproteomics data sets. Although MS is capable of identifying thousands of proteins in a given sample with high accuracy and reproducibility, their spatiotemporal relationships are typically lost in

such experiments. Imaging techniques are typically employed for spatial and temporal analysis of molecules in cells and tissues and are widely employed for protein and, more recently, glycan analysis.[400,401] However, image analysis of specific protein glycoforms remains a significant challenge. Several laboratories have combined oligosaccharide metabolic labeling with protein labeling to image protein glycoforms using FRET-based microscopies.[402–406] In addition, protein-specific aptamers have been used to direct selective labeling to nearby glycans.[407] These methods have yet to be scaled beyond a single protein target, but with multiplexable platforms such as MALDI imaging MS,[408,409] secondary-ion MS,[410] and mass cytometry (CyTOF),[411–413] there could be a rich future for the expansion of spatially resolved MS across the glycoproteome.

Acknowledgments

References

1. Cravatt BF, Simon GM, Yates JR III. The Biological Impact of Mass-Spectrometry-Based Proteomics. Nature. 2007; 450:991–1000. [PubMed: 18075578]

2. Moellering RE, Cravatt BF. How Chemoproteomics Can Enable Drug Discovery and Development. Chem Biol. 2012; 19:11–22. [PubMed: 22284350]

3. Sabido E, Selevsek N, Aebersold R. Mass Spectrometry-Based Proteomics for Systems Biology. Curr Opin Biotechnol. 2012; 23:591–597. [PubMed: 22169889]

4. Beck M, Claassen M, Aebersold R. Comprehensive Proteomics. Curr Opin Biotechnol. 2011; 22:3–8. [PubMed: 20888217]

5. Yates JR 3rd. The Revolution and Evolution of Shotgun Proteomics for Large-Scale Proteome Analysis. J Am Chem Soc. 2013; 135:1629–1640. [PubMed: 23294060]

6. Collins BC, Gillet LC, Rosenberger G, Rost HL, Vichalkovski A, Gstaiger M, Aebersold R. Quantifying Protein Interaction Dynamics by SWATH Mass Spectrometry: Application to the 14–3-3 System. Nat Methods. 2013; 10:1246–1253. [PubMed: 24162925]

7. Domon B, Aebersold R. Mass Spectrometry and Protein Analysis. Science. 2006; 312:212–217. [PubMed: 16614208]

8. Han X, Aslanian A, Yates JR 3rd. Mass Spectrometry for Proteomics. Curr Opin Chem Biol. 2008; 12:483–490. [PubMed: 18718552]

9. Aebersold R, Mann M. Mass Spectrometry-Based Proteomics. Nature. 2003; 422:198–207. [PubMed: 12634793]

10. Steen H, Mann M. The Abc's (and Xyz's) of Peptide Sequencing. Nat Rev Mol Cell Biol. 2004; 5:699–711. [PubMed: 15340378]

11. Eng JK, Searle BC, Clauser KR, Tabb DL. A Face in the Crowd: Recognizing Peptides through Database Search. Mol Cell Proteomics. 2011; 10:R111.009522.

12. Witze ES, Old WM, Resing KA, Ahn NG. Mapping Protein Post-Translational Modifications with Mass Spectrometry. Nat Methods. 2007; 4:798–806. [PubMed: 17901869]

13. Jensen ON. Interpreting the Protein Language Using Proteomics. Nat Rev Mol Cell Biol. 2006; 7:391–403. [PubMed: 16723975]

14. Sanman LE, Bogyo M. Activity-Based Profiling of Proteases. Annu Rev Biochem. 2014; 83:249–273. [PubMed: 24905783]

15. Olsen JV, Mann M. Status of Large-Scale Analysis of Post-Translational Modifications by Mass Spectrometry. Mol Cell Proteomics. 2013; 12:3444–3452. [PubMed: 24187339]

16. Heal WP, Tate EW. Getting a Chemical Handle on Protein Post-Translational Modification. Org Biomol Chem. 2010; 8:731–738. [PubMed: 20135026]

17. Tate EW. Recent Advances in Chemical Proteomics: Exploring the Post-Translational Proteome. J Chem Biol. 2008; 1:17–26. [PubMed: 19568795]

18. Chuh KN, Pratt MR. Chemical Methods for the Proteome-Wide Identification of Posttranslationally Modified Proteins. Curr Opin Chem Biol. 2015; 24:27–37. [PubMed: 25461721]

19. Chuh KN, Batt AR, Pratt MR. Chemical Methods for Encoding and Decoding of Posttranslational Modifications. Cell Chem Biol. 2016; 23:86–107. [PubMed: 26933738]

20. Bertozzi CR, Kiessling LL. Chemical Glycobiology. Science. 2001; 291:2357–2364. [PubMed: 11269316]

21. Prescher JA, Bertozzi CR. Chemical Technologies for Probing Glycans. Cell. 2006; 126:851–854. [PubMed: 16959565]

22. Kiessling LL, Splain RA. Chemical Approaches to Glycobiology. Annu Rev Biochem. 2010; 79:619–653. [PubMed: 20380561]

23. Rakus JF, Mahal LK. New Technologies for Glycomic Analysis: Toward a Systematic Understanding of the Glycome. Annu Rev Anal Chem. 2011; 4:367–392.

24. Whitman, CM., Bond, MR., Kohler, JJ. Chemical glycobiology. In: Liu, H-W., editor. Comprehensive Natural Products II: Chemistry and Biology. Elsevier; Oxford, U.K: 2010. p. 175-224.

25. Khoury GA, Baliban RC, Floudas CA. Proteome-Wide Post-Translational Modification Statistics: Frequency Analysis and Curation of the Swiss-Prot Database. Sci Rep. 2011; 1:90.

26. Stanley P. Golgi Glycosylation. Cold Spring Harbor Perspect Biol. 2011; 3:a005199.

27. Hang HC, Bertozzi CR. The Chemistry and Biology of Mucin-Type O-Linked Glycosylation. Bioorg Med Chem. 2005; 13:5021–5034. [PubMed: 16005634]

28. Marino K, Bones J, Kattla JJ, Rudd PM. A Systematic Approach to Protein Glycosylation Analysis: A Path through the Maze. Nat Chem Biol. 2010; 6:713–723. [PubMed: 20852609]

29. Raman R, Raguram S, Venkataraman G, Paulson JC, Sasisekharan R. Glycomics: An Integrated Systems Approach to Structure-Function Relationships of Glycans. Nat Methods. 2005; 2:817–824. [PubMed: 16278650]

30. Helenius A, Aebi M. Intracellular Functions of N-Linked Glycans. Science. 2001; 291:2364–2369. [PubMed: 11269317]

31. Rudd PM, Elliott T, Cresswell P, Wilson IA, Dwek RA. Glycosylation and the Immune System. Science. 2001; 291:2370–2376. [PubMed: 11269318]

32. Varki, A., Lowe, JB. Biological Roles of Glycans. In: Varki, A.Cummings, RD.Esko, JD.Freeze, HH.Stanley, P.Bertozzi, CR.Hart, GW., Etzler, ME., editors. Essentials of Glycobiology. 2. Vol. Chapter 6. Cold Spring Harbor Laboratory Press; Cold Spring Harbor, NY: 2009.

33. Woods RJ, Edge CJ, Dwek RA. Protein Surface Oligosaccharides and Protein Function. Nat Struct Biol. 1994; 1:499–501. [PubMed: 7664073]

34. Tabak LA. The Role of Mucin-Type O-Glycans in Eukaryotic Development. Semin Cell Dev Biol. 2010; 21:616–621. [PubMed: 20144722]

35. Haltiwanger RS, Lowe JB. Role of Glycosylation in Development. Annu Rev Biochem. 2004; 73:491–537. [PubMed: 15189151]

36. Ohtsubo K, Marth JD. Glycosylation in Cellular Mechanisms of Health and Disease. Cell. 2006; 126:855–867. [PubMed: 16959566]

37. Dube DH, Bertozzi CR. Glycans in Cancer and Inflammation–Potential for Therapeutics and Diagnostics. Nat Rev Drug Discovery. 2005; 4:477–488. [PubMed: 15931257]

38. Drake PM, Cho W, Li B, Prakobphol A, Johansen E, Anderson NL, Regnier FE, Gibson BW, Fisher SJ. Sweetening the Pot: Adding Glycosylation to the Biomarker Discovery Equation. Clin Chem. 2010; 56:223–236. [PubMed: 19959616]

39. Pinho SS, Reis CA. Glycosylation in Cancer: Mechanisms and Clinical Implications. Nat Rev Cancer. 2015; 15:540–555. [PubMed: 26289314]

40. Kim EH, Misek DE. Glycoproteomics-Based Identification of Cancer Biomarkers. Int J Proteomics. 2011; 2011:601937. [PubMed: 22084691]

41. Gabius HJ. Glycobiomarkers by Glycoproteomics and Glycan Profiling (Glycomics): Emergence of Functionality. Biochem Soc Trans. 2011; 39:399–405. [PubMed: 21265812]

42. Pan S, Wang Y, Quinn JF, Peskind ER, Waichunas D, Wimberger JT, Jin J, Li JG, Zhu D, Pan C, Zhang J. Identification of Glycoproteins in Human Cerebrospinal Fluid with a Complementary Proteomic Approach. J Proteome Res. 2006; 5:2769–2779. [PubMed: 17022648]

43. Ludwig JA, Weinstein JN. Biomarkers in Cancer Staging, Prognosis and Treatment Selection. Nat Rev Cancer. 2005; 5:845–856. [PubMed: 16239904]

44. Rini, J., Esko, J., Varki, A. Glycosyltransferases and Glycan-Processing Enzymes. In: Varki, A.Cummings, RD.Esko, JD.Freeze, HH.Stanley, P.Bertozzi, CR.Hart, GW., Etzler, ME., editors. Essentials of Glycobiology. 2. Vol. Chapter 5. Cold Spring Harbor Laboratory Press; Cold Spring Harbor, NY: 2009.

45. Varki, A., Esko, JD., Colley, KJ. Cellular Organization of Glycosylation. In: Varki, A.Cummings, RD.Esko, JD.Freeze, HH.Stanley, P.Bertozzi, CR.Hart, GW., Etzler, ME., editors. Essentials of Glycobiology. 2. Vol. Chapter 3. Cold Spring Harbor Laboratory Press; Cold Spring Harbor, NY: 2009.

46. Hofsteenge J, Muller DR, de Beer T, Loffler A, Richter WJ, Vliegenthart JF. New Type of Linkage between a Carbohydrate and a Protein: C-Glycosylation of a Specific Tryptophan Residue in Human Rnase Us. Biochemistry. 1994; 33:13524–13530. [PubMed: 7947762]

47. Jensen PH, Kolarich D, Packer NH. Mucin-Type O-Glycosylation–Putting the Pieces Together. FEBS J. 2010; 277:81–94. [PubMed: 19919547]

48. Burda P, Aebi M. The Dolichol Pathway of N-Linked Glycosylation. Biochim Biophys Acta, Gen Subj. 1999; 1426:239–257.

49. Lafite P, Daniellou R. Rare and Unusual Glycosylation of Peptides and Proteins. Nat Prod Rep. 2012; 29:729–738. [PubMed: 22511359]

50. Weerapana E, Imperiali B. Asparagine-Linked Protein Glycosylation: From Eukaryotic to Prokaryotic Systems. Glycobiology. 2006; 16:91R–101R.

51. Kukuruzinska MA, Lennon K. Protein N-Glycosylation: Molecular Genetics and Functional Significance. Crit Rev Oral Biol Med. 1998; 9:415–448. [PubMed: 9825220]

52. Thaysen-Andersen M, Packer NH. Site-Specific Glycoproteomics Confirms That Protein Structure Dictates Formation of N- Glycan Type, Core Fucosylation and Branching. Glycobiology. 2012; 22:1440–1452. [PubMed: 22798492]

53. Yamauchi M, Sricholpech M. Lysine Post-Translational Modifications of Collagen. Essays Biochem. 2012; 52:113–133. [PubMed: 22708567]

54. Halim A, Brinkmalm G, Ruetschi U, Westman-Brinkmalm A, Portelius E, Zetterberg H, Blennow K, Larson G, Nilsson J. Site-Specific Characterization of Threonine, Serine, and Tyrosine Glycosylations of Amyloid Precursor Protein/Amyloid Beta-Peptides in Human Cerebrospinal Fluid. Proc Natl Acad Sci U S A. 2011; 108:11848–11853. [PubMed: 21712440]

55. Zarschler K, Janesch B, Pabst M, Altmann F, Messner P, Schaffer C. Protein Tyrosine O-Glycosylation–a Rather Unexplored Prokaryotic Glycosylation System. Glycobiology. 2010; 20:787–798. [PubMed: 20200052]

56. Hart GW, Housley MP, Slawson C. Cycling of O-Linked β-N-Acetylglucosamine on Nucleocytoplasmic Proteins. Nature. 2007; 446:1017–1022. [PubMed: 17460662]

57. Love DC, Hanover JA. The Hexosamine Signaling Pathway: Deciphering the "O-GlcNAc Code". Sci Signaling. 2005; 2005:re13.

58. Banerjee PS, Hart GW, Cho JW. Chemical Approaches to Study O-GlcNAcylation. Chem Soc Rev. 2013; 42:4345–4357. [PubMed: 23247267]

59. Wang Z, Gucek M, Hart GW. Cross-Talk between GlcNAcylation and Phosphorylation: Site-Specific Phosphorylation Dynamics in Response to Globally Elevated O-GlcNAc. Proc Natl Acad Sci U S A. 2008; 105:13793–13798. [PubMed: 18779572]

60. Hart GW. Minireview Series on the Thirtieth Anniversary of Research on O-GlcNAcylation of Nuclear and Cytoplasmic Proteins: Nutrient Regulation of Cellular Metabolism and Physiology by O-GlcNAcylation. J Biol Chem. 2014; 289:34422–34423. [PubMed: 25336646]

61. Mulloy, B., Hart, GW., Stanley, P. Structural Analysis of Glycans. In: Varki, A.Cummings, RD.Esko, JD.Freeze, HH.Stanley, P.Bertozzi, CR.Hart, GW., Etzler, ME., editors. Essentials of

Glycobiology. 2. Vol. Chapter 47. Cold Spring Harbor Laboratory Press; Cold Spring Harbor, NY: 2009.

62. Morelle W, Canis K, Chirat F, Faid V, Michalski JC. The Use of Mass Spectrometry for the Proteomic Analysis of Glycosylation. Proteomics. 2006; 6:3993–4015. [PubMed: 16786490]

63. Budnik BA, Lee RS, Steen JA. Global Methods for Protein Glycosylation Analysis by Mass Spectrometry. Biochim Biophys Acta, Proteins Proteomics. 2006; 1764:1870–1880.

64. Wuhrer M, Catalina MI, Deelder AM, Hokke CH. Glycoproteomics Based on Tandem Mass Spectrometry of Glycopep-tides. J Chromatogr B: Anal Technol Biomed Life Sci. 2007; 849:115–128.

65. Wei X, Li L. Comparative Glycoproteomics: Approaches and Applications. Briefings Funct Genomics Proteomics. 2009; 8:104–113.

66. Pan S, Chen R, Aebersold R, Brentnall TA. Mass Spectrometry Based Glycoproteomics–from a Proteomics Perspective. Mol Cell Proteomics. 2011; 10:R110.003251.

67. Zhang Y, Yin H, Lu H. Recent Progress in Quantitative Glycoproteomics. Glycoconjugate J. 2012; 29:249–258.

68. Nilsson J, Halim A, Grahn A, Larson G. Targeting the Glycoproteome. Glycoconjugate J. 2013; 30:119–136.

69. Novotny MV, Alley WR Jr, Mann BF. Analytical Glycobiology at High Sensitivity: Current Approaches Directions. Glycoconjugate J. 2013; 30:89–117.

70. Lai ZW, Nice EC, Schilling O. Glycocapture-Based Proteomics for Secretome Analysis. Proteomics. 2013; 13:512–525. [PubMed: 23197367]

71. Lazar IM, Deng J, Ikenishi F, Lazar AC. Exploring the Glycoproteomics Landscape with Advanced MS Technologies. Electrophoresis. 2015; 36:225–237. [PubMed: 25311661]

72. Ahn YH, Kim JY, Yoo JS. Quantitative Mass Spectrometric Analysis of Glycoproteins Combined with Enrichment Methods. Mass Spectrom Rev. 2015; 34:148–165. [PubMed: 24889823]

73. McDonald WH, Yates JR III. Shotgun Proteomics: Integrating Technologies to Answer Biological Questions. Curr Opin Mol Ther. 2003; 5:302–309. [PubMed: 12870441]

74. Zhang Y, Fonslow BR, Shan B, Baek MC, Yates JR 3rd. Protein Analysis by Shotgun/Bottom-up Proteomics. Chem Rev. 2013; 113:2343–2394. [PubMed: 23438204]

75. Eng JK, McCormack AL, Yates JR 3rd. An Approach to Correlate Tandem Mass Spectral Data of Peptides with Amino Acid Sequences in a Protein Database. J Am Soc Mass Spectrom. 1994; 5:976–989. [PubMed: 24226387]

76. Bruce, C., Stone, K., Gulcicek, E., Williams, K. Current Protocols in Bioinformatics. Vol. Chapter 13. John Wiley & Sons, Inc; New York: 2013. Proteomics and the Analysis of Proteomic Data: 2013 Overview of Current Protein-Profiling Technologies.

77. Wiesner J, Premsler T, Sickmann A. Application of Electron Transfer Dissociation (Etd) for the Analysis of Posttranslational Modifications. Proteomics. 2008; 8:4466–4483. [PubMed: 18972526]

78. Mechref, Y. Current Protocols in Protein Science. Vol. Chapter 12. John Wiley & Sons, Inc; New York: 2012. Use of CID/ETD Mass Spectrometry to Analyze Glycopeptides.

