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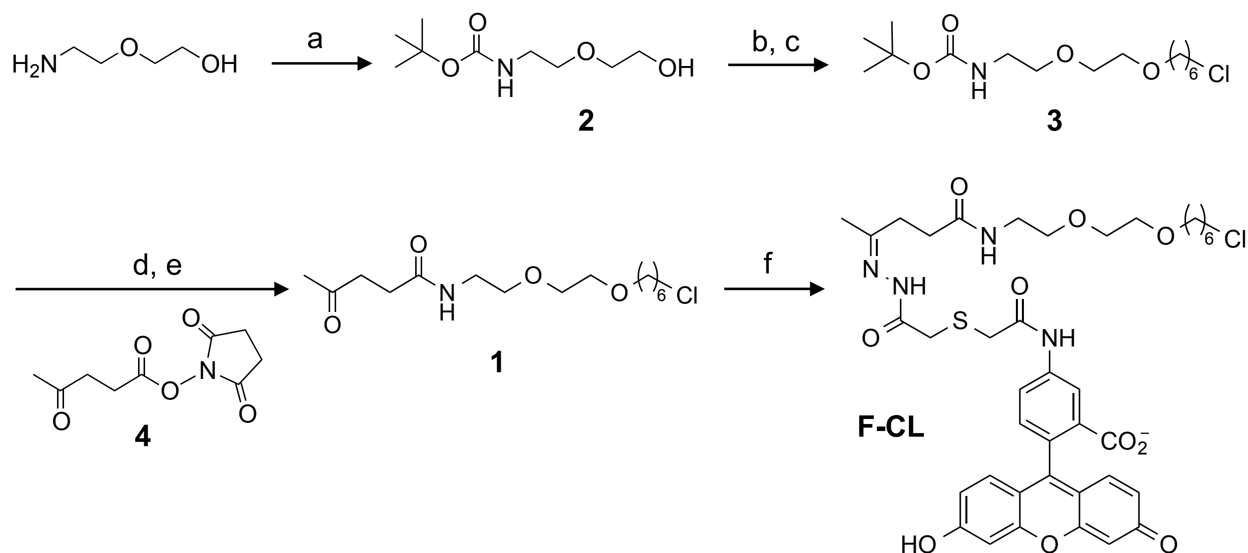
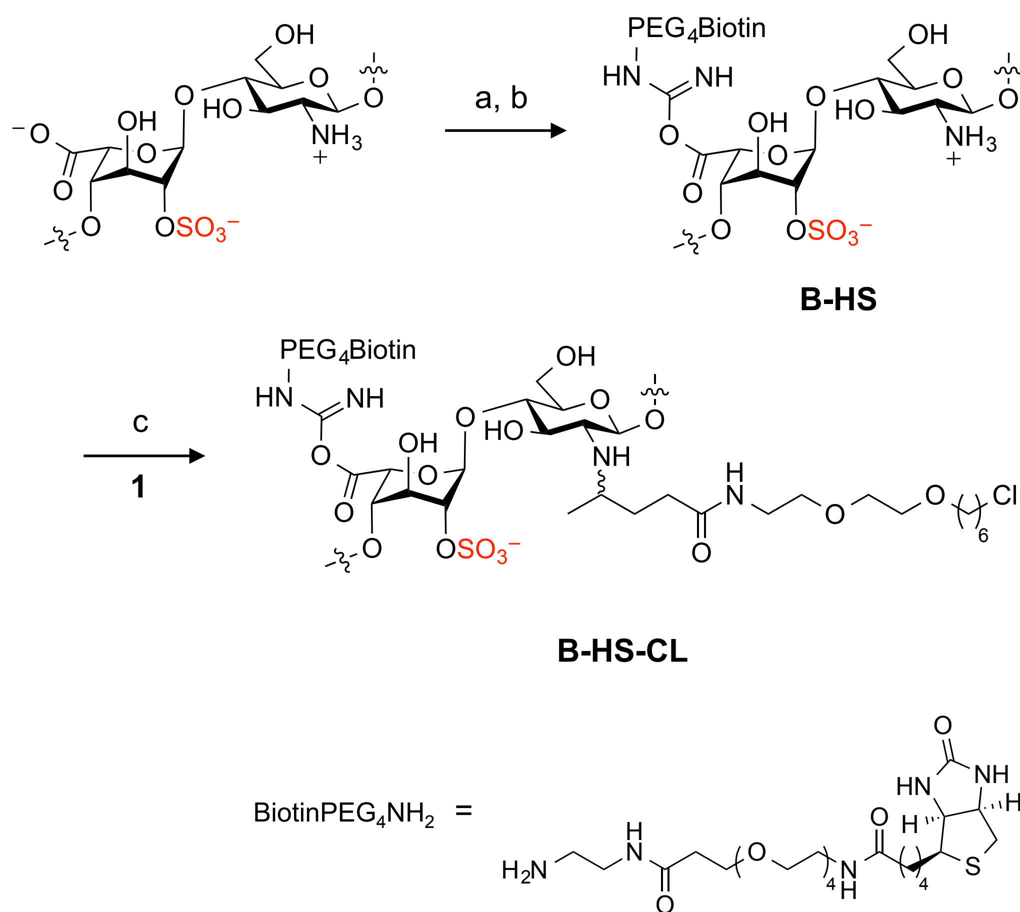


Scheme S1. Synthesis of chloroalkane linker (CL) **1** and chloroalkane-conjugated fluorescein (F-CL). Conditions: a) Boc_2O (2 eq), MeOH, 0 °C, 2 h, 91%; b) 60% NaH (1.5 eq), DMF, 0 °C, 1 h; c) 1-chloro-6-iodohexane (1.2 eq), 24 h, 37% over two steps; d) TFA (6 eq), DCM, 4 h; e) **4** (1 eq), DCM, 24 h, 67% over two steps; f) 5-(((2-(carbohydrazino)methyl)thio)acetyl)-aminofluorescein (F) (0.5 eq), MeOH, 16 h, 75%. MeOH = methanol, NaH = sodium hydride, DMF = *N,N*-dimethylformamide, TFA = trifluoroacetic acid, DCM = dichloromethane, EDC = 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide.



Scheme S2. Synthesis of biotinylated heparan sulfate with and without the chloroalkane linker (B-HS and B-HS-CL). Conditions: a) BrCN (excess), 0.2 M NaOH, pH 11, 10 min; b) BiotinPEG$_4$NH$_2$ (excess), 0.2 M sodium borate, pH 8, 12 h; c) **1** (excess), NaBH$_3$CN (excess), MeOH, ddH$_2$O, 16 h. Step (c) was also used to synthesize all HS-CL derivatives listed in Figure 1B. PEG = polyethylene glycol, ddH$_2$O = deionized water.

