CHAPTER 4

The Chemoenzymatic Detection of Fucose-α(1,2)-Galactose Glycans*

Introduction

As we have described in previous chapters, metabolic labeling is a powerful approach to detect glycans *in vivo* and to enrich glycoproteins for proteomic analysis (1, 2). However, one limitation of metabolic labeling is that it tracks only monosaccharides, each of which can be found on a large number of different cellular glycans. For example, although we are interested in Fucα(1-2)Gal structures, the metabolic labeling approach tracks fucosylated glycans of any linkage. Disaccharides or trisaccharides of specific sugar composition and glycosidic linkage are not uniquely accessible by hijacking the biosynthetic machinery with non-natural monosaccharide analogs. Moreover, the non-natural sugar is often incorporated sub-stoichiometrically into glycoconjugates, depending on its extent of cellular uptake and ability to compete with natural sugars.

The complementary approach of chemoenzymatic labeling exploits an exogenous glycosyltransferase to covalently tag specific glycans of interest with a non-natural sugar analog. Similar to metabolic labeling, the non-natural analog contains a bioorthogonal group that can be subsequently reacted with a variety of chemical reporters. Recently, this strategy has been used to detect and image *O*-linked β-*N*-acetylglucosamine (*O*-GlcNAc) glycosylated proteins (3, 4), to identify the *O*-GlcNAc proteome (5, 6), to quantify *O*-GlcNAc glycosylation stoichiometries (7), and to monitor the dynamics of *O*-GlcNAc glycosylation in response to cellular stimuli (3, 6-8). Because chemoenzymatic labeling is not limited by endogenous biosynthetic machinery and has the potential to proceed in quantitative yield, stoichiometric addition of the non-natural sugar can be achieved, resulting in high detection sensitivity relative to antibodies and lectins (3). Additionally, chemoenzymatic approaches have the ability to detect specific glycan motifs of a defined structure,

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depending on the specificity of the exogenous tagging enzyme. For instance, Wu and coworkers recently demonstrated the detection of *N*-acetyllactosamine (LacNAc) on living cells and in developing zebrafish *in vivo* (9). However, the development of a successful chemoenzymatic strategy requires the identification and characterization of suitable enzymes for tagging specific glycans. For this reason, chemoenzymatic methods have only been developed in the few instances described above.

In the following chapter, we describe a new chemoenzymatic strategy for the detection of Fucα(1- 2)Gal glycans within the glycome. As described in Chapter 1, Fucα(1−2)Gal structures are found on *N*- and *O*-linked glycoproteins and glycolipids (10), and the Fucα(1-2)Gal epitope is an attractive biomarker and potential therapeutic target for cancer (11-13). However, the extent to which this sugar epitope serves as a marker of disease progression and its precise contributions to cancer pathogenesis are not well understood. The development of new methods to detect and study Fucα(1-2)Gal glycans would accelerate an understanding of their roles in vital biological processes and disease. We describe a chemoenzymatic approach that exploits the bacterial homologue to the blood group transferase A (BgtA), a recently reported bacterial glyscosyltransferases that synthesizes the blood group A antigen by transferring a GalNAc to the Fucα(1−2)Gal structure (Figure 4.1) (14).

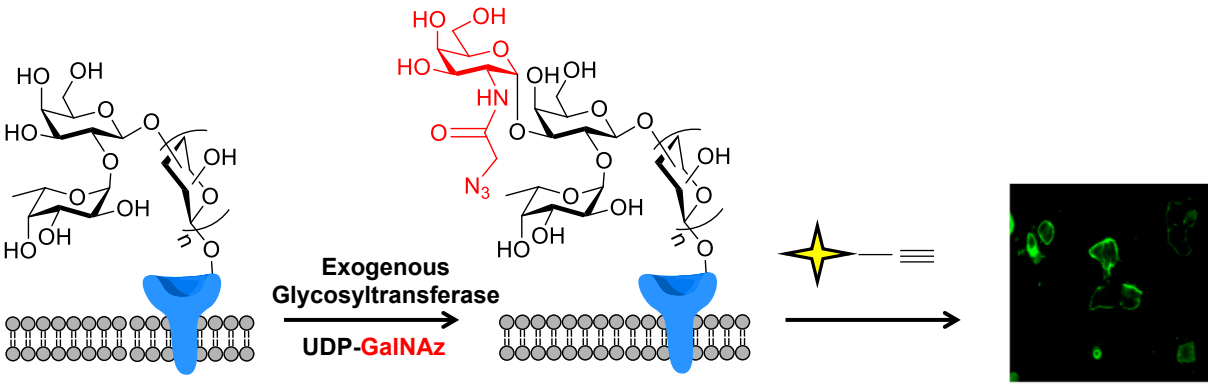

Figure 4.1 The chemoenzymatic strategy allows for the specific detection of Fucα(1-2)Gal glycans.

We take advantage of the specificity of BgtA for Fucα(1−2)Gal structures to develop a rapid, chemoselective, detection of the Fucα(1-2)Gal epitope on a wide range of oligosaccharide structures. We demonstrate that this approach can be used to selectively label glycoproteins in neuronal cell lysates and to track Fucα(1-2)Gal glycoconjugates on the surfaces of living cells. Moreover, we apply this approach to quantify the relative expression levels of Fucα(1−2)Gal glycans on normal and tumorigenic cells.

Results and Discussion

Expression and Purification of BgtA

The bacterial homologue of the human blood group A antigen glycosyltransferase (BgtA) was acquired as a His tagged construct in the pET28A vector from the laboratory of George Peng Wang (14). The enzyme was expressed and purified from Bl21 cells on a Ni-NTA column. Cells were cultured at 16 °C and protein expression was induced with 0.8 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) when at an optical density (O.D) of 0.8 units. Protein was subsequently expressed at 16 °C for 16 h, after which the cells were pelleted and lysed for protein purification. Induction and expression of the enzyme was confirmed via SDS-PAGE prior to purification. The enzyme was purified on Ni-NTA resin over the period of two days, and eluted in increasing concentrations of imidazole. Fractions were analyzed via SDS-PAGE (Figure 4.2); the purified enzyme appears at a molecular weight of 37 kDa. Fractions containing pure enzyme were concentrated to 1 mg/mL and stored at 4 °C. Typically, 10 L of bacterial culture yielded 2-4 mg of purified enzyme.

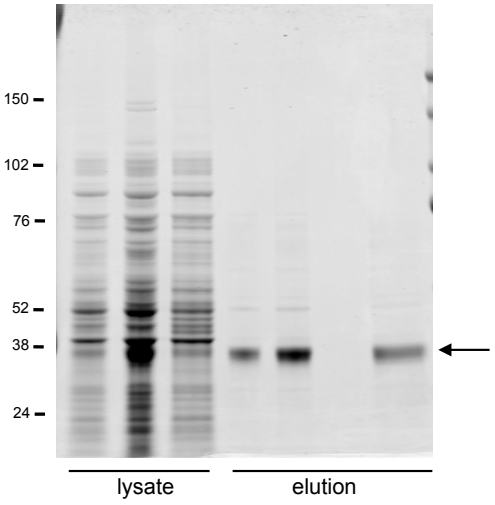

Figure 4.2 The expression and purification of BgtA

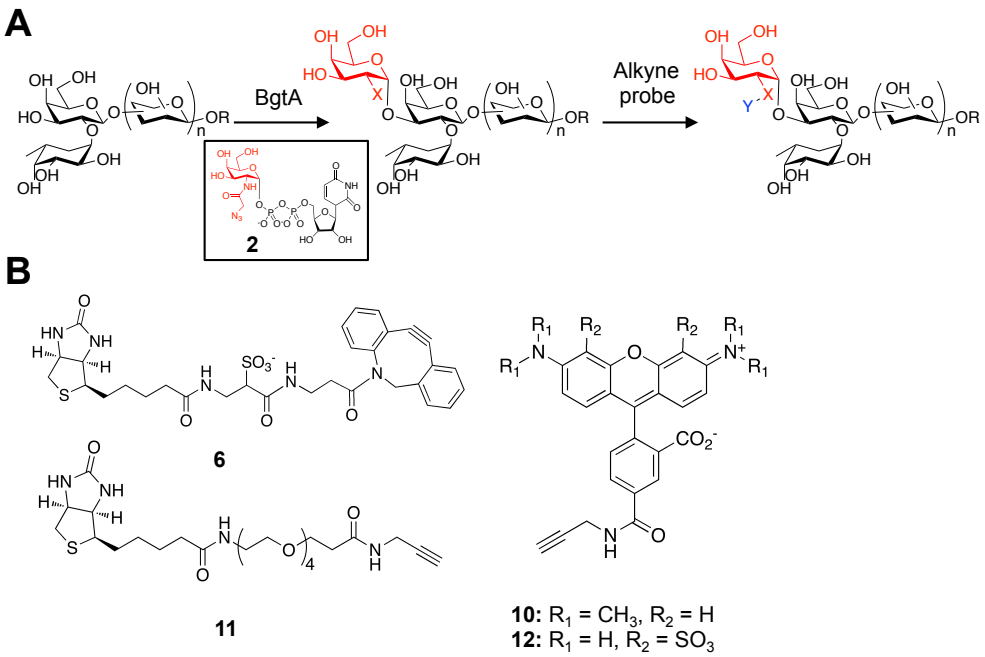

Figure 4.3 Chemoenzymatic strategy to detect Fucα(1-2)Gal glycans. (A) The chemoenzymatic detection strategy with the non-natural nucleotide sugar analog **2**, UDP-GalNAz (B) Alkyne probes for the detection of chemoenzymatically tagged glycans.