79. Anderson NL, Anderson NG. The Human Plasma Proteome: History, Character, and Diagnostic Prospects. Mol Cell Proteomics. 2002; 1:845–867. [PubMed: 12488461]

80. Picotti P, Aebersold R, Domon B. The Implications of Proteolytic Background for Shotgun Proteomics. Mol Cell Proteomics. 2007; 6:1589–1598. [PubMed: 17533221]

81. Tissot B, North SJ, Ceroni A, Pang PC, Panico M, Rosati F, Capone A, Haslam SM, Dell A, Morris HR. Glycoproteomics: Past, Present and Future. FEBS Lett. 2009; 583:1728–1735. [PubMed: 19328791]

82. Dallas DC, Martin WF, Hua S, German JB. Automated Glycopeptide Analysis–Review of Current State and Future Directions. Briefings Bioinf. 2013; 14:361–374.

83. Li F, Glinskii OV, Glinsky VV. Glycobioinformatics: Current Strategies and Tools for Data Mining in MS-Based Glycoproteomics. Proteomics. 2013; 13:341–354. [PubMed: 23175233]

84. Leymarie N, Griffin PJ, Jonscher K, Kolarich D, Orlando R, McComb M, Zaia J, Aguilan J, Alley WR, Altmann F, Ball LE, Basumallick L, Bazemore-Walker CR, Behnken H, Blank MA, Brown

KJ, Bunz SC, Cairo CW, Cipollo JF, Daneshfar R, Desaire H, Drake RR, Go EP, Goldman R, Gruber C, Halim A, Hathout Y, Hensbergen PJ, Horn DM, Hurum D, Jabs W, Larson G, Ly M, Mann BF, Marx K, Mechref Y, Meyer B, Moginger U, Neususs C, Nilsson J, Novotny MV, Nyalwidhe JO, Packer NH, Pompach P, Reiz B, Resemann A, Rohrer JS, Ruthenbeck A, Sanda M, Schulz JM, Schweiger-Hufnagel U, Sihlbom C, Song E, Staples GO, Suckau D, Tang H, Thaysen-Andersen M, Viner RI, An Y, Valmu L, Wada Y, Watson M, Windwarder M, Whittal R, Wuhrer M, Zhu Y, Zou C. Interlaboratory Study on Differential Analysis of Protein Glycosylation by Mass Spectrometry: The Abrf Glycoprotein Research Multi-Institutional Study 2012. Mol Cell Proteomics. 2013; 12:2935–2951. [PubMed: 23764502]

85. Chandler KB, Costello CE. Glycomics and Glycoproteomics of Membrane Proteins and Cell-Surface Receptors: Present Trends and Future Opportunities. Electrophoresis. 2016; 37:1407–1419. [PubMed: 26872045]

86. Thaysen-Andersen M, Packer NH. Advances in LC-MS/MS-Based Glycoproteomics: Getting Closer to System-Wide Site-Specific Mapping of the N- and O-Glycoproteome. Biochim Biophys Acta, Proteins Proteomics. 2014; 1844:1437–1452.

87. Thaysen-Andersen M, Packer NH, Schulz BL. Maturing Glycoproteomics Technologies Provide Unique Structural Insights into the N-Glycoproteome and Its Regulation in Health and Disease. Mol Cell Proteomics. 2016; 15:1773–1790. [PubMed: 26929216]

88. An HJ, Froehlich JW, Lebrilla CB. Determination of Glycosylation Sites and Site-Specific Heterogeneity in Glycoproteins. Curr Opin Chem Biol. 2009; 13:421–426. [PubMed: 19700364]

89. Dreyfuss JM, Jacobs C, Gindin Y, Benson G, Staples GO, Zaia J. Targeted Analysis of Glycomics Liquid Chromatography/Mass Spectrometry Data. Anal Bioanal Chem. 2011; 399:727–735. [PubMed: 20953780]

90. Bielik AM, Zaia J. Historical Overview of Glycoanalysis. Methods Mol Biol. 2010; 600:9–30. [PubMed: 19882118]

91. Ruhaak LR, Miyamoto S, Lebrilla CB. Developments in the Identification of Glycan Biomarkers for the Detection of Cancer. Mol Cell Proteomics. 2013; 12:846–855. [PubMed: 23365456]

92. Abbott KL, Pierce JM. Lectin-Based Glycoproteomic Techniques for the Enrichment and Identification of Potential Biomarkers. Methods Enzymol. 2010; 480:461–476. [PubMed: 20816222]

93. Fanayan S, Hincapie M, Hancock WS. Using Lectins to Harvest the Plasma/Serum Glycoproteome. Electrophoresis. 2012; 33:1746–1754. [PubMed: 22740463]

94. Tang H, Hsueh P, Kletter D, Bern M, Haab B. The Detection and Discovery of Glycan Motifs in Biological Samples Using Lectins and Antibodies: New Methods and Opportunities. Adv Cancer Res. 2015; 126:167–202. [PubMed: 25727148]

95. Haab BB. Using Lectins in Biomarker Research: Addressing the Limitations of Sensitivity and Availability. Proteomics: Clin Appl. 2012; 6:346–350. [PubMed: 22927350]

96. Cummings, RD., Etzler, ME. R-Type Lectins. In: Varki, A.Cummings, RD.Esko, JD.Freeze, HH.Stanley, P.Bertozzi, CR.Hart, GW., Etzler, ME., editors. Essentials of Glycobiology. 2. Vol. Chapter 28. Cold Spring Harbor Laboratory Press; Cold Spring Harbor, NY: 2009.

97. Drake PM, Schilling B, Niles RK, Braten M, Johansen E, Liu H, Lerch M, Sorensen DJ, Li B, Allen S, Hall SC, Witkowska HE, Regnier FE, Gibson BW, Fisher SJ. A Lectin Affinity Workflow Targeting Glycosite-Specific, Cancer-Related Carbohydrate Structures in Trypsin-Digested Human Plasma. Anal Biochem. 2011; 408:71–85. [PubMed: 20705048]

98. Zielinska DF, Gnad F, Wisniewski JR, Mann M. Precision Mapping of an in Vivo N-Glycoproteome Reveals Rigid Topological and Sequence Constraints. Cell. 2010; 141:897–907. [PubMed: 20510933]

99. Zielinska DF, Gnad F, Schropp K, Wisniewski JR, Mann M. Mapping N-Glycosylation Sites across Seven Evolutionarily Distant Species Reveals a Divergent Substrate Proteome Despite a Common Core Machinery. Mol Cell. 2012; 46:542–548. [PubMed: 22633491]

100. Deeb SJ, Cox J, Schmidt-Supprian M, Mann M. N-Linked Glycosylation Enrichment for in-Depth Cell Surface Proteomics of Diffuse Large B-Cell Lymphoma Subtypes. Mol Cell Proteomics. 2014; 13:240–251. [PubMed: 24190977]

101. Durham M, Regnier FE. Targeted Glycoproteomics: Serial Lectin Affinity Chromatography in the Selection of O-Glycosylation Sites on Proteins from the Human Blood Proteome. J Chromatogr. 2006; 1132:165–173.

102. Roquemore EP, Chou TY, Hart GW. Detection of O-Linked N-Acetylglucosamine (O-GlcNAc) on Cytoplasmic and Nuclear Proteins. Methods Enzymol. 1994; 230:443–460. [PubMed: 8139512]

103. Vosseller K, Trinidad JC, Chalkley RJ, Specht CG, Thalhammer A, Lynn AJ, Snedecor JO, Guan S, Medzihradszky KF, Maltby DA, Schoepfer R, Burlingame AL. O-Linked N-Acetylglucosamine Proteomics of Postsynaptic Density Preparations Using Lectin Weak Affinity Chromatography and Mass Spectrometry. Mol Cell Proteomics. 2006; 5:923–934. [PubMed: 16452088]

104. Ma ZY, Skorobogatko Y, Vosseller K. Tandem Lectin Weak Affinity Chromatography for Glycoprotein Enrichment. Methods Mol Biol. 2013; 951:21–31. [PubMed: 23296521]

105. Li Y, Wen T, Zhu M, Li L, Wei J, Wu X, Guo M, Liu S, Zhao H, Xia S, Huang W, Wang P, Wu Z, Zhao L, Shui W, Li Z, Yin Z. Glycoproteomic Analysis of Tissues from Patients with Colon Cancer Using Lectin Microarrays and Nanolc-MS/MS. Mol BioSyst. 2013; 9:1877–1887. [PubMed: 23567825]

106. Teo CF, Ingale S, Wolfert MA, Elsayed GA, Not LG, Chatham JC, Wells L, Boons GJ. Glycopeptide-Specific Monoclonal Antibodies Suggest New Roles for O-GlcNAc. Nat Chem Biol. 2010; 6:338–343. [PubMed: 20305658]

107. Zachara NE, Vosseller K, Hart GW. Detection and Analysis of Proteins Modified by O-Linked N-Acetylglucosamine John Wiley & Sons, Inc. Current protocols in protein science. 2011; Chapter 12:1–18.

108. Fujiki R, Hashiba W, Sekine H, Yokoyama A, Chikanishi T, Ito S, Imai Y, Kim J, He HH, Igarashi K, Kanno J, Ohtake F, Kitagawa H, Roeder RG, Brown M, Kato S. GlcNAcylation of Histone H2B Facilitates Its Monoubiquitination. Nature. 2011; 480:557–560. [PubMed: 22121020]

109. Nilsson J. Liquid Chromatography-Tandem Mass Spectrometry-Based Fragmentation Analysis of Glycopeptides. Glycoconjugate J. 2016; 33:261–272.

110. Song E, Mechref Y. Defining Glycoprotein Cancer Biomarkers by MS in Conjunction with Glycoprotein Enrichment. Biomarkers Med. 2015; 9:835–844.

111. Zauner G, Deelder AM, Wuhrer M. Recent Advances in Hydrophilic Interaction Liquid Chromatography (HILIC) for Structural Glycomics. Electrophoresis. 2011; 32:3456–3466. [PubMed: 22180202]

112. Hagglund P, Bunkenborg J, Elortza F, Jensen ON, Roepstorff P. A New Strategy for Identification of N-Glycosylated Proteins and Unambiguous Assignment of Their Glycosylation Sites Using HILIC Enrichment and Partial Deglycosylation. J Proteome Res. 2004; 3:556–566. [PubMed: 15253437]

113. Mysling S, Palmisano G, Hojrup P, Thaysen-Andersen M. Utilizing Ion-Pairing Hydrophilic Interaction Chromatography Solid Phase Extraction for Efficient Glycopeptide Enrichment in Glycoproteomics. Anal Chem. 2010; 82:5598–5609. [PubMed: 20536156]

114. Jensen PH, Mysling S, Hojrup P, Jensen ON. Glycopeptide Enrichment for MALDI-TOF Mass Spectrometry Analysis by Hydrophilic Interaction Liquid Chromatography Solid Phase Extraction (HILIC SPE). Methods Mol Biol. 2013; 951:131–144. [PubMed: 23296529]

115. Palmisano G, Lendal SE, Engholm-Keller K, Leth-Larsen R, Parker BL, Larsen MR. Selective Enrichment of Sialic Acid-Containing Glycopeptides Using Titanium Dioxide Chromatography with Analysis by HILIC and Mass Spectrometry. Nat Protoc. 2010; 5:1974–1982. [PubMed: 21127490]

116. Bull SD, Davidson MG, van den Elsen JM, Fossey JS, Jenkins AT, Jiang YB, Kubo Y, Marken F, Sakurai K, Zhao J, James TD. Exploiting the Reversible Covalent Bonding of Boronic Acids: Recognition, Sensing, and Assembly. Acc Chem Res. 2013; 46:312–326. [PubMed: 23148559]

117. Leitner A, Lindner W. Current Chemical Tagging Strategies for Proteome Analysis by Mass Spectrometry. J Chromatogr B: Anal Technol Biomed Life Sci. 2004; 813:1–26.

118. Beatty KE. Chemical Strategies for Tagging and Imaging the Proteome. Mol BioSyst. 2011; 7:2360–2367. [PubMed: 21691667]

119. Gygi SP, Rist B, Gerber SA, Turecek F, Gelb MH, Aebersold R. Quantitative Analysis of Complex Protein Mixtures Using Isotope-Coded Affinity Tags. Nat Biotechnol. 1999; 17:994–999. [PubMed: 10504701]

120. Ross PL, Huang YLN, Marchese JN, Williamson B, Parker K, Hattan S, Khainovski N, Pillai S, Dey S, Daniels S, Purkayastha S, Juhasz P, Martin S, Bartlet-Jones M, He F, Jacobson A, Pappin DJ. Multiplexed Protein Quantitation in *Saccharomyces cerevisiae* Using Amine-Reactive Isobaric Tagging Reagents. Mol Cell Proteomics. 2004; 3:1154–1169. [PubMed: 15385600]

121. Willems LI, van der Linden WA, Li N, Li KY, Liu N, Hoogendoorn S, van der Marel GA, Florea BI, Overkleeft HS. Bioorthogonal Chemistry: Applications in Activity-Based Protein Profiling. Acc Chem Res. 2011; 44:718–729. [PubMed: 21797256]

122. Heal WP, Dang TH, Tate EW. Activity-Based Probes: Discovering New Biology and New Drug Targets. Chem Soc Rev. 2011; 40:246–257. [PubMed: 20886146]

123. Jessani N, Cravatt BF. The Development and Application of Methods for Activity-Based Protein Profiling. Curr Opin Chem Biol. 2004; 8:54–59. [PubMed: 15036157]

124. Uttamchandani M, Li J, Sun H, Yao SQ. Activity-Based Protein Profiling: New Developments and Directions in Functional Proteomics. ChemBioChem. 2008; 9:667–675. [PubMed: 18283695]

125. Grammel M, Hang HC. Chemical Reporters for Biological Discovery. Nat Chem Biol. 2013; 9:475–484. [PubMed: 23868317]

126. Sletten EM, Bertozzi CR. Bioorthogonal Chemistry: Fishing for Selectivity in a Sea of Functionality. Angew Chem, Int Ed. 2009; 48:6974–6998.

127. Prescher JA, Bertozzi CR. Chemistry in Living Systems. Nat Chem Biol. 2005; 1:13–21. [PubMed: 16407987]

128. Ramil CP, Lin Q. Bioorthogonal Chemistry: Strategies and Recent Developments. Chem Commun (Cambridge, U K). 2013; 49:11007–11022.

129. Patterson DM, Prescher JA. Orthogonal Bioorthogonal Chemistries. Curr Opin Chem Biol. 2015; 28:141–149. [PubMed: 26276062]

130. Debets MF, van Hest JC, Rutjes FP. Bioorthogonal Labelling of Biomolecules: New Functional Handles and Ligation Methods. Org Biomol Chem. 2013; 11:6439–6455. [PubMed: 23969529]

131. King M, Wagner A. Developments in the Field of Bioorthogonal Bond Forming Reactions-Past and Present Trends. Bioconjugate Chem. 2014; 25:825–839.

132. Rawn JD, Lienhard GE. The Binding of Boronic Acids to Chymotrypsin. Biochemistry. 1974; 13:3124–3130. [PubMed: 4858346]

133. Siegel D. Applications of Reversible Covalent Chemistry in Analytical Sample Preparation. Analyst. 2012; 137:5457–5482. [PubMed: 23013801]

134. Sparbier K, Koch S, Kessler I, Wenzel T, Kostrzewa M. Selective Isolation of Glycoproteins and Glycopeptides for MALDI-TOF MS Detection Supported by Magnetic Particles. J Biomol Technol. 2005; 16:407–413.

135. Xu YW, Wu ZX, Zhang LJ, Lu HJ, Yang PY, Webley PA, Zhao DY. Highly Specific Enrichment of Glycopeptides Using Boronic Acid-Functionalized Mesoporous Silica. Anal Chem. 2009; 81:503–508. [PubMed: 19117470]

136. Sparbier K, Wenzel T, Kostrzewa M. Exploring the Binding Profiles of ConA, Boronic Acid and WGA by MALDI-TOF/TOF MS and Magnetic Particles. J Chromatogr B: Anal Technol Biomed Life Sci. 2006; 840:29–36.