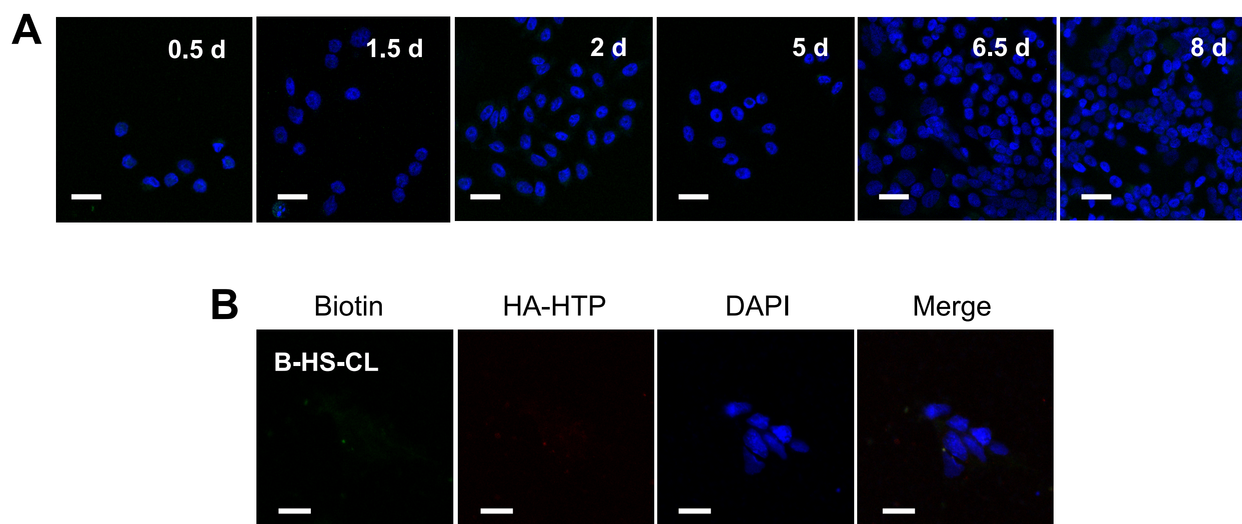


Figure S1. Specificity controls for the HTP anchoring strategy. A) CHO cells stably expressing HTP do not incorporate fluorescein lacking the chloroalkane linker. Cells were incubated with 5-(((2-(carbohydrazino)methyl)thio)acetyl)-aminofluorescein (F) for 1 h at 37 °C. Cultures were then stained with DAPI (blue) and imaged at the indicated time points. No appreciable fluorescein (green) signal was observed. B) CHO cells lacking HTP do not incorporate B-HS-CL into their membranes. Cells were transfected with an empty pDisplay vector (pD; no HTP insert), incubated with B-HS-CL for 6 h at 37 °C, and stained with DAPI (blue), streptavidin-IRDye800 (green), and an anti-HA antibody (red). No appreciable biotin signal (green) was observed. HTP = HaloTag protein, HA = hemagglutinin, DAPI = 4',6-diamidino-2-phenylindole. Scale bars represent 20 μm.

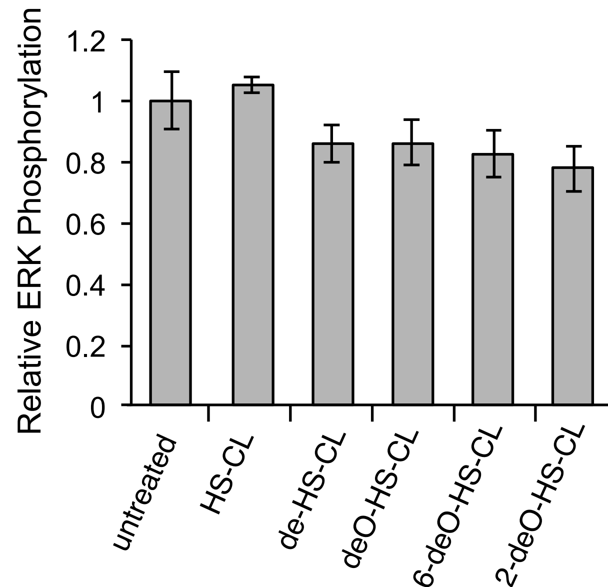


Figure S2. Cell-surface presentation of HS GAGs on ESCs does not induce FGF2-mediated ERK1/2 phosphorylation in the absence of HTP expression. Mouse ESCs were mock transfected with an empty pDisplay vector (pD; no HTP insert), incubated with the indicated HS-CL derivatives, washed, stimulated with FGF2, and analyzed for phospho-ERK levels as described. No significant differences were observed between untreated cells and cells treated with the HS-CL derivatives, suggesting that cell-surface anchoring of HS-CL onto HTPs is required for inducing ERK activation. Data represent the mean ± S.E.M. from three independent experiments ($n = 3$). $P > 0.05$ for all conditions when compared to the untreated control.