BgtA Accepts Non-Natural Substrates with Azido- Functionalities.

In order to design a method for the detection of Fucα(1-2)Gal glycans, we first investigated whether BgtA could be exploited to transfer a non-natural sugar analog to the Fucα(1-2)Gal structure. BgtA has been shown to transfer *N*-acetylgalactosamine (GalNAc) from UDP-GalNAc onto the C-3 position of Gal in Fucα(1-2)Gal structures (14). As other *N*-acetylgalactosaminyltransferases have been shown to tolerate substitutions at the C-2 position of GalNAc (15), we hypothesized that BgtA might also tolerate a substitution in the donor nucleotide sugar, allowing for the incorporation of an azido tag onto Fucα(1-2)Gal (Figure 4.3A). The labeled glycoconjugates could subsequently be detected with alkyne-functionalized probes using CuAAC chemistry (Figure 4.3B) (16-18). To test this approach, we synthesized the Fucα(1-2)Gal substrate **1** via reductive amination of 2'-fucosyllactose with *p*-nitrobenzylamine and sodium cyanoborohydride (Figure 4.4).

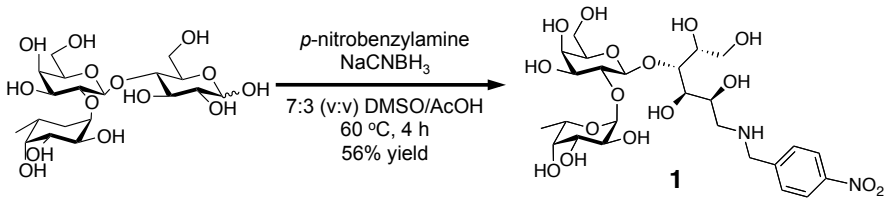

Figure 4.4 Synthesis of Fucα(1-2)Gal substrate **1**

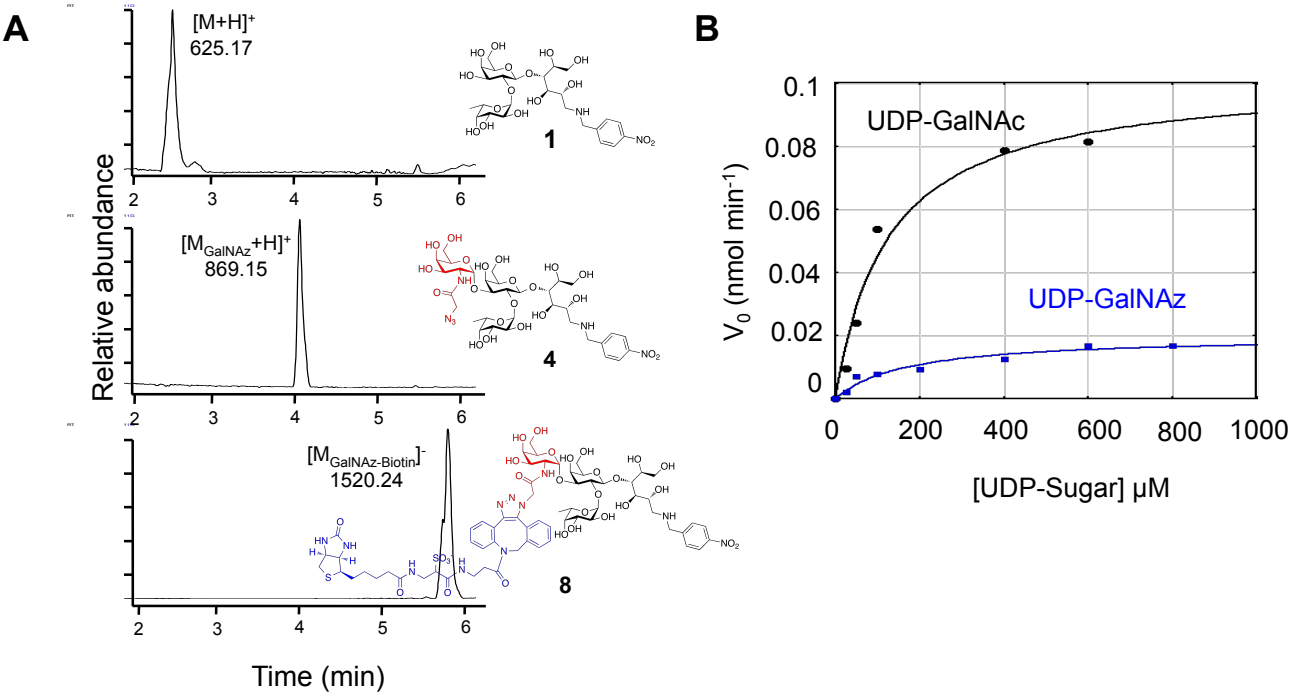

Figure 4.5 Labeling of substrate 1. (A) LC−MS traces monitoring the reaction progress at time 0 (top), 12 h after the addition of BgtA and 2 (middle), and 3 h after the addition of ADIBO-biotin 6 (bottom). (B) Kinetic comparison of the BgtA-catalyzed reaction of 1 with UDP-GalNAc (black) and UDP-GalNAz (blue). Reactions were performed in duplicate using 100 μM of acceptor 1 and varying concentrations of the donor. Initial rates as a function of substrate concentration were plotted and revealed apparent kcat/Km values of 5.7 nM-1min-1 and 40.4 nM-1min-1, respectively, and apparent Km values of 127 ± 36 μM and 168 ± 55 μM, respectively. The apparent Vmax value for UDP-GalNAc (0.100 ± 0.010 nmol•min-1) is approximately 5-fold higher than that of UDP-GalNAz (0.020 ± 0.002 nmol•min-1).