137. Xu Y, Bailey UM, Punyadeera C, Schulz BL. Identification of Salivary N-Glycoproteins and Measurement of Glycosylation Site Occupancy by Boronate Glycoprotein Enrichment and Liquid Chromatography/Electrospray Ionization Tandem Mass Spectrometry. Rapid Commun Mass Spectrom. 2014; 28:471–482. [PubMed: 24497285]

138. Chen W, Smeekens JM, Wu R. A Universal Chemical Enrichment Method for Mapping the Yeast N-Glycoproteome by Mass Spectrometry (MS). Mol Cell Proteomics. 2014; 13:1563–1572. [PubMed: 24692641]

139. Lu YW, Chien CW, Lin PC, Huang LD, Chen CY, Wu SW, Han CL, Khoo KH, Lin CC, Chen YJ. BAD-Lectins: Boronic Acid-Decorated Lectins with Enhanced Binding Affinity for the Selective Enrichment of Glycoproteins. Anal Chem. 2013; 85:8268–8276. [PubMed: 23895469]

140. Zhang H, Li XJ, Martin DB, Aebersold R. Identification and Quantification of N-Linked Glycoproteins Using Hydrazide Chemistry, Stable Isotope Labeling and Mass Spectrometry. Nat Biotechnol. 2003; 21:660–666. [PubMed: 12754519]

141. Tian Y, Zhou Y, Elliott S, Aebersold R, Zhang H. Solid-Phase Extraction of N-Linked Glycopeptides. Nat Protoc. 2007; 2:334–339. [PubMed: 17406594]

142. Liu T, Qian WJ, Gritsenko MA, Camp DG 2nd, Monroe ME, Moore RJ, Smith RD. Human Plasma N-Glycoproteome Analysis by Immunoaffinity Subtraction, Hydrazide Chemistry, and Mass Spectrometry. J Proteome Res. 2005; 4:2070–2080. [PubMed: 16335952]

143. Sun B, Ranish JA, Utleg AG, White JT, Yan X, Lin B, Hood L. Shotgun Glycopeptide Capture Approach Coupled with Mass Spectrometry for Comprehensive Glycoproteomics. Mol Cell Proteomics. 2007; 6:141–149. [PubMed: 17074749]

144. Zhou Y, Aebersold R, Zhang H. Isolation of N-Linked Glycopeptides from Plasma. Anal Chem. 2007; 79:5826–5837. [PubMed: 17591751]

145. Danzer C, Eckhardt K, Schmidt A, Fankhauser N, Ribrioux S, Wollscheid B, Muller L, Schiess R, Zullig R, Lehmann R, Spinas G, Aebersold R, Krek W. Comprehensive Description of the N-Glycoproteome of Mouse Pancreatic β-Cells and Human Islets. J Proteome Res. 2012; 11:1598–1608. [PubMed: 22148984]

146. Lewandrowski U, Moebius J, Walter U, Sickmann A. Elucidation of N-Glycosylation Sites on Human Platelet Proteins: A Glycoproteomic Approach. Mol Cell Proteomics. 2006; 5:226–233. [PubMed: 16263699]

147. Ramachandran P, Boontheung P, Xie Y, Sondej M, Wong DT, Loo JA. Identification of N-Linked Glycoproteins in Human Saliva by Glycoprotein Capture and Mass Spectrometry. J Proteome Res. 2006; 5:1493–1503. [PubMed: 16740002]

148. Liu Y, Huttenhain R, Surinova S, Gillet LC, Mouritsen J, Brunner R, Navarro P, Aebersold R. Quantitative Measurements of N-Linked Glycoproteins in Human Plasma by SWATH-MS. Proteomics. 2013; 13:1247–1256. [PubMed: 23322582]

149. Zhang H, Yi EC, Li XJ, Mallick P, Kelly-Spratt KS, Masselon CD, Camp DG, Smith RD, Kemp CJ, Aebersold R. High Throughput Quantitative Analysis of Serum Proteins Using Glycopeptide Capture and Liquid Chromatography Mass Spectrometry. Mol Cell Proteomics. 2005; 4:144–155. [PubMed: 15608340]

150. Sun B, Ma L, Yan X, Lee D, Alexander V, Hohmann LJ, Lorang C, Chandrasena L, Tian Q, Hood L. N-Glycoproteome of E14. Tg2a Mouse Embryonic Stem Cells. PLoS One. 2013; 8:e55722. [PubMed: 23405203]

151. Shah P, Wang X, Yang W, Toghi Eshghi S, Sun S, Hoti N, Chen L, Yang S, Pasay J, Rubin A, Zhang H. Integrated Proteomic and Glycoproteomic Analyses of Prostate Cancer Cells Reveal Glycoprotein Alteration in Protein Abundance and Glyco-sylation. Mol Cell Proteomics. 2015; 14:2753–2763. [PubMed: 26256267]

152. Huang J, Wan H, Yao Y, Li J, Cheng K, Mao J, Chen J, Wang Y, Qin H, Zhang W, Ye M, Zou H. Highly Efficient Release of Glycopeptides from Hydrazide Beads by Hydroxylamine Assisted Pngase F Deglycosylation for N-Glycoproteome Analysis. Anal Chem. 2015; 87:10199–10204. [PubMed: 26399494]

153. Zhang Z, Sun D, Cong Y, Mao J, Huang J, Qin H, Liu J, Huang G, Wang L, Ye M, Zou H. Amine Chemistry Method for Selective Enrichment of N-Linked Glycopeptides for Glycoproteomics Analysis. J Proteome Res. 2015; 14:3892–3899. [PubMed: 26257013]

154. Huang J, Qin H, Sun Z, Huang G, Mao J, Cheng K, Zhang Z, Wan H, Yao Y, Dong J, Zhu J, Wang F, Ye M, Zou H. A Peptide N-Terminal Protection Strategy for Comprehensive Glycoproteome Analysis Using Hydrazide Chemistry Based Method. Sci Rep. 2015; 5:10164. [PubMed: 25959593]

155. Wollscheid B, Bausch-Fluck D, Henderson C, O'Brien R, Bibel M, Schiess R, Aebersold R, Watts JD. Mass-Spectrometric Identification and Relative Quantification of N-Linked Cell Surface Glycoproteins. Nat Biotechnol. 2009; 27:378–386. [PubMed: 19349973]

156. Gundry RL, Raginski K, Tarasova Y, Tchernyshyov I, Bausch-Fluck D, Elliott ST, Boheler KR, Van Eyk JE, Wollscheid B. The Mouse C2c12 Myoblast Cell Surface N-Linked Glycoproteome:

Identification, Glycosite Occupancy, and Membrane Orientation. Mol Cell Proteomics. 2009; 8:2555–2569. [PubMed: 19656770]

157. Gundry RL, Riordon DR, Tarasova Y, Chuppa S, Bhattacharya S, Juhasz O, Wiedemeier O, Milanovich S, Noto FK, Tchernyshyov I, Raginski K, Bausch-Fluck D, Tae HJ, Marshall S, Duncan SA, Wollscheid B, Wersto RP, Rao S, Van Eyk JE, Boheler KR. A Cell Surfaceome Map for Immunophenotyping and Sorting Pluripotent Stem Cells. Mol Cell Proteomics. 2012; 11:303–316. [PubMed: 22493178]

158. Bausch-Fluck D, Hofmann A, Wollscheid B. Cell Surface Capturing Technologies for the Surfaceome Discovery of Hepatocytes. Methods Mol Biol. 2012; 909:1–16. [PubMed: 22903705]

159. Hofmann A, Gerrits B, Schmidt A, Bock T, Bausch-Fluck D, Aebersold R, Wollscheid B. Proteomic Cell Surface Phenotyping of Differentiating Acute Myeloid Leukemia Cells. Blood. 2010; 116:e26–e34. [PubMed: 20570859]

160. Schiess R, Mueller LN, Schmidt A, Mueller M, Wollscheid B, Aebersold R. Analysis of Cell Surface Proteome Changes via Label-Free, Quantitative Mass Spectrometry. Mol Cell Proteomics. 2009; 8:624–638. [PubMed: 19036722]

161. Boheler KR, Bhattacharya S, Kropp EM, Chuppa S, Riordon DR, Bausch-Fluck D, Burridge PW, Wu JC, Wersto RP, Chan GC, Rao S, Wollscheid B, Gundry RL. A Human Pluripotent Stem Cell Surface N-Glycoproteome Resource Reveals Markers, Extracellular Epitopes, and Drug Targets. Stem Cell Rep. 2014; 3:185–203.

162. Sun Z, Dong J, Zhang S, Hu Z, Cheng K, Li K, Xu B, Ye M, Nie Y, Fan D, Zou H. Identification of Chemoresistance-Related Cell-Surface Glycoproteins in Leukemia Cells and Functional Validation of Candidate Glycoproteins. J Proteome Res. 2014; 13:1593–1601. [PubMed: 24467213]

163. Tyleckova J, Valekova I, Zizkova M, Rakocyova M, Marsala S, Marsala M, Gadher SJ, Kovarova H. Surface N-Glycoproteome Patterns Reveal Key Proteins of Neuronal Differentiation. J Proteomics. 2016; 132:13–20. [PubMed: 26581640]

164. Bausch-Fluck D, Hofmann A, Bock T, Frei AP, Cerciello F, Jacobs A, Moest H, Omasits U, Gundry RL, Yoon C, Schiess R, Schmidt A, Mirkowska P, Hartlova A, Van Eyk JE, Bourquin JP, Aebersold R, Boheler KR, Zandstra P, Wollscheid B. A Mass Spectrometric-Derived Cell Surface Protein Atlas. PLoS One. 2015; 10:e0121314. [PubMed: 25894527]

165. Nilsson J, Ruetschi U, Halim A, Hesse C, Carlsohn E, Brinkmalm G, Larson G. Enrichment of Glycopeptides for Glycan Structure and Attachment Site Identification. Nat Methods. 2009; 6:809–811. [PubMed: 19838169]

166. Halim A, Nilsson J, Ruetschi U, Hesse C, Larson G. Human Urinary Glycoproteomics; Attachment Site Specific Analysis of N- and O-Linked Glycosylations by CID and ECD. Mol Cell Proteomics. 2012; 11:M111.013649.

167. Kurogochi M, Matsushista T, Amano M, Furukawa J, Shinohara Y, Aoshima M, Nishimura S. Sialic Acid-Focused Quantitative Mouse Serum Glycoproteomics by Multiple Reaction Monitoring Assay. Mol Cell Proteomics. 2010; 9:2354–2368. [PubMed: 20571061]

168. Zeng Y, Ramya TN, Dirksen A, Dawson PE, Paulson JC. High-Efficiency Labeling of Sialylated Glycoproteins on Living Cells. Nat Methods. 2009; 6:207–209. [PubMed: 19234450]

169. Ramya TN, Weerapana E, Cravatt BF, Paulson JC. Glycoproteomics Enabled by Tagging Sialic Acid or Galactose Terminated Glycans. Glycobiology. 2013; 23:211–221. [PubMed: 23070960]

170. Klement E, Lipinszki Z, Kupihar Z, Udvardy A, Medzihradszky KF. Enrichment of O-GlcNAc Modified Proteins by the Periodate Oxidation-Hydrazide Resin Capture Approach. J Proteome Res. 2010; 9:2200–2206. [PubMed: 20146544]

171. Taga Y, Kusubata M, Ogawa-Goto K, Hattori S. Development of a Novel Method for Analyzing Collagen O-Glycosylations by Hydrazide Chemistry. Mol Cell Proteomics. 2012; 11:M111.010397.

172. Rannes JB, Ioannou A, Willies SC, Grogan G, Behrens C, Flitsch SL, Turner NJ. Glycoprotein Labeling Using Engineered Variants of Galactose Oxidase Obtained by Directed Evolution. J Am Chem Soc. 2011; 133:8436–8439. [PubMed: 21526835]

173. McCombs JE, Kohler JJ. Pneumococcal Neuraminidase Substrates Identified through Comparative Proteomics Enabled by Chemoselective Labeling. Bioconjugate Chem. 2016; 27:1013–1022.

174. Bond MR, Kohler JJ. Chemical Methods for Glycoprotein Discovery. Curr Opin Chem Biol. 2007; 11:52–58. [PubMed: 17174139]

175. Campbell CT, Sampathkumar SG, Yarema KJ. Metabolic Oligosaccharide Engineering: Perspectives, Applications, and Future Directions. Mol BioSyst. 2007; 3:187–194. [PubMed: 17308665]

176. Rouhanifard SH, Nordstrom LU, Zheng T, Wu P. Chemical Probing of Glycans in Cells and Organisms. Chem Soc Rev. 2013; 42:4284. [PubMed: 23257905]

177. Nischan N, Kohler JJ. Advances in Cell Surface Glycoengineering Reveal Biological Function. Glycobiology. 2016; 26:789–796. [PubMed: 27066802]

178. Kayser H, Zeitler R, Kannicht C, Grunow D, Nuck R, Reutter W. Biosynthesis of a Nonphysiological Sialic Acid in Different Rat Organs, Using *N*-Propanoyl-D-hexosamines as Precursors. J Biol Chem. 1992; 267:16934–16938. [PubMed: 1512235]

179. Keppler OT, Horstkorte R, Pawlita M, Schmidt C, Reutter W. Biochemical Engineering of the N-Acyl Side Chain of Sialic Acid: Biological Implications. Glycobiology. 2001; 11:11R–18R. [PubMed: 11181557]

180. Saxon E, Bertozzi CR. Cell Surface Engineering by a Modified Staudinger Reaction. Science. 2000; 287:2007–2010. [PubMed: 10720325]

181. Mahal LK, Yarema KJ, Bertozzi CR. Engineering Chemical Reactivity on Cell Surfaces through Oligosaccharide Biosynthesis. Science. 1997; 276:1125–1128. [PubMed: 9173543]

182. Laughlin ST, Baskin JM, Amacher SL, Bertozzi CR. In Vivo Imaging of Membrane-Associated Glycans in Developing Zebrafish. Science. 2008; 320:664–667. [PubMed: 18451302]

183. Prescher JA, Dube DH, Bertozzi CR. Chemical Remodelling of Cell Surfaces in Living Animals. Nature. 2004; 430:873–877. [PubMed: 15318217]

184. Chang PV, Chen X, Smyrniotis C, Xenakis A, Hu T, Bertozzi CR, Wu P. Metabolic Labeling of Sialic Acids in Living Animals with Alkynyl Sugars. Angew Chem, Int Ed. 2009; 48:4030–4033.

185. Rostovtsev VV, Green LG, Fokin VV, Sharpless KB. A Stepwise Huisgen Cycloaddition Process: Copper(I)-Catalyzed Regioselective "Ligation" of Azides and Terminal Alkynes. Angew Chem, Int Ed. 2002; 41:2596–2599.

186. Tornoe CW, Christensen C, Meldal M. Peptidotriazoles on Solid Phase: [1,2,3]-Triazoles by Regiospecific Copper(I)-Catalyzed 1,3-Dipolar Cycloadditions of Terminal Alkynes to Azides. J Org Chem. 2002; 67:3057–3064. [PubMed: 11975567]

187. Agard NJ, Baskin JM, Prescher JA, Lo A, Bertozzi CR. A Comparative Study of Bioorthogonal Reactions with Azides. ACS Chem Biol. 2006; 1:644–648. [PubMed: 17175580]

188. Agard NJ, Prescher JA, Bertozzi CR. A Strain-Promoted [3 + 2] Azide-Alkyne Cycloaddition for Covalent Modification of Biomolecules in Living Systems. J Am Chem Soc. 2004; 126:15046–15047. [PubMed: 15547999]

189. Hsu TL, Hanson SR, Kishikawa K, Wang SK, Sawa M, Wong CH. Alkynyl Sugar Analogs for the Labeling and Visualization of Glycoconjugates in Cells. Proc Natl Acad Sci U S A. 2007; 104:2614–2619. [PubMed: 17296930]

190. Han S, Collins BE, Bengtson P, Paulson JC. Homomultimeric Complexes of Cd22 in B Cells Revealed by Protein-Glycan Cross-Linking. Nat Chem Biol. 2005; 1:93–97. [PubMed: 16408005]

191. Luchansky SJ, Goon S, Bertozzi CR. Expanding the Diversity of Unnatural Cell-Surface Sialic Acids. ChemBioChem. 2004; 5:371–374. [PubMed: 14997530]

192. Hang HC, Yu C, Kato DL, Bertozzi CR. A Metabolic Labeling Approach toward Proteomic Analysis of Mucin-Type O-Linked Glycosylation. Proc Natl Acad Sci U S A. 2003; 100:14846–14851. [PubMed: 14657396]

193. Boyce M, Carrico IS, Ganguli AS, Yu SH, Hangauer MJ, Hubbard SC, Kohler JJ, Bertozzi CR. Metabolic CrossTalk Allows Labeling of O-Linked *β-N*-Acetylglucosamine-Modified Proteins via the *N*-Acetylgalactosamine Salvage Pathway. Proc Natl Acad Sci U S A. 2011; 108:3141–3146. [PubMed: 21300897]

194. Dube DH, Prescher JA, Quang CN, Bertozzi CR. Probing Mucin-Type O-Linked Glycosylation in Living Animals. Proc Natl Acad Sci U S A. 2006; 103:4819–4824. [PubMed: 16549800]

195. Rabuka D, Hubbard SC, Laughlin ST, Argade SP, Bertozzi CR. A Chemical Reporter Strategy to Probe Glycoprotein Fucosylation. J Am Chem Soc. 2006; 128:12078–12079. [PubMed: 16967952]

196. Sawa M, Hsu TL, Itoh T, Sugiyama M, Hanson SR, Vogt PK, Wong CH. Glycoproteomic Probes for Fluorescent Imaging of Fucosylated Glycans in Vivo. Proc Natl Acad Sci U S A. 2006; 103:12371–12376. [PubMed: 16895981]

197. Vocadlo DJ, Hang HC, Kim EJ, Hanover JA, Bertozzi CR. A Chemical Approach for Identifying O-GlcNAc-Modified Proteins in Cells. Proc Natl Acad Sci U S A. 2003; 100:9116–9121. [PubMed: 12874386]

198. Zaro BW, Yang YY, Hang HC, Pratt MR. Chemical Reporters for Fluorescent Detection and Identification of O-GlcNAc-Modified Proteins Reveal Glycosylation of the Ubiquitin Ligase Nedd4–1. Proc Natl Acad Sci U S A. 2011; 108:8146–8151. [PubMed: 21540332]

199. Gurcel C, Vercoutter-Edouart AS, Fonbonne C, Mortuaire M, Salvador A, Michalski JC, Lemoine J. Identification of New O-GlcNAc Modified Proteins Using a Click-Chemistry-Based Tagging. Anal Bioanal Chem. 2008; 390:2089–2097. [PubMed: 18369606]

200. Sarkar AK, Fritz TA, Taylor WH, Esko JD. Disaccharide Uptake and Priming in Animal Cells: Inhibition of Sialyl Lewis X by Acetylated Gal β1 → 4GlcNAcβ-O-Naphthalenemethanol. Proc Natl Acad Sci U S A. 1995; 92:3323–3327. [PubMed: 7724561]

201. Varki A. Sialic Acids in Human Health and Disease. Trends Mol Med. 2008; 14:351–360. [PubMed: 18606570]

202. Cohen M, Varki A. The Sialome–Far More Than the Sum of Its Parts. OMICS. 2010; 14:455–464. [PubMed: 20726801]

203. Hanson SR, Hsu TL, Weerapana E, Kishikawa K, Simon GM, Cravatt BF, Wong CH. Tailored Glycoproteomics and Glycan Site Mapping Using Saccharide-Selective Bioorthogonal Probes. J Am Chem Soc. 2007; 129:7266–7267. [PubMed: 17506567]

204. Yang L, Nyalwidhe JO, Guo S, Drake RR, Semmes OJ. Targeted Identification of Metastasis-Associated Cell-Surface Sialoglycoproteins in Prostate Cancer. Mol Cell Proteomics. 2011; 10:M110.007294.

