SUPPLEMENTARY DATA

Metabolic Inhibition of Sialyl-Lewis^x Biosynthesis by 5-Thiofucose Remodels the Cell Surface and Impairs Selectin-Mediated Cell Adhesion

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Supplementary Experimental Procedures

Synthesis of GDP-5-thiofucose (GDP-5T-Fuc). 5T-Fuc, synthesized exactly as reported previously (1) was used to chemoenzymatically prepare GDP-5T-Fuc using the bifunctional *Bacteroides fragilis* enzyme L-fucokinase-GDP-L-fucose pyrophosphorylase (FKP)(2). This enzyme, expressed in *Escherichia coli* BL21 cells transformed with recombinant *fkp* in the pET16b plasmid, was kindly provided by Dr. Laurie Comstock. Protein expression in these cells was induced at 16 °C for 22 h upon the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to final concentration of 100 μM. FKP was further purified from cell lysates by Ni-affinity chromatography and dialyzed against 50 mM Tris, pH7.5. FKP was concentrated using Centricon centrifugal filtration tube (Millipore) and stored at -20 °C in 30% glycerol. GDP-5T-Fuc was produced according to the protocol used by Zhao *et al*. (3) with some modifications. Briefly, 5T-Fuc (3 mM) was dissolved in 50 mM Tris, pH 7.5 containing 10 mM $MgSO_4$ in a 50 mL centrifuge tube. Solid ATP (Bioshop) and GTP (Sigma), 4 mM each, were added to the monosaccharide solution and the reaction was initiated upon the addition of FKP and inorganic pyrophosphatase (Sigma) to final concentrations of 3.3 mg/mL and 10 U/mL, respectively. After reacting for 18 h at 37 °C excess nucleotide mono, di and triphosphates were digested by adding 30 U/mL alkaline phosphatase (Roche) to the reaction mixture. Once nucleotides, most notably AMP, were no longer visible in the reaction mixture when analyzed by capillary electrophoresis (CE), the GDP-5T-Fuc was purified by ion exchange (AG 1X4, formate-form) and ion-paired reverse phase high performance liquid chromatography (HPLC) exactly as previously described (4). To facilitate accurate mass determination, GDP-5T-Fuc was converted into its disodium salt by passing the HPLC-purified sample through a column containing a 1 mL bed volume of the AG 50W-X8 (Na^+ form) ion-exchange resin. L-fucose (Sigma) was used to prepare GDP-Fuc in exactly the same way as GDP-5T-Fuc. Both GDP-Fuc (5) and GDP-5T-Fuc (6) were fully characterized by NMR spectroscopy and the data match those reported in the literature.

Guanosine- 5'-(β-L-fucopyranosyl) diphosphate disodium salt (GDP-Fuc). ^{1}H NMR (600 MHz, D_2O): δ (ppm) 1.24 (d, 3H, $J_{6",5"}$ = 6.6 Hz, H-6"), 3.58 (dd, 1H, $J_{2",3"}$ = 10.1 Hz, $J_{2",1"}$ = 7.8Hz, H-2"), 3.67 (dd, 1H, $J_{3",4"}$ = 3.5 Hz, H-3"), 3.71-3.73 (m, 1H, H-4"), 3.76-3.79 (m, 1H, H-5"), 4.21-4.24 (m, 2H, H-5a'/5b'), 4.36-4.38 (m, 1H, H-4'), 4.55 (dd, 1H, $J_{3',2'}$ = 5.9 Hz, $J_{3',4'}$ = 2.8 Hz), 4.83 (dd, 1H, H-2'), 4.94 (dd, 1H, $J_{1",P}$ = 8.2 Hz, H-1"), 5.96 (d, 1H, $J_{1',2'}$ = 6.4 Hz, H-1'), 8.12 (s, 1H, H-8); ^{13}C NMR (150 MHz, D_2O) δ (ppm) 18.21 (C-6"), 68.14 (C-5'), 73.30 (C-3'), 73.86 (C-2"), 73.96 (C-4"), 74.22 (C-5"), 75.28 (C-3"), 76.30 (C-2'), 86.64 (C-4'), 89.46 (C-1') , 101.25 (C-1"), 119.26 (C-5), 140.27 (C-8), 154.68 (C-4), 157.63 (C-2), 162.88 (C-6); ^{31}P NMR (242 MHz, D_2O): δ (ppm) -11.07 (d, $J_{p,p}$ = 26.6 Hz), -12.91 (d).

Guanosine- 5'-(5-thio-β-L-fucopyranosyl) diphosphate disodium salt (GDP-5T-Fuc). ^{1}H NMR (600 MHz, D_2O): δ (ppm) 1.18 (d, 3H, $J_{6",5"}$ = 6.7 Hz, H-6"), 3.20-3.22 (m, 1H, H-5"), 3.49 (dd, 1H, $J_{2",3"}$ = 10.1 Hz, H-3"), 3.81 (dd, 1H, $J_{2",1"}$ = 9.4Hz, H-2"), 3.90-3.93 (m, 1H, H-4"), 4.23-4.27 (m, 2H, H-5'), 4.36-4.38 (m, 1H, H-4'), 4.56-4.58 (m, 1H, H-3'), 4.83 (dd, 1H, , $J_{2',3'}$ = 5.6 Hz, H-2'), 5.12 (dd, 1H, $J_{1",P}$ = 8.9 Hz, H-1"), 5.96 (d, 1H, $J_{1',2'}$ = 6.2 Hz, H-1'), 8.14 (s, 1H, H-8); ^{13}C NMR (150 MHz, D_2O) δ (ppm) 18.09 (C-6"), 41.48 (C-5"), 73.15 (C-3'), 75.78 (C-4"), 76.23 (C-2'), 76.52 (C-3"), 79.98 (C-2"), 80.23 (C-1"), 86.57 (C-4'), 89.37 (C-1'), 119.03 (C-5), 140.27 (C-8), 154.50 (C-4), 157.04 (C-2), 162.22 (C-6); ^{31}P NMR (242 MHz, D_2O): δ (ppm) -11.06 (d, $J_{p,p}$ = 21.3 Hz), -12.45 (d).

Nucleotide sugar analysis by CE and AGX1 and FKP digestions. HepG2 cells were grown in the presence of varying concentrations of 5T-Fuc for 24 h and nucleotide sugars were extracted exactly as previously described (4). The CE analysis method has also been reported in detail elsewhere (4,7). The comigration of GDP-Man and GDP-5T-Fuc necessitated the need to develop a means to resolve between these two compounds, particularly in light of the fact that GDP-Man is a precursor of GDP-Fuc in the *de novo* biosynthetic pathway. The pyrophosphylase-catalyzed condensation between Fuc-1-PO_4 and GTP is a readily reversible reaction (8). Therefore, GDP-Man and GDP-5T-Fuc could be distinguished on the basis of the latter's conversion into 5T-Fuc-1-PO_4 by FKP in the presence of excess pyrophosphate (PPi). Trial reactions were optimized before this procedure was utilized for cell-derived nucleotide sugars. Final conditions for the FKP digestion of GDP-Fuc/5T-Fuc were 50 mM Tris, pH 7.5, 10 mM $MgSO_4$, 200 mM PPi (Sigma) and 1.1 mg / mL FKP in a total volume of 150 µL. Both GDP-Fuc and GDP-5T-Fuc were effectively converted to their corresponding sugar-phosphates by this procedure; other GDP-sugars (Glc or Man) were not affected by this enzyme. All samples were digested for 2 h at 37 °C and were terminated /cleaned-up by applying them to 50 mg Hypersep Hypercarb (Thermo Scientific) solid-phase extraction (SPE) columns. After washing with the SPE columns with 1 x 750 µL H_2O, nucleotide sugars were eluted with 90% CH_3CN containing 50 mM triethylammonium acetate, pH 7.0. Using a vacuum manifold capable of holding up to 6 SPE tubes, all samples were rapidly desalted in less than 10 min. Samples were partially concentrated on a SpeedVac (Thermo Scientific) and lyophilized.

