4-deoxy-4-fluoro-GalNAz (4FGalNAz) is a metabolic chemical reporter of O-GlcNAc modifications, highlighting the notable substrate flexibility of O-GlcNAc transferase.

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Figure S1. Synthesis of Ac₄4FGalNAz.

Figure S2. 4FGalNAz is not particularly toxic to mammalian cells. CHO cells were incubated with MCR or DMSO vehicle under the indicated conditions before cell viability was measured using an MTT assay.
Figure S3. Characterization of 4FGalNAz in Jurkat cells. a) 4FGalNAz does not inhibit O-GlcNAc modifications. Jurkat cells were treated under the indicated conditions before visualization of O-GlcNAc levels by western blotting. b) 4FGalNAz labeling can be detected by flow cytometry. Jurkat cells were treated with individual MCRs (50 μM) for 3 d before the live cells were subjected to SPACC with DBCO-biotin, followed by FITC-Avidin and detection of fluorescence by flow-cytometry.

Figure S4. 4FGalNAz is a substrate for GalK2 and AGX1. a) 4FGalNAz is accepted by GALK2. Michaelis-Menten enzyme curves were measured using recombinant GalK2 and the indicated concentrations of either GalNAc or 4FGalNAz. Enzyme constants were determined using line fitting in Graphpad Prism 9. b) 4FGalNAz-1-phosphate is accepted by AGX1. Michaelis-Menten enzyme curves were measured using recombinant AGX1 and the indicated concentrations of either GalNAc or 4FGalNAz. Enzyme constants were determined using line fitting in Graphpad Prism 9.
Experimental Methods

Chemical synthesis

General information

All reagents used for chemical synthesis were purchased from Sigma-Aldrich, Alfa Aesar or EMD Millipore unless otherwise specified and used without further purification. All anhydrous reactions were performed under argon or nitrogen atmosphere. Analytical thin-layer chromatography (TLC) was conducted on EMD Silica Gel 60 F254 plates with detection by ceric ammonium molybdate (CAM), anisaldehyde or UV. For flash chromatography, 60 Å silica gel (EMD) was utilized. 1H spectra were obtained at 400, 500, or 600 MHz on Varian spectrometers Mercury 400, VNMRS-500, or -600. Chemical shifts are recorded in ppm (δ) relative to solvent. Coupling constants (J) are reported in Hz. 13C spectra were obtained at 100, 125, or 150 MHz on the same instruments.

1-O-Benzyl-N-acetyl-α-D-glucosamine (2)

To a stirred suspension of N-acetylglucosamine 1 (10.0 g, 45.2 mmol) in benzyl alcohol (125 mL) at 0 °C, acetyl chloride (10.9 mL, 12.0 g, 152 mmol) was added dropwise. The reaction mixture was stirred for 30 min at rt and further stirred for 24 h at 65 °C. The benzyl glucoside was precipitated using cold Et2O and the liquid phase was discarded. The resulting syrup corresponding to the benzyl glucoside was washed with cold Et2O, solubilized in MeOH and neutralized with NaHCO3 (solid) until pH 7 was achieved. The suspension was filtered through a short pad of Celite™, further washed with MeOH, and the solvent was removed under reduced pressure. Recrystallization from EtOH yielded 2 (11.0 g, 78%) as a white solid. 1H NMR (400 MHz, CD3OD) δ 7.43 – 7.20 (m, 5H), 4.74 (d, J = 12.0 Hz, 1H), 4.59 (s, 1H), 4.49 (d, J = 12.0 Hz, 1H), 4.00 – 3.56 (m, 4H), 3.41 – 3.27 (m, 2H), 1.94 (s, 3H). HRMS: calc’d. for C15H21NaO6 (M+Na)+ 334.1267, found 334.1262.