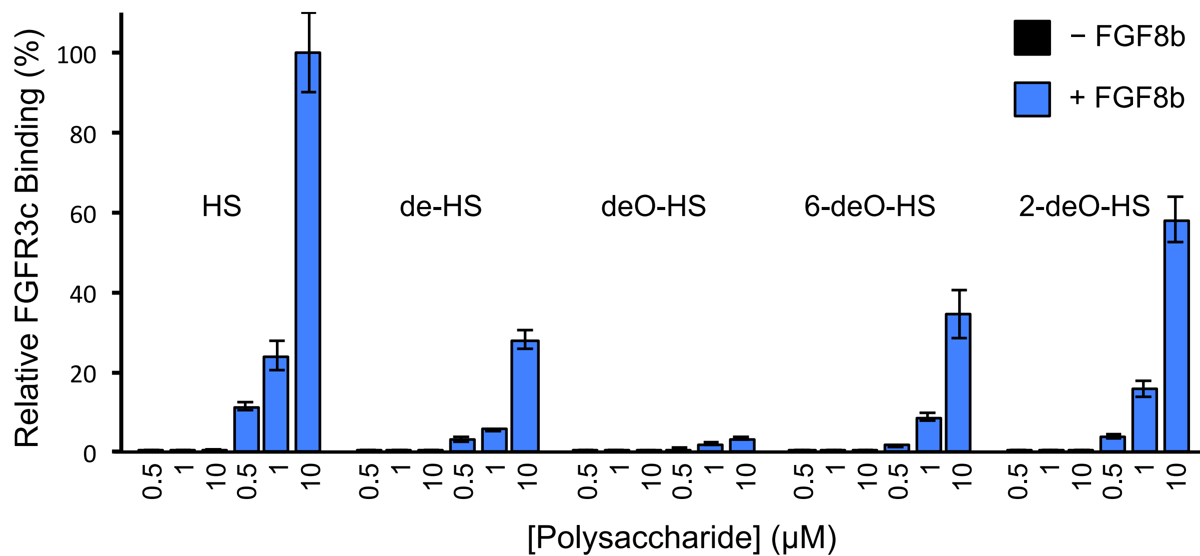


Figure S3. FGF8b and its receptor FGFR3c are capable of forming ternary complexes with heparin/highly sulfated HS. Microarrays printed with various concentrations of the HS derivatives (0.5 to 10 μM) were incubated with an FGFR3c-Fc fusion protein in the presence (blue bars) or absence (black bars) of FGF8b. Binding of FGFR3c-Fc was visualized using an AF647-conjugated goat anti-human Fc antibody and quantified by measuring the fluorescence intensity at each spot. Data represent the mean ± S.E.M. from ten replicate microarray spots and were normalized with respect to the maximum fluorescence intensity on the array.

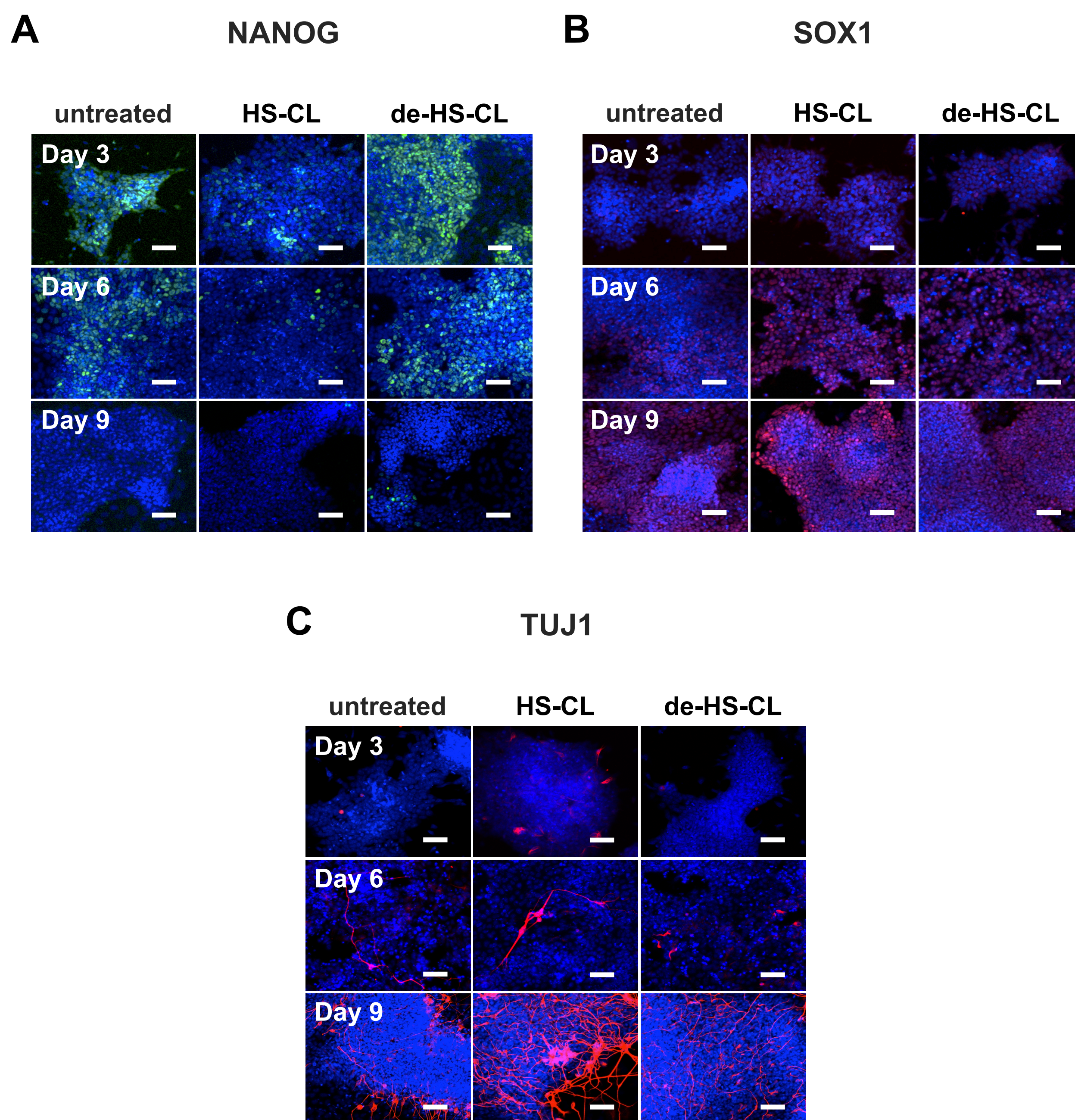


Figure S4. Surface display of highly sulfated HS induces accelerated self-renewal exit, neural lineage commitment, and differentiation into mature, neuronal cells. mESCs were fixed at the indicated time points and stained by immunocytochemistry for A) pluripotent marker NANOG, B) neuroectoderm marker SOX1, or C) neuronal marker TUJ1. NANOG is shown in green, SOX1 and TUJ1 in red, and DAPI-stained nuclei in blue. Scale bars represent 20 µm.