205. Pan PW, Zhang Q, Hou J, Liu Z, Bai F, Cao MR, Sun T, Bai G. Cell Surface Glycoprotein Profiling of Cancer Cells Based on Bioorthogonal Chemistry. Anal Bioanal Chem. 2012; 403:1661–1670. [PubMed: 22526661]

206. Roper SM, Zemskova M, Neely BA, Martin A, Gao P, Jones EE, Kraft AS, Drake RR. Targeted Glycoprotein Enrichment and Identification in Stromal Cell Secretomes Using Azido Sugar Metabolic Labeling. Proteomics: Clin Appl. 2013; 7:367–371. [PubMed: 23687070]

207. Rong J, Han J, Dong L, Tan Y, Yang H, Feng L, Wang QW, Meng R, Zhao J, Wang SQ, Chen X. Glycan Imaging in Intact Rat Hearts and Glycoproteomic Analysis Reveal the Upregulation of Sialylation During Cardiac Hypertrophy. J Am Chem Soc. 2014; 136:17468–17476. [PubMed: 25313651]

208. Kuhn PH, Koroniak K, Hogl S, Colombo A, Zeitschel U, Willem M, Volbracht C, Schepers U, Imhof A, Hoffmeister A, Haass C, Rossner S, Brase S, Lichtenthaler SF. Secretome Protein Enrichment Identifies Physiological Bace1 Protease Substrates in Neurons. EMBO J. 2012; 31:3157–3168. [PubMed: 22728825]

209. Haun RS, Quick CM, Siegel ER, Raju I, Mackintosh SG, Tackett AJ. Bioorthogonal Labeling Cell-Surface Proteins Expressed in Pancreatic Cancer Cells to Identify Potential Diagnostic/Therapeutic Biomarkers. Cancer Biol Ther. 2015; 16:1557–1565. [PubMed: 26176765]

210. Autelitano F, Loyaux D, Roudieres S, Deon C, Guette F, Fabre P, Ping Q, Wang S, Auvergne R, Badarinarayana V, Smith M, Guillemot JC, Goldman SA, Natesan S, Ferrara P, August P. Identification of Novel Tumor-Associated Cell Surface Sialoglycoproteins in Human Glioblastoma Tumors Using Quantitative Proteomics. PLoS One. 2014; 9:e110316. [PubMed: 25360666]

211. Delcourt N, Quevedo C, Nonne C, Fons P, O'Brien D, Loyaux D, Diez M, Autelitano F, Guillemot JC, Ferrara P, Muriana A, Callol C, Herault JP, Herbert JM, Favre G, Bono F. Targeted

Identification of Sialoglycoproteins in Hypoxic Endothelial Cells and Validation in Zebrafish Reveal Roles for Proteins in Angiogenesis. J Biol Chem. 2015; 290:3405–3417. [PubMed: 25384978]

212. Tian Y, Almaraz RT, Choi CH, Li QK, Saeui C, Li D, Shah P, Bhattacharya R, Yarema KJ, Zhang H. Identification of Sialylated Glycoproteins from Metabolically Oligosaccharide Engineered Pancreatic Cells. Clin Proteomics. 2015; 12:11. [PubMed: 25987888]

213. Xie R, Dong L, Huang R, Hong S, Lei R, Chen X. Targeted Imaging and Proteomic Analysis of Tumor-Associated Glycans in Living Animals. Angew Chem, Int Ed. 2014; 53:14082–14086.

214. Xie R, Hong S, Feng L, Rong J, Chen X. Cell-Selective Metabolic Glycan Labeling Based on Ligand-Targeted Liposomes. J Am Chem Soc. 2012; 134:9914–9917. [PubMed: 22646989]

215. Xie R, Dong L, Du Y, Zhu Y, Hua R, Zhang C, Chen X. In Vivo Metabolic Labeling of Sialoglycans in the Mouse Brain by Using a Liposome-Assisted Bioorthogonal Reporter Strategy. Proc Natl Acad Sci U S A. 2016; 113:5173–5178. [PubMed: 27125855]

216. Becker DJ, Lowe JB. Fucose: Biosynthesis and Biological Function in Mammals. Glycobiology. 2003; 13:41R–53R.

217. Okajima T, Irvine KD. Regulation of Notch Signaling by O-Linked Fucose. Cell. 2002; 111:893–904. [PubMed: 12526814]

218. Harvey BM, Rana NA, Moss H, Leonardi J, Jafar-Nejad H, Haltiwanger RS. Mapping Sites of O-Glycosylation and Fringe Elongation on Drosophila Notch. J Biol Chem. 2016; 291:16348–16360. [PubMed: 27268051]

219. Okeley NM, Alley SC, Anderson ME, Boursalian TE, Burke PJ, Emmerton KM, Jeffrey SC, Klussman K, Law CL, Sussman D, Toki BE, Westendorf L, Zeng W, Zhang X, Benjamin DR, Senter PD. Development of Orally Active Inhibitors of Protein and Cellular Fucosylation. Proc Natl Acad Sci U S A. 2013; 110:5404–5409. [PubMed: 23493549]

220. Liu TW, Kaji H, Togayachi A, Ito H, Sato T, Narimatsu H. A Chemoenzymatic Approach toward the Identification of Fucosylated Glycoproteins and Mapping of N-Glycan Sites. Glycobiology. 2012; 22:630–637. [PubMed: 22203233]

221. Al-Shareffi E, Chaubard JL, Leonhard-Melief C, Wang SK, Wong CH, Haltiwanger RS. 6-Alkynyl Fucose Is a Bioorthogonal Analog for O-Fucosylation of Epidermal Growth Factor-Like Repeats and Thrombospondin Type-1 Repeats by Protein O-Fucosyltransferases 1 and 2. Glycobiology. 2013; 23:188–198. [PubMed: 23045360]

222. Hubbard SC, Boyce M, McVaugh CT, Peehl DM, Bertozzi CR. Cell Surface Glycoproteomic Analysis of Prostate Cancer-Derived Pc-3 Cells. Bioorg Med Chem Lett. 2011; 21:4945–4950. [PubMed: 21798741]

223. Slade PG, Hajivandi M, Bartel CM, Gorfien SF. Identifying the Cho Secretome Using Mucin-Type O-Linked Glycosylation and Click-Chemistry. J Proteome Res. 2012; 11:6175–6186. [PubMed: 23140450]

224. Smeekens JM, Chen W, Wu R. Mass Spectrometric Analysis of the Cell Surface N-Glycoproteome by Combining Metabolic Labeling and Click Chemistry. J Am Soc Mass Spectrom. 2015; 26:604–614. [PubMed: 25425172]

225. Xiao H, Tang GX, Wu R. Site-Specific Quantification of Surface N-Glycoproteins in Statin-Treated Liver Cells. Anal Chem. 2016; 88:3324–3332. [PubMed: 26894747]

226. Unlu M, Morgan ME, Minden JS. Difference Gel Electrophoresis: A Single Gel Method for Detecting Changes in Protein Extracts. Electrophoresis. 1997; 18:2071–2077. [PubMed: 9420172]

227. Osterman IA, Ustinov AV, Evdokimov DV, Korshun VA, Sergiev PV, Serebryakova MV, Demina IA, Galyamina MA, Govorun VM, Dontsova OA. A Nascent Proteome Study Combining Click Chemistry with 2de. Proteomics. 2013; 13:17–21. [PubMed: 23161590]

228. Palaniappan KK, Hangauer MJ, Smith TJ, Smart BP, Pitcher AA, Cheng EH, Bertozzi CR, Boyce M. A Chemical Glycoproteomics Platform Reveals O-GlcNAcylation of Mitochondrial Voltage-Dependent Anion Channel 2. Cell Rep. 2013; 5:546–552. [PubMed: 24120863]

229. Sprung R, Nandi A, Chen Y, Kim SC, Barma D, Falck JR, Zhao Y. Tagging-via-Substrate Strategy for Probing O-GlcNAc Modified Proteins. J Proteome Res. 2005; 4:950–957. [PubMed: 15952742]

230. Nandi A, Sprung R, Barma DK, Zhao Y, Kim SC, Falck JR, Zhao Y. Global Identification of O-GlcNAc-Modified Proteins. Anal Chem. 2006; 78:452–458. [PubMed: 16408927]

231. Hahne H, Sobotzki N, Nyberg T, Helm D, Borodkin VS, van Aalten DM, Agnew B, Kuster B. Proteome Wide Purification and Identification of O-GlcNAc-Modified Proteins Using Click Chemistry and Mass Spectrometry. J Proteome Res. 2013; 12:927–936. [PubMed: 23301498]

232. Gurel Z, Zaro BW, Pratt MR, Sheibani N. Identification of O-GlcNAc Modification Targets in Mouse Retinal Pericytes: Implication of P53 in Pathogenesis of Diabetic Retinopathy. PLoS One. 2014; 9:e95561. [PubMed: 24788674]

233. Chuh KN, Zaro BW, Piller F, Piller V, Pratt MR. Changes in Metabolic Chemical Reporter Structure Yield a Selective Probe of O-GlcNAc Modification. J Am Chem Soc. 2014; 136:12283–12295. [PubMed: 25153642]

234. Longwell SA, Dube DH. Deciphering the Bacterial Glycocode: Recent Advances in Bacterial Glycoproteomics. Curr Opin Chem Biol. 2013; 17:41–48. [PubMed: 23276734]

235. Champasa K, Longwell SA, Eldridge AM, Stemmler EA, Dube DH. Targeted Identification of Glycosylated Proteins in the Gastric Pathogen Helicobacter Pylori (Hp). Mol Cell Proteomics. 2013; 12:2568–2586. [PubMed: 23754784]

236. Koenigs MB, Richardson EA, Dube DH. Metabolic Profiling of Helicobacter Pylori Glycosylation. Mol BioSyst. 2009; 5:909–912. [PubMed: 19668851]

237. Almaraz RT, Aich U, Khanna HS, Tan E, Bhattacharya R, Shah S, Yarema KJ. Metabolic Oligosaccharide Engineering with N-Acyl Functionalized ManNAc Analogs: Cytotoxicity, Metabolic Flux, and Glycan-Display Considerations. Biotechnol Bioeng. 2012; 109:992–1006. [PubMed: 22068462]

238. Hayes BK, Greis KD, Hart GW. Specific Isolation of O-Linked N-Acetylglucosamine Glycopeptides from Complex Mixtures. Anal Biochem. 1995; 228:115–122. [PubMed: 8572267]

239. Torres CR, Hart GW. Topography and Polypeptide Distribution of Terminal *N*-Acetylglucosamine Residues on the Surfaces of Intact Lymphocytes. Evidence for *O*-Linked GlcNAc. J Biol Chem. 1984; 259:3308–3317. [PubMed: 6421821]

240. Ramakrishnan B, Qasba PK. Structure-Based Design of β1,4-Galactosyltransferase I (β4Gal-T1) with Equally Efficient *N*-Acetylgalactosaminyltransferase Activity: Point Mutation Broadens β4Gal-T1 Donor Specificity. J Biol Chem. 2002; 277:20833–20839. [PubMed: 11916963]

241. Clark PM, Dweck JF, Mason DE, Hart CR, Buck SB, Peters EC, Agnew BJ, Hsieh-Wilson LC. Direct in-Gel Fluorescence Detection and Cellular Imaging of O-GlcNAc-Modified Proteins. J Am Chem Soc. 2008; 130:11576–11577. [PubMed: 18683930]

242. Khidekel N, Arndt S, Lamarre-Vincent N, Lippert A, Poulin-Kerstien KG, Ramakrishnan B, Qasba PK, Hsieh-Wilson LC. A Chemoenzymatic Approach toward the Rapid and Sensitive Detection of O-GlcNAc Posttranslational Modifications. J Am Chem Soc. 2003; 125:16162–16163. [PubMed: 14692737]

243. Tai HC, Khidekel N, Ficarro SB, Peters EC, Hsieh-Wilson LC. Parallel Identification of O-GlcNAc-Modified Proteins from Cell Lysates. J Am Chem Soc. 2004; 126:10500–10501. [PubMed: 15327282]

244. Khidekel N, Ficarro SB, Peters EC, Hsieh-Wilson LC. Exploring the O-GlcNAc Proteome: Direct Identification of O-GlcNAc-Modified Proteins from the Brain. Proc Natl Acad Sci U S A. 2004; 101:13132–13137. [PubMed: 15340146]

245. Khidekel N, Ficarro SB, Clark PM, Bryan MC, Swaney DL, Rexach JE, Sun YE, Coon JJ, Peters EC, Hsieh-Wilson LC. Probing the Dynamics of O-GlcNAc Glycosylation in the Brain Using Quantitative Proteomics. Nat Chem Biol. 2007; 3:339–348. [PubMed: 17496889]

246. Wang Z, Park K, Comer F, Hsieh-Wilson LC, Saudek CD, Hart GW. Site-Specific GlcNAcylation of Human Erythrocyte Proteins: Potential Biomarker(S) for Diabetes. Diabetes. 2009; 58:309–317. [PubMed: 18984734]

247. Parker BL, Gupta P, Cordwell SJ, Larsen MR, Palmisano G. Purification and Identification of O-GlcNAc-Modified Peptides Using Phosphate-Based Alkyne Click Chemistry in Combination with Titanium Dioxide Chromatography and Mass Spectrometry. J Proteome Res. 2011; 10:1449–1458. [PubMed: 21158410]

248. Sakabe K, Wang Z, Hart GW. *β-N*-Acetylglucosamine (O-GlcNAc) Is Part of the Histone Code. Proc Natl Acad Sci U S A. 2010; 107:19915–19920. [PubMed: 21045127]

249. Wang Z, Udeshi ND, O'Malley M, Shabanowitz J, Hunt DF, Hart GW. Enrichment and Site Mapping of O-Linked N-Acetylglucosamine by a Combination of Chemical/Enzymatic Tagging, Photochemical Cleavage, and Electron Transfer Dissociation Mass Spectrometry. Mol Cell Proteomics. 2010; 9:153–160. [PubMed: 19692427]

250. Wang Z, Udeshi ND, Slawson C, Compton PD, Sakabe K, Cheung WD, Shabanowitz J, Hunt DF, Hart GW. Extensive Crosstalk between O-GlcNAcylation and Phosphorylation Regulates Cytokinesis. Sci Signaling. 2010; 3:ra2.

251. Alfaro JF, Gong CX, Monroe ME, Aldrich JT, Clauss TR, Purvine SO, Wang Z, Camp DG 2nd, Shabanowitz J, Stanley P, Hart GW, Hunt DF, Yang F, Smith RD. Tandem Mass Spectrometry Identifies Many Mouse Brain O-GlcNAcylated Proteins Including EGF Domain-Specific O-GlcNAc Transferase Targets. Proc Natl Acad Sci U S A. 2012; 109:7280–7285. [PubMed: 22517741]

252. Nishikaze T, Kawabata S, Iwamoto S, Tanaka K. Reversible Hydrazide Chemistry-Based Enrichment for O-GlcNAc-Modified Peptides and Glycopeptides Having Non-Reducing GlcNAc Residues. Analyst. 2013; 138:7224–7232. [PubMed: 24131013]

253. Tsumoto H, Ogasawara D, Hashii N, Suzuki T, Akimoto Y, Endo T, Miura Y. Enrichment of O-GlcNAc-Modified Peptides Using Novel Thiol-Alkyne and Thiol-Disulfide Exchange. Bioorg Med Chem Lett. 2015; 25:2645–2649. [PubMed: 25980911]

254. Griffin ME, Jensen EH, Mason DE, Jenkins CL, Stone SE, Peters EC, Hsieh-Wilson LC. Comprehensive Mapping of O-GlcNAc Modification Sites Using a Chemically Cleavable Tag. Mol BioSyst. 2016; 12:1756–1759. [PubMed: 27063346]

255. Muia RP, Yu H, Prescher JA, Hellman U, Chen X, Bertozzi CR, Campetella O. Identification of Glycoproteins Targeted by Trypanosoma Cruzi Trans-Sialidase, a Virulence Factor That Disturbs Lymphocyte Glycosylation. Glycobiology. 2010; 20:833–842. [PubMed: 20354005]

256. Lantos AB, Carlevaro G, Araoz B, Ruiz Diaz P, Camara MdlM, Buscaglia CA, Bossi M, Yu H, Chen X, Bertozzi CR, Mucci J, Campetella O. Sialic Acid Glycobiology Unveils Trypanosoma Cruzi Trypomastigote Membrane Physiology. PLoS Pathog. 2016; 12:e1005559. [PubMed: 27058585]

257. Pasek M, Ramakrishnan B, Boeggeman E, Manzoni M, Waybright TJ, Qasba PK. Bioconjugation and Detection of Lactosamine Moiety Using Alpha1,3-Galactosyltransferase Mutants That Transfer C2-Modified Galactose with a Chemical Handle. Bioconjugate Chem. 2009; 20:608–618.

258. Zheng T, Jiang H, Gros M, Soriano del Amo D, Sundaram S, Lauvau G, Marlow F, Liu Y, Stanley P, Wu P. Tracking N-Acetyllactosamine on Cell-Surface Glycans in Vivo. Angew Chem, Int Ed. 2011; 50:4113–4118.

259. Wagner GK, Pesnot T, Field RA. A Survey of Chemical Methods for Sugar-Nucleotide Synthesis. Nat Prod Rep. 2009; 26:1172–1194. [PubMed: 19693414]

260. Chicooree N, Unwin RD, Griffiths JR. The Application of Targeted Mass Spectrometry-Based Strategies to the Detection and Localization of Post-Translational Modifications. Mass Spectrom Rev. 2015; 34:595–626. [PubMed: 24737647]

261. Stavenhagen K, Hinneburg H, Thaysen-Andersen M, Hartmann L, Varon Silva D, Fuchser J, Kaspar S, Rapp E, Seeberger PH, Kolarich D. Quantitative Mapping of Glycoprotein Micro-Heterogeneity and Macro-Heterogeneity: An Evaluation of Mass Spectrometry Signal Strengths Using Synthetic Peptides and Glycopeptides. J Mass Spectrom. 2013; 48:627–639. [PubMed: 23722953]

262. Wang Z, Hart GW. Glycomic Approaches to Study GlcNAcylation: Protein Identification, Site-Mapping, and Site-Specific O-GlcNAc Quantitation. Clin Proteomics. 2008; 4:5–13.