Benzy 2-Acetamido-4,6-O-benzylidene-2-deoxy-α-D-glucopyranoside (3)

Benzaldehyde (3.46 ml, 23.0 mmol) and p-toluenesulfonic acid (2 g, 11.5 mmol) were added to a suspension of the starting material 2 (6.5 g, 20.88 mmol) in anhydrous DMF (20 ml). The mixture was stirred at 65°C for 20 h. After this time the solvent was evaporated under reduced pressure, then the solid residue was washed and triturated with hexane. The residue was then triturated with a warm NaHCO3 saturated solution. After cooling to room temperature, the solid was filtered and the solvent evaporated to afford the compound 3 (7.6 g, 91%) as a solid. 1H NMR (400 MHz, CDCl3) δ 7.56 – 7.45 (m, 5H), 7.43 – 7.31 (m, 5H), 5.85 (d, J = 8.9 Hz, 1H), 5.56 (s, 1H), 4.93 (d, J = 3.8 Hz, 1H), 4.77 – 4.67 (m, 1H), 4.49 (d, J = 11.8 Hz, 1H), 4.28 – 4.17 (m, 2H), 3.95 (t, J = 9.6 Hz, 1H), 3.90 – 3.83 (m, 1H), 3.76 (t, J = 10.2 Hz, 1H), 3.65 – 3.55 (m, 2H), 1.99 (s, 3H). HRMS: calc’d. for C22H25NaO6 (M+Na)+ 422.1580, found 422.1564.

Benzy 2-Acetamido-3-O-benzyl-4,6-O-benzylidene-2-deoxy-α-D-glucopyranoside (4)

NaH 60% suspension in mineral oil (1 g, 25.0 mmol) was added portionwise to a solution of the derivative 3 (5 g, 12.5 mmol) and benzyl bromide (3 mL, 25.0 mmol) in THF (150 mL) at 0°C. The reaction mixture was stirred for 24 h, then the reaction was quenched in cold water and neutralized with formic acid 10%. The solid was filtered, washed with water and dried under reduced pressure to yield the compound 4 (4.60 g, 75%). 1H NMR (400 MHz, CDCl3) δ 7.58 – 7.19 (m, 15H), 5.60 (s, 1H), 5.35 (d, J = 9.3 Hz, 1H), 4.92 (dd, J = 8.1, 4.2 Hz, 2H), 4.67 (dd, J = 27.8, 12.0 Hz, 2H), 4.46 (d, J = 11.8 Hz, 1H), 4.31 (td, J = 9.3, 3.8 Hz, 1H), 4.25 (dd, J = 10.1, 4.7 Hz, 1H), 3.94 – 3.85 (m, 1H), 3.83 – 3.71 (m, 2H), 1.87 (s, 3H).

Benzy 2-Acetamido-3,6-di-O-benzyl-2-deoxy-α-D-glucopyranoside (5)

BF3/Et2O (1.55 mL, 12.6 mmol) was added dropwise to a solution of Et3SiH (6.7 mL, 42.0 mmol) and compound 4 (4.11 g, 8.40 mmol) in dry CH2Cl2 (47 mL) at 0 °C. After stirring at 0 °C for 2 h, the reaction mixture was quenched with triethylamine until neutralization, and then purified through flash silica gel column (toluene:acetone 7:3) to yield compound 5 (2.6 g, 63%) as a white solid. 1H NMR (400 MHz, CDCl3) δ 7.43 – 7.23 (m, 15H), 5.42 (d, J = 9.3 Hz, 1H), 4.89 (d, J = 3.8, 1.2 Hz, 1H), 4.79 – 4.67 (m, 3H), 4.59 (q, J = 11.9 Hz, 2H), 4.45 (d, J = 11.7 Hz, 1H), 4.27 (td, J = 10.4, 9.9, 3.7 Hz, 1H), 3.84 – 3.59 (m, 4H), 1.85 (d, J = 1.2 Hz, 3H).
Benzyl 2-acetamido-3,6-di-O-benzyl-2,4-dideoxy-4-fluoro-α-D-galactopyranoside (6)