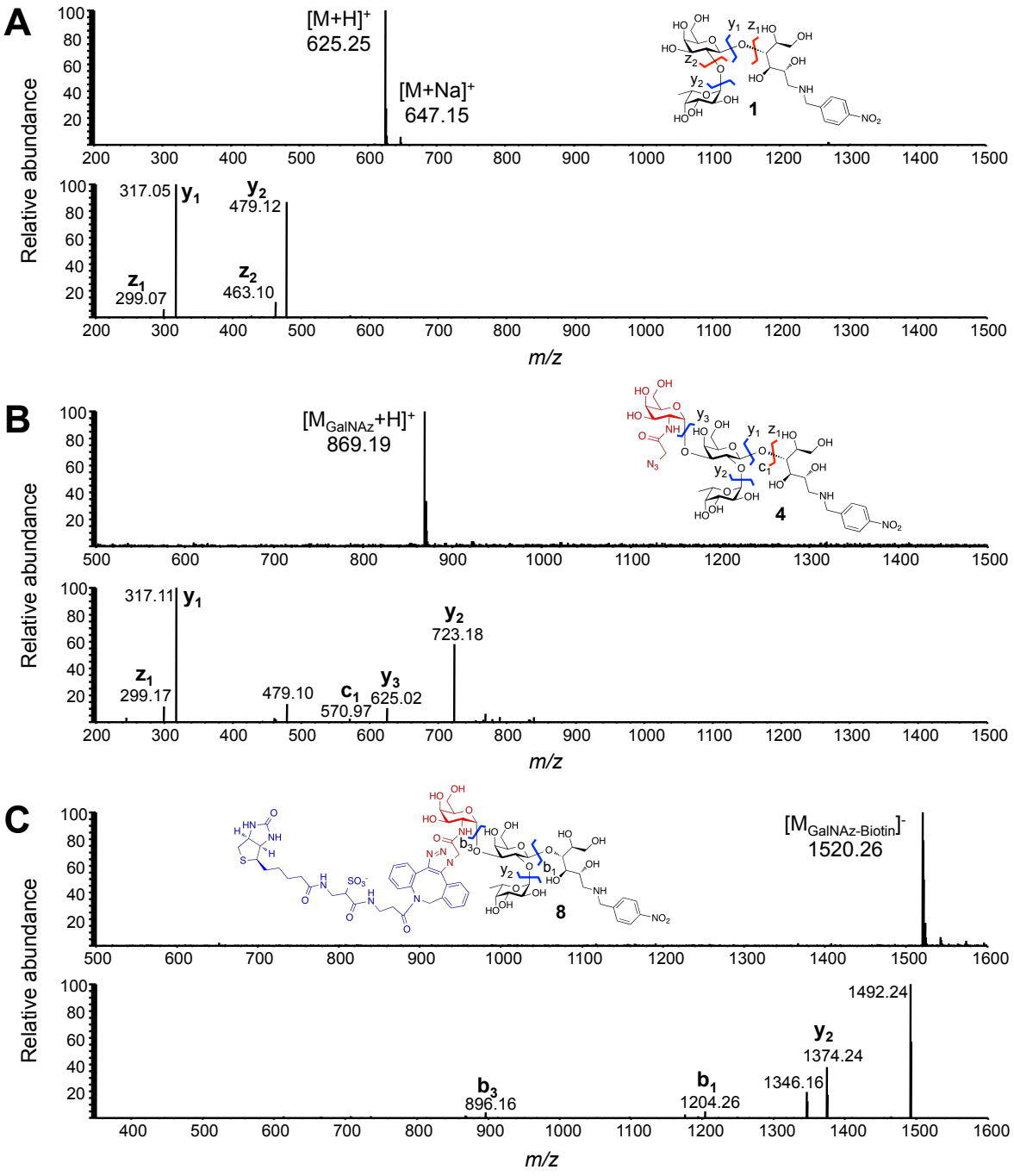

Figure 4.6 LC-MS/MS analysis of 1, 4 and 8 during the chemoenzymatic labeling reaction. (A) Compound **1** at time 0. (B) Compound **4**, generated 12 h after the addition of BgtA and UDP-GalNAz **2**. (C) Biotinylated glycan **8**, generated 3 h after reaction with ADIBO-biotin **6**.

BgtA was expressed in *E. coli* and purified to homogeneity as described above. Treatment of **1** with BgtA and UDP-*N*-azidoacetylgalactosamine (UDP-GalNAz, **2**; Figure 4.3A) led to complete conversion to the desired product **4**, after 12 h at 4 °C, as determined by liquid chromatography-mass spectrometry analysis (LC-MS; Figure 4.5A). Kinetic analysis revealed an apparent K_{cat}/K_m value of 5.7 nm^{-1} min^{-1} for UDP-GalNAz, approximately 7 fold lower than the value obtained for UDP-GalNAc, 40.4 nm^{-1} min^{-1} (Figure 4.5B). Subsequent reaction with an aza-dibenzocyclooctyne-biotin conjugate (ADIBO-biotin, **6**; Figure 4.3B) using copper-free click chemistry (3 h, rt) afforded the biotinylated product **8** (Figure 4.5). LC-MS/MS analysis of compounds **1**, **4**, and **8** identified masses and fragmentation patterns consistent with the expected structures (Figure 4.6). Thus, we determined that BgtA accepts the non-natural substrate UDP-GalNAz and can be exploited for efficient chemoenzymatic labeling of Fucα(1-2)Gal glycans.

BgtA Specifically Modifies Fucα(1-2)Gal Glycans on Carbohydrate Microarrays.[†]

A major advantage of chemoenzymatic tagging over metabolic labeling approaches is their ability tqo detect a specific subset of glycan structures, such as specific disaccharides. In order to profile the glycan specificity of BgtA, we utilized glycan microarrays provided by the Consortium for Functional Glycomics (19). The glycan microarrays are printed with 611 distinct glycan structures, and have been utilized to test the specificities of lectins and or antibodies, and profile the reactivity of glycosyltransferases (19-22). Glycosylation reactions were carried with BgtA and UDP-GalNAz on the microarray at 3 different time points (0.5, 2, and 12 h). A control reaction, with the UDP-GalNAz omitted, was carried out at 12 h. Subsequent to the chemoenzymatic labeling, the labeled glycans were reacted with ADIBO-Biotin and the biotinylated glycans were detected with Cy5-conjugated streptavidin. Strong fluorescence labeling of Fucα(1-2)Gal structures was observed within 0.5 h (Figure 4.7). The top 26 glycans labeled consisted of terminal Fucα(1-2)Gal structures, including the Globo H hexasaccharide (60; Figure 4.7), highlighting the specificity

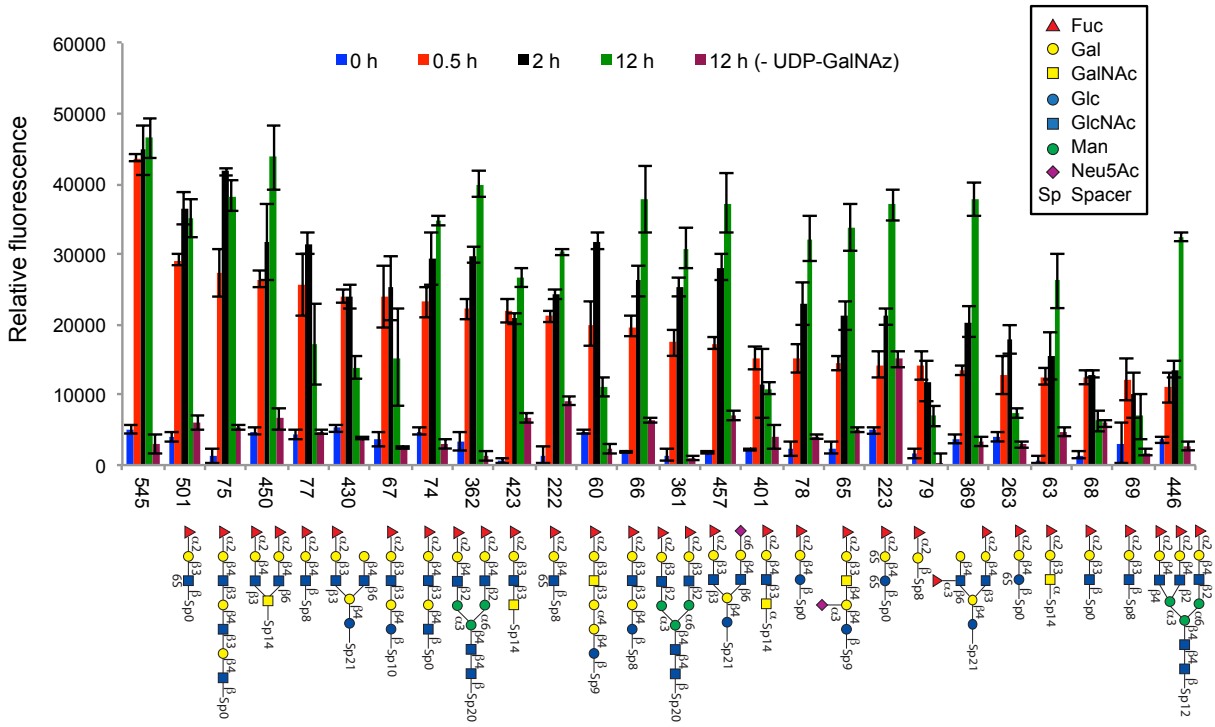

Figure 4.7 Time course analysis using glycan microarrays. Representative structures from the top 26 glycans with the highest relative fluorescence intensity after 0.5 h are plotted, all of which represent terminal Fucα(1-2)Gal structures. UDP-GalNAz was omitted as a control (12 h, -UDP-GalNAz).

of our chemoenzymatic approach. Notably, a wide variety of structures containing Fucα(1-2)Gal were efficiently labeled, including both linear (e.g., 501, 74, and 66; Figure 4.8A) as well as branched structures (e.g., 450, 362, and 457; Figure 4.8B). Moreover, ~91% of the Fucα(1-2)Gal glycans containing a free C-3 hydroxyl group on Gal were labeled on the array, demonstrating the sensitivity of the approach. In summary, our results demonstrate that chemoenzymatic labeling with BgtA is both sensitive and selective for glycans containing terminal Fucα(1-2)Gal structures, including the Lewis blood group antigens (type I and II) and the cancer-relevant Globo H antigen (23, 24)

Labeling of Fucα(1-2)Gal Proteins in Cell Lysates.