263. Chalkley RJ, Clauser KR. Modification Site Localization Scoring: Strategies and Performance. Mol Cell Proteomics. 2012; 11:3–14. [PubMed: 22328712]

264. Xu SL, Medzihradszky KF, Wang ZY, Burlingame AL, Chalkley RJ. N-Glycopeptide Profiling in *Arabidopsis* Inflorescence. Mol Cell Proteomics. 2016; 15:2048–2054. [PubMed: 27067053]

265. Medzihradszky KF, Kaasik K, Chalkley RJ. Tissue-Specific Glycosylation at the Glycopeptide Level. Mol Cell Proteomics. 2015; 14:2103–2110. [PubMed: 25995273]

266. Huddleston MJ, Bean MF, Carr SA. Collisional Fragmentation of Glycopeptides by Electrospray Ionization LC/MS and LC/MS/MS: Methods for Selective Detection of Glycopeptides in Protein Digests. Anal Chem. 1993; 65:877–884. [PubMed: 8470819]

267. Medzihradszky KF, Gillece-Castro BL, Settineri CA, Reid Townsend R, Masiarz FR, Burlingame AL. Structure Determination of O-Linked Glycopeptides by Tandem Mass Spectrometry. Biol Mass Spectrom. 1990; 19:777–781.

268. Hinneburg H, Stavenhagen K, Schweiger-Hufnagel U, Pengelley S, Jabs W, Seeberger PH, Silva DV, Wuhrer M, Kolarich D. The Art of Destruction: Optimizing Collision Energies in Quadrupole-Time of Flight (Q-TOF) Instruments for Glycopeptide-Based Glycoproteomics. J Am Soc Mass Spectrom. 2016; 27:507–519. [PubMed: 26729457]

269. Wuhrer M, Catalina MI, Deelder AM, Hokke CH. Glycoproteomics Based on Tandem Mass Spectrometry of Glycopeptides. J Chromatogr B: Anal Technol Biomed Life Sci. 2007; 849:115–128.

270. Wu SW, Liang SY, Pu TH, Chang FY, Khoo KH. Sweet-Heart - an Integrated Suite of Enabling Computational Tools for Automated MS2/MS3 Sequencing and Identification of Glycopeptides. J Proteomics. 2013; 84:1–16. [PubMed: 23568021]

271. Plummer TH Jr, Elder JH, Alexander S, Phelan AW, Tarentino AL. Demonstration of Peptide:N-Glycosidase F Activity in Endo-β-N-acetylglucosaminidase F Preparations. J Biol Chem. 1984; 259:10700–10704. [PubMed: 6206060]

272. Bunkenborg J, Pilch BJ, Podtelejnikov AV, Wisniewski JR. Screening for N-Glycosylated Proteins by Liquid Chromatography Mass Spectrometry. Proteomics. 2004; 4:454–465. [PubMed: 14760718]

273. Weng Y, Sui Z, Jiang H, Shan Y, Chen L, Zhang S, Zhang L, Zhang Y. Releasing N-Glycan from Peptide N-Terminus by N-Terminal Succinylation Assisted Enzymatic Deglycosylation. Sci Rep. 2015; 5:9770. [PubMed: 25902405]

274. Kaji H, Saito H, Yamauchi Y, Shinkawa T, Taoka M, Hirabayashi J, Kasai K, Takahashi N, Isobe T. Lectin Affinity Capture, Isotope-Coded Tagging and Mass Spectrometry to Identify N-Linked Glycoproteins. Nat Biotechnol. 2003; 21:667–672. [PubMed: 12754521]

275. Gonzalez J, Takao T, Hori H, Besada V, Rodriguez R, Padron G, Shimonishi Y. A Method for Determination of N-Glycosylation Sites in Glycoproteins by Collision-Induced Dissociation Analysis in Fast Atom Bombardment Mass Spectrometry: Identification of the Positions of Carbohydrate-Linked Asparagine in Recombinant Alpha-Amylase by Treatment with Peptide-N-Glycosidase F in 18o-Labeled Water. Anal Biochem. 1992; 205:151–158. [PubMed: 1443554]

276. Atwood JA 3rd, Minning T, Ludolf F, Nuccio A, Weatherly DB, Alvarez-Manilla G, Tarleton R, Orlando R. Glycoproteomics of Trypanosoma Cruzi Trypomastigotes Using Subcellular Fractionation, Lectin Affinity, and Stable Isotope Labeling. J Proteome Res. 2006; 5:3376–3384. [PubMed: 17137339]

277. Angel PM, Lim JM, Wells L, Bergmann C, Orlando R. A Potential Pitfall in 18o-Based N-Linked Glycosylation Site Mapping. Rapid Commun Mass Spectrom. 2007; 21:674–682. [PubMed: 17279607]

278. Robinson NE, Robinson AB. Deamidation of Human Proteins. Proc Natl Acad Sci U S A. 2001; 98:12409–12413. [PubMed: 11606750]

279. Palmisano G, Melo-Braga MN, Engholm-Keller K, Parker BL, Larsen MR. Chemical Deamidation: A Common Pitfall in Large-Scale N-Linked Glycoproteomic Mass Spectrometry-Based Analyses. J Proteome Res. 2012; 11:1949–1957. [PubMed: 22256963]

280. Hagglund P, Bunkenborg J, Elortza F, Jensen ON, Roepstorff P. A New Strategy for Identification of N-Glycosylated Proteins and Unambiguous Assignment of Their Glycosylation Sites Using HILIC Enrichment and Partial Deglycosylation. J Proteome Res. 2004; 3:556–566. [PubMed: 15253437]

281. Zhang W, Wang H, Zhang L, Yao J, Yang P. Large-Scale Assignment of N-Glycosylation Sites Using Complementary Enzymatic Deglycosylation. Talanta. 2011; 85:499–505. [PubMed: 21645732]

282. Hong JC, Kim YS. Alkali-Catalyzed β-Elimination of Periodate-Oxidized Glycans: A Novel Method of Chemical Deglycosylation of Mucin Gene Products in Paraffin Embedded Sections. Glycoconjugate J. 2000; 17:691–703.

283. Gerken TA, Gupta R, Jentoft N. A Novel Approach for Chemically Deglycosylating O-Linked Glycoproteins. The Deglycosylation of Submaxillary and Respiratory Mucins. Biochemistry. 1992; 31:639–648. [PubMed: 1310044]

284. Hanisch FG, Teitz S, Schwientek T, Muller S. Chemical De-O-Glycosylation of Glycoproteins for Application in LC-Based Proteomics. Proteomics. 2009; 9:710–719. [PubMed: 19132687]

285. Patel TP, Parekh RB. Release of Oligosaccharides from Glycoproteins by Hydrazinolysis. Methods Enzymol. 1994; 230:57–66. [PubMed: 8139515]

286. Edge AS, Faltynek CR, Hof L, Reichert LE Jr, Weber P. Deglycosylation of Glycoproteins by Trifluoromethanesulfonic. Acid Anal Biochem. 1981; 118:131–137. [PubMed: 6175244]

287. Edge AS. Deglycosylation of Glycoproteins with Trifluor-omethanesulphonic Acid: Elucidation of Molecular Structure and Function. Biochem J. 2003; 376:339–350. [PubMed: 12974674]

288. Rademaker GJ, Haverkamp J, Thomasoates J. Determination of Glycosylation Sites in O-Linked Glycopeptides - a Sensitive Mass-Spectrometric Protocol. Org Mass Spectrom. 1993; 28:1536–1541.

289. Greis KD, Hayes BK, Comer FI, Kirk M, Barnes S, Lowary TL, Hart GW. Selective Detection and Site-Analysis of O-GlcNAc-Modified Glycopeptides by β-Elimination and Tandem Electrospray Mass Spectrometry. Anal Biochem. 1996; 234:38–49. [PubMed: 8742080]

290. Hanisch FG, Jovanovic M, Peter-Katalinic J. Glycoprotein Identification and Localization of O-Glycosylation Sites by Mass Spectrometric Analysis of Deglycosylated/Alkylaminylated Peptide Fragments. Anal Biochem. 2001; 290:47–59. [PubMed: 11180936]

291. Rademaker GJ, Pergantis SA, Blok-Tip L, Langridge JI, Kleen A, Thomas-Oates JE. Mass Spectrometric Determination of the Sites of O-Glycan Attachment with Low Picomolar Sensitivity. Anal Biochem. 1998; 257:149–160. [PubMed: 9514784]

292. Zheng Y, Guo Z, Cai Z. Combination of β-Elimination and Liquid Chromatography/Quadrupole Time-of-Flight Mass Spectrometry for the Determination of O-Glycosylation Sites. Talanta. 2009; 78:358–363. [PubMed: 19203595]

293. Wells L, Vosseller K, Cole RN, Cronshaw JM, Matunis MJ, Hart GW. Mapping Sites of O-GlcNAc Modification Using Affinity Tags for Serine and Threonine Post-Translational Modifications. Mol Cell Proteomics. 2002; 1:791–804. [PubMed: 12438562]

294. Overath T, Kuckelkorn U, Henklein P, Strehl B, Bonar D, Kloss A, Siele D, Kloetzel PM, Janek K. Mapping of O-GlcNAc Sites of 20 S Proteasome Subunits and Hsp90 by a Novel Biotin-Cystamine Tag. Mol Cell Proteomics. 2012; 11:467–477. [PubMed: 22556278]

295. Nika H, Nieves E, Hawke DH, Angeletti RH. Optimization of the β-Elimination/Michael Addition Chemistry on Reversed-Phase Supports for Mass Spectrometry Analysis of O-Linked Protein Modifications. J Biomol Technol. 2013; 24:132–153.

296. Cao W, Cao J, Huang J, Yao J, Yan G, Xu H, Yang P. Discovery and Confirmation of O-GlcNAcylated Proteins in Rat Liver Mitochondria by Combination of Mass Spectrometry and Immunological Methods. PLoS One. 2013; 8:e76399. [PubMed: 24098488]

297. Vosseller K, Hansen KC, Chalkley RJ, Trinidad JC, Wells L, Hart GW, Burlingame AL. Quantitative Analysis of Both Protein Expression and Serine/Threonine Post-Translational Modifications through Stable Isotope Labeling with Dithiothreitol. Proteomics. 2005; 5:388–398. [PubMed: 15648052]

298. Ma J, Liu T, Wei AC, Banerjee P, O'Rourke B, Hart GW. O-GlcNAcomic Profiling Identifies Widespread O-GlcNAcylation in Oxidative Phosphorylation System Regulating Cardiac Mitochondrial Function. J Biol Chem. 2015; 290:29141–29153. [PubMed: 26446791]

299. Ma J, Banerjee P, Whelan SA, Liu T, Wei AC, Ramirez-Correa G, McComb ME, Costello CE, O'Rourke B, Murphy A, Hart GW. Comparative Proteomics Reveals Dysregulated Mitochondrial O-GlcNAcylation in Diabetic Hearts. J Proteome Res. 2016; 15:2254–2264. [PubMed: 27213235]

300. Zubarev RA, Horn DM, Fridriksson EK, Kelleher NL, Kruger NA, Lewis MA, Carpenter BK, McLafferty FW. Electron Capture Dissociation for Structural Characterization of Multiply Charged Protein Cations. Anal Chem. 2000; 72:563–573. [PubMed: 10695143]

301. Zubarev RA, Kelleher NL, McLafferty FW. Electron Capture Dissociation of Multiply Charged Protein Cations. A Nonergodic Process. J Am Chem Soc. 1998; 120:3265–3266.

302. Syka JE, Coon JJ, Schroeder MJ, Shabanowitz J, Hunt DF. Peptide and Protein Sequence Analysis by Electron Transfer Dissociation Mass Spectrometry. Proc Natl Acad Sci U S A. 2004; 101:9528–9533. [PubMed: 15210983]

303. Huang TY, Emory JF, O'Hair RA, McLuckey SA. Electron-Transfer Reagent Anion Formation via Electrospray Ionization and Collision-Induced Dissociation. Anal Chem. 2006; 78:7387–7391. [PubMed: 17073403]

304. Kelleher NL, Zubarev RA, Bush K, Furie B, Furie BC, McLafferty FW, Walsh CT. Localization of Labile Posttranslational Modifications by Electron Capture Dissociation: The Case of Gamma-Carboxyglutamic Acid. Anal Chem. 1999; 71:4250–4253. [PubMed: 10517147]

305. Coon JJ, Ueberheide B, Syka JEP, Dryhurst DD, Ausio J, Shabanowitz J, Hunt DF. Protein Identification Using Sequential Ion/Ion Reactions and Tandem Mass Spectrometry. Proc Natl Acad Sci U S A. 2005; 102:9463–9468. [PubMed: 15983376]

306. Mirgorodskaya E, Roepstorff P, Zubarev RA. Localization of O-Glycosylation Sites in Peptides by Electron Capture Dissociation in a Fourier Transform Mass Spectrometer. Anal Chem. 1999; 71:4431–4436. [PubMed: 10546526]

307. Kim MS, Pandey A. Electron Transfer Dissociation Mass Spectrometry in Proteomics. Proteomics. 2012; 12:530–542. [PubMed: 22246976]

308. McAlister GC, Berggren WT, Griep-Raming J, Horning S, Makarov A, Phanstiel D, Stafford G, Swaney DL, Syka JEP, Zabrouskov V, Coon JJ. A Proteomics Grade Electron Transfer Dissociation-Enabled Hybrid Linear Ion Trap-Orbitrap Mass Spectrometer. J Proteome Res. 2008; 7:3127–3136. [PubMed: 18613715]

309. Zhao P, Viner R, Teo CF, Boons GJ, Horn D, Wells L. Combining High-Energy C-Trap Dissociation and Electron Transfer Dissociation for Protein O-GlcNAc Modification Site Assignment. J Proteome Res. 2011; 10:4088–4104. [PubMed: 21740066]

310. Chalkley RJ, Thalhammer A, Schoepfer R, Burlingame AL. Identification of Protein O-GlcNAcylation Sites Using Electron Transfer Dissociation Mass Spectrometry on Native Peptides. Proc Natl Acad Sci U S A. 2009; 106:8894–8899. [PubMed: 19458039]

311. Myers SA, Panning B, Burlingame AL. Polycomb Repressive Complex 2 Is Necessary for the Normal Site-Specific O-GlcNAc Distribution in Mouse Embryonic Stem Cells. Proc Natl Acad Sci U S A. 2011; 108:9490–9495. [PubMed: 21606357]

312. Trinidad JC, Barkan DT, Gulledge BF, Thalhammer A, Sali A, Schoepfer R, Burlingame AL. Global Identification and Characterization of Both O-GlcNAcylation and Phosphorylation at the Murine Synapse. Mol Cell Proteomics. 2012; 11:215–229. [PubMed: 22645316]

313. Nagel AK, Schilling M, Comte-Walters S, Berkaw MN, Ball LE. Identification of O-Linked N-Acetylglucosamine (O-GlcNAc)-Modified Osteoblast Proteins by Electron Transfer Dissociation Tandem Mass Spectrometry Reveals Proteins Critical for Bone Formation. Mol Cell Proteomics. 2013; 12:945–955. [PubMed: 23443134]

314. Scott NE, Parker BL, Connolly AM, Paulech J, Edwards AVG, Crossett B, Falconer L, Kolarich D, Djordjevic SP, Hojrup P, Packer NH, Larsen MR, Cordwell SJ. Simultaneous Glycan-Peptide Characterization Using Hydrophilic Interaction Chromatography and Parallel Fragmentation by CID, Higher Energy Collisional Dissociation, and Electron Transfer Dissociation MS Applied to the *N*-Linked Glycoproteome of *Campylobacter jejuni*. Mol Cell Proteomics. 2011; 10:M000031-MCP201. [PubMed: 20360033]

315. Trinidad JC, Schoepfer R, Burlingame AL, Medzihradszky KF. N- and O-Glycosylation in the Murine Synaptosome. Mol Cell Proteomics. 2013; 12:3474–3488. [PubMed: 23816992]

316. Smith GT, Sweredoski MJ, Hess S. O-Linked Glycosylation Sites Profiling in Mycobacterium Tuberculosis Culture Filtrate Proteins. J Proteomics. 2014; 97:296–306. [PubMed: 23702328]

317. Wang X, Yuan ZF, Fan J, Karch KR, Ball LE, Denu JM, Garcia BA. A Novel Quantitative Mass Spectrometry Platform for Determining Protein O-GlcNAcylation Dynamics. Mol Cell Proteomics. 2016; 15:2462–2475. [PubMed: 27114449]

318. Swaney DL, McAlister GC, Wirtala M, Schwartz JC, Syka JE, Coon JJ. Supplemental Activation Method for High-Efficiency Electron-Transfer Dissociation of Doubly Protonated Peptide Precursors. Anal Chem. 2007; 79:477–485. [PubMed: 17222010]

319. Swaney DL, McAlister GC, Coon JJ. Decision Tree-Driven Tandem Mass Spectrometry for Shotgun Proteomics. Nat Methods. 2008; 5:959–964. [PubMed: 18931669]

320. Baker PR, Medzihradszky KF, Chalkley RJ. Improving Software Performance for Peptide Electron Transfer Dissociation Data Analysis by Implementation of Charge State- and Sequence-Dependent Scoring. Mol Cell Proteomics. 2010; 9:1795–1803. [PubMed: 20513802]

321. Darula Z, Chalkley RJ, Lynn A, Baker PR, Medzihradszky KF. Improved Identification of O-Linked Glycopep-tides from Etd Data with Optimized Scoring for Different Charge States and Cleavage Specificities. Amino Acids. 2011; 41:321–328. [PubMed: 20652609]

322. Darula Z, Medzihradszky KF. Glycan Side Reaction May Compromise ETD-Based Glycopeptide Identification. J Am Soc Mass Spectrom. 2014; 25:977–987. [PubMed: 24664807]

323. Perdivara I, Petrovich R, Allinquant B, Deterding LJ, Tomer KB, Przybylski M. Elucidation of O-Glycosylation Structures of the β-Amyloid Precursor Protein by Liquid Chromatography-Mass Spectrometry Using Electron Transfer Dissociation and Collision Induced Dissociation. J Proteome Res. 2009; 8:631–642. [PubMed: 19093876]

324. Sihlbom C, van Dijk Hard I, Lidell ME, Noll T, Hansson GC, Backstrom M. Localization of O-Glycans in MUC1 Glycoproteins Using Electron-Capture Dissociation Fragmentation Mass Spectrometry. Glycobiology. 2009; 19:375–381. [PubMed: 19095697]

325. Takahashi K, Wall SB, Suzuki H, Smith ADt, Hall S, Poulsen K, Kilian M, Mobley JA, Julian BA, Mestecky J, Novak J, Renfrow MB. Clustered O-Glycans of Iga1: Defining Macro- and Microheterogeneity by Use of Electron Capture/Transfer Dissociation. Mol Cell Proteomics. 2010; 9:2545–2557. [PubMed: 20823119]

326. Ali L, Flowers SA, Jin C, Bennet EP, Ekwall AK, Karlsson NG. The O-Glycomap of Lubricin, a Novel Mucin Responsible for Joint Lubrication, Identified by Site-Specific Glycopeptide Analysis. Mol Cell Proteomics. 2014; 13:3396–3409. [PubMed: 25187573]

327. Darula Z, Medzihradszky KF. Affinity Enrichment and Characterization of Mucin Core-1 Type Glycopeptides from Bovine Serum. Mol Cell Proteomics. 2009; 8:2515–2526. [PubMed: 19674964]

328. Darula Z, Sherman J, Medzihradszky KF. How to Dig Deeper? Improved Enrichment Methods for Mucin Core-1 Type Glycopeptides. Mol Cell Proteomics. 2012; 11:O111.016774.