To a solution of the compound 5 (4.6 g, 9.36 mmol) in anhydrous DCM (93 mL) and anhydrous pyridine (15.5 mL) stirred at 0 °C was added triflic anhydride (3.26 mL, 19.65 mmol) dropwise. The solution was stirred under the same conditions for 1 h. After that time, the reaction was diluted with DCM, washed with HCl 1M x 2, saturated aqueous NaHCO₃, and brine solution. The organic phase was dried over Na₂SO₄, and evaporated under reduced pressure. The residue was engaged in the next step without further purification. Tetra-N-butylammonium fluoride (19.6 g, 74.91 mmol) was added to a solution of the crude triflate derivative (5.84 g, 9.36 mmol) in anhydrous MeCN (172 mL). The mixture was stirred at room temperature for 24 h. After evaporation of the solvent, the crude reaction mixture was subjected to flash chromatography on silica gel (hexane:acetone 6:4). The resulting impure glycoside was further purified by reversed phase C-18 column chromatography (H₂O:ACN 60:40 to 0:100 0.1% TFA in 24 min) to afford the compound 6 (2.86 g, 62% over 2 steps). ¹H NMR (400 MHz, CDCl₃) δ 7.37 – 7.19 (m, 15H), 5.36 (d, J = 9.0 Hz, 1H), 4.97 (d, J = 3.7 Hz, 1H), 4.77 – 4.62 (m, 3H), 4.54 (s, 3H), 4.46 (dd, J = 12.0, 7.5 Hz, 2H), 3.74 – 3.55 (m, 3H), 1.87 (s, 3H). HRMS: calc’d. for C₂₉H₅₅FNO₅ (M+H)⁺ 494.2343, found 494.2324.

2-Acetamido-2,4-dideoxy-4-fluoro-D-galactopyranose (7)

Pd/C (10%, 2.4 g) was added to a solution of 6 (2.86 g, 5.79 mmol) in acetic acid (83 mL). The mixture was vigorously stirred under H₂ atmosphere for 48 h. After completion by TLC the mixture was filtered over a celite pad and the solvent evaporated in vacuo. The mixture was dissolved on a RP C-18 column chromatography (H₂O:ACN 100:0 to 0:100 0.1% TFA in 15 min) to yield the compound 7 (1.09 g, 84%). ¹H NMR (400 MHz, CDCl₃) δ 5.12 (d, J = 3.5 Hz, 1H), 4.17 (dd, J = 11.1, 3.5 Hz, 1H), 4.12 – 3.98 (m, 1H), 3.95 – 3.83 (m, 1H), 3.72 – 3.59 (m, 3H). ¹⁹F NMR (376 MHz, CD₃OD) δ -223.29 – -223.68 (m).

2-Amino-2,4-dideoxy-4-fluoro-D-galactose hydrochloride (8)

A stirred solution of 7 (1.25 g, 5.60 mmol) in 3M HCl (56 mL) was heated at 95-100°C for 3h. After this time the solvent was evaporated, and the crude was eluted on a RP C-18 flash chromatography (H₂O:ACN 100:0 to 0:100 0.1% TFA over 15 min) to yield 8 (1.1 g, 90%). ¹H NMR (400 MHz, CD₃OD) δ 5.43 (d, J = 3.5 Hz, 1H), 4.19 – 4.01 (m, 2H), 3.74 – 3.63 (m, 3H), 3.42 – 3.34 (m, 1H). ¹⁹F NMR (376 MHz, CD₃OD) δ -223.89 – -224.34 (m). HRMS: calc’d. for C₆H₁₃FNO₄ (M-Cl)⁺ 182.0829, found 182.0828.