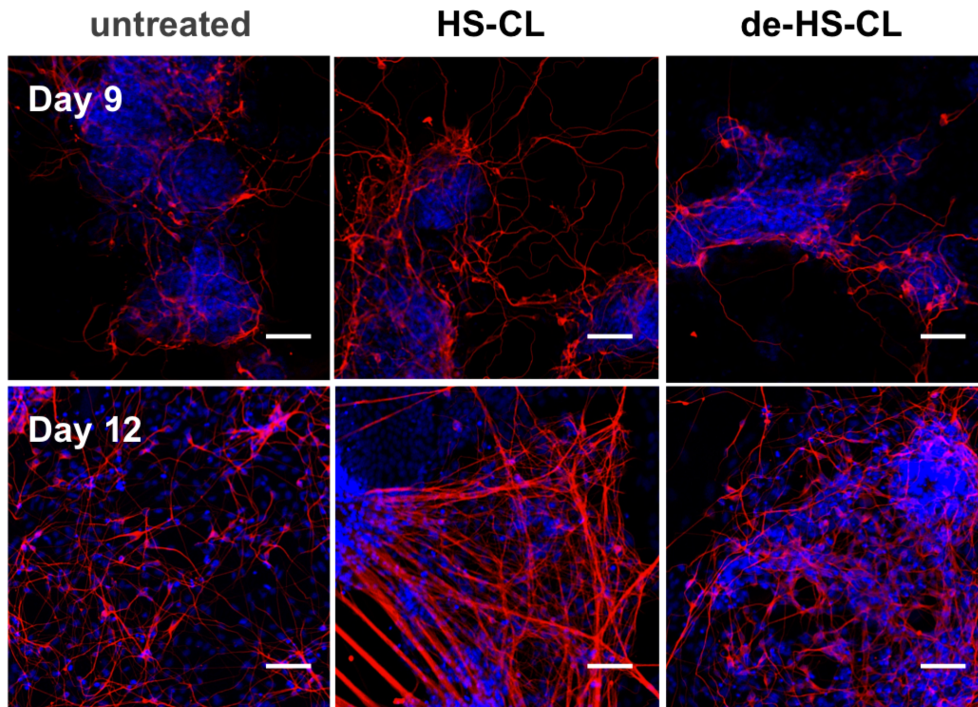


Figure S5. ESCs treated with HS-CL develop more extensive neuronal processes compared to untreated cells and de-HS-CL treated cells. Cells were stained with an anti-TUJ1 antibody (red) and DAPI (a nuclei marker, blue) 9 and 12 days after treatment with the indicated HS derivative. As indicated in red, more elaborate neurite processes were observed for cells treated with HS-CL compared to untreated cells and cells treated with de-HS-CL at both time points. Scale bars represent 50 μm.

Table S1. qRT-PCR primers used in this study.

Gene	Forward Primer	Reverse Primer	Amplicon (bp)
GAPDH	AAC AGA AAC TCC CAC TCT TC	CCT GTT GCT GTA GCC GTA TT	111
SDHA	GCT GGA GAA GAA TCG GTT ATG A	GCA TCG ACT TCT GCA TGT TTA G	97
NANOG	TTT GGA AGC CAC TAG GGA AAG	CCA GAT GTT GCG TAA GTC TCA TA	115
SOX1	ACA CAC ACA CAC ACA CTC TC	CCT CAA GAT CTG GTC AGG AAT G	101
TUJ1	CCA TTC TGG TGG ACT TGG AA	GCA CCA CTC TGA CCA AAG ATA	103

Materials and Methods

Reagents and materials.

Chemicals and biochemical reagents. All chemicals and reagents were analytical grade, obtained from Sigma-Aldrich (St. Louis, MO), and used without further purification unless specified. Heparin/HS derivatives were purchased from Neoparin, Inc. (Alameda, CA). Primary antibodies against HA (rabbit mAb, 3724), Myc (mouse mAb, 2276), p44/42 MAP kinase (ERK, mouse mAb, 4696), phospho-p44/42 MAP kinase (phospho-ERK, rabbit mAb, 4370), NANOG (rabbit mAb, 8842), and βIII-tubulin/TUJ1 (rabbit mAb, 5568) and the IRDye800 goat anti-rabbit IgG antibody were purchased from Cell Signaling Technology, Inc. (Beverly, MA). Secondary antibodies (AlexaFluor (AF) 680 goat anti-rabbit, AF680 goat anti-mouse, AF568 donkey anti-goat, AF568 goat anti-mouse, and AF488 goat anti-rabbit), AF488 streptavidin, and 5-(((2-(carbohydrazino)methyl)thio)acetyl)-aminofluorescein (F) were obtained from Life Technologies, Inc. (Carlsbad, CA). The AF647 goat anti-human Fc antibody was purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). Recombinant human basic FGF (FGF2), FGF8b, FGFR1 alpha IIIc Fc chimera (FGFR1-Fc), FGFR3c IIIc Fc chimera (FGFR3c-Fc), and polyclonal goat anti-SOX1 antibody (AF3369) were purchased from R&D Systems, Inc. (Minneapolis, MN).

Cell culture materials. All cell culture-related medium and supplements (TrypLE Express, Hank's balanced salt solution (HBSS), phosphate-buffer saline (PBS), Dulbecco's modified eagle's medium (DMEM), GlutaMAX, trypsin, fetal bovine serum (FBS), penicillin/streptomycin (P/S), N2, and B27) were received from Life Technologies Inc. unless otherwise specified. Xfect™ mESC Transfection Reagent was obtained from Clontech Laboratories, Inc. (Mountain View, CA). Mitomycin C from *Streptomyces caespitosus* (475820) was purchased from EMD Millipore Corp. (Temecula, CA). Mouse embryonic stem cells (mESCs; ES-E14TG2a, CRL-1821) and a Chinese hamster ovary (CHO; pgsB-618, CRL-2241) cell line deficient in galactosyltransferase I were obtained from American Type Culture Collection (ATCC; Manassas, VA). F12 and DMEM culture medium and non-heat-inactivated FBS used for mESC culture were also purchased from ATCC. Mouse embryonic fibroblasts (MEFs; GSC-6001) were purchased from AMSBIO Biotechnology, Ltd. (Irvine, CA). PerfeCTa® SYBR® Green FastMix®, ROX™ (95073-05K) was purchased from Quanta Biosciences (Gaithersburg, MD).

General synthetic procedures.

Unless specifically stated, all reactions were conducted in flame-dried glassware under an argon (Ar) atmosphere using anhydrous solvents. All solvents and commercially available reagents were obtained from Sigma-Aldrich or Thermo Fisher Scientific, Inc. (Pittsburgh, PA) and used as received, unless specified otherwise. Thin-layer chromatography was performed using silica gel 60 F254 pre-coated plates (0.25 mm) (E. Merck KG, Darmstadt, Germany), and ICN silica gel (0.032-0.063 mm particle diameter) (Analtech Inc., Newark, DE) was employed for flash chromatography. Molecular masses of intermediate and final compounds were analyzed using an Agilent 1100 LC-MS (Agilent, Santa Clara, CA), and ^{1}H NMR data were obtained using Varian Mercury 300 (300 MHz), Varian Inova 500 (500 MHz), or Varian Inova 600 (600 MHz) spectrometers and are reported in parts per million (δ) relative to $CDCl_3$ (7.26 ppm) or D_2O (4.79 ppm). For small molecules, ^{1}H NMR data are reported with the following: multiplicity (s = singlet, bs = broad singlet, d = doublet, dd = doublet of doublets, t = triplet, q = quartet, and m = multiplet), coupling constant (Hz), and integration values. For polysaccharides, ^{1}H NMR data are reported in two separate sections: the disaccharide unit and substoichiometric components (*i.e.*, *N*-acetyl, biotin$(PEG)_4$, chloroalkane linker (CL)). Peaks for substoichiometric components within the disaccharide region were broad and partially obscured and therefore are not reported. Integration values are reported relative to each component and percent incorporation per disaccharide unit. Incorporation of biotin$(PEG)_4$ or CL is estimated using NMR peak intensities compared to the GlcN C-2 proton peak (~3.29 ppm) and is reported as the average number of molecules per polysaccharide, assuming polysaccharides (12-13 kDa) had an average length of 22 disaccharide units.

Synthesis of chloroalkane linker (CL) 1.

The chloroalkane linker **1** was synthesized using procedures adapted from So *et al.*[1] and Huang *et al.*[2] The ^{1}H NMR and MS values were in good agreement with previously reported values.[1]

N-Boc-2-(2-hydroxyethoxy)ethylamine (**2**).[1] A solution of di-*tert*-butyl dicarbonate (Boc_2O) (2.18 g, 10.0 mmol, 1 eq) in anhydrous methanol (2 mL) was added dropwise to a stirring solution of 2-(2-aminoethoxy)ethanol (1.05 g, 9.99 mmol) in anhydrous methanol (20 mL) at 0 °C. The mixture was stirred for 30 min at 0 °C and then warmed to RT. After 2 h, the solution was diluted with DCM (30 mL), washed with ddH_2O (3 x 25 mL) and brine (1 x 25 mL), dried over $MgSO_4$, filtered, and concentrated to afford a colorless oil **2** (1.85 g, 91%). ^{1}H NMR (500 MHz, $CDCl_3$): δ 1.42 (s, 9H, CH_3), 3.30 (t, *J* = 5.1 Hz, 2H, CH_2N), 3.49-3.58 (m, 4H, CH_2OCH_2), and 3.68-3.75 (m, 2H, CH_2OH). MS (ESI) calcd. for $C_4H_{11}NO_2^+$ [M + H^+] 206.14, found 206.10.

N-Boc-1-(2-(2-aminoethoxy)ethoxy-6-chlorohexane (**3**).[1] A solution of **2** (0.34 g, 1.7 mmol) in DMF (2 mL) was added dropwise to a stirring solution of 60% NaH (0.095 g, 2.4 mmol, 1.4 eq) in DMF (30 mL) at 0 °C. The mixture was stirred for 1 h at 0 °C, after which 1-chloro-6-iodohexane (0.30 mL, 2.0 mmol, 1.2 eq) was added. Stirring was continued for 24 h at RT, and then the reaction was quenched with 1 M HCl and extracted with DCM, ddH_2O (6 x 25 mL) and brine (1 x 25 mL), dried over $MgSO_4$, filtered, and concentrated to afford a yellow oil. Purification by silica gel flash chromatography using 3:1 Hex/EtOAc gave **3** as a colorless oil (0.20 g, 37%). ^{1}H NMR (500 MHz, $CDCl_3$): δ 1.33-1.40 (m, 2H, CH_2), 1.43 (s, 9H, CH_3), 1.44-1.48 (m, 2H, CH_2), 1.55-1.64 (m, 2H, CH_2), 1.73-1.80 (m, 2H, CH_2), 3.30 (t, *J* = 5.2 Hz, 2H, CH_2N), 3.45 (t, *J* = 6.7 Hz, 2H, CH_2Cl), 3.49-3.57 (m, 6H, CH_2O), and 3.57-3.61 (m, 2H, CH_2O). MS (ESI) calcd. for $C_{14}H_{29}ClNO_5^+$ [M + H^+] 324.20, found 324.21.

2,5-Dioxopyrrolidin-1-yl 4-oxopentanoate (**4**).[2] *N*-Hydroxysuccinimide (NHS; 0.55 g, 4.8 mmol, 1.1 eq) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) hydrochloride (0.91 g, 4.8 mmol, 1.1 eq) were added to a stirring solution of levulinic acid (0.50 g, 4.3 mmol) in anhydrous DCM at 0 °C. The mixture was warmed to RT and stirred for 2 h, at which time the reaction was diluted with DCM, extracted with ddH_2O (3 x 25 mL) and brine (1 x mL), dried over $MgSO_4$, filtered, and concentrated to afford a white fluffy solid **4** (0.69 g, 75%). ^{1}H NMR (500

MHz, $CDCl_3$): δ 2.21 (s, 3H, CH_3), 2.83 (s, 4H, CH_2CH_2-succinimide), and 2.86-2.92 (m, 4H, CH_2CH_2-levulinate). MS (ESI) calcd. for $C_9H_{11}NO_5^+$ [M + H^+] 214.08, found 214.10.

N-(2-(2-(6-Chlorohexyloxy)ethoxy)ethyl)-4-oxopentanamide (**1**). Trifluoroacetic acid (TFA) (0.14 mL, 1.8 mmol, 6 eq) was added dropwise to a stirring solution of **3** (0.10 g, 0.31 mmol) in anhydrous DCM (5 mL). The mixture was stirred at RT for 4 h, then azeotroped with toluene, and concentrated to afford the free amine as a yellow oil, which was used without further purification. This intermediate (0.025 g, 0.074 mmol) was stirred with *N*,*N*-diisopropylethylamine (DIPEA; 0.076 mL, 0.436 mmol, 6 eq) in anhydrous DCM (3 mL) for 20 min at RT, to which **4** (0.016 g, 0.075 mmol, 1 eq) was added and stirred overnight. After 24 h, the reaction was diluted with DCM (20 mL) and extracted with ddH_2O (3 x 25 mL) and brine (1 x 25 mL), dried over $MgSO_4$, filtered, and concentrated to afford a yellow oil. Purification by silica gel flash chromatography using 97:3 DCM/MeOH gave **1** as a colorless oil (0.016 g, 67%). ^{1}H NMR (500 MHz, $CDCl_3$): δ 1.33-1.41 (m, 2H, CH_2), 1.42-1.49 (m, 2H, CH_2), 1.56-1.65 (m, 2H, CH_2), 1.73-1.81 (m, 2H, CH_2), 2.18 (s, 3H, CH_3), 2.43 (t, *J* = 6.6 Hz, 2H, $CH_2C(O)$), 2.79 (t, *J* = 6.6 Hz, 2H, CH-$_2$C(O)), 3.41-3.49 (m, 4H, CH_2Cl and CH_2N), 3.51-3.56 (m, 4H, CH_2O), 3.56-3.63 (m, 4H, CH_2O), 6.15 (bs, 1H, NH). MS (ESI) calcd. for $C_{15}H_{28}ClNO_4^+$ [M + H^+] 322.18, found 322.20.

Synthesis of compounds for method validation.

Fluorescein-chloroalkane linker conjugate (F-CL). To a solution of **1** (2.6 mg, 8.1 μmol) in 0.5 mL anhydrous MeOH was added 5-(((2-(carbohydrazino)methyl)thio)acetyl)aminofluorescein (F) in ddH_2O (4.0 mg, 8.1 μmol, 1 eq; C-356, Life Technologies). The mixture was stirred for 12 h at RT in the dark, concentrated, and purified by silica gel flash chromatography in the dark using a 97:3 DCM/MeOH mixture to afford the desired compound as an orange solid (4.8 mg, 75%). ^{1}H NMR (500 MHz, D_2O): δ 1.26-1.33 (m, 2H, CH_2), 1.33-1.39 (m, 2H, CH_2), 1.40 (s, 3H, CH_3), 1.44-1.52 (m, 2H, CH_2), 1.63-1.70 (m, 2H, CH_2), 3.32-3.55 (m, 20H, CH_2), 6.42-6.48 (m, 2H, Ar-H) 6.49-6.60 (m, 4H, Ar-H), 7.07 (dd, *J* = 8.3, 2.9 Hz, 1H, Ar-H), 7.73-7.81 (m, 1H, Ar-H), and 8.21-8.30 (m, 1H, Ar-H). MS (ESI) calcd. for $C_{39}H_{43}ClN_4O_{10}S^{-2}Na^+$ [M^{-2} + Na^+] 817.23, found 817.25.

Biotinylated heparan sulfate (B-HS). To a stirring solution of EZ-Link NHS-PEG_4-biotin (0.10 g, 0.17 mmol; 21363, Thermo Scientific) in dry DCM (5 mL) was added a solution of ethylenediamine (0.022 mL, 0.33 mmol, 2 eq) and triethylamine (TEA; 0.15 mL, 1.1 mmol, 6 eq) in dry DCM (1 mL). The mixture immediately turned cloudy upon addition and was then stirred for 1.5 h at RT. The precipitate was filtered, and the reaction was concentrated to afford the conjugated amine as a white solid (0.068 g, 76%), which was used without further purification. De-6-*O*-sulfated HS (7.0 mg, 0.58 μmol) was dissolved in ddH_2O (1 mL), followed by the addition of cyanogen bromide in ddH_2O (5.0 mg, 47 μmol, excess). The pH was adjusted to 11.0 using 0.2 M NaOH and stirred for 10 min. The mixture was then desalted on a PD-10 Sephadex column using 0.2 M sodium borate (pH 8.0). The HS fractions were pooled (3 mL) and immediately stirred with the biotin-conjugated amine (5.0 mg, 9.4 μmol, excess) overnight (12 h). The mixture was then flash frozen, lyophilized, redissolved in ddH_2O (0.5 mL), and purified using a G-25 Sephadex column. The desired fractions were pooled, flash frozen, and lyophilized to afford B-HS as a white solid (99% recovery). ^1H NMR (600 MHz, D_2O): δ 3.29 (bs, 1H), 3.72-3.91 (m, 5H), 4.04-4.13 (m, 1H), 4.18-4.32 (m, 1H), 4.33-4.44 (m, 1H), 4.84 (bs, 1H), 5.26 (bs, 1H, IdoA C-1), 5.42 (bs, 1H, GlcN C-1); substoichiometric: 1.30 (bs, 2H, CH_2-biotinPEG_4), 1.43 (bs, 2H, CH_2-biotinPEG_4), 1.50-1.52 (m, 2H, CH_2-biotinPEG_4), 2.05 (bs, 3H, NHAc), 2.29 (t, 2H, CH_2-biotinPEG_4), 2.52-2.60 (m, 2H, CH_2-biotinPEG_4), 2.78-2.82 (m, 2H, C(O)CH_2-biotinPEG_4) 2.99-3.03 (m, 1H, CH-biotinPEG_4), 3.32-3.54 (m, 22H, CH_2O- and CH_2N-biotinPEG_4). Biotin$(PEG)_4$ incorporation was estimated to be 0.9 molecules per polysaccharide.

Biotinylated heparan sulfate with chloroalkane linker (B-HS-CL). A solution of **1** (4.0 mg, 12 μmol, excess) and $NaBH_3CN$ (2.0 mg, 32 μmol, excess) in 1:1 MeOH:ddH_2O (400 μL) was added to B-HS (2.0 mg, 0.17 μmol) in ddH_2O (500 μL). MeOH (approximately 300 μL) was added until the reaction turned from cloudy to colorless, and the mixture was stirred for 12 h at RT, concentrated, and purified by gel filtration chromatography using G-25 Sephadex resin. The pooled fractions containing the polysaccharide were lyophilized to yield a white powder (99% recovery). ^1H NMR (600 MHz, D_2O): δ 3.24 (bs, 1H), 3.62-3.89 (m, 5H), 4.04-4.14 (m, 1H), 4.17-4.32 (m, 1H), 4.33-4.44 (m, 1H), 5.24-5.45 (m, 2H, IdoA C-1, GlcN C-1); substoichiometric: 1.07 (d, J = 6.3 Hz, 3H, CH_3-CL), 1.50-1.54 (m, 2H), 2.01 (s, 3H, NHAc), 2.18-2.23 (m, 6H), 2.34-2.45 (m, 2H), 2.75 (s, 2H), 2.99-3.10 (m, 2H). CL incorporation was estimated to be 1.8 CL molecules per polysaccharide.

Synthesis of compounds for biological assays.

General procedure for chloroalkane heparin/heparan sulfate derivatives. In a typical procedure, **1** (4.0 mg, 12 μmol, excess) was dissolved in MeOH and ddH_2O (1:1, 400 μL) with $NaBH_3CN$ (2.0 mg, 32 μmol, excess) and added to HS, de-HS, deO-HS, 6-deO-HS, or 2-deO-HS (2.0 mg, 0.17 μmol) in ddH_2O (500 μL). MeOH (approximately 300 μL) was added until the reaction turned from cloudy to colorless, and the mixture was stirred for 12 h at RT, concentrated, and purified by gel filtration chromatography using G-25 Sephadex resin. The pooled fractions containing the polysaccharides were lyophilized to yield white powders (99% recovery of polysaccharide in all cases). CL incorporation was estimated to be between 0.4 to 4 molecules per polysaccharide.