A similar strategy was used to remove UDP-GlcNAc from the mixtures of nucleotide sugars extracted from cells. Though UDP-GlcNAc could be resolved from GDP-Man (and hence GDP-5T-Fuc) its relatively high abundance complicated accurate peak area measurements as GDP-Man tends to appear as a shoulder on a much (about 12-fold) larger UDP-GlcNAc peak. Trial experiments using cell-derived nucleotide sugars were performed with the UDP-GlcNAc pyrophosphorylase AGX1; a similar procedure has been reported for the AGX1 isoform AGX2 (9). Optimized reaction conditions for the elimination of UDP-GlcNAc from cell-derived nucleotide sugars were to dissolved samples in a solution (200 µL) containing 50 mM Tris, pH 7.5, 2 mM $MgCl_2$, 200 mM PPi, 22 µg/mL AGX1 and 5 U/mL alkaline phosphatase (Roche). Samples were reacted for 2 h at 37 °C and desalted prior to CE analysis as described for FKP above. Typically the nucleotide sugars from 0.5-1x10^6 cells were extracted and analyzed by CE for each condition tested. These were digested with AGX1 and analyzed by CE after which each samples were further treated with FKP and analyzed a second time. For each sample the difference in the peak area corresponding to the co-migrating GDP-Man/5T-Fuc before and after FKP treatment was taken to be the peak area of GDP-5T-Fuc alone. GDP-Man levels were assessed directly from the FKP-treated samples, while GDP-Fuc was measured from the AGX-1-digested mixture or from non-treated samples. All peak areas were corrected such that GDP-Glc (which was added to samples to a final concentration of 5 µM prior to nucleotide sugar extractions from cell lysates) peak areas were normalized.

Analysis of HepG2-derived *N*-glycans. *N*-glycans from HepG2 cells grown in the presence or absence of 50 µM 5T-Fuc (1.5x10^6 cells for each condition) for 24h, were obtained after the PNGaseF (25 U/mL)-digestion of lysates as essentially described above. After a 20h digestion, protein was removed by adding of 3 volumes -20 °C EtOH to each sample and storing them at -20 °C for 1h before the precipitated protein was pelleted by centrifugation (15 min, 4 °C, 15,000 rcf). The supernatant, containing released *N*-glycans, was concentrated on a SpeedVac and lyophilized. The dried *N*-glycans were desalted by passing them through 3mL HyperSep

HyperCarb SPE cartridges; appropriate fractions were lyophilized, labelled by reductive amination with 8-aminopyrene-1,3,6-trisulfonate (APTS) (Sigma), diluted with H_2O, and passed through columns (1 mL bed volume) containing Sephadex G-10 (Amersham Biosciences). The earliest eluting fractions were collected and lyophilized in the dark. A portion of the liberated glycans from each sample was digested for 4 h at 37 ° C with 406 mU/mL bovine kidney α-fucosidase (Prozyme) in 50 mM sodium citrate, pH 5.6. The activity of the fucosidase was confirmed with a control sample containing 2 mM paranitrophenol-α-L-fucopyranoside. All samples were analyzed by CE on a ProteomeLab PA800 exactly previously described (7). After analysis, the fucosidase-treated samples were desalted using Sephadex G-10, dried, and treated with 0.3 mg/mL purified *Micromonospora viridifaciens* neuraminidase (a gift from Andrew Bennet, SFU) in PBS for 20 h at 37 ° C. These desialylated and defucosylated samples were analyzed a second time by CE.

Fucosyltransferase assays.

The ability of recombinant human FUT7 to transfer GDP-5T-Fuc relative to GDP-Fuc was assessed using a CE based assay. An acceptor substrate was prepared by the reductive amination of 3 μg of a triantenary, trisialylated *N*-glycan (A3) (Prozyme) with APTS (Sigma) exactly as described above for cell-derived *N*-glycans. Note that A3 is typically obtained from bovine fetuin, a commonly utilized substrate for FUT assays (10). This reaction was quenched with 100 μL H_2O and excess APTS and salts were removed by passing the sample through a 18 x 0.5 cm column of Sephadex G-10 packed in H_2O. Appropriate A3-containg fractions were pooled and lyophilized. This glycan is composed of nearly equal amounts of two major isomers which appear to differ in the linkage of a single Neu5Ac residue when analyzed by CE before and after neuraminidase-treatment. A3 was mixed with recombinant human FUT7 (R&D Systems) in freshly prepared reaction buffer and this mixture was aliquoted into 200 μL PCR tubes containing either GDP-Fuc or GDP-5T-Fuc to initiate reactions. Each 150 μL reaction contained 100 mM Tris, pH7.5, 5 mM $CaCl_2$, 20 mM $MnCl_2$, 100 μM nucleotide sugar and 2.7 μg/mL FUT7. Reactions were incubated for 40 (GDP-Fuc) or 80 min (GDP-5T-Fuc) at 37 ° C before they were terminated by snap freezing them in liquid nitrogen. In contrast with the cell-derived *N*-glycans described above, these samples were resolved by CE at a constant current of -50 μA on a polyvinylalcohol-coated capillary (Beckman-Coulter) in a background electrolyte of 50 mM Tris-acetate, pH 5.5. Under these conditions starting material and products were resolved and detected by laser-induced fluorescence (LIF) using a 488 nm Ar ion laser (Beckman-Coulter). Peak areas were calculated by integration of the LIF signal using 32 Karat, Version 7.0 software (Beckman-Coulter) and relative percent conversions were calculated by dividing the peak area of the fucosylated glycan product (which was background-corrected by subtracting the signal obtained from a sample that had been incubated in the absence of any nucleotide sugars) by the total area of all A3 peaks. Each condition was repeated in triplicate and average values are reported ± SEM.

No new products were observed by CE when similar reactions were performed with A3 as a FUT3 substrate. HepG2 cells contained significant levels of Le^X, and given that these cells express FUT3(11) which is capable of fucosylating neutral acceptors (12), we tested the ability of FUT3 to use desialylated A3 (NA3) as a substrate. A3 was treated with neuraminidase (0.16 mg/mL) in 50 mM NaOAc, pH 5.5 at 37 ° C. Complete desialation under these conditions was achieved in less than 3 min. NA3 was desalted using Sephadex G-10 and lyophilized. Recombinant human FUT3 (R&D Systems) mixed with NA3 in freshly prepared reaction buffer and this mixture was added to tubes containing either GDP-Fuc or GDP-5T-Fuc to initiate

reactions. Each 150 μL reaction contained 20 mM Tris, pH 7.0, 20 mM $MnCl_2$, 100 μM nucleotide sugar and 6.6 μg/mL FUT3. Reactions were analyzed after 1.5 h as described for FUT7.