2-azidoacetamido-2,4-dideoxy-4-fluoro-D-galactopyranose (9)

Pentafluorophenyl Trifluoromethanesulfonate (3.4 mL, 19.79 mmol) was added to a solution of azido acetic acid (1 g, 9.89 mmol) and anhydrous pyridine (3.6 mL, 44.53 mmol) in DMF (20 mL), then the solution was stirred at room temperature for 18h. The solvent was partially evaporated and the residue was diluted with Et₂O and washed successively with 2x aqueous saturated NaHCO₃ and brine. The organic phase was dried over Na₂SO₄, and the solvent evaporated under reduced pressure. The crude mixture was then subjected to silica gel flash column chromatography (Hexane:Acetone 8:2) to afford the azido acetate pentafluorophenyl ester (2 g, 73%). The azido acetate pentafluorophenyl ester (1.96 g, 7.35 mmol) was added to a mixture of the compound 8 (800 mg, 3.68 mmol), and triethylamine (1.3 mL, 9.19 mmol) in MeOH (20 mL). The mixture was stirred at room temperature for 24 h. The solvent was then evaporated under reduced pressure and the crude mixture purified by RP C-18 flash chromatography (H₂O:ACN 100:0 to 0:100 0.1% TFA in 25 min) to afford the compound 9 (950 mg, 98%). ¹H NMR (400 MHz, CD₃OD) δ 5.13 (d, J = 3.5 Hz, 1H), 4.20 (dd, J = 11.0, 3.6 Hz, 1H), 4.06 (dt, J = 30.3, 6.7 Hz, 1H), 3.94 – 3.83 (m, 4H), 3.72 – 3.62 (m, 2H). ¹⁹F NMR (376 MHz, CD₃OD) δ -222.77 (dt, J = 50.8, 29.6 Hz). ¹³C NMR (101 MHz, CD₃OD) δ 169.15, 91.28, 88.15, 71.28, 69.35, 66.68, 61.33, 51.38. HRMS: calc’d. for C₆H₁₃FN₃O₅ (M+Na⁺) 287.0768, found 287.0766.

1, 3, 6-tri-O-acetyl-2-azidoacetamido-2,4-dideoxy-4-fluoro-D-galactopyranose (10)

Acetic anhydride (4 mL) was added to a solution of the compound 9 (950 mg, 3.60 mmol) in pyridine (8 mL); the solution was stirred at room temperature for 24 h. The residue was co-evaporated with toluene, then purified by silica gel column chromatography (hexane:acetone 7:3) to afford the compound 10 (900 mg, 61%) as a yellow-orange amorphous solid. ¹H NMR (400 MHz, CDCl₃) δ 6.41 (d, J = 8.9 Hz, 1H), 6.23 (d, J = 3.6 Hz,
1H), 5.31 – 5.14 (m, 2H), 4.79 – 4.68 (m, 1H), 4.31 – 4.13 (m, 2H), 3.95 (s, 2H), 2.17 (s, 3H), 2.14 (s, 3H), 2.06 (s, 3H). 19F NMR (376 MHz, CDCl3) δ -213.65 – -214.08 (m). 13C NMR (101 MHz, CDCl3) δ 171.35, 170.47, 168.81, 167.20, 90.73, 86.66, 69.07, 67.93, 61.37, 52.40, 47.02, 20.87, 20.78, 20.70. HRMS: calc’d. for C13H23FN3O8 (M+H)+ 391.1265, found 391.1268.