329. Bagdonaite I, Norden R, Joshi HJ, Dabelsteen S, Nystrom K, Vakhrushev SY, Olofsson S, Wandall HH. A Strategy for O-Glycoproteomics of Enveloped Viruses–the O-Glycoproteome of Herpes Simplex Virus Type 1. PLoS Pathog. 2015; 11:e1004784. [PubMed: 25830354]

330. Levery SB, Steentoft C, Halim A, Narimatsu Y, Clausen H, Vakhrushev SY. Advances in Mass Spectrometry Driven O-Glycoproteomics. Biochim Biophys Acta, Gen Subj. 2015; 1850:33–42.

331. Steentoft C, Vakhrushev SY, Vester-Christensen MB, Schjoldager KT, Kong Y, Bennett EP, Mandel U, Wandall H, Levery SB, Clausen H. Mining the O-Glycoproteome Using Zinc-Finger Nuclease-Glycoengineered Simplecell Lines. Nat Methods. 2011; 8:977–982. [PubMed: 21983924]

332. Steentoft C, Bennett EP, Clausen H. Glycoengineering of Human Cell Lines Using Zinc Finger Nuclease Gene Targeting: Simplecells with Homogeneous GalNAc O-Glycosylation Allow Isolation of the O-Glycoproteome by One-Step Lectin Affinity Chromatography. Methods Mol Biol. 2013; 1022:387–402. [PubMed: 23765677]

333. Vakhrushev SY, Steentoft C, Vester-Christensen MB, Bennett EP, Clausen H, Levery SB. Enhanced Mass Spectrometric Mapping of the Human GalNAc-Type O-Glycoproteome with Simplecells. Mol Cell Proteomics. 2013; 12:932–944. [PubMed: 23399548]

334. Yang Z, Halim A, Narimatsu Y, Jitendra Joshi H, Steentoft C, Gram Schjoldager KTB, Alder Schulz M, Sealover NR, Kayser KJ, Paul Bennett E, Levery SB, Vakhrushev SY, Clausen H. The

GalNAc-Type O-Glycoproteome of Cho Cells Characterized by the Simplecell Strategy. Mol Cell Proteomics. 2014; 13:3224–3235. [PubMed: 25092905]

335. Pedersen NB, Wang S, Narimatsu Y, Yang Z, Halim A, Schjoldager KT, Madsen TD, Seidah NG, Bennett EP, Levery SB, Clausen H. Low Density Lipoprotein Receptor Class a Repeats Are O-Glycosylated in Linker Regions. J Biol Chem. 2014; 289:17312–17324. [PubMed: 24798328]

336. Steentoft C, Vakhrushev SY, Joshi HJ, Kong Y, Vester-Christensen MB, Schjoldager KT, Lavrsen K, Dabelsteen S, Pedersen NB, Marcos-Silva L, Gupta R, Bennett EP, Mandel U, Brunak S, Wandall HH, Levery SB, Clausen H. Precision Mapping of the Human O-GalNAc Glycoproteome through Simplecell Technology. EMBO J. 2013; 32:1478–1488. [PubMed: 23584533]

337. Kong Y, Joshi HJ, Schjoldager KT, Madsen TD, Gerken TA, Vester-Christensen MB, Wandall HH, Bennett EP, Levery SB, Vakhrushev SY, Clausen H. Probing Polypeptide GalNAc-Transferase Isoform Substrate Specificities by in Vitro Analysis. Glycobiology. 2015; 25:55–65. [PubMed: 25155433]

338. Campos D, Freitas D, Gomes J, Magalhaes A, Steentoft C, Gomes C, Vester-Christensen MB, Ferreira JA, Afonso LP, Santos LL, Pinto de Sousa J, Mandel U, Clausen H, Vakhrushev SY, Reis CA. Probing the O-Glycoproteome of Gastric Cancer Cell Lines for Biomarker Discovery. Mol Cell Proteomics. 2015; 14:1616–1629. [PubMed: 25813380]

339. Schjoldager KT, Vakhrushev SY, Kong Y, Steentoft C, Nudelman AS, Pedersen NB, Wandall HH, Mandel U, Bennett EP, Levery SB, Clausen H. Probing Isoform-Specific Functions of Polypeptide GalNAc-Transferases Using Zinc Finger Nuclease Glycoengineered Simplecells. Proc Natl Acad Sci U S A. 2012; 109:9893–9898. [PubMed: 22566642]

340. Vester-Christensen MB, Halim A, Joshi HJ, Steentoft C, Bennett EP, Levery SB, Vakhrushev SY, Clausen H. Mining the O-Mannose Glycoproteome Reveals Cadherins as Major O-Mannosylated Glycoproteins. Proc Natl Acad Sci U S A. 2013; 110:21018–21023. [PubMed: 24101494]

341. Halim A, Larsen IS, Neubert P, Joshi HJ, Petersen BL, Vakhrushev SY, Strahl S, Clausen H. Discovery of a Nucleocytoplasmic O-Mannose Glycoproteome in Yeast. Proc Natl Acad Sci U S A. 2015; 112:15648–15653. [PubMed: 26644575]

342. Neubert P, Halim A, Zauser M, Essig A, Joshi HJ, Zatorska E, Larsen IS, Loibl M, Castells-Ballester J, Aebi M, Clausen H, Strahl S. Mapping the O-Mannose Glycoproteome in Saccharomyces Cerevisiae. Mol Cell Proteomics. 2016; 15:1323–1337. [PubMed: 26764011]

343. Huttenhain R, Malmstrom J, Picotti P, Aebersold R. Perspectives of Targeted Mass Spectrometry for Protein Biomarker Verification. Curr Opin Chem Biol. 2009; 13:518–525. [PubMed: 19818677]

344. Schmidt A, Claassen M, Aebersold R. Directed Mass Spectrometry: Towards Hypothesis-Driven Proteomics. Curr Opin Chem Biol. 2009; 13:510–517. [PubMed: 19775930]

345. Picotti P, Aebersold R. Selected Reaction Monitoring-Based Proteomics: Workflows, Potential, Pitfalls and Future Directions. Nat Methods. 2012; 9:555–566. [PubMed: 22669653]

346. Peterson AC, Russell JD, Bailey DJ, Westphall MS, Coon JJ. Parallel Reaction Monitoring for High Resolution and High Mass Accuracy Quantitative, Targeted Proteomics. Mol Cell Proteomics. 2012; 11:1475–1488. [PubMed: 22865924]

347. Ebhardt HA, Root A, Sander C, Aebersold R. Applications of Targeted Proteomics in Systems Biology and Translational Medicine. Proteomics. 2015; 15:3193–3208. [PubMed: 26097198]

348. Picotti P, Rinner O, Stallmach R, Dautel F, Farrah T, Domon B, Wenschuh H, Aebersold R. High-Throughput Generation of Selected Reaction-Monitoring Assays for Proteins and Proteomes. Nat Methods. 2010; 7:43–46. [PubMed: 19966807]

349. Whiteaker JR, Halusa GN, Hoofnagle AN, Sharma V, MacLean B, Yan P, Wrobel JA, Kennedy J, Mani DR, Zimmerman LJ, Meyer MR, Mesri M, Rodriguez H, Paulovich AG. Clinical Proteomic Tumor Analysis Consortium. CPTAC Assay Portal: A Repository of Targeted Proteomic Assays. Nat Methods. 2014; 11:703–704. [PubMed: 24972168]

350. MacLean B, Tomazela DM, Shulman N, Chambers M, Finney GL, Frewen B, Kern R, Tabb DL, Liebler DC, MacCoss MJ. Skyline: An Open Source Document Editor for Creating and Analyzing Targeted Proteomics Experiments. Bioinformatics. 2010; 26:966–968. [PubMed: 20147306]

351. Goldman R, Sanda M. Targeted Methods for Quantitative Analysis of Protein Glycosylation. Proteomics: Clin Appl. 2015; 9:17–32. [PubMed: 25522218]

352. Thomas SN, Harlan R, Chen J, Aiyetan P, Liu Y, Sokoll LJ, Aebersold R, Chan DW, Zhang H. Multiplexed Targeted Mass Spectrometry-Based Assays for the Quantification of N-Linked Glycosite-Containing Peptides in Serum. Anal Chem. 2015; 87:10830–10838. [PubMed: 26451657]

353. Huttenhain R, Surinova S, Ossola R, Sun Z, Campbell D, Cerciello F, Schiess R, Bausch-Fluck D, Rosenberger G, Chen J, Rinner O, Kusebauch U, Hajduch M, Moritz RL, Wollscheid B, Aebersold R. N-Glycoprotein Srmatlas: A Resource of Mass Spectrometric Assays for N-Glycosites Enabling Consistent and Multiplexed Protein Quantification for Clinical Applications. Mol Cell Proteomics. 2013; 12:1005–1016. [PubMed: 23408683]

354. Sjostrom M, Ossola R, Breslin T, Rinner O, Malmstrom L, Schmidt A, Aebersold R, Malmstrom J, Nimeus E. A Combined Shotgun and Targeted Mass Spectrometry Strategy for Breast Cancer Biomarker Discovery. J Proteome Res. 2015; 14:2807–2818. [PubMed: 25944384]

355. Liu Y, Chen J, Sethi A, Li QK, Chen L, Collins B, Gillet LC, Wollscheid B, Zhang H, Aebersold R. Glycoproteomic Analysis of Prostate Cancer Tissues by SWATH Mass Spectrometry Discovers N-Acylethanolamine Acid Amidase and Protein Tyrosine Kinase 7 as Signatures for Tumor Aggressiveness. Mol Cell Proteomics. 2014; 13:1753–1768. [PubMed: 24741114]

356. Zacchi LF, Schulz BL. SWATH-MS Glycoproteomics Reveals Consequences of Defects in the Glycosylation Machinery. Mol Cell Proteomics. 2016; 15:2435–2447. [PubMed: 27094473]

357. Xu Y, Bailey UM, Schulz BL. Automated Measurement of Site-Specific N-Glycosylation Occupancy with SWATH-MS. Proteomics. 2015; 15:2177–2186. [PubMed: 25737293]

358. Palaniappan KK, Pitcher AA, Smart BP, Spiciarich DR, Iavarone AT, Bertozzi CR. Isotopic Signature Transfer and Mass Pattern Prediction (Isostamp): An Enabling Technique for Chemically-Directed Proteomics. ACS Chem Biol. 2011; 6:829–836. [PubMed: 21604797]

359. Woo CM, Iavarone AT, Spiciarich DR, Palaniappan KK, Bertozzi CR. Isotope-Targeted Glycoproteomics (IsoTaG): A Mass-Independent Platform for Intact N- and O-Glycopeptide Discovery and Analysis. Nat Methods. 2015; 12:561–567. [PubMed: 25894945]

360. Breidenbach MA, Palaniappan KK, Pitcher AA, Bertozzi CR. Mapping Yeast N-Glycosites with Isotopically Recoded Glycans. Mol Cell Proteomics. 2012; 11:M111.015339.

361. Goodlett DR, Bruce JE, Anderson GA, Rist B, Pasa-Tolic L, Fiehn O, Smith RD, Aebersold R. Protein Identification with a Single Accurate Mass of a Cysteine-Containing Peptide and Constrained Database Searching. Anal Chem. 2000; 72:1112–1118. [PubMed: 10740847]

362. Woo CM, Bertozzi CR. Isotope Targeted Glycoproteomics (IsoTaG) to Characterize Intact, Metabolically Labeled Glycopeptides from Complex Proteomes. Curr Protoc Chem Biol. 2016; 8:59–82. [PubMed: 26995354]

363. Sheta R, Woo CM, Roux-Dalvai F, Fournier F, Bourassa S, Droit A, Bertozzi CR, Bachvarov D. A Metabolic Labeling Approach for Glycoproteomic Analysis Reveals Altered Glycoprotein Expression Upon GALNT3 Knockdown in Ovarian Cancer Cells. J Proteomics. 2016; 145:91–102. [PubMed: 27095597]

364. Breidenbach MA, Gallagher JE, King DS, Smart BP, Wu P, Bertozzi CR. Targeted Metabolic Labeling of Yeast N-Glycans with Unnatural Sugars. Proc Natl Acad Sci U S A. 2010; 107:3988–3993. [PubMed: 20142501]

365. Leitner A, Walzthoeni T, Kahraman A, Herzog F, Rinner O, Beck M, Aebersold R. Probing Native Protein Structures by Chemical Cross-Linking, Mass Spectrometry, and Bioinformatics. Mol Cell Proteomics. 2010; 9:1634–1649. [PubMed: 20360032]

366. Tanaka Y, Bond MR, Kohler JJ. Photocrosslinkers Illuminate Interactions in Living Cells. Mol BioSyst. 2008; 4:473–480. [PubMed: 18493640]

367. Frei AP, Jeon OY, Kilcher S, Moest H, Henning LM, Jost C, Pluckthun A, Mercer J, Aebersold R, Carreira EM, Wollscheid B. Direct Identification of Ligand-Receptor Interactions on Living Cells and Tissues. Nat Biotechnol. 2012; 30:997–1001. [PubMed: 22983091]

368. Collins BE, Paulson JC. Cell Surface Biology Mediated by Low Affinity Multivalent Protein-Glycan Interactions. Curr Opin Chem Biol. 2004; 8:617–625. [PubMed: 15556405]

369. Pham ND, Parker RB, Kohler JJ. Photocrosslinking Approaches to Interactome Mapping. Curr Opin Chem Biol. 2013; 17:90–101. [PubMed: 23149092]

370. Lauc G, Lee RT, Dumic J, Lee YC. Photoaffinity Glycoprobes - a New Tool for the Identification of Lectins. Glycobiology. 2000; 10:357–364. [PubMed: 10764823]

371. Ilver D, Arnqvist A, Ogren J, Frick IM, Kersulyte D, Incecik ET, Berg DE, Covacci A, Engstrand L, Boren T. *Helicobacter pylori* Adhesin Binding Fucosylated Histo-Blood Group Antigens Revealed by Retagging. Science. 1998; 279:373–377. [PubMed: 9430586]

372. Pacuszka T, Fishman PH. Synthesis of a Photoreactive, Radiolabelled Derivative of the Oligosaccharide of Gm1 Ganglioside. Glycobiology. 1992; 2:251–255. [PubMed: 1498422]

373. Ramya TNC, Weerapana E, Liao L, Zeng Y, Tateno H, Liao L, Yates JR III, Cravatt BF, Paulson JC. *In Situ* Trans Ligands of CD22 Identified by Glycan-Protein Photocross-Linking-Enabled Proteomics. Mol Cell Proteomics. 2010; 9:1339–1351. [PubMed: 20172905]

374. Kelm S, Brossmer R, Isecke R, Gross HJ, Strenge K, Schauer R. Functional Groups of Sialic Acids Involved in Binding to Siglecs (Sialoadhesins) Deduced from Interactions with Synthetic Analogues. Eur J Biochem. 1998; 255:663–672. [PubMed: 9738906]

375. Luchansky SJ, Bertozzi CR. Azido Sialic Acids Can Modulate Cell-Surface Interactions. ChemBioChem. 2004; 5:1706–1709. [PubMed: 15568180]

376. Yu SH, Wands AM, Kohler JJ. Photoaffinity Probes for Studying Carbohydrate Biology. J Carbohydr Chem. 2012; 31:325–352. [PubMed: 23239902]

377. Bond MR, Zhang HC, Vu PD, Kohler JJ. Photocrosslinking of Glycoconjugates Using Metabolically Incorporated Diazirine-Containing Sugars. Nat Protoc. 2009; 4:1044–1063. [PubMed: 19536272]

378. Tanaka Y, Kohler JJ. Photoactivatable Crosslinking Sugars for Capturing Glycoprotein Interactions. J Am Chem Soc. 2008; 130:3278–3279. [PubMed: 18293988]

379. Bond MR, Whitman CM, Kohler JJ. Metabolically Incorporated Photocrosslinking Sialic Acid Covalently Captures a Ganglioside-Protein Complex. Mol BioSyst. 2010; 6:1796–1799. [PubMed: 20625600]

380. Bond MR, Zhang H, Kim J, Yu SH, Yang F, Patrie SM, Kohler JJ. Metabolism of Diazirine-Modified N-Acetylmannosamine Analogues to Photo-Cross-Linking Sialosides. Bioconjugate Chem. 2011; 22:1811–1823.