Having demonstrated the specificity of the approach, we next sought to determine whether the chemoenzymatic strategy could be used to detect Fucα(1-2)Gal glycoproteins in cell lysates. As it had been previously reported that Fucα(1-2)Gal glycoproteins are highly enriched in the olfactory

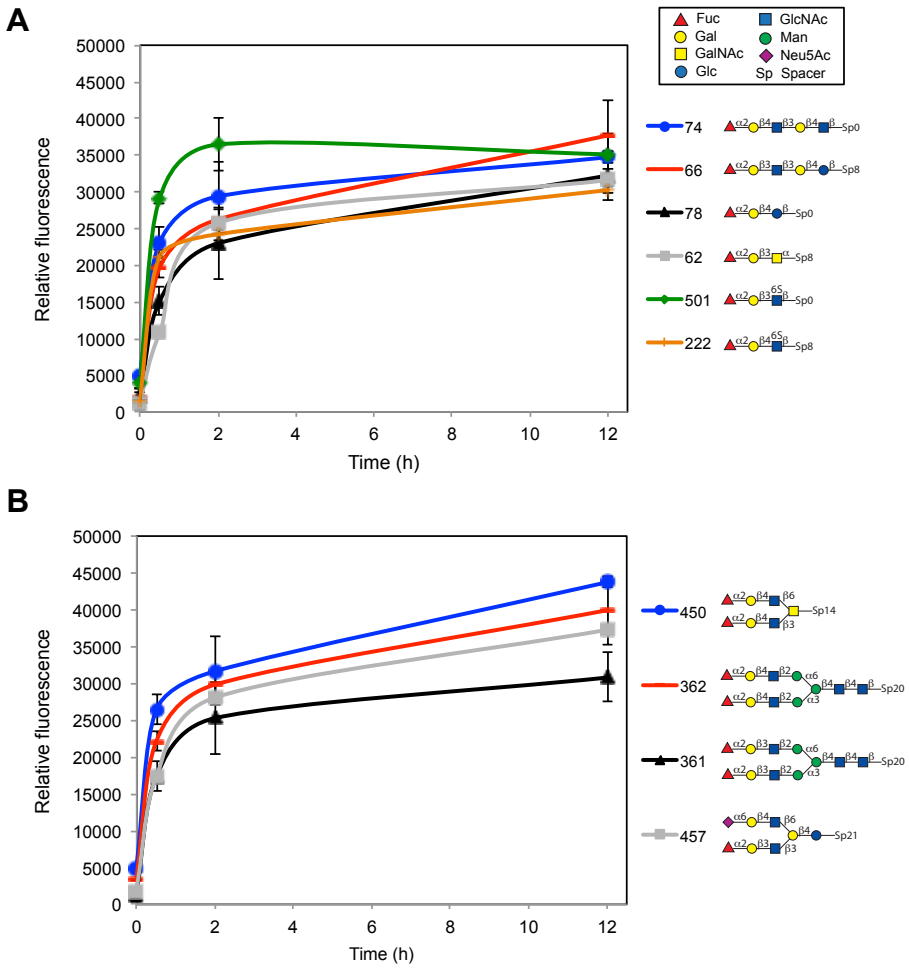

Figure 4.8: The chemoenzymatic approach labels a variety of linear (*A*) and branched (*B*) Fucα(1-2)Gal structures.

bulb of postnatal day 3 (P3) rat pups (25, 26), we elected to test the chemoenzymatic strategy in olfactory bulb lysates. Olfactory bulb lysates were chemoenzymatically labeled with BgtA and UDP-GalNAz, and then subjected to a CuAAC reaction with an alkyne-functionalized tetramethyl-6-carboxyrhodamine dye (alkyne-TAMRA, **10**; Figure 4.3B). Labeling with alkyne-TAMRA enabled direct visualization of the glycoproteins by in-gel fluorescence. As shown in Figure 4.9, we observed strong fluorescence labeling of Fucα(1-2)Gal glycoproteins (lane 1). Minimal non-specific labeling was detected in the absence of BgtA, UDP-GalNAz, or alkyne-TAMRA (lanes 2-4). Interestingly, BgtA itself was labeled, suggesting that it may bear a Fucα(1-2)Gal glycan and undergo autoglycosylation.

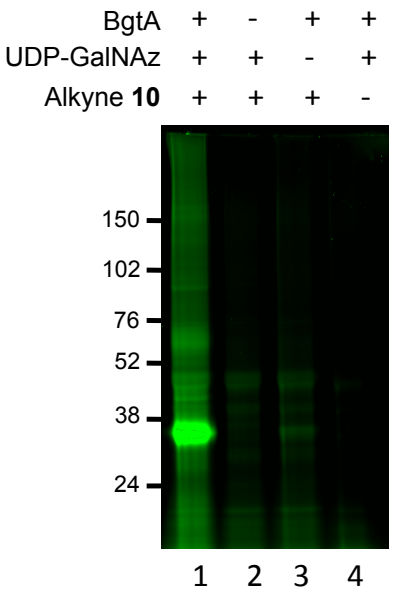

Figure 4.9 In-gel fluorescence detection of Fucα(1-2)Gal glycoproteins from olfactory bulb lysates. Low background fluorescence was observed in the absence of BgtA, UDP-GalNAz 2, or alkyne-TAMRA 10. The strong band at approximately 35 kDa is likely autoglycosylated BgtA.

Labeling and Enrichment of Specific Fucα(1-2)Gal Proteins

To confirm the specific labeling of Fucα(1-2)Gal glycoproteins, we biotinylated the chemoenzymatically labeled olfactory bulb lysates using the alkyne-biotin derivative **11** (Figure 4.4B), isolated the biotinylated proteins on streptavidin resin, and probed for the presence of known Fucα(1-2)Gal glycoproteins. Neural cell adhesion molecule (NCAM), synapsin I (syn I), and munc18-1 were all detected by immunoblotting, confirming the specific chemoenzymatic labeling of known Fucα(1-2)Gal glycoproteins (Figure 4.10A). None of the target proteins were detected in the streptavidin enriched fraction of control lysates labeled in the absence of BgtA. In addition to endogenous proteins, overexpressed synapsin I could also be labeled via the chemoenzymatic strategy. Glycosylated synapsin I could be readily detected following overexpression of Flag-tagged synapsin I in HeLa cells, chemoenzymatic labeling of the lysates with alkyne-TAMRA, synapsin immunoprecipitation, and visualization of the labeled sugar using an anti-TAMRA antibody (Figure 4.10B). Together, these results demonstrate that the chemoenzymatic tagging strategy can be utilized to label both endogenous and overexpressed Fucα(1-2)Gal glycoproteins from cells and tissues.

We also investigated whether the chemoenzymatic strategy was more sensitive than a lectin affinity chromatography method to detect Fucα(1-2)Gal glycoproteins. Olfactory bulb lysate was enriched over UEAI conjugated resin or control agarose resin, and probed for the enrichment of synapsin I. Importantly, UEAI lectin affinity chromatography failed to pull-down and detect glycosylated synapsin I, though the chemoenzymatic strategy detected a significant fraction of glycosylated synapsin I, when performed on the same scale (Figure 4.11). Moreover, previous studies have reported that the Fucα(1-2)Gal-specific antibody A46-B/B10 does not immunoprecipitate glycosylated synapsin I from the same neuronal lysates (25), indicating that the chemoenzymatic strategy is more sensitive than a fucose-specific lectin and antibody.

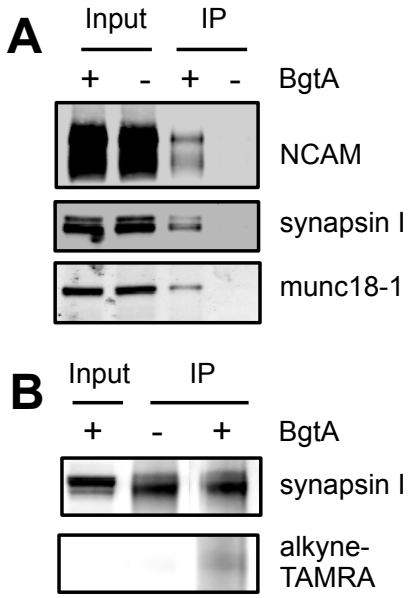

Figure 4.10 Chemoenzymatic Detection of Fucα(1-2)Gal Glycoproteins (A) Chemoenzymatic detection of endogenous Fucα(1-2)Gal glycoproteins from neuronal lysates. (B) Chemoenzymatic detection of FLAG-tagged synapsin I expressed in HeLa cells.