Supplementary Figures

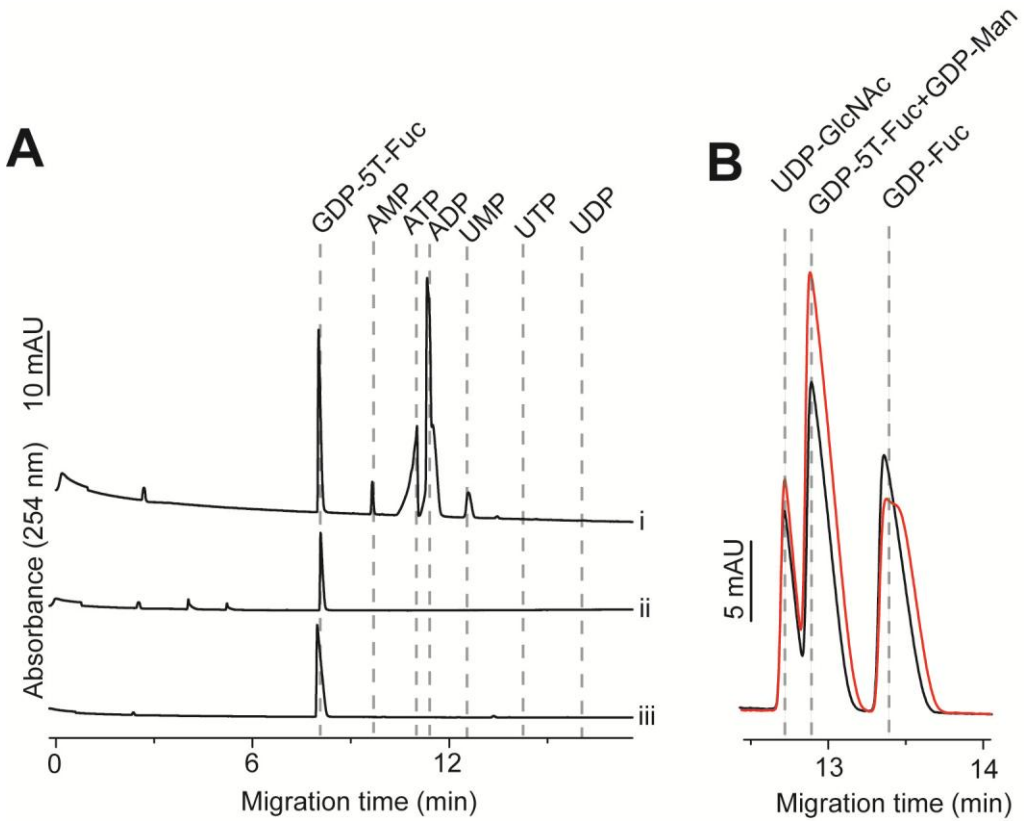

Figure S1. Chemoenzymatic synthesis of GDP-5T-Fuc. A. 5T-Fuc, ATP, GTP, FKP and PPA were incubated at 37°C for 18 h after which an aliquot of the reaction solution was analyzed directly by CE (i). Treatment of this reaction mixture with alkaline phosphatase was carried out until mono, di, or trinucleotides were no longer detected (ii). GDP-5T-Fuc was further purified by ion exchange chromatography and ion-paired HPLC (iii). **B.** The electrophoretic mobility of GDP-5T-Fuc relative to other common nucleotide sugars was assessed and it was discovered that GDP-5T-Fuc co-migrates with GDP-Man. A mixture UDP-GlcNAc, GDP-5T-Fuc and GDP-Fuc (black) was spiked with GDP-Man and immediately reanalyzed (red). Note that the differences in migration time between A and B was because the latter set of electropherograms were acquired at -25 kV (rather than -30 kV) to provide better resolution between UDP-GlcNAc and GDP-Man.

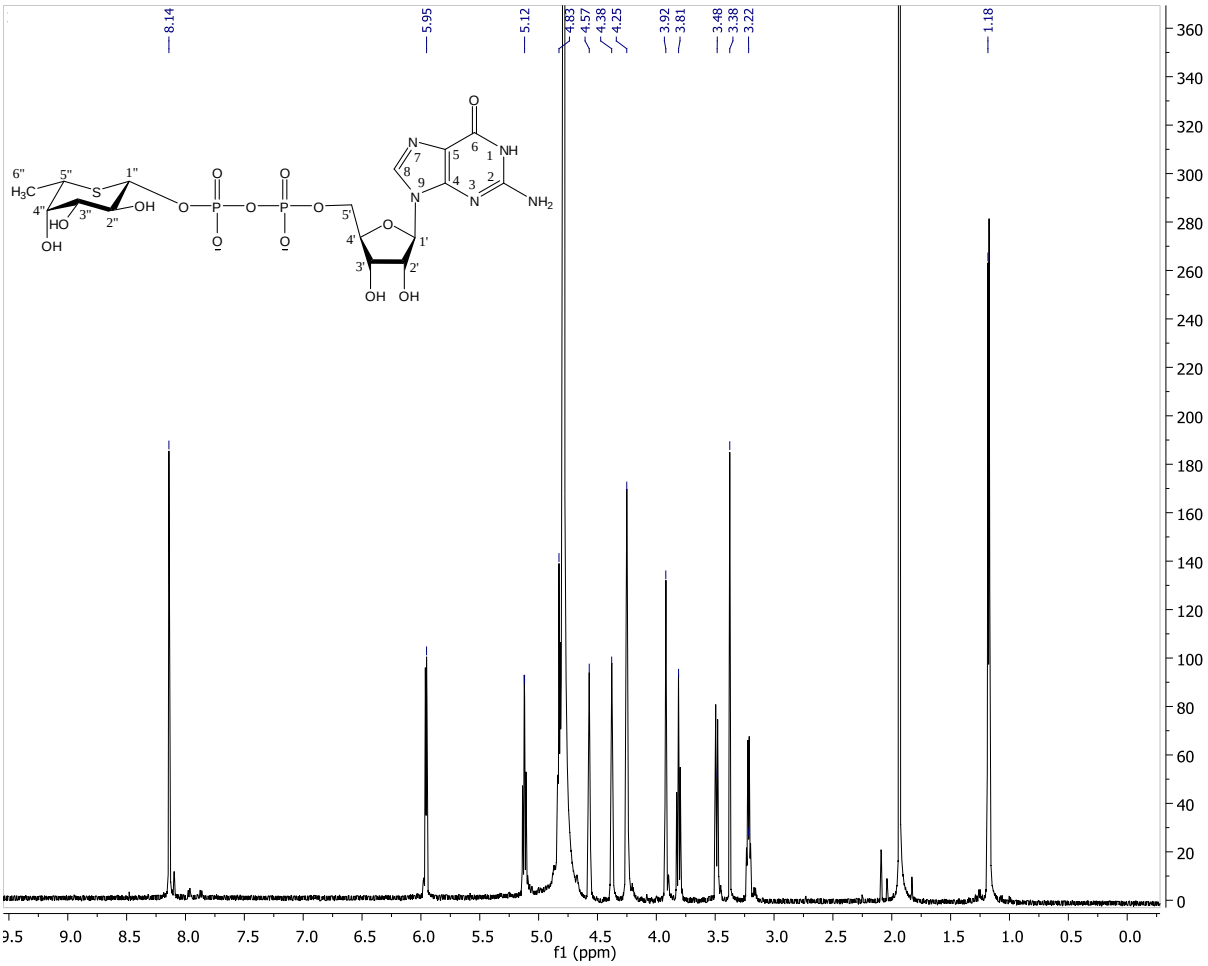

Figure S2. ^{1}H NMR spectrum of GDP-5T-Fuc. Peaks at 1.93 and 3.38 ppm arise from NaOAc and CH_3OH respectively.