HS-CL: ^{1}H NMR (600 MHz, D_2O): δ 3.29 (bs, 1H), 3.78-3.91 (m, 2H), 4.06 (bs, 1H), 4.12 (bs, 1H), 4.20-4.31 (m, 2H), 4.33-4.44 (m, 2H), 4.91 (bs, 1H), 5.27-5.46 (m, 2H, IdoA C-1, GlcN C-1); substoichiometric: 1.19 (dd, *J* = 6.3, 0.9 Hz, 3H, CH_3-CL), 1.67-1.77 (m, 2H), 2.06 (bs, NHAc), 2.20-2.31 (m, 6H), 2.38-2.45 (m, 2H), 2.73 (s, 2H), 2.77-2.86 (m, 2H).

de-HS-CL: ^{1}H NMR (600 MHz, D_2O): δ 3.77-3.94 (m, 4H), 3.96-4.12 (m, 2H), 4.19-4.38 (m, 2H), 4.92 (bs, 1H), 5.08-5.23 (m, 2H, IdoA C-1), 5.40 (bs, 1H, GlcN C-1); substoichiometric: 1.19 (d, *J* = 6.2 Hz, 3H, CH_3-CL), 1.68-1.77 (m, 2H), 2.02 (bs, 3H, NHAc), 2.41 (t, *J* = 6.8 Hz, 2H), 2.66 (s, 2H), 2.79 (t, *J* = 6.8 Hz, 2H).

deO-HS-CL: ^{1}H NMR (600 MHz, D_2O): δ 3.26 (bs, 1H), 3.62-3.77 (m, 4H), 3.78-3.93 (m, 3H), 4.06 (bs, 1H), 4.12 (bs, 1H), 4.95 (bs, 1H, IdoA C-1), 5.38 (bs, 1H, GlcN C-1); substoichiometric: 1.19 (d, *J* = 6.3 Hz, 3H, CH_3-CL), 1.70-1.75 (m, 2H), 2.04 (bs, 3H, NHAc), 2.21-2.26 (m, 6H), 2.40-2.43 (m, 2H), 2.66 (s, 2H), 2.78 (t, *J* = 6.9 Hz, 2H).

6-deO-HS-CL: ^{1}H NMR (600 MHz, D_2O): δ 3.26 (bs, 1H), 3.64-3.95 (m, 5H), 4.05 (bs, 1H), 4.26 (bs, 1H), 4.36 (bs, 1H), 4.93 (bs, 1H), 5.28-5.41 (m, 2H, IdoA C-1, GlcN C-1); substoichiometric: 1.19 (dd, *J* = 6.3, 1.7 Hz, 3H, CH_3-CL), 1.69-1.76 (m, 2H), 2.06 (bs, 3H, NHAc), 2.18-2.30 (m, 6H), 2.41 (td, *J* = 6.9, 1.7 Hz, 3H), 2.66 (d, *J* = 1.7 Hz, 2H), 2.79 (td, *J* = 6.9, 1.5 Hz, 2H).

2-deO-HS-CL: ^{1}H NMR (600 MHz, D_2O): δ 3.26 (bs, 1H), 3.60-3.93 (m, 5H), 3.94-4.29 (m, 3H), 4.35 (bs, 1H), 5.30-5.45 (m, 2H, GlcN C-1, IdoA C-1); substoichiometric: 1.19 (d, J = 6.3 Hz, 3H, CH_3-CL), 1.69-1.77 (m, 2H), 2.05 (bs, 3H, NHAc), 2.21-2.34 (m, 6H), 2.44 (t, J = 6.8 Hz, 2H), 2.72-2.83 (m, 4H).

General biological procedures.

Transmembrane HaloTag protein DNA construct. The sequence encoding the HaloTag protein (HTP) was PCR amplified from the pFC14K HT7 CMV Flexi Vector (Promega) using the primers 5'-TT ATC CGC GGT GGA TCC GAA ATC GGT ACT GGC TTT-3' (upstream) and 5'-A CTA GTC GAC ACC GGA AAT CTC CAG AGT AGA CAG-3' (downstream) to introduce a 5' SacII restriction site and a 3' SalI restriction site. The PCR product was digested with SalI and SacII according to standard procedures and gel purified. The pDisplay vector (pD; Life Technologies) was linearized with SalI and SacII according to standard procedures and purified using a QIAquick PCR purification kit (Qiagen, Valencia, CA). The insert was ligated into the linearized vector using T4 DNA ligase. The HTP gene was inserted between the sequence encoding the N-terminal murine Ig κ-chain leader and the C-terminal PDGFR transmembrane domain to generate the final HTP construct. The plasmid was confirmed by Sanger sequencing (Laragen Inc.).

Cell culture. All cell cultures were maintained at 37 °C and 5% CO_2 unless otherwise indicated. CHO cells were cultured on tissue culture plates in F12 medium supplemented with 10% FBS and 1% P/S and were passaged after reaching ~80% confluency. mESCs were cultured strictly according to ATCC protocols to maintain a homogeneous population of pluripotent cells. Confluent MEFs were treated with mitomycin C (10 μg/mL in DMEM with 10 % FBS and 1% P/S) for 3 h at 37 °C to provide a feeder cell layer for mESCs. mESCs were cultured directly on the MEF feeder layer in DMEM (ATCC) with 10% non-heat-inactivated FBS and 0.1 mM β-mercaptoethanol (defined henceforth as complete DMEM). To passage or isolate mESCs for transfection, collagenase IV (10 μg/mL) in DPBS (with $CaCl_2$ and $MgCl_2$) was equilibrated to 37 °C and 5% CO_2 and added to mESCs (3 mL) and allowed to detach the cells for 3-5 min at 37 °C. The cells were gently agitated to remove the large colonies, leaving the majority of remaining MEFs attached to the plate. The released mESCs were then added to 6 mL of pre-warmed complete DMEM and centrifuged (1,200 rpm for 2.5 min). The collagenase IV-containing medium was aspirated, and the

cells were resuspended in 10 mL of fresh pre-warmed complete DMEM. At this time, the cells were either transferred to new tissue culture plates on top of a confluent layer of mitomycin C-treated MEFs for further culture or to plates pre-coated with 0.2% gelatin for transfection. mESCs were passaged every 3 to 5 days, depending on their colony sizes and proximity to one another, and the medium was replaced every 2 days.