2-azidoacetamido-4-fluoro-2, 4-dideoxy-D-galactopyranose 1-(dihydrogen phosphate)
A 1750 μL solution of trGlmU– NahK⁵ (3.5 mg), 4FGalNAz (9) (9.3 mg, 20 mM), ATP (10 mM) and MgCl₂ (5 mM), in 200 mM Tris/HCl buffer (pH 8.0) was incubated at 37 °C for 24 h. The pH of the solution was verified using pH indicator paper before the addition of the enzyme. After 24 h, the reaction was lyophilized, then resuspended in 1:1 ACN:H₂O and purified by HPLC SeQuant ZIC-HILIC chromatography column (5 μm, 200A, 150 x 10 mm, EMD Milipore), using a gradient 10% to 40% B over 35 min, (buffer A: ACN, buffer B: 20 mM NH₄OAc in H₂O) to give the 1-phosphate product (2.7 mg, 40%). 1H NMR (600 MHz, D₂O) δ 5.37 – 5.33 (m, 1H), 4.83 (d, J = 50.8 Hz, 1H), 4.21 – 4.10 (m, 2H), 4.01 (d, J = 16.3 Hz, 1H), 3.94 (d, J = 16.1 Hz, 1H), 3.71 – 3.66 (m, 1H), 1.79 (s, 2H). 31P NMR (243 MHz, D₂O) δ 0.86. 19F NMR (564 MHz, D₂O) δ -220.66 (dt, J = 50.7, 30.5 Hz). 13C NMR (151 MHz, D₂O) δ 181.35, 171.05, 92.99, 90.23, 70.01, 60.06, 51.68, 23.16. HRMS: calc’d. for C₃H₁₃FN₃O₈ (M-H)⁻ 343.0455, found 343.0459.

Uridine 5'- Diphospho-(2-azidoacetamido-4-fluoro-1, 2, 4-dideoxy-D-galactopyranosyl)
A 2500 μL solution of trGlmU– NahK (5 mg), 4FGalNAz-1-phosphate (17.2 mg, 20 mM), ATP (10 mM), UTP (10 mM), inorganic pyrophosphatase (5 Units) and MgCl₂ (5 mM), in 200 mM Tris/HCl buffer (pH 8.0) was incubated at 37 °C for 24 h. The pH of the solution was verified using pH indicator paper before the addition of the enzyme. After 24 h, the reaction was lyophilized, then resuspended in 5:5 ACN:H₂O and purified by HPLC SeQuant ZIC-HILIC chromatography column (5 μm, 200A, 150 x 10 mm, EMD Milipore), using a gradient 10% to 40% B over 35 min, (buffer A: ACN, buffer B: 20 mM NH₄OAc in H₂O) to give UDP-4FGalNAz (3 mg, 28%). 1H NMR (400 MHz, D₂O) δ 7.80 (d, J = 8.1 Hz, 1H), 5.85 – 5.78 (m, 1H), 4.30 – 3.87 (m, 4H), 3.72 – 3.55 (m, 2H), 3.53 – 3.35 (m, 2H), 3.20 (s, 2H), 1.76 (s, 6H), 1.19 – 1.08 (m, 1H). 31P NMR (162 MHz, D₂O) δ -11.40 (d, J = 20.5 Hz), -13.23 (d, J = 21.1 Hz). 19F NMR (376 MHz, D₂O) δ -220.79 (dt, J = 50.0, 30.2 Hz). 13C NMR (151 MHz, D₂O) δ 171.13, 166.21, 151.79, 141.65, 102.60, 94.34, 89.22 (d, J = 210.6 Hz), 83.15, 83.09, 73.69, 72.02, 69.61, 65.00, 62.44, 59.84, 51.57, 50.03. HRMS: calc’d. for C₃₁H₅₄F₄N₈O₁₆P₂ (M-H)⁻ 649.0708, found 649.0706.
2-azidoacetamido-2,4-dideoxy-4-fluoro-D-galactopyranose (9)
1, 3, 6-tri-O-acetyl-2-azidoacetamido-2,4-dideoxy-4-fluoro-D galactopyranose (Ac₃FGalNAz)
2-azidoacetamido-4-fluoro-2, 4-dideoxy-D-galactopyranose 1-(dihydrogen phosphate) (4FGalNAz-1-phosphate)
Uridine 5’- Diphospho-(2-azidoacetamido-4-fluoro-1, 2, 4-dideoxy-D-galactopyranosyl) (UDP-4FGalNAz)
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