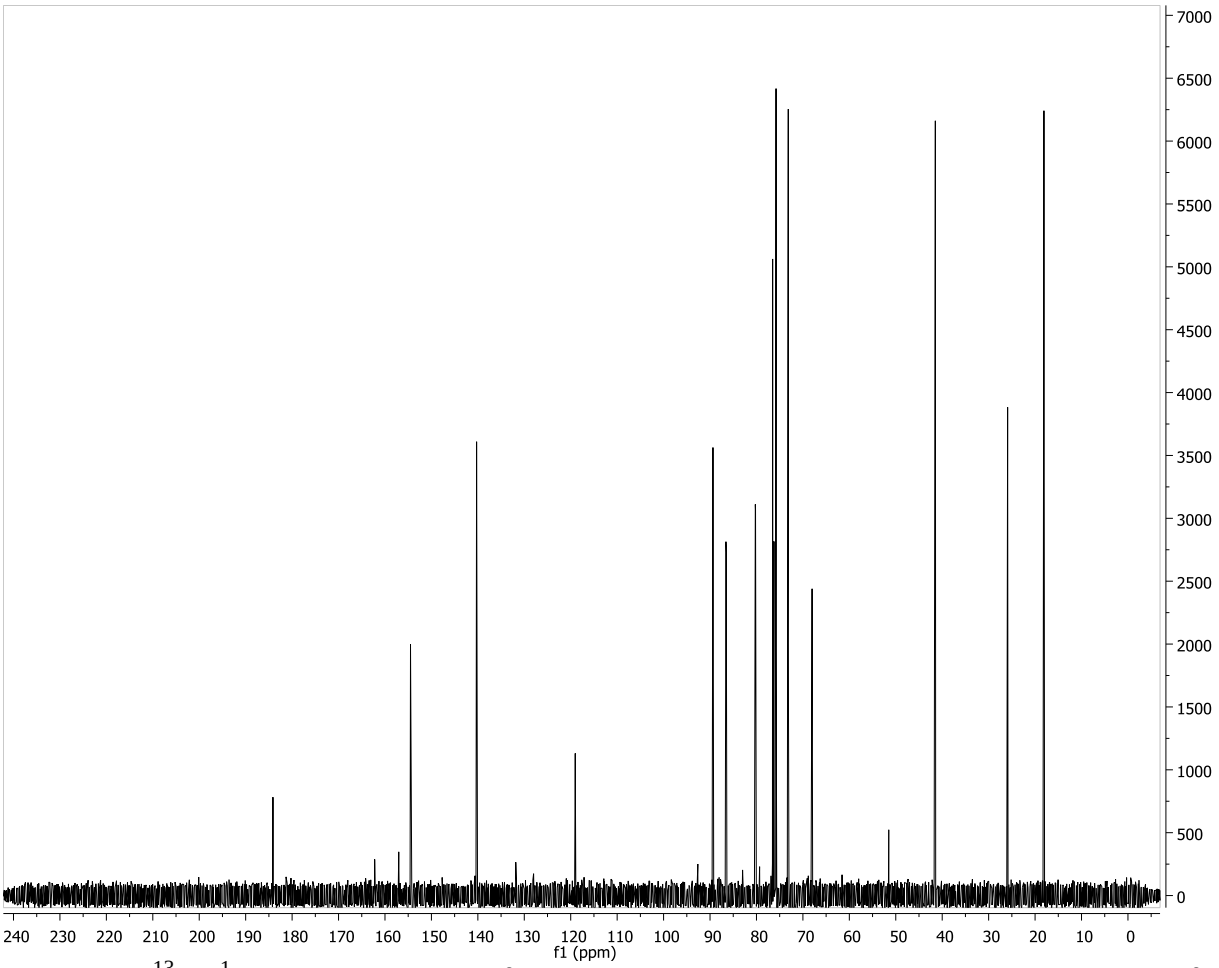

Figure S3. ^{13}C {^{1}H} NMR spectrum of GDP-5T-Fuc. Peaks at 184.13 and 51.51ppm arise from NaOAc and CH_3OH respectively.

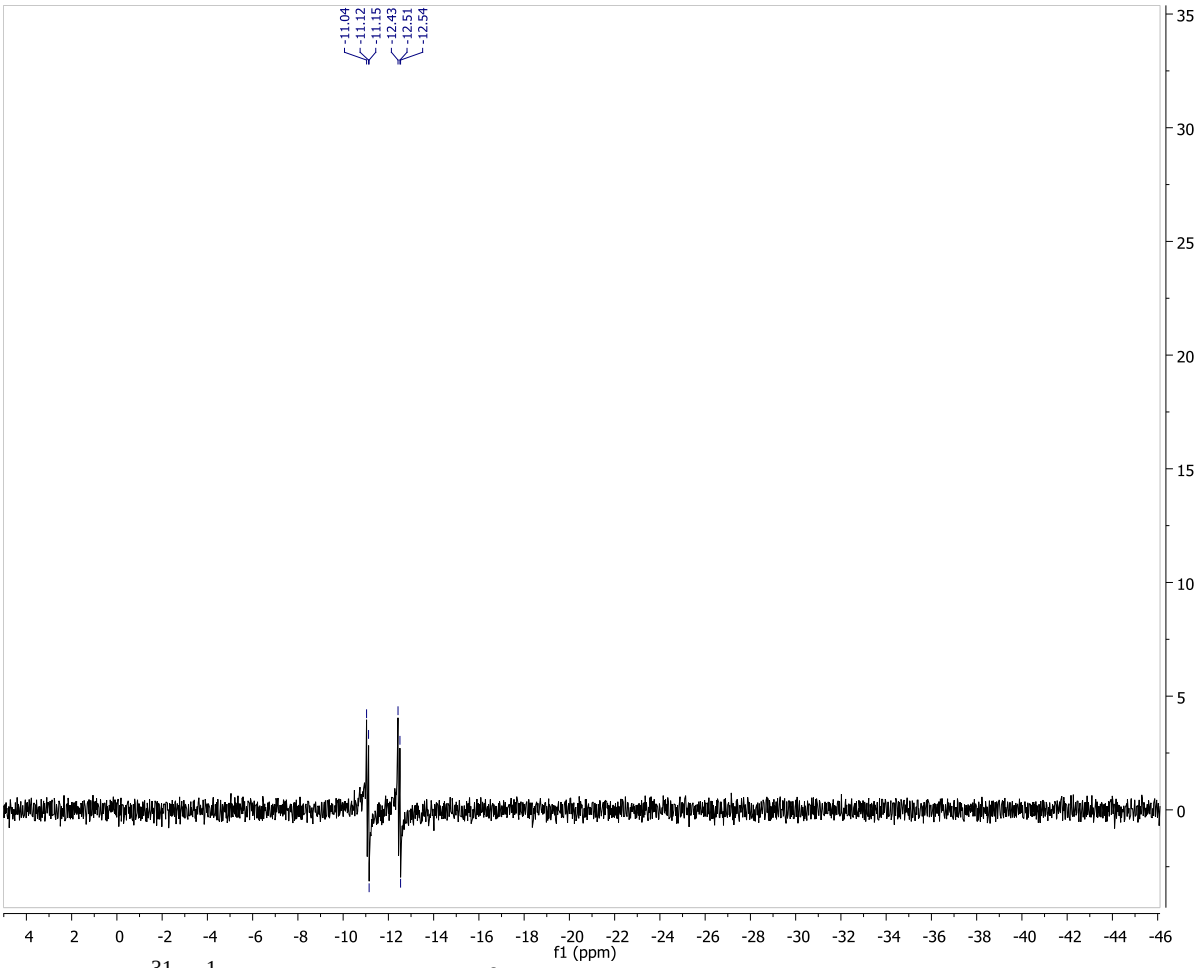

Figure S4. $^{31}P\{^{1}H\}$ NMR spectrum of GDP-5T-Fuc.

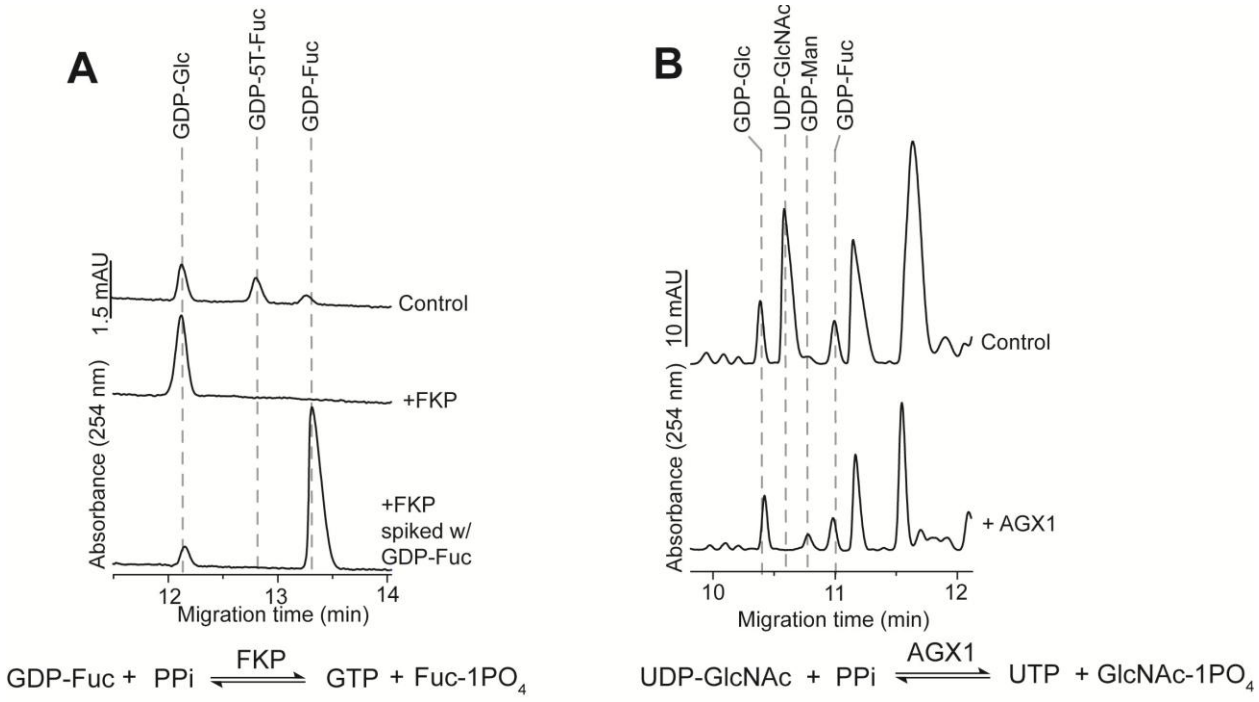

Figure S5. Nucleotide sugar pyrophosphorylase digestions simplified nucleotide sugar analysis: Test reactions. A, A mixture of GDP-Glc, GDP-5T-Fuc and GDP-Fuc was incubated with 200 mM PPi in the presence of FKP for 2h. Control reactions were treated identically except the FKP was omitted. After the reaction samples were desalted using 1 mL HyperSep cartridges, dried, dissolved again in H_2O and analyzed by CE. Immediately after analysis the FKP-treated sample was spiked with GDP-Fuc and reanalyzed. **B**, The same procedure was used to test the pyrophosphorylase ability of AGX1. Nucleotide sugars extracted from HepG2 cells were split into two equal portions, one of which was incubated with AGX1 in the presence of 200 mM pyrophosphate (PPi) for 2 h. Because these samples contain significant quantities of nucleotide triphosphates (ATP for instance is several orders of magnitude more abundant than UDP-GlcNAc) alkaline phosphatase was included to hydrolyze the existing UTP and that formed by the AGX1.