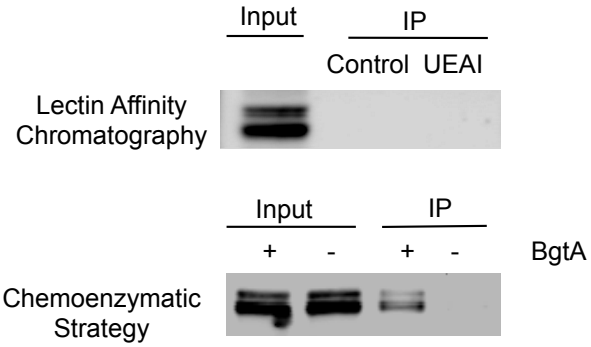

Figure 4.11 Lectin affinity chromatography with UEAI was performed on 500 µg of olfactory bulb lysate. No synapsin was detected in the enriched fraction, whereas synapsin was detected when the chemoenzymatic strategy was performed on 500 µg of olfactory bulb lysate, demonstrating that the chemoenzymatic strategy detects Fucα(1-2)Gal glycoproteins with greater sensitivity.

Imaging and Quantification of Fucα(1-2)Gal Glycans on Cells.

Finally, we investigated whether our chemoenzymatic labeling strategy could be used to image Fucα(1-2)Gal glycans in intact cells. We first sought to detect a known Fucα(1-2)Gal glycoprotein, and thus transfected HeLa cells with a Flag-tagged synapsin I construct. Expression of Flag-synapsin I was detected 24 h post-transfection using an anti-Flag antibody (Figure 4.12, red). To detect glycosylated synapsin, the cells were fixed, permeabilized, and chemoenzymatically labeled on coverslip with BgtA and UDPGalNAz. CuAAC chemistry was then performed using an alkyne-functionalized Alexa Fluor 488 dye (**12**; Figure 4.3B) to install a fluorescent reporter onto the Fucα(1-2)Gal glycans. Strong fluorescence labeling of cells transfected with synapsin I was observed (Figure 4.13, green), and the labeling showed excellent overlap with Flag-synapsin I expression (yellow). Cells expressing Flag-synapsin I that were not treated with BgtA did not exhibit strong labeling, suggesting the specific labeling of a Fucα(1-2)Gal structure on synapsin I (Figure 4.13, middle row). Chemoenzymatic labeling of cells not expressing Flag-synapsin I showed only weak labeling (Figure 4.13, bottom row), suggesting labeling of endogenous Fucα(1-2)Gal glycans.

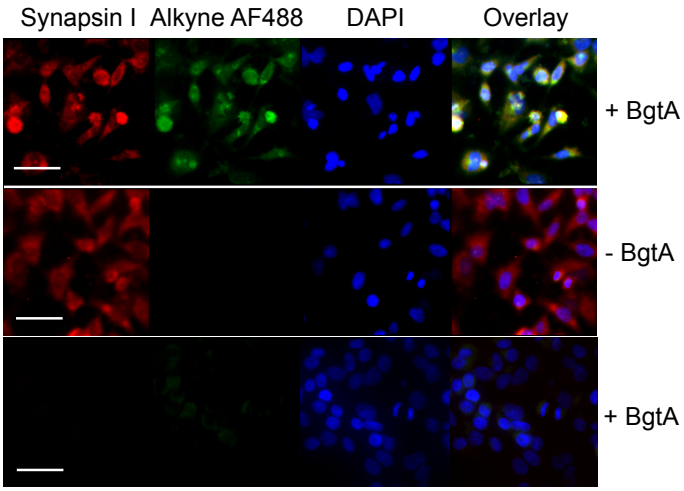

Figure 4.12 Fluorescence detection of Fucα(1-2)Gal glycans (green) shows significant overlay (yellow) with overexpressed FLAG-Synapsin I (red) in HeLa cells. Low levels of endogenous Fucα(1-2)Gal glycans are labeled in mock-transfected cells (bottom row.)

The Fucα(1-2)Gal epitope has been identified on the cell surface of breast, prostate, and ovarian cancer cells and may be a useful biomarker for carcinoma progression and prognosis.(23, 27) Therefore, we sought to apply our chemoenzymatic approach toward the detection of endogenous Fucα(1-2)Gal glycans on live cancer cells. Cells from the human breast adenocarcinoma cell line MCF-7, known to express high levels of the Globo H hexasaccharide (24), were first chemoenzymatically labeled with BgtA and the natural donor-sugar UDP-GalNAc, and detected with a anti-blood group A antibody conjugated to fluorescein to confirm the enzymatic labeling of surface Fucα(1-2)Gal glycans (Figure 4.13). Cell surface fluorescence was apparent, indicating that BgtA successfully labeled cell-surface glycans. MCF-7 cells were then labeled with BgtA and UDP-GalNAz for 1 h at 37 °C. We attempted to detect cell surface labeling with a variety of fluorescent strained-alkyne probes, including biarylazacycloocynone-fluorescein (BARAC-Fluor) and difluorinated cyclooctyne–Alexa Fluor 488 (DIFO-488) (28-30). However, low signal complicated our efforts and we were unable to successfully detect chemoenzymatically labeled Fucα(1-2)Gal glycans with the fluorescent strained alkyne probes. We resorted to a two-step detection strategy, consisting of tagging the chemoenzymatically labeled glycans with ADIBO-Biotin **6**, followed by

visualization with a fluorescent streptavidin conjugate. After reaction with ADIBO-biotin (1 h, rt), Fucα(1-2)Gal glycans were successfully detected using streptavidin conjugated to Alexa Fluor 488. Membrane-associated fluorescence was observed for cells treated with both BgtA and UDP-GalNAz, whereas no labeling was detected for control cells labeled in the absence of BgtA (Figure 4.14).

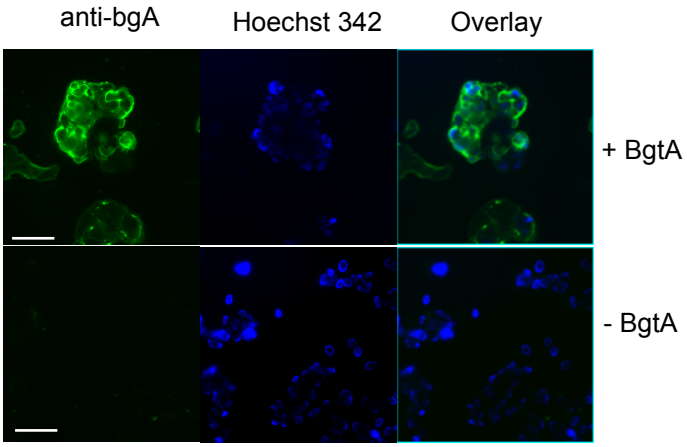

Figure 4.13 Chemoenzymatic Labeling with UDP-GalNAc. Fluorescence detection of Fucα(1-2)Gal glycans (green) on live MCF-7 cells with anti-BgA conjugated to flourescein. Cells were chemoenzymatically labeled, utilizing the natural donor UDP-GalNAc and detected with an antibody recognizing the blood group A antigen, anti-bgA. Nuclei were stained with Hoechst 342 (blue).

The ability to image Fucα(1-2)Gal glycans on living cells could facilitate comparisons of the expression levels of Fucα(1-2)Gal glycans across various cancers and the development of diagnostic tools to detect cancer progression. In order to assess the potential of the chemoenzymatic strategy for biomarker detection, several cancer cell lines were chemoenzymatically labeled to detect surface Fucα(1-2)Gal expression. MCF-7 (breast cancer), MDA-mb-231 (highly invasive breast cancer), H1299 (lung cancer), and LnCAP (prostate cancer) cell lines were chemoenzymatically labeled in suspension with BgtA and UDP-GalNAz (2 h, 37 °C), reacted with ADIBO-biotin (1 h, rt), and stained with streptavidin- Alexa Fluor 488 (20 min, 4 °C). Control reactions were undertaken with the UDP-GalNAz omitted, and cell surface fluorescence of viable cells was detected by flow cytometry. As shown by flow cytometry analysis, LnCaP cells displayed the highest levels of

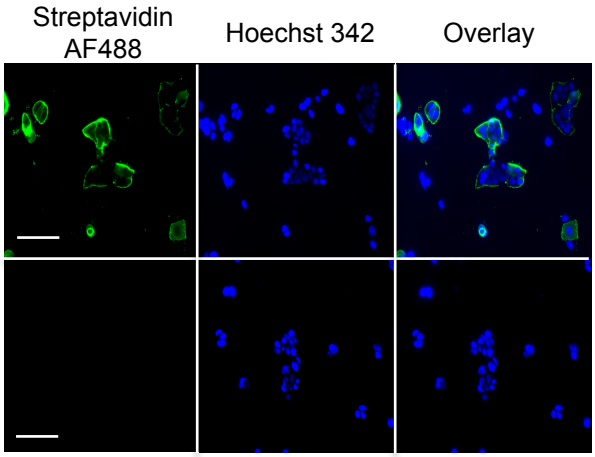

Figure 4.14 Fluorescence detection of Fucα(1-2)Gal glycans (green) on live MCF-7 cells. Nuclei were stained with Hoechst 342 (blue).

fluorescence, followed by the mammary cancer cells lines MCF-7 and MDA-mb-231, with H1299 cells showing lower fluorescence signal (Figure 4.14). MCF-7, MDA-mb-231 and LnCAP cells exhibited expression of Fucα(1-2)Gal, consistent with reports of high Globo H expression on mammary and prostate tumors (24, 31). H1299 cells, a model for non-small cell lung carcinoma and also reported to express Globo H (32), showed lower levels of Fucα(1-2)Gal expression as determined by chemoenzymatic labeling. As the Fucα(1-2)Gal antigen is a potential target for cancer therapeutics (11-13), the rapid profiling of Fucα(1-2)Gal expression levels on specific cancer cells or tissue may assist in determining whether the antigen is a useful therapeutic target for a particular cancer. Primary prostate epithelial cells (PrEC) were then labeled to determine whether our chemoenzymatic strategy could be used to detect changes in Fucα(1-2)Gal expression levels in normal versus cancerous cells. Flow cytometry analysis showed a strong difference in the fluorescence labeling of LnCAP cells compared to PrEC cells, with approximately a 50% increase in Fucα(1-2)Gal expression on the surface of LnCAP cells (Figure 4.15A and 4.15B). These results demonstrate that the chemoenzymatic approach can handily distinguish between cancerous cells and normal cells, providing a new potential strategy for biomarker detection. The method could be

particularly useful for the detection of prostate cancer from tissue biopsies, as the current standard of PSA detection to diagnose prostate cancer has a significant false-positive rate, leading to overtreatment (33). In addition to histological detection, our chemoenzymatic technique could provide a new potential strategy to distinguish normal PSA from tumorigenic PSA, which has higher levels of Fucα(1-2)Gal glycosylation (34). Future studies will optimize PSA detection strategies and explore the diagnostic applications of this approach to human disease.

In conclusion, we have developed a robust chemoenzymatic strategy for the rapid and sensitive detection of Fucα(1-2)Gal glycans, a disaccharide motif implicated in cognitive processes and a potential cancer biomarker. The approach enables the specific, covalent labeling of Fucα(1-2)Gal glycans in cell lysates and on the cell surface with a variety of chemical reporters. Given the versatility of the azide and ketone functionalities that can be installed onto the Fucα(1-2)Gal epitope, we envision a variety of diverse applications for this strategy, including biomarker detection, affinity enrichment, and isotopic labeling for comparative proteomics of Fucα(1-2)Gal glycoproteins. The ability to specifically detect Fucα(1-2)Gal glycans should facilitate investigations into the physiological importance of the Fucα(1-2)Gal epitope in both neurobiology and cancer. Such tools will expand the technologies available for glycomic studies and further our understanding of the roles of specific carbohydrate structures in physiology and disease

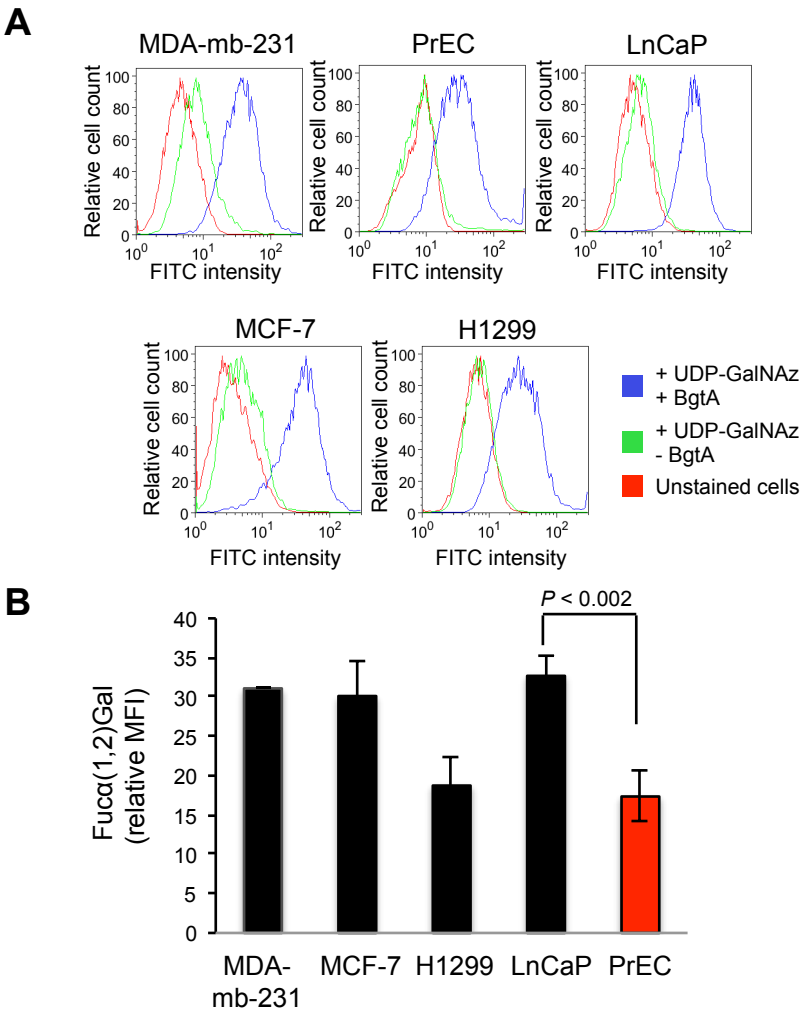

Figure 4.14 (A) Flow cytometry analysis of the relative expression levels of Fucα(1-2)Gal glycans across various cancer cell lines, with comparison to non-cancerous PrEC cells. Cells were untreated (red) or chemoenzymatically labeled in the presence (blue) or absence (green) of BgtA. (B) Quantification of the mean fluorescence intensity (MFI) relative to cells labeled in the absence of BgtA. Error bars represent data from duplicate (MCF-7, MDA-mb- 231, H1299) or triplicate (LnCAP, PrEC) experiments.

Experimental Methods

General Methods for Chemical Synthesis. Unless otherwise stated, all starting materials and reagents were purchased from Sigma-Aldrich and used without further purification. All ^{1}H and ^{13}C NMR spectra were recorded on a Varian Innova 600 spectrometer and referenced to solvent peaks. Data for ^{1}H NMR spectra are reported as follows: chemical shift (δ ppm), multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet), coupling constant in Hz, and integration. Low-resolution mass spectra were recorded on an 1100 Agilent Liquid Chromatograph Mass Spectrometer with an Agilent SB-C18 reverse-phase column (3.5 µm, 4.6 x 250 mm) with monitoring at 280 and 310 nm. High-resolution mass spectra (HRMS) were obtained using an Agilent 6200 Series Time of Flight Mass Spectrometer with an Agilent G1978A Multimode source using mixed electrospray ionization/atmospheric pressure chemical ionization (MultiMode ESI/APCI).

Synthesis of 1-*p*-Nitrobenzyl-(2-fucosyl)-lactose (1). A 0.35 M solution of *p*-nitrobenzylamine in 7:3 (*v/v*) DMSO/AcOH (50 µL, 18 µmol) was added slowly to 2'-fucosyllactose (1.0 mg, 2.0 µmol) at rt. $NaCNBH_3$ (50 µL of a 1 M solution in 7:3 (*v/v*) DMSO/AcOH, 50 µmol) was then added slowly at rt, and the solution was stirred at 65 °C for 4 h. The reaction was quenched by adding 10 volumes of MeCN and incubated at -20 °C for 2 h. The precipitated mixture was then centrifuged at 10,000 x *g* for 5 min at 4 °C, and the supernatant was discarded. Ten additional volumes of MeCN were added to the pellet, and the vortexed mixture was incubated at -20 °C for 2 h and centrifuged as above. This step was repeated two more times to remove the excess *p*-nitrobenzylamine. The pellet was then resuspended in 5% MeCN and the product purified by semi-preparative HPLC (Agilent 1100) using two preparative reverse-phase columns (Agilent Eclipse XDB-C18; 5 µm, 9.4 x 250 mm) connected in series and a gradient of 5-20% B over 20 min at 4 mL/min (A, 0.5% aqueous AcOH; B, 100% MeCN). The product eluted at approximately 9.5 min. Lyophilization afforded a fluffy white solid (0.72 mg; 56% yield): ^{1}H NMR (600 MHz, D_2O) δ 8.33 (d, J = 8.6 Hz, 2H), 7.72 (d, J = 8.7 Hz, 2H),

5.31 (s, 1H), 4.60 (d, *J* = 7.8 Hz, 1H), 4.33 (q, *J* = 13.7 Hz, 2H), 4.23 (t, *J* = 6.3 Hz, 2H), 3.93 (d, *J* = 3.4 Hz, 1H), 3.91–3.86 (m, 4H), 3.83 (t, *J* = 4.4 Hz, 4H), 3.79–3.71 (m, 4H), 3.67 (dd, *J* = 9.5, 7.9 Hz, 1H), 3.32 (d, *J* = 11.7 Hz, 1H), 3.10 (t, *J* = 11.1 Hz, 1H), 1.23 (d, *J* = 6.6 Hz, 3H). ^{13}C NMR (151 MHz, D_2O) δ 147.86, 130.43, 124.09, 109.99, 107.14, 100.42, 99.65, 76.94, 76.65, 75.19, 73.42, 71.69, 70.48, 70.19, 69.45, 68.97, 68.51, 68.21, 67.18, 61.93, 60.90, 50.51, 49.52, 23.19, 15.29. HRMS: $[M+H]^+$ calculated for $C_{25}H_{40}N_2O_{16}$ 625.5996, found 625.2451.

Expression and Purification of BgtA. *E. coli* BL21 (DE3) harboring the recombinant plasmid vector pET28a-BtgA-His was kindly provided by Dr. Peng George Wang (Ohio State University). The protein was expressed and purified as described (14). Briefly, the cells were grown in LB medium (1L) at 37 °C. Isopropyl-1-thio-β-D-galactospyranoside (IPTG, 0.8 mM final concentration; Sigma) was added when the cells reached an OD_{600} of 0.8, and the cells were incubated for an additional 16 h at 16 °C. The pelleted cells were lysed in Cell Lytic B Lysis Reagent (Sigma-Aldrich) supplemented with EDTA-free Complete™ protease inhibitors (Roche), 0.5 M NaCl, and 20 mM imidazole (Sigma-Aldrich) by rotating end-over-end for 20 min at rt. After centrifugation, the clarified lysate was added to prewashed Ni-NTA beads and incubated at 4 °C for 1 h, washed in 20 mM Tris•HCl pH 7.5, 0.5 M NaCl and 50 mM imidazole, and eluted in a step gradient with the elution buffer (20 mM Tris•HCl pH 7.5, 0.5 M NaCl, and 100, 200 or 500 mM imidazole). After SDS–PAGE analysis, the purified protein was concentrated with 10,000 Da molecular weight cut-off (MWCO) spin filters (Millipore) and dialyzed into 20 mM Tris•HCl pH 7.5 containing 10% glycerol and stored at 4 °C.

BgtA Activity Assay and Monitoring of Chemoenzymatic Labeling Reactions by LC-MS/MS. The Fucα(1-2)Gal substrate **1** (10 μM) was dissolved in 20 mM Tris•HCl pH 7.5, 50 mM NaCl, and 5 mM $MnCl_2$. BgtA enzyme(14) and UDP-ketoGal **3(3)** or UDP-GalNAz **2** (Invitrogen) were added to final concentrations of 0.16 mg/mL and 50 μM, respectively, in a final volume of 100 μL. The

reaction was incubated at 4 °C in the dark for 12-16 h, and the reaction progress was monitored by LC-MS/MS. To label with aminooxy-biotin **7**, the reactions were diluted 5-fold with saturated urea, 2.7 M NaOAc pH 3.9 (50 mM final concentration and pH 4.8), and **7** (5 mM final concentration, Dojindo) and incubated for 20–24 h at rt. To label with ADIBO-biotin **6**, 250 μM of **6** (Click Chemistry Tools) was added, and the reaction was incubated for 3 h at rt. Following the labeling steps, the azido-labeled samples were filtered through a 3,000 Da MWCO Vivaspin 500 spin filter (GE Lifesciences) and injected on a reverse-phase HPLC column (Phenomenex Gemini; 5 m, 2.0 x 100 mm), fitted with a C8 guard column, using a ThermoScientific Accela 600 HPLC pump interfaced with a ThermoScientific LTQ mass spectrometer. A linear, 3-90% gradient of B (A: H_2O/0.1% aqueous formic acid B: MeCN/0.1% formic acid in MeCN) over 7 min was used to resolve peaks with a flow rate of 0.21 mL/min. Mass analysis was performed in positive ion mode except in the case of sulfated compound **8**, where the analysis was performed in negative ion mode.

Chemoenzymatic Labeling on the Glycan Array. Glycan Array version 5.0 was provided by the Consortium for Functional Glycomics. Pre-equilibrated arrays were treated with BgtA enzyme (0.16 mg/mL) and 500 μM UDP-GalNAz **2** in 20 mM Tris•HCl pH 7.4, 50 mM NaCl, 2 mM $MnCl_2$ containing 1% bovine serum albumin (BSA) for various times (0, 0.5, 2, and 12 h) at rt, washed 4 times with wash buffer (20 mM Tris•HCl pH 7.4, 50 mM NaCl, 0.1% Triton X-100) and then 4 times with rinse buffer (20 mM Tris•HCl pH 7.4, 50 mM NaCl). Arrays were then incubated with ADIBO-biotin (5 μM) in 20 mM Tris•HCl pH 7.4, 50 mM NaCl for 2 h at rt. After washing as described above, the arrays were washed further with 20% aqueous MeOH and then incubated with streptavidin Cy-5 (0.5 μg/mL; eBioSciences) in 20 mM Tris•HCl pH 7.4, 50 mM NaCl, 0.05% Tween-20, 1% BSA, for 1 h at rt. Arrays were then washed 4 times with wash buffer (containing 0.05% Tween-20 instead of 0.1% Triton X-100), 4 times with rinse buffer, and 4 times with water. Arrays were dried with a low stream of filtered air and analyzed using a PerkinElmer ScanArray Express.

Chemoenzymatic Labeling of Cell Lysates. The olfactory bulbs of postnatal day 3 rat pups were dissected on ice and lysed in boiling 1% SDS (5 volumes/weight) with sonication until the mixture was homogeneous. Protein was precipitated using methanol/chloroform/water. Briefly, protein was diluted to 200 µL and precipitated by sequential mixing with 600 µL of MeOH, 200 µL of $CHCl_3$ and 450 µL H_2O, after which the mixture was centrifuged at 23,000 x *g* for 15 min. Precipitated protein was washed with 450 µL of MeOH and centrifuged at 23,000 x *g* for 10 min. After the protein pellet was allowed to dry briefly, the pellet was re-dissolved at 5 mg/mL in 20 mM HEPES pH 7.9 containing 1% SDS, and diluted 5-fold into a buffer with the following final concentrations: 20 mM HEPES pH 7.9, 50 mM NaCl, 2% NP-40, 5 mM $MnCl_2$. UDP-GalNAz **2** (25 µM; Invitrogen) and BgtA (0.16 mg/mL) were added, and the samples were incubated at 4 °C for 16-20 h. The labeled proteins were precipitated as above and resuspended in 50 mM Tris pH 7.4 containing 1% SDS at 4 mg/mL. The resuspended proteins were subsequently reacted with alkyne-TAMRA **10** (Invitrogen) or alkyne-biotin **11** (Invitrogen) as per the Click-It™ TAMRA and Biotin Glycoprotein Detection Kit (Invitrogen) instructions, except that EDTA-free Complete™ protease inhibitors were added during the reaction. For TAMRA labeling, negative controls were performed under identical conditions except that BgtA, UDP-GalNAz **2**, or alkyne-TAMRA **10** was omitted from the labeling reaction. TAMRA-labeled proteins were resolved by SDS-PAGE and visualized in-gel using a Typhoon Scanner (GE Healthcare). For biotin labeling, negative controls were performed under identical conditions except that BgtA was omitted from the labeling reaction.

Purification of Biotin-Labeled Fuc α (1-2)Gal Proteins. Chemoenzymatically labeled samples were precipitated using methanol/chloroform/water as described above and re-dissolved in boiling 1% SDS plus Complete™ protease inhibitors at a concentration of 2 mg/mL. The SDS was quenched with 1 volume of NETFD buffer (100 mM NaCl, 50 mM Tris•HCl pH 7.4, 5 mM EDTA, 6% NP-40) plus protease inhibitors. The samples were incubated with pre-washed streptavidin resin (Pierce; 100

μL/1 mg protein) for 2 h at 4 °C. The resin was washed twice with 10 column volumes each of low salt buffer (0.1 M Na_2HPO_4 pH 7.5, 0.15 M NaCl, 1% Triton-X100, 0.1% SDS), twice with 10 column volumes each of high salt buffer (0.1 M Na_2HPO_4 pH 7.5, 0.5 M NaCl, 0.2% Triton-X100), and once with 10 column volumes of 50 mM Tris•HCl pH 7.4. Captured protein was eluted in boiling 2X sample buffer (100 mM Tris pH 6.8, 4% SDS, 200 mM DTT, 20% glycerol, 0.1% bromophenol blue; 50 μL /100 μL resin) for 5 min.

Western Blotting for Parallel Identification of Fuc α (1-2)Gal Glycoproteins. The purified, labeled material from above was resolved on a NuPAGE 4-12% Bis-Tris gel (Invitrogen) and transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore). The membrane was blocked in 5% milk (BioRad) in TBST (50 mM Tris•HCl, 150 mM NaCl, 0.05% Tween 20, pH 7.4) for 1 h at rt. Primary antibodies in 5% milk in TBST were added overnight at 4 °C at the following concentrations: mouse anti-NCAM monoclonal antibody (Abcam) at 1 μg/mL, mouse anti-synapsin I ascites (Synaptic Systems) at 0.1 μg/ml, or mouse anti-munc18-1 (Synaptic Systems) at 0.1 μg/mL. Membranes were washed with TBST, and incubated with the appropriate Alexa Fluor 680-conjugated (Invitrogen) or IR800-conjugated (Rockland) secondary antibody, and visualized using a LiCOR Odyssey Imaging System.

Cell Culture. HeLa, MCF-7, and MDA-mb-231 cells were grown in DMEM medium supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin, and 0.1 mg/mL streptomycin (Gibco). LNCaP and H1299 cells were grown in RPMI medium 1640 supplemented with 10% FBS, 100 units/mL penicillin, and 0.1 mg/mL streptomycin (Gibco). The PrEC line was maintained in PrEBM medium (Lonza). All transfections were carried out in antibiotic-free media. In all cases, cells were incubated in a 5% CO_2 humidified chamber at 37 °C. The PrEC line was obtained from Lonza; all other cell lines were obtained from ATCC.

Immunoprecipitation of TAMRA-Labeled Synapsin I from HeLa Cell Lysates. HeLa cells were transfected with pCMV-FLAG-synapsin Ia(25) using Lipofectamine LTX reagent (Invitrogen). The cells were lysed and chemoenzymatically labeled and protein was precipitated as described above. After the protein pellet was allowed to dry briefly, the pellet was re-dissolved in boiling 1% SDS plus Complete™ protease inhibitors at a concentration of 2 mg/mL. The SDS was quenched with 1 volume of NETFD buffer plus protease inhibitors, and the lysate was incubated with 40 μL of prewashed anti-Flag M2 Affinity Gel (Sigma-Aldrich) for 90 min at 4 °C. The resin was washed once with 4 column volumes of NETFD buffer and three times with 4 column volumes of NETF buffer (100 mM NaCl, 50 mM Tris•HCl pH 7.4, 5 mM EDTA). Captured protein was eluted in boiling 2X sample buffer (50 μL buffer/100 μL resin). Purified, labeled material was resolved by SDS-PAGE and transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore). Western blotting was performed as above except the primary anti-TAMRA rabbit antibody (0.1 μg/μL; Invitrogen) was used.

Detection of Cell-Surface Fucα(1-2)Gal Glycans on Live MCF-7 Cells by Fluorescence Microscopy. MCF-7 cells (ATCC) were seeded at 2 x 10^5 cells/coverslip. Twelve hours after plating, the cells were washed twice with 1% FBS, 10 mM HEPES in calcium and magnesium free Hank's Balanced Salt Solution (CMF HBSS, Gibco) and incubated in the chemoenzymatic labeling buffer (2% FBS, 10 mM HEPES pH 7.9 in HBSS) with UDP-GalNAz **2** (500 μM) and BgtA (0.17 mg/mL) in a total volume of 100 μL for 2 h at 37 ºC. Mock reactions were performed without the addition of BgtA. After chemoenzymatic labeling, the cells were washed twice with 100% FBS and twice with the chemoenzymatic labeling buffer. Enzymatic addition of GalNAz onto Fucα(1-2)Gal glycans was detected by incubating the cells with ADIBO-biotin (20 μM in the chemoenzymatic labeling buffer; 500 μL) for 1 h at rt, washing the coverslips as described, and then incubation with

streptavidin-Alexa Fluor 488 (1 μg/mL in PBS containing 3% BSA; Invitrogen) for 30 min at rt. Cells were washed once with PBS, after which nuclei were stained with Hoechst-33342 (1 μg/μL; Invitrogen) in PBS for 15 min at rt. Coverslips were washed twice with 100% FBS and mounted in media (on ice), sealed with paraffin, and imaged immediately using a 40x Plan-Achromat objective on a Zeiss Meta510 inverted microscope.

Detection of Fucα(1-2)-Gal Glycans on Synapsin I in Fixed HeLa Cells by Fluorescence Microscopy. HeLa cells were plated onto 15 mm coverslips (Carolina Biologicals) at a density of 75 cells/mm^2. After 12 h, cells were transfected with pCMV-Flag-synapsin Ia (0.5 μg DNA/coverslip) using Lipofectamine LTX (4 μL in 200 μL Optimem; Invitrogen). After 24 h, the media was removed, and the cells were rinsed one time with PBS, fixed in 4% paraformaldehyde in PBS, pH 7.5 for 20 min at rt, washed twice with PBS, permeabilized in 0.3% Triton X-100 in PBS for 10 min at rt, and washed twice with the enzymatic labeling buffer (50 mM HEPES, 125 mM NaCl, pH 7.9). Reaction mixtures and negative controls (without BgtA) were prepared by adding 100 μL of 20 mM HEPES pH 7.9, 50 mM NaCl, 2% NP-40, 5 mM $MnCl_2$, UDP-GalNAz **1** (25 μM), and BgtA (0.17 mg/mL) at 4 °C for 24 h (100 μL/coverslip) in a humidified chamber. After chemoenzymatic labeling, the cells were washed twice with the chemoenzymatic labeling buffer. Enzymatic addition of GalNAz onto Fucα(1-2)Gal glycans was detected by treating the cells with 5 μM alkyne-functionalized Alexa Fluor 488 (Invitrogen), 0.1 mM triazoleamine ligand (Invitrogen), 2 mM sodium ascorbate (Sigma-Aldrich), and 1 mM $CuSO_4$ (Sigma-Aldrich) in 2% FBS (Gibco) in PBS at rt for 1 h. The coverslips were mounted onto glass slides using Vectashield mounting medium with DAPI (4 μL; Vector Labs) and sealed with clear nail polish. Cells were imaged using a Nikon Eclipse TE2000-S inverted microscope, and images were captured with Metamorph software using a 20x Plan Fluor objective.

Detection of Cell-Surface Fucα(1-2)Gal Glycans on Live Cancer Cells by Flow Cytometry. All cells were seeded at 4 x 10^6 cells per 10-cm plate in 10 mL of the appropriate media. On the day of analysis, cells were lifted off the plate with DNase (0.4 mg/mL; Worthington) and 1 mM EDTA and washed with 1% FBS, 10 mM HEPES in CMF HBSS. One million cells were chemoenzymatically labeled with UDP-GalNAz (500 μM) and BgtA (0.17 μg/μL) in 1% FBS, 10 mM HEPES in CMF HBSS (100 μL) for 2 h at 37 °C. Cells were spun twice through 100% FBS (1 mL) to remove excess reagent (500 x *g*, 5 min) and resupended in 1% FBS, 10 mM HEPES in CMF HBSS (100 μL) containing ADIBO-biotin (20 μM) and incubated for 1 h at rt. Cells were again spun twice through 100% FBS (1 mL), and washed with 3% BSA in PBS (1 mL). Cells were then resuspended in 3% BSA in PBS (100 μL) containing streptavidin-Alexa Fluor 488 (1 μg/mL) and incubated for 20 min at 4 °C. Cells were subsequently spun twice through 100% FBS (1 mL) and resuspended in 2% FBS, 10 mM HEPES in CMF HBSS (750 μL) for flow cytometry analysis. Immediately before analysis, 7-amino-actinomycin D (7-AAD, 5 μL; eBioscience) was added to measure cell viability. Cells were analyzed for FITC intensity on a Beckman Dickenson FACSCalibur flow cytometer equipped with a 488-nm argon laser. For each experiment, 10,000 live cells were analyzed, and data analysis was performed on FlowJo (Tristar Inc.). Data points for LnCAP and PrEC cells were collected in triplicate, and for all other cells, in duplicate.

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