HTP transfections. CHO cells were plated and transfected with the HTP plasmid using Lipofectamine LTX (Life Technologies) according to the manufacturer's protocol. For transfecting mESCs, the protocol for Xfect^{TM} mESC Transfection Reagent was followed. Briefly, mESCs were plated on 0.2% gelatin-coated 6-well plates at 1 x 10^6 cells/well in complete DMEM. After 5 h, the HTP plasmid-lipid transfection complexes were generated and added according to the manufacturer's instructions. The mESCs were then incubated for 3 h with the transfection complexes at 37 °C and 5% CO_2, and the medium was replaced with 2 mL of pre-warmed complete DMEM.

Generation of an HTP-expressing stable cell line. CHO cells lacking galactosyltransferase I (and therefore lacking glycosaminoglycans) that had been transfected with HTP were selected using complete F12 medium supplemented with 700 μL of G418 solution (0.8 mg/mL). The medium was replaced every 2 d for 3 weeks. After 3 weeks, the cells were incubated with fresh F12 medium containing 5 μM F-CL for 1 h (37 °C, 5% CO_2) and then sorted via fluorescence-activated cell-sorting (FACS) using a FACSAria Flow Cytometer Cell Sorter (BD Biosciences) with an excitation wavelength of 488 nm.

HTP labeling for fluorescent imaging analyses. CHO cells were plated and cultured on poly-ornithine-coated (10 μg/mL) glass coverslips in complete F12 medium (10% FBS, 1% P/S) at 37 °C and 5% CO_2. For labeling, the medium was replaced with fresh F12 medium that contained 5 μM F or F-CL for 1 h (37 °C, 5% CO_2). The cells were then washed with PBS (2 x 1 mL), fixed with 4% paraformaldehyde in PBS (15 min at RT), rinsed with PBS (2 x 1 mL), permeabilized with a solution containing 1% BSA and 0.1% Triton X-100 (20 min at RT), incubated with a monoclonal mouse anti-Myc antibody (1:1000; 12 h at 4 °C), washed with PBS (2 x 5 min), incubated with a goat anti-mouse AF568 secondary antibody (1:1000; 1 h at RT), and washed with PBS (2 x 5 min). The coverslips were mounted with VECTASHIELD mounting medium containing DAPI (Vector Laboratories Inc., Burlingame, CA) and imaged using a Zeiss LSM 700 inverted confocal microscope.

HTP labeling for Western blot analyses. CHO cells stably expressing HTP were cultured on 10-cm plates in complete F12 medium until approximately 85% confluent. The medium was then replaced with fresh F12 medium (4 mL) that contained 5 μg/mL B-HS, or B-HS-CL, and the cells were incubated for 6 h at 37 °C. After labeling, the medium was removed, and the cells were scraped and collected in cold PBS (1 mL), centrifuged (3,000 rpm for 2.5 min at 4 °C), and homogenized in 150 μL of lysis buffer (50 mM Tris/HCl pH 7.4, 250 mM mannitol, 50 mM NaF, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM Na_2VO_4, 1% Triton X-100, and 1x cOmplete protease inhibitor cocktail (Roche, Pleasanton, CA) via 10 passages through a 26g x 3/8" needle on ice. The lysates were then clarified by centrifugation (15,000 rpm for 15 min at 4 °C) and transferred to clean microcentrifuge tubes, after which the total protein concentration was determined for each sample using the Bradford assay (Bio-Rad Laboratories). Samples were boiled for 5 min with SDS-PAGE loading buffer and resolved by SDS-PAGE on 4-12% Bis-Tris gels (Life Technologies). Proteins were transferred to PVDF membranes and blocked with blocking buffer (5% milk in a solution of 50 mM Tris pH 7.6, 150 mM NaCl, and 0.5% Tween-20 (TBST)) for 1 h at RT. Blots were incubated with IRDye800 streptavidin (1:1000; LI-COR Biosciences, Lincoln, NE) and an anti-HA rabbit monoclonal antibody (1:1000) overnight at 4 °C in blocking buffer, rinsed twice with TBST, and incubated again with an AF680-conjugated goat anti-rabbit IgG antibody (1:10000) for 1 h at RT. Membranes washed three times with TBST for 5 min at RT and were then imaged and quantified using an Odyssey scanner (LI-COR Biosciences).

HTP-conjugate lifetime assay. CHO cells were plated onto 15-mm coverslips, cultured, and labeled with 5 μM F or F-CL in F12 medium for 1 h (37 °C, 5% CO_2). Cells were rinsed once with prewarmed PBS and subsequently cultured with fresh F12 medium (37 °C, 5% CO_2). At each time point (between 0.5 and 8 d), the coverslips were removed from culture, submerged in PBS for 2 min, fixed with 4% paraformaldehyde in PBS for 15 min, and then washed in PBS for 5 min. The coverslips were blotted dry, mounted onto glass microscope slides using VECTASHIELD mounting medium containing DAPI, and imaged using a Zeiss LSM 700 inverted confocal microscope.

Microarray analyses. Glycosaminoglycan microarrays were generated as previously described.[4] Slides were blocked with 10% FBS in PBS for 1 h at RT. Stock solutions of FGF2, FGF8b, FGFR1-Fc (2 μM), and FGFR3c-Fc were prepared in sterile 1x PBS containing 1% BSA. Blocked slides were washed once with PBS and slowly rocked with

150 μL of 1% BSA/PBS containing 1 μM receptor with or without 1 μM ligand (*i.e.*, 1 μM FGFR1-Fc ± 1 μM FGF2; 1 μM FGFR3-Fc ± 1 μM FGF8b) for 2 h at RT. Slides were washed three times with PBS and incubated with an AF647-conjugated goat anti-human Fc antibody (1:5000) in 1% BSA/PBS for 1 h at RT in the dark with gentle rocking. Slides were then washed three times with PBS and twice with ddH_2O and blown dry under a stream of filtered air. Arrays were scanned using a G2565BA DNA Microarray Scanner (Agilent), and fluorescence was quantified using GenePix 5.0 software (Molecular Devices) with normalization against local background. The data represent the average of 10 spots per concentration of polysaccharide.

ERK activation assays. Pluripotent mESCs were transfected with the HTP plasmid as described above. After 48 h, the cells were detached with collagenase IV (10 μg/mL, 3 mL) in DPBS, pelleted, and resuspended in 2 mL of complete DMEM containing