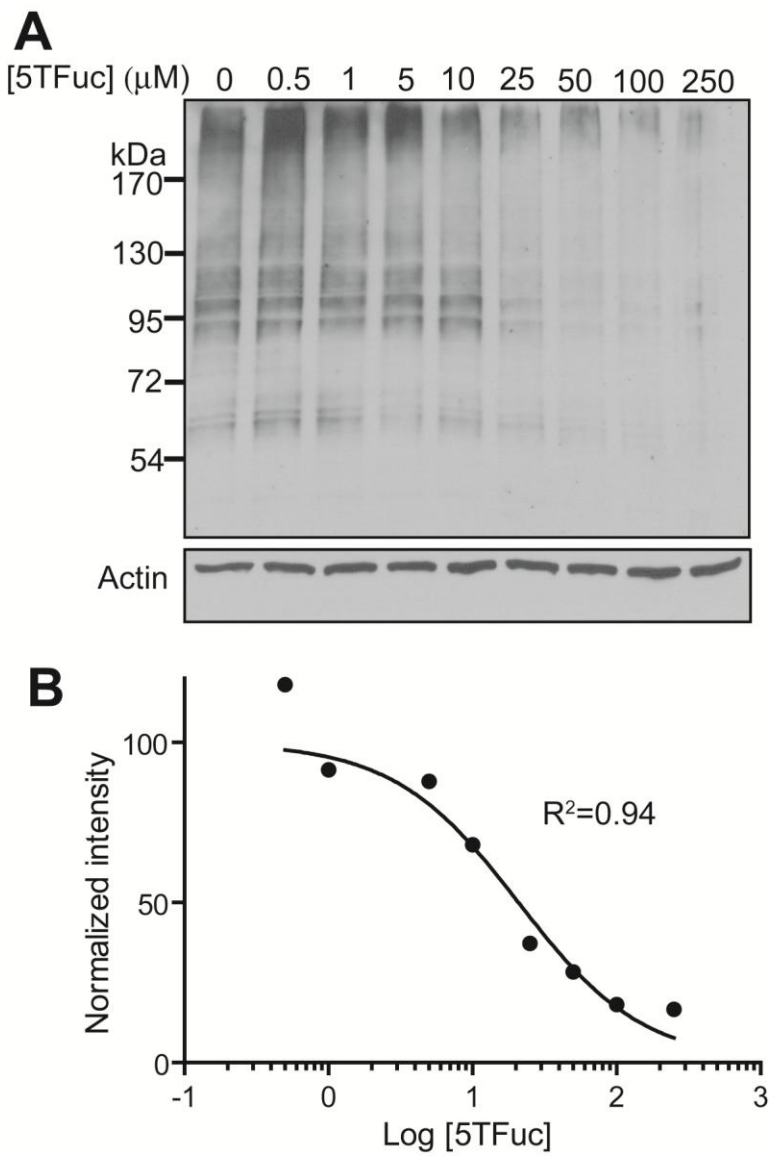

Figure S6. EC_{50} determination for the 5T-Fuc-induced decrement of sLe^X expression. A, Immunoblot of HepG2 cell lysates following treatment with increasing (0-250 μM) amounts of peracetylated 5T-Fuc for 24 h. The upper blot was probed with the sLe^X-specific SNH3 antibody while a parallel blot was probed with an anti-actin antibody (JLA20). **B**, Densitometry analysis was used to determine an EC_{50} for 5T-Fuc. The reactivity of lysates probed with the SNH3 was corrected for actin levels and plotted as a percentage relative to the untreated control sample as a function of the LOG 5T-Fuc concentration. This analysis gave an EC_{50} of 21 ± 1 μM.

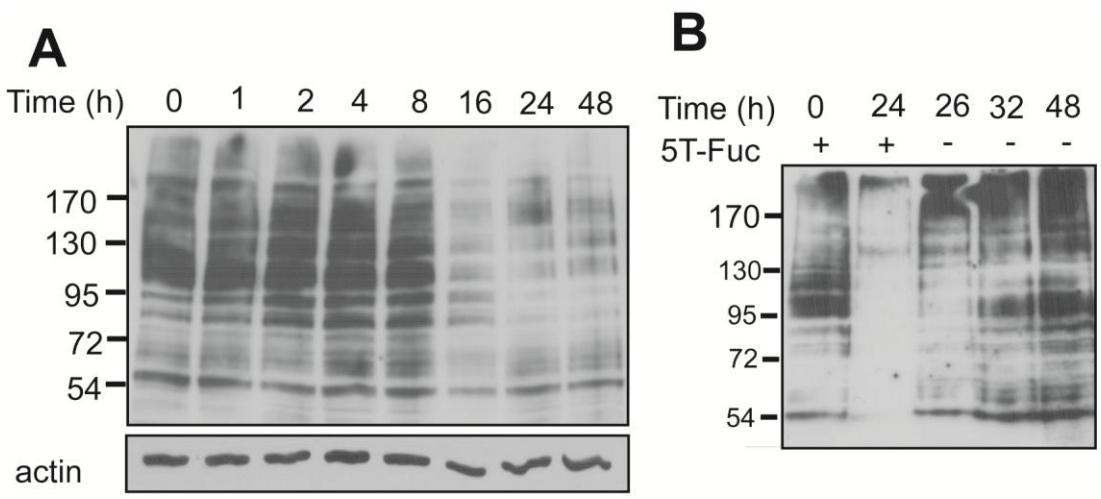

Figure S7. Time-dependant inhibition of sLe^X expression by 5T-Fuc. A, HepG2 cells were incubated in the presence of 50 μM 5T-Fuc for an increasing length of time (0 – 48h). Immunoblots of cell lyates were probed with the sLe^X-specific CSLEX-1 antibody while a parallel blot was probed with an anti-actin antibody. **B,** The rate at which 5T-Fuc was washed out of cells was also assessed. Cells were exposed to 5T-Fuc for 24 h after which the media were replaced. Cells were grown in inhibitor-free media for an additional 2, 8 or 24h prior to harvesting and analysis.