381. Wands AM, Fujita A, McCombs JE, Cervin J, Dedic B, Rodriguez AC, Nischan N, Bond MR, Mettlen M, Trudgian DC, Lemoff A, Quiding-Jarbrink M, Gustavsson B, Steentoft C, Clausen H, Mirzaei H, Teneberg S, Yrlid U, Kohler JJ. Fucosylation and Protein Glycosylation Create Functional Receptors for Cholera Toxin. eLife. 2015; 4:e09545. [PubMed: 26512888]

382. Feng L, Hong S, Rong J, You Q, Dai P, Huang R, Tan Y, Hong W, Xie C, Zhao J, Chen X. Bifunctional Unnatural Sialic Acids for Dual Metabolic Labeling of Cell-Surface Sialylated Glycans. J Am Chem Soc. 2013; 135:9244–9247. [PubMed: 23725545]

383. McCombs JE, Zou C, Parker RB, Cairo CW, Kohler JJ. Enhanced Cross-Linking of Diazirine-Modified Sialylated Glycoproteins Enabled through Profiling of Sialidase Specificities. ACS Chem Biol. 2016; 11:185–192. [PubMed: 26541974]

384. Yu SH, Boyce M, Wands AM, Bond MR, Bertozzi CR, Kohler JJ. Metabolic Labeling Enables Selective Photo-crosslinking of O-GlcNAc-Modified Proteins to Their Binding Partners. Proc Natl Acad Sci U S A. 2012; 109:4834–4839. [PubMed: 22411826]

385. Lanucara F, Holman SW, Gray CJ, Eyers CE. The Power of Ion Mobility-Mass Spectrometry for Structural Characterization and the Study of Conformational Dynamics. Nat Chem. 2014; 6:281–294. [PubMed: 24651194]

386. Both P, Green AP, Gray CJ, Sardzik R, Voglmeir J, Fontana C, Austeri M, Rejzek M, Richardson D, Field RA, Widmalm G, Flitsch SL, Eyers CE. Discrimination of Epimeric Glycans and Glycopeptides Using IM-MS and Its Potential for Carbohydrate Sequencing. Nat Chem. 2014; 6:65–74. [PubMed: 24345949]

387. Creese AJ, Cooper HJ. Separation and Identification of Isomeric Glycopeptides by High Field Asymmetric Waveform Ion Mobility Spectrometry. Anal Chem. 2012; 84:2597–2601. [PubMed: 22280549]

388. Hofmann J, Hahm HS, Seeberger PH, Pagel K. Identification of Carbohydrate Anomers Using Ion Mobility-Mass Spectrometry. Nature. 2015; 526:241–244. [PubMed: 26416727]

389. Seo Y, Andaya A, Leary JA. Preparation, Separation, and Conformational Analysis of Differentially Sulfated Heparin Octasaccharide Isomers Using Ion Mobility Mass Spectrometry. Anal Chem. 2012; 84:2416–2423. [PubMed: 22283665]

390. Guttman M, Lee KK. Site-Specific Mapping of Sialic Acid Linkage Isomers by Ion Mobility Spectrometry. Anal Chem. 2016; 88:5212–5217. [PubMed: 27089023]

391. Hu H, Khatri K, Klein J, Leymarie N, Zaia J. A Review of Methods for Interpretation of Glycopeptide Tandem Mass Spectral Data. Glycoconjugate J. 2016; 33:285–296.

392. Bern, M., Kil, YJ., Becker, C. Current Protocols in Bioinformatics. Vol. Chapter 13. John Wiley & Sons, Inc; New York: 2012. Byonic: Advanced Peptide and Protein Identification Software.

393. Apte A, Meitei NS. Bioinformatics in Glycomics: Glycan Characterization with Mass Spectrometric Data Using Simglycan. Methods Mol Biol. 2010; 600:269–281. [PubMed: 19882135]

394. Nasir W, Toledo AG, Noborn F, Nilsson J, Wang M, Bandeira N, Larson G. SweetNET: A Bioinformatics Workflow for Glycopeptide MS/MS Spectral Analysis. J Proteome Res. 2016; 15:2826–2840. [PubMed: 27399812]

395. Cooper CA, Joshi HJ, Harrison MJ, Wilkins MR, Packer NH. GlycoSuiteDB: A Curated Relational Database of Glycoprotein Glycan Structures and Their Biological Sources. 2003 Update. Nucleic Acids Res. 2003; 31:511–513. [PubMed: 12520065]

396. Campbell MP, Peterson R, Mariethoz J, Gasteiger E, Akune Y, Aoki-Kinoshita KF, Lisacek F, Packer NH. UniCarbKB: Building a Knowledge Platform for Glycoproteomics. Nucleic Acids Res. 2014; 42:D215–D221. [PubMed: 24234447]

397. Campbell MP, Packer NH. Unicarbkb: New Database Features for Integrating Glycan Structure Abundance, Compositional Glycoproteomics Data, and Disease Associations. Biochim Biophys Acta, Gen Subj. 2016; 1860:1669–1675.

398. Wu TJ, Shamsaddini A, Pan Y, Smith K, Crichton DJ, Simonyan V, Mazumder R. A Framework for Organizing Cancer-Related Variations from Existing Databases, Publications and Ngs Data Using a High-Performance Integrated Virtual Environment (Hive). Database. 2014; 2014:bau022. [PubMed: 24667251]

399. Ritchie MD, Holzinger ER, Li R, Pendergrass SA, Kim D. Methods of Integrating Data to Uncover Genotype-Phenotype Interactions. Nat Rev Genet. 2015; 16:85–97. [PubMed: 25582081]

400. Li B, Mock F, Wu P. Imaging the Glycome in Living Systems. Methods Enzymol. 2012; 505:401–419. [PubMed: 22289465]

401. Laughlin ST, Bertozzi CR. Imaging the Glycome. Proc Natl Acad Sci U S A. 2009; 106:12–17. [PubMed: 19104067]

402. Haga Y, Ishii K, Hibino K, Sako Y, Ito Y, Taniguchi N, Suzuki T. Visualizing Specific Protein Glycoforms by Transmembrane Fluorescence Resonance Energy Transfer. Nat Commun. 2012; 3:907. [PubMed: 22713749]

403. Belardi B, de la Zerda A, Spiciarich DR, Maund SL, Peehl DM, Bertozzi CR. Imaging the Glycosylation State of Cell Surface Glycoproteins by Two-Photon Fluorescence Lifetime Imaging Microscopy. Angew Chem, Int Ed. 2013; 52:14045–14049.

404. Lin W, Du Y, Zhu Y, Chen X. A Cis-Membrane Fret-Based Method for Protein-Specific Imaging of Cell-Surface Glycans. J Am Chem Soc. 2014; 136:679–687. [PubMed: 24308457]

405. Lin W, Gao L, Chen X. Protein-Specific Imaging of O-GlcNAcylation in Single Cells. ChemBioChem. 2015; 16:2571–2575. [PubMed: 26488919]

406. Doll F, Buntz A, Spate AK, Schart VF, Timper A, Schrimpf W, Hauck CR, Zumbusch A, Wittmann V. Visualization of Protein-Specific Glycosylation inside Living Cells. Angew Chem, Int Ed. 2016; 55:2262–2266.

407. Robinson PV, de Almeida-Escobedo G, de Groot AE, McKechnie JL, Bertozzi CR. Live-Cell Labeling of Specific Protein Glycoforms by Proximity-Enhanced Bioorthogonal Ligation. J Am Chem Soc. 2015; 137:10452–10455. [PubMed: 26280358]

408. Watrous JD, Dorrestein PC. Imaging Mass Spectrometry in Microbiology. Nat Rev Microbiol. 2011; 9:683–694. [PubMed: 21822293]

409. Schwamborn K, Caprioli RM. Molecular Imaging by Mass Spectrometry–Looking Beyond Classical Histology. Nat Rev Cancer. 2010; 10:639–646. [PubMed: 20720571]

410. Boxer SG, Kraft ML, Weber PK. Advances in Imaging Secondary Ion Mass Spectrometry for Biological Samples. Annu Rev Biophys. 2009; 38:53–74. [PubMed: 19086820]

411. Bandura DR, Baranov VI, Ornatsky OI, Antonov A, Kinach R, Lou X, Pavlov S, Vorobiev S, Dick JE, Tanner SD. Mass Cytometry: Technique for Real Time Single Cell Multitarget Immunoassay Based on Inductively Coupled Plasma Time-of-Flight Mass Spectrometry. Anal Chem. 2009; 81:6813–6822. [PubMed: 19601617]

412. Bendall SC, Simonds EF, Qiu P, Amir E-aD, Krutzik PO, Finck R, Bruggner RV, Melamed R, Trejo A, Ornatsky OI, Balderas RS, Plevritis SK, Sachs K, Pe'er D, Tanner SD, Nolan GP. Single-Cell Mass Cytometry of Differential Immune and Drug Responses across a Human Hematopoietic Continuum. Science. 2011; 332:687–696. [PubMed: 21551058]

413. Giesen C, Wang HA, Schapiro D, Zivanovic N, Jacobs A, Hattendorf B, Schuffler PJ, Grolimund D, Buhmann JM, Brandt S, Varga Z, Wild PJ, Gunther D, Bodenmiller B. Highly Multiplexed Imaging of Tumor Tissues with Subcellular Resolution by Mass Cytometry. Nat Methods. 2014; 11:417–422. [PubMed: 24584193]

Biographies

Krishnan Palaniappan received his undergraduate degree in Chemistry from Carnegie Mellon University in 2005. He worked with Professor Bruce Armitage to study the behavior of DNA intercalating dyes. He then spent a year at the National Institutes of Health in Bethesda, MD, as a Postbaccalaureate fellow. Working in the lab of Dr. Kenneth Jacobson, he probed the binding sites of G-protein-coupled receptors for orthogonal activation using tailored ligands. In 2006, he attended graduate school at the University of California, Berkeley, where he worked jointly with Professors Carolyn Bertozzi and Matthew Francis. He was awarded his Ph.D. in Chemistry in 2012 for his research on the development of chemical tools and computational strategies for glycoproteomics and the development of viral capsid-based contrast agents for molecular imaging using xenon MRI. He is currently a Senior Scientist at Verily Life Sciences (formerly Google Life Sciences).

Carolyn Bertozzi is the Anne T. and Robert M. Bass Professor of Chemistry and Professor of Chemical & Systems Biology and Radiology (by courtesy) at Stanford University, as well as an Investigator of the Howard Hughes Medical Institute. She completed her undergraduate degree in Chemistry at Harvard University in 1988 and her Ph.D. in Chemistry from UC Berkeley in 1993. After completing postdoctoral work at UCSF in the field of cellular immunology, she joined the UC Berkeley faculty in 1996 and rose through the ranks to become the T. Z. and Armgard Chu Distinguished Professor of Chemistry and Professor of Cell and Molecular Biology. In June 2015, she joined the faculty at Stanford University coincident with the launch of Stanford's ChEM-H institute.
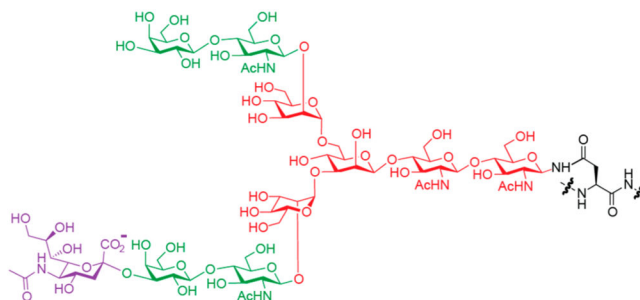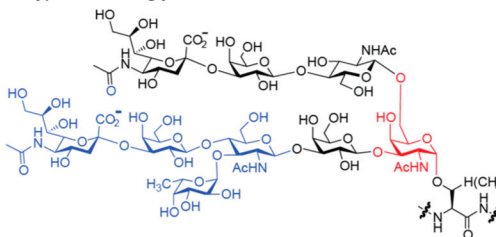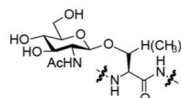
Figure 1.