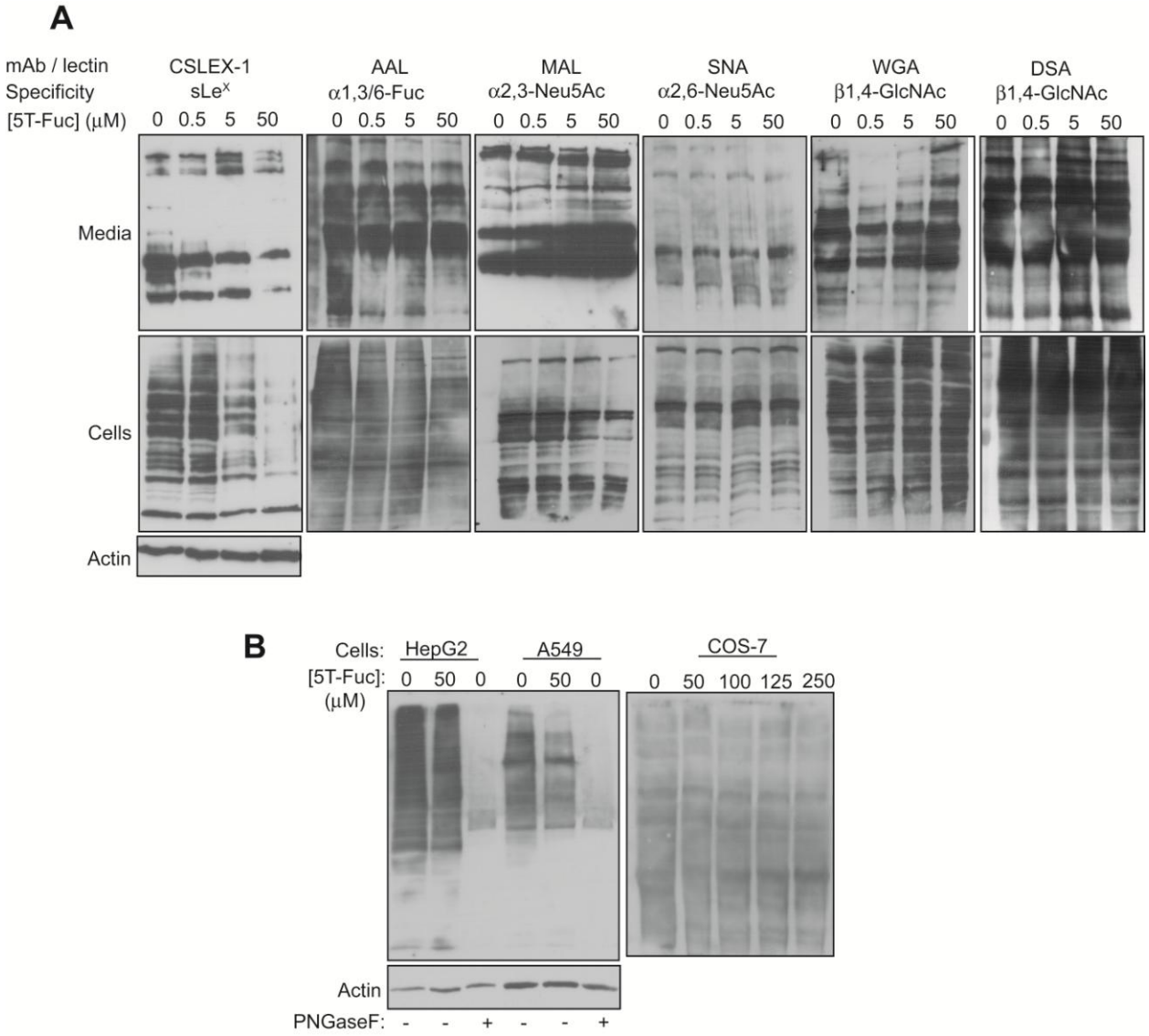

Figure S8. Lectin-blot analysis of the intrecellular and secreted glycoproteins of HepG2 cells demonstrates no large changes in glycosylation upon 5T-Fuc-treatment. A. HepG2 cells were grown for 48h in serum-free media the presence of increasing concentrations of 5T-Fuc after which the media was collected, concentrated and analyzed along with the cell lysates by lectin-blotting. Protein concentrations were normalized by adjusting the volume each sample and all blots contain the same amount of protein (cells, 20 μg/lane; media, 10 μg/lane). **B.** 5T-Fuc did not greatly affect AAL-binding to cells expressing only α1,6-Fuc residues.

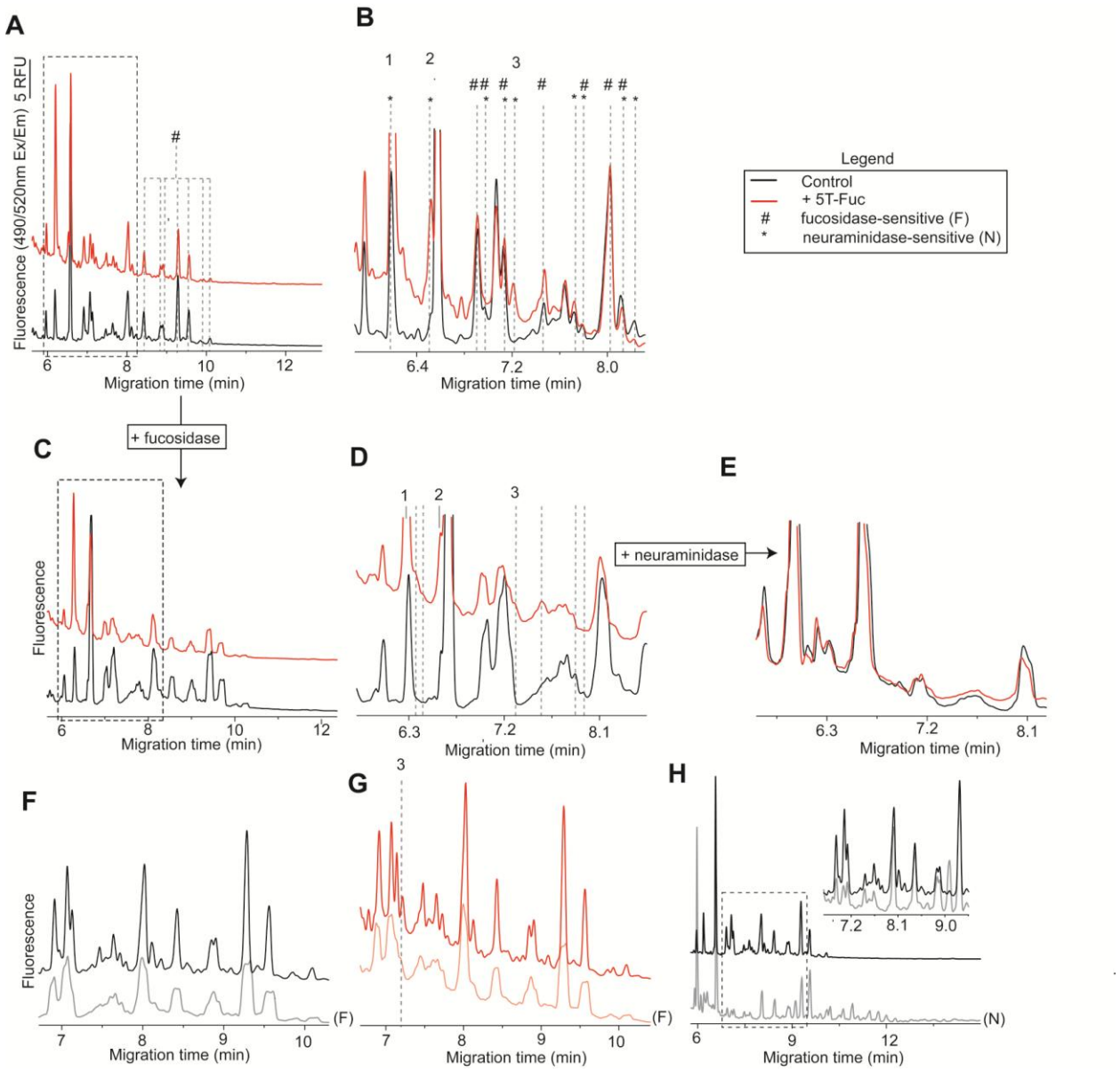

Fig. S9. 5T-Fuc causes changes in the fucosylation of HepG2 *N*-glycans: CE analysis. Since PNGaseF-treatment effectively eliminates the detection of sLe^X on immunoblots probed with the monoclonal antibodies CSLEX-1 and SNH3 (Fig. 3B and S7), it can be presumed that there should be some differences observed in the *N*-glycans derived from 5T-Fuc- and untreated cells. HepG2 cells have previously been shown to synthesize *N*-glycans containing on average 2 and up to 4 Fuc residues per glycan. **A**, The *N*-glycans obtained upon the PNGaseF-digestion of HepG2 lysates were fluorescently labelled with APTS and resolved by CE. The CE method utilized separates labelled glycans based on both size and charge with smaller and/or more highly charged samples migrating the fastes. Peaks before 6 min in electropherograms are due to excess APTS (and trace impurities therein) which were not completely removed from samples by size-exclusion chromatography. **B,** The boxed region of the electropherograms shown in A are

expanded to demonstrate slight differences between untreated (black) and 5T-Fuc-treated cells (red). The glycans from treated and untreated cells are notably similar, consistent with lectin blotting data (Fig. 3D and S7), however, several differences were observed (numbered 1-3): 1 and 2 are substantially larger, while 1 is only observed in 5T-Fuc-treated cells. **C,** Each sample was digested with α1,3/4/6-fucosidase. **D,** The boxed region of C is expanded and reveals that the glycans of 5T-Fuc-treated cells differ from control even after fucosidase digestion (some of these have been indicated with dotted lines); however, **E,** neuraminidase digestion of these samples renders them indistinguishable. Notice that peaks 1-3 do not appear to be to be fucosidase substrates. **F, G,** demonstrate the affect of α-fucosidase (F) on *N*-glycans from non- and 5T-Fuc-treated cells, respectively. The majority of glycans from HepG2 cells appeared to be sensitive to this enzyme (labelled # in A and B). Notice that fucosidase-treatment causes most peaks to appear as doublets, with the earlier peak corresponding to the defucosylated glycan. Complete removal of fucose residues from glycans was not observed even after doubling the enzyme concentration. **H,** Neuraminic (sialic) acid (Neu5Ac)-containing glycans could be identified based on their neuraminidase (N)-sensitivity (these are labelled * in A and B). Because of the negative charge of Neu5Ac, glycans containing this residue migrate between 7 and 9 min (inset) and shift to >9-15 min upon their hydrolysis.

Conclusions: Slight differences are observed in the *N*-glycans obtained from 5T-Fuc cells; in particular, an accumulation of three fucosidase-resistant peaks was observed in these samples. The differences in *N*-glycans observed after fucosidase-digestion suggests that 5T-Fuc, by blocking the activity of α1,3-fucosyltransferases, allows for the *N*-glycans of treated cells to be differentially modified by sialyl-transferases. This is supported by the observation that further neuraminidase treatment renders both samples equivalent. These data indicate that the new (or larger, *i.e.*, more abundant) glycan peaks observed in the 5T-Fuc-treated sample are due to the lack of Fuc residues and not to the transfer of hydrolytically-resistant 5T-Fuc moieties.

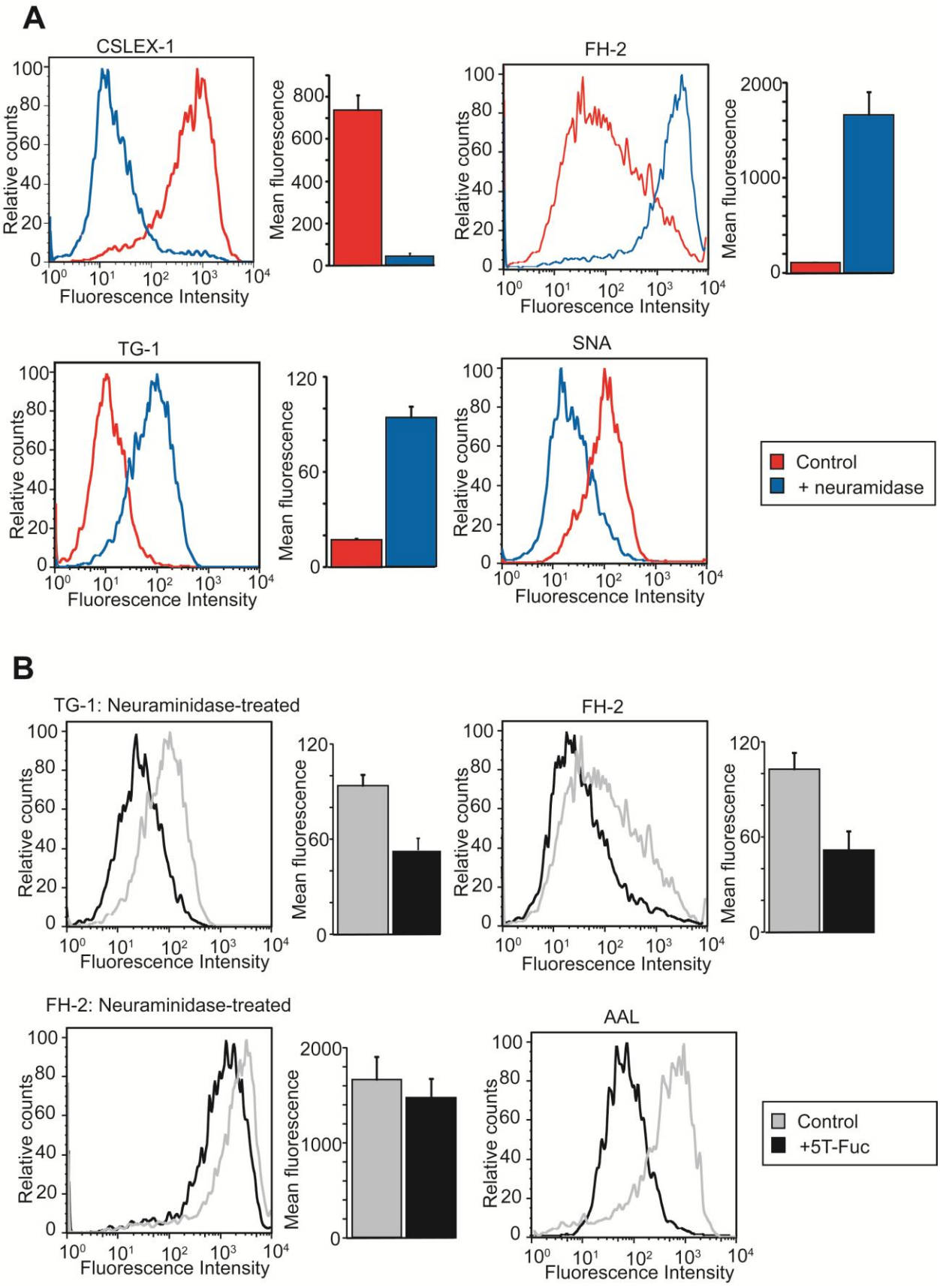
A
CSLEX-1
FH-2
TG-1
SNA
Relative counts
Fluorescence Intensity
Mean fluorescence
Control
+ neuramidase
B
TG-1: Neuraminidase-treated
FH-2
FH-2: Neuraminidase-treated
AAL
Control
+5T-Fuc

Fig. S10. HepG2 cell analysis by flow cytometry. A, Neuraminidase-digestion of cell surface glycans was used to confirm the specificity of the anti-sLe^X (CSLEX-1) and anti-Le^X (FH2 and TG-1) antibodies used in these experiments. Neuraminidase-treatment was used as a control for all experiments although the representative histograms in A are all derived from untreated HepG2 cells. Experiments were performed in triplicate and the averaged mean fluorescence intensities (MFI) are plotted ± SEM. Exposing cells to neuraminidase (blue) greatly diminishes their ability to bind to CSLEX-1 relative to untreated cells (red), however, since this treatment converts sLe^X to Le^X a concomitant increase in affinity for FH2 and TG-1 was observed for neuraminidase-treated cells. The Neu5Ac-specific lectin SNA was also used to confirm desialylation. **B,** Representative histograms. All data were from cells that were not exposed to neuraminidase unless otherwise indicated.

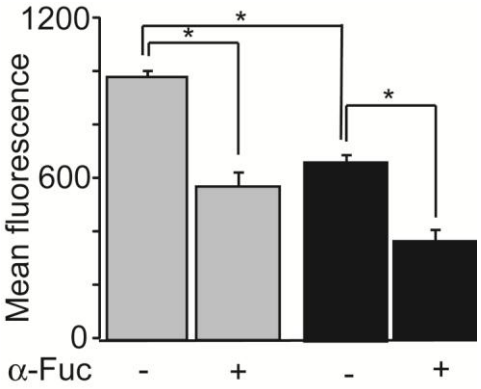

Fig. S11. Alpha-fucosidase exposure reduces CSLEX-1 binding to HepG2 cells. HepG2 cells were harvested and exposed to 52 mU/mL α-fucosidase (from bovine kidney) in 50 mM sodium citrate, pH 5.6 for 30 min. After the enzyme was removed cells were stained using CSLEX-1 and analyzed by flow cytometry. The mean fluorescent intensities of triplicate samples were calculated and the averaged data are presented ± SEM. (**P*<0.005).

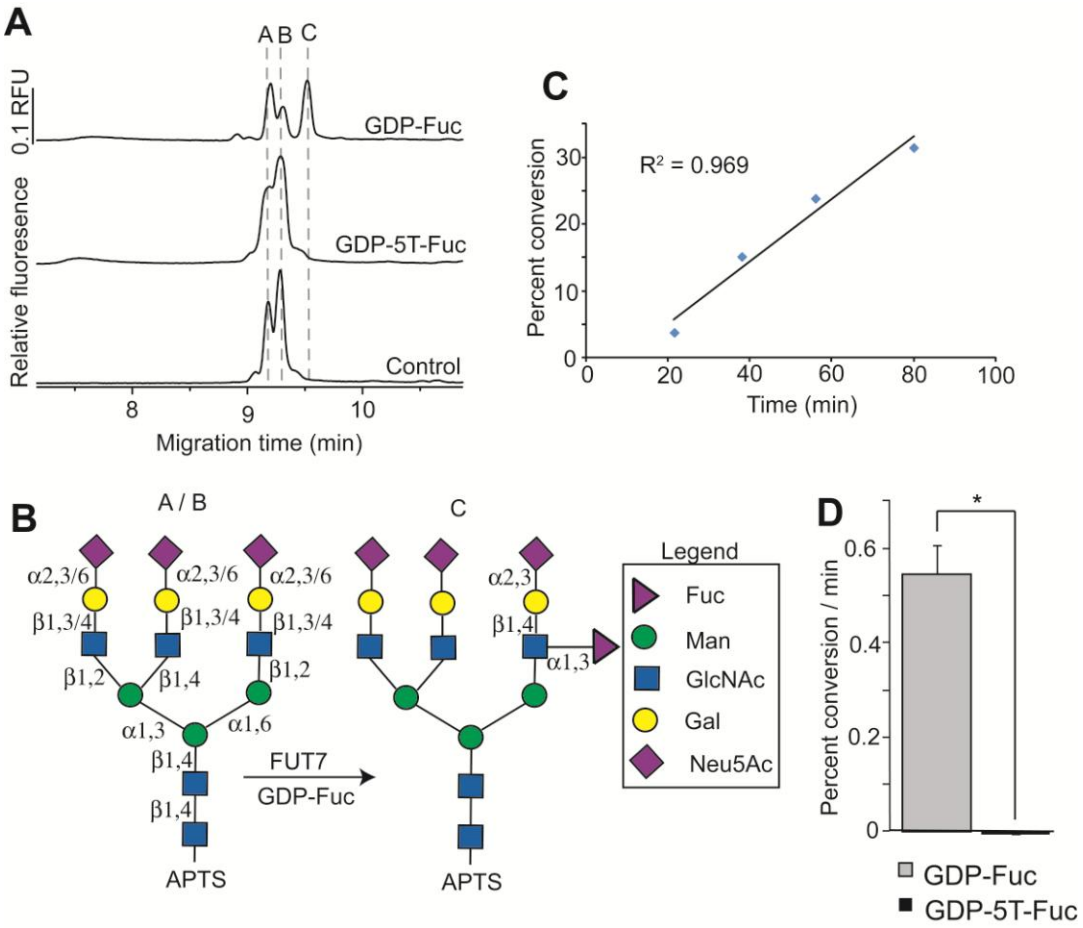

Figure S12. GDP-5T-Fuc is a poor substrate for FUT7. A. A CE-based assay was used to monitor the relative rates of fucose or 5T-Fuc transfer by FUT7. **B.** The acceptor glycan for this assay was a fluorescently-labelled triantenary, trisialylated complex *N*-glycan, comprised mostly of two isomers (A/B) differing in the linkage of a single Neu5Ac residue. Glycan B accepted a fucose residue from FUT7 to form a sLe^X-containing product (glycan C). The specific antenna which was fucosylated was not determined. **C.** The fucosylation of *N*-glycan B by FUT7 was linear over 1.5 h. **D.** Over this period A/B was converted to glycan C at a rate of 0.545±0.062 % converted/min in the presence of GDP-Fuc and -0.008±0.001 % converted/min when GDP-5T-Fuc was used as a donor (**P*=0.0009, *n*=3).

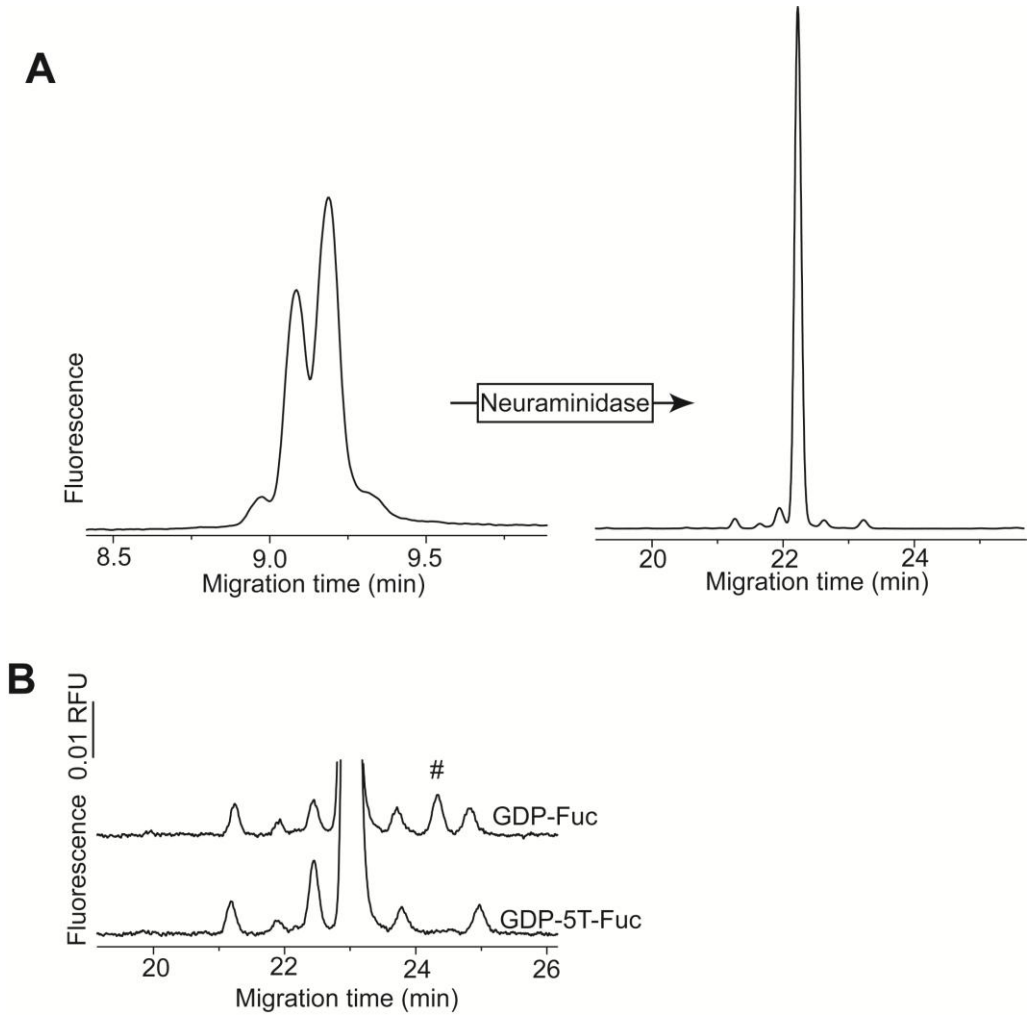

Fig. S13. GDP-5T-Fuc is a poor FUT3 substrate. A, The trisialylated *N*-glycan A3 did not appear to be a suitable acceptor substrate for FUT3, however, unlike FUT7, FUT3 is able to fucosylate desialylated glycans. Neuramindase-digestion of A3 generates six regioisomeric products (NA3) which could be resolved by CE. **B,** A new glycan peak was observed (#) in electropherograms acquired from FUT3 assays when GDP-Fuc but not GDP-5T-Fuc was used as a donor sugar.

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