Glycan structures display a range of complexity. (a) Example of an N-glycan, with the conserved pentasaccharide ($Man_3GlcNAc_2$) core structure highlighted in red. This core structure can be further elaborated with N-acetyllactosamine (GalGlcNAc, green), sialic acid (purple), or other monosaccharides. (b) Example of a mucin-type O-glycan, with the conserved α-linked GalNAc residue highlighted in red. This monosaccharide can also be elaborated to generate higher-order glycans; depicted is the sialyl Lewis X antigen (blue). (c) Example of the O-GlcNAc modification, characterized by a single β-linked GlcNAc monosaccharide.
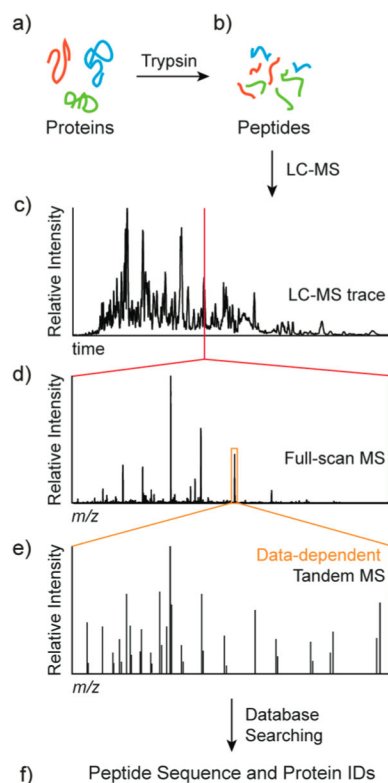
Figure 2.
Schematic of the bottom-up shotgun proteomics method. (a) A mixture of proteins is subjected to proteolytic digestion, producing (b) a mixture of peptides. (c) The peptides are separated using LC, and (d) full-scan mass spectra are collected at regular intervals. (e) Typically, ions are selected from each full scan for fragmentation (i.e., tandem MS) in a data-dependent fashion (i.e., based on ion abundance). (f) These tandem mass spectra are analyzed using a database search to generate peptide and protein identifications.
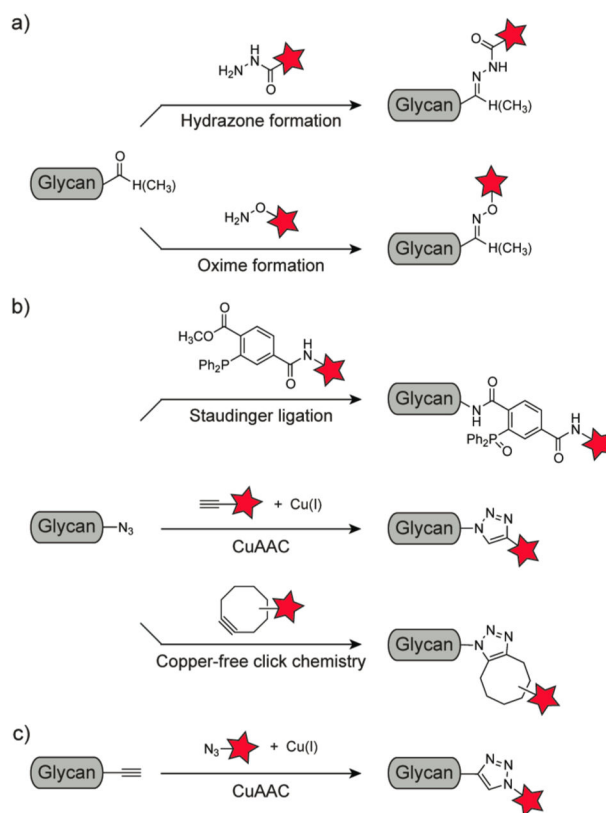
Figure 3.
Bioorthogonal reactions with aldehyde-, ketone-, azide-, or alkyne-functionalized monosaccharides. These chemical handles can be introduced into glycans through chemical, metabolic, or enzymatic methods. After an enrichment probe (red star) has been appended to glycoproteins or glycopeptides, these compounds can be enriched from complex mixtures and identified by MS. (a) Aldehydes and ketones are condensed with amine nucleophiles, including hydrazide compounds (top) and aminooxy compounds (bottom), to form stable hydrazone or oxime linkages, respectively. (b) Azides undergo the Staudinger ligation (top) with triarylphosphines or a [3 + 2] cycloaddition with terminal alkynes through the Cu-catalyzed azide–alkyne cycloaddition (CuAAC, middle) or strained alkynes in the absence of Cu (copper-free click chemistry, bottom). (c) Alkynes undergo a [3 + 2] cycloaddition with azides through the CuAAC reaction.
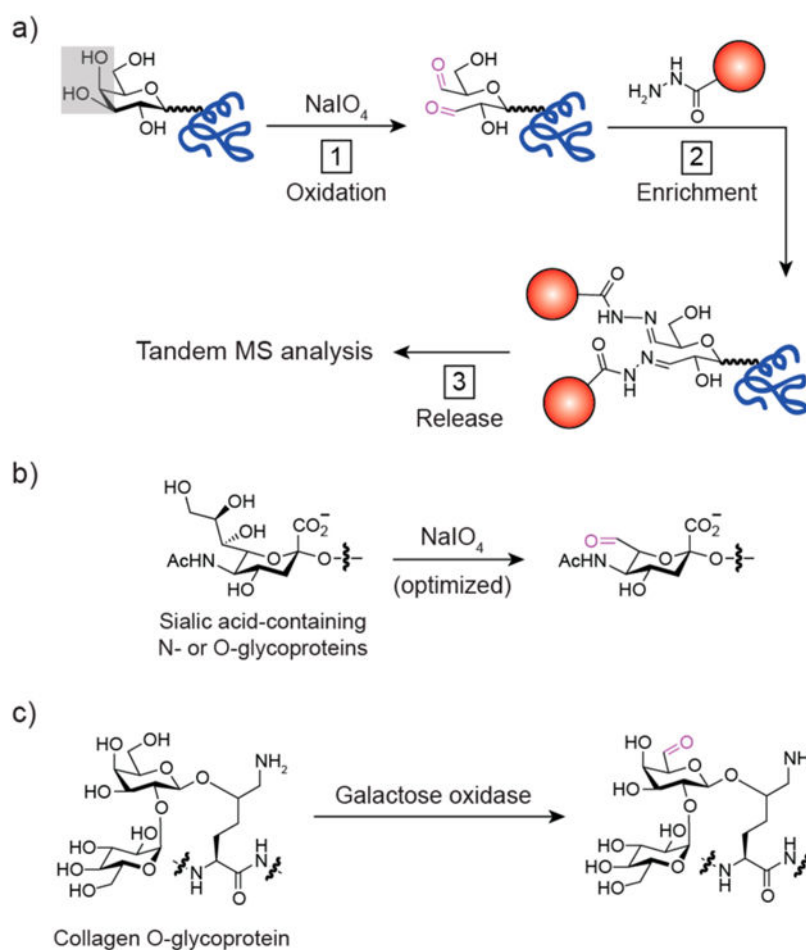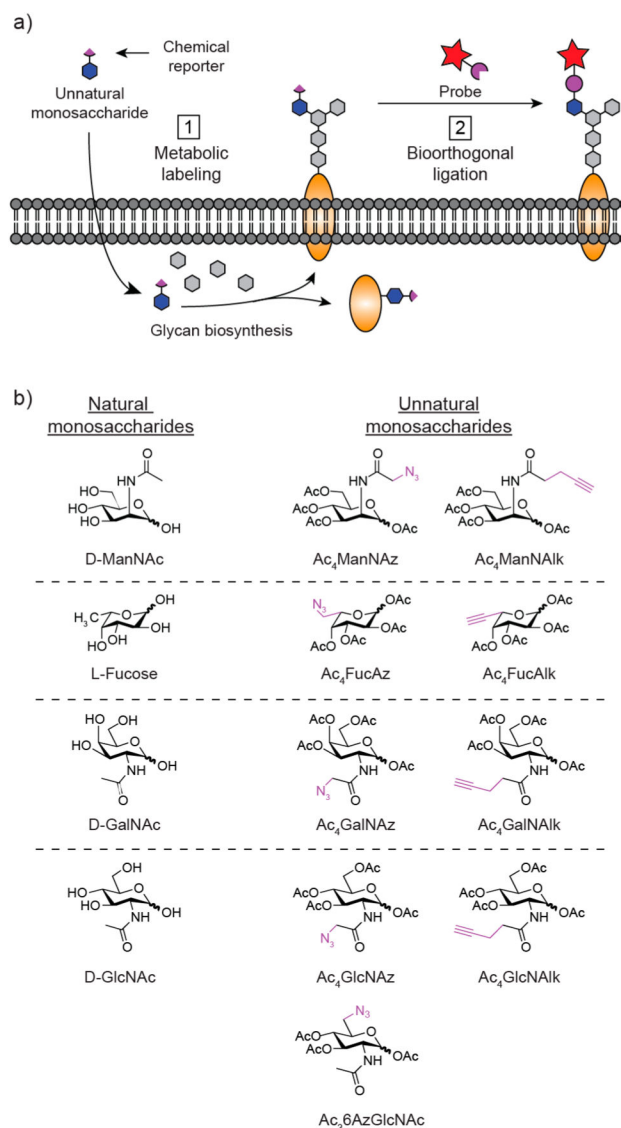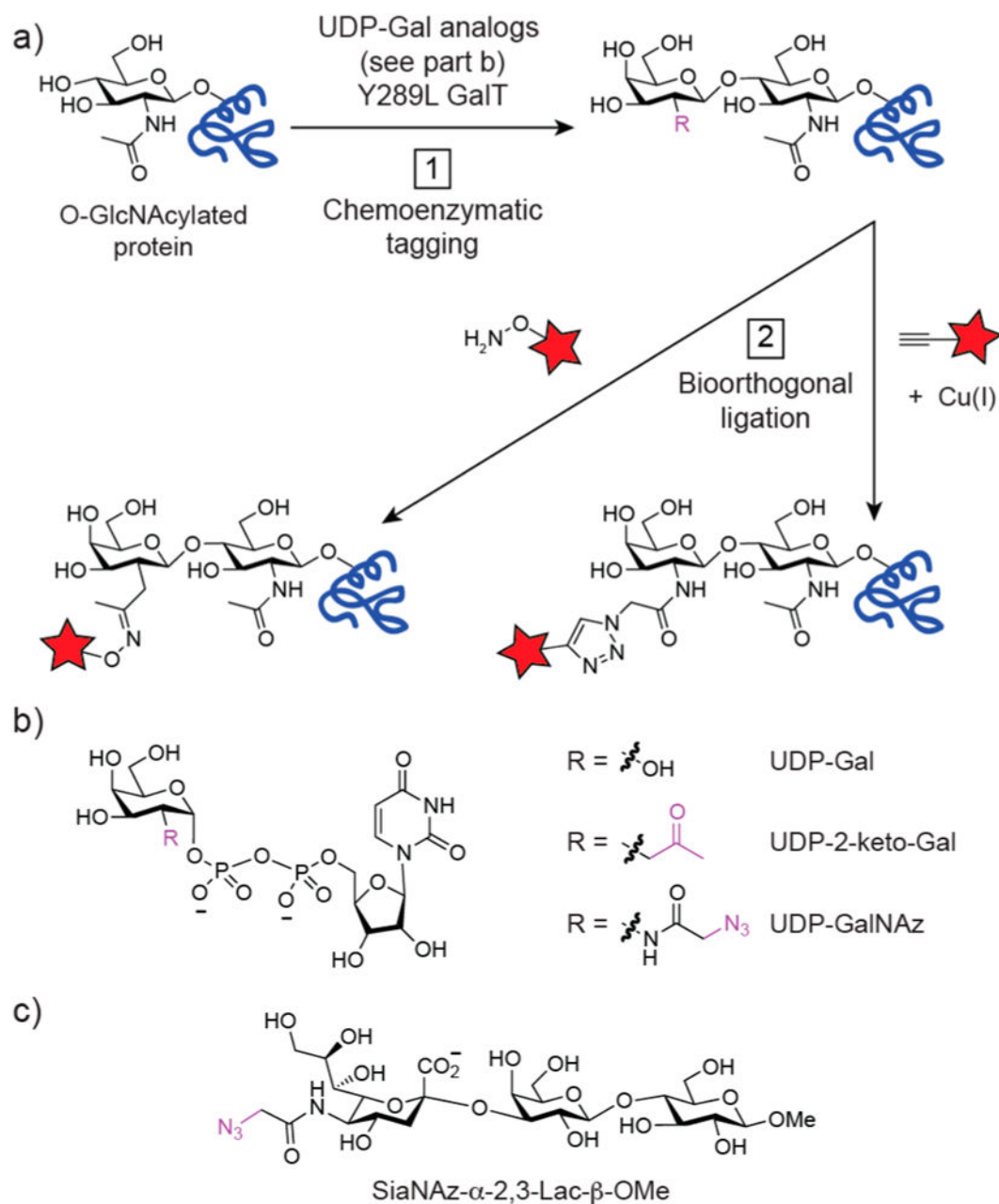
Figure 4.
Enrichment of glycoproteins using carbonyl chemistry. (a) Schematic of hydrazide capture technology. (1) Glycoproteins are subjected to mild oxidants, most commonly sodium periodate, which oxidatively cleaves the cis diols (highlighted in a gray box) in glycans into an aldehyde group (highlighted in purple). (2) The aldehyde is condensed with a hydrazide-functionalized solid support to covalently capture and enrich glycoproteins. (3) The protein is released by deglycosylation from the solid support for identification by LC-MS. (b,c) Hydrazide capture technology can also target specific monosaccharides, including (b) sialic acid using optimized oxidation conditions and (c) galactose using galactose oxidase.
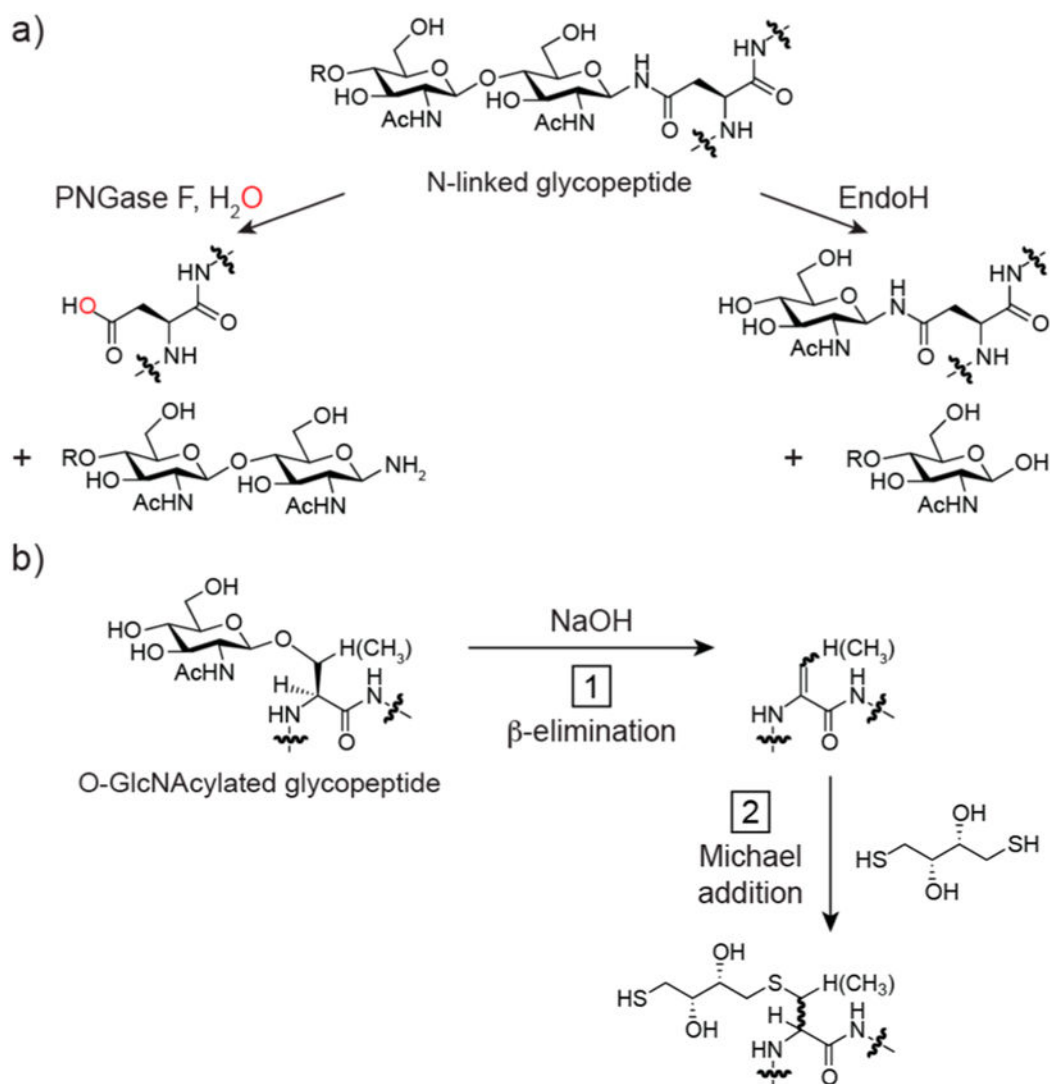
Figure 5.
Enrichment of glycoproteins using metabolic labeling followed by bioorthogonal ligation. (a) Schematic of metabolic labeling to introduce an unnatural monosaccharide carrying a chemical reporter (i.e., azide or alkyne; purple triangle) directly into glycans. (1) Unnatural monosaccharides (blue hexagons) are accepted by the cell's natural machinery and incorporated into the cell surface, secreted, and/or attached to intracellular glycoproteins (orange ovals). The natural monosaccharides are depicted as gray hexagons. (2) A bioorthogonal chemical reaction is performed to label glycoproteins with a probe molecule (red star). (b) Chemical structures of natural monosaccharides (left column) and their azide and alkyne analogues (functional groups highlighted in purple) that have been used for metabolic labeling and enrichment of glycoproteins or glycopeptides. Although the unnatural monosaccharides are depicted in a peracetylated form, after they have entered cells by passive diffusion, cytosolic esterases will cleave the acetyl groups.

Figure 6.
Enrichment of glycoproteins using chemoenzymatic tagging followed by bioorthogonal
ligation. (a) Schematic showing how chemoenzymatic tagging can be used to introduce a
chemical reporter directly onto glycoproteins in vitro. (1) UDP-galactose analogues are
appended onto O-GlcNAc conjugates using a mutant galactosyl transferase (e.g., Y298L
GalT). (2) A bioorthogonal chemical reaction is performed to label glycans with a probe
molecule (red star). (b) Chemical structures of Y298L GalT substrates, including UDP-
galactose (UDP-Gal) and two analogues, UDP-2-keto-Gal and UDP-GalNAz. (c) Structure
of an azide-functionalized trisaccharide used to identify glycoprotein targets of a viral trans-
sialidase.

Figure 7.
Examples of enzymatic and chemical deglycosylation. (a) N-glycans can be enzymatically removed using N-glycosidases or endoglycosidases, most commonly PNGase F (top), which converts asparagine into aspartic acid after cleaving off the entire glycan. If performed in the presence of ^{18}O-labeled water, the heavy oxygen (highlighted in red) will mark the site of glycosylation. Alternatively, Endo H (bottom) cleaves between the two innermost GlcNAc resides, leaving a single peptide-bound GlcNAc residue to mark the site of glycosylation. (b) O-glycans can be chemically deglycosylated. Illustrated is a schematic of the *β*-elimination followed by Michael addition with dithiothreitol (DTT) (BEMAD) method. (1) *β*-elimination, most commonly with NaOH, converts glycosylated serine or threonine residues into dehydroalanine or dehydrobutyric acid, respectively. (2) Michael addition with amine or thiol nucleophiles, most commonly DTT, covalently marks the site of glycosylation.
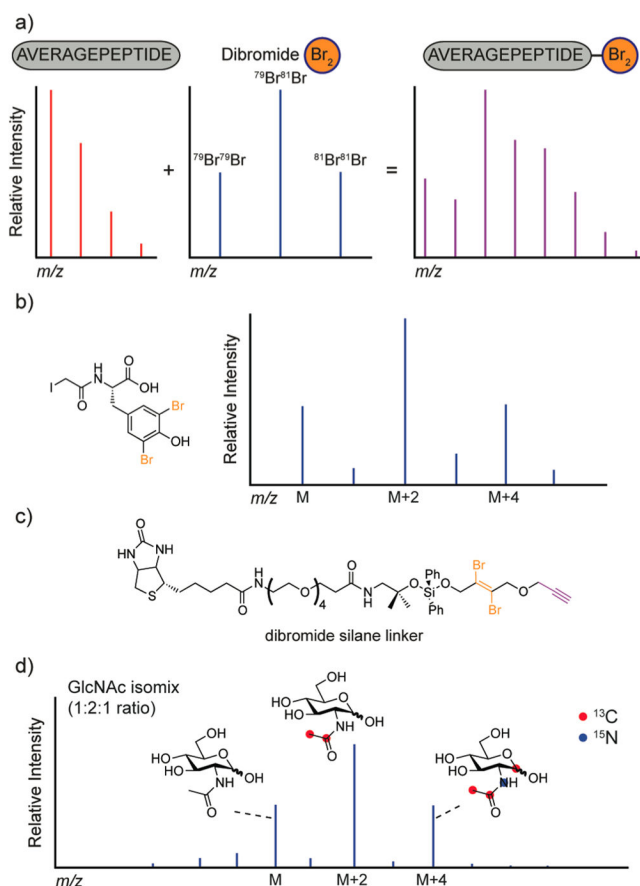
Figure 8.
Chemically directed glycoproteomics using chemical or metabolic methods to embed a dibromide or dibromide-like motif into glycans. (a) Two bromine atoms perturb a peptide's isotopic envelope in a predictable way. Illustrated are simulated isotopic envelopes of an AVERAGEPEPTIDE, a dibromide (Br_2), and a dibromide-labeled AVERAGEPEPTIDE, the last of which can be computationally recognized with high sensitivity and specificity in complex mass spectra. (b) Chemical structure and mass spectrum of an iodoacetamide-derivatized dibromide tag that was used to chemically modify peptides by alkylating cysteine residues. (c) Chemical structure of a cleavable dibromide enrichment tag for isotope-targeted glycoproteomics. The silane linkage can be cleaved using 2% formic acid, producing a low-molecular-weight tag that remains on the labeled glycopeptide. (d) A GlcNAc isomix was used to metabolically install a dibromide-like motif directly into glycoproteins for high-confidence glycosite mapping without chemical tagging. The GlcNAc isomix consisted of three components and was designed to mimic the 1:2:1 peak intensity distribution of the dibromide triplet pattern using heavy nitrogen and carbon instead of bromine atoms.
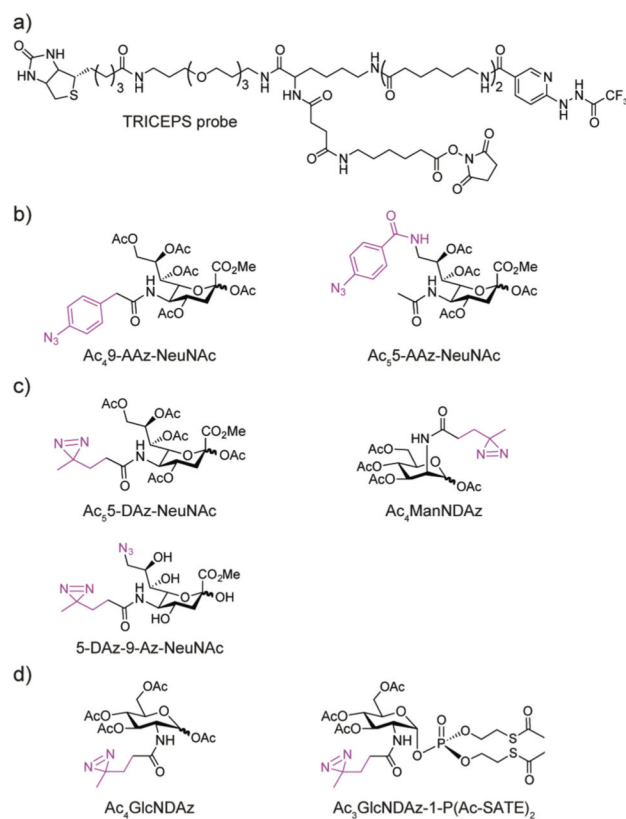
Figure 9.
Chemical tools for covalently capturing glycoproteins and glycan-binding proteins. (a) Chemical structure of the trifunctional chemoproteomics (TRICEPS) probe that was used to capture cell-surface glycoprotein receptors for a specific ligand. (b) Chemical structures of sialic acid derivatives bearing an aryl azide photo-activatable cross-linking functional group. (c) Chemical structures of sialic acid derivatives bearing a diazirine photoactivatable cross-linking functional group. (d) Chemical structures of GlcNAc derivatives bearing a diazirine photoactivatable cross-linking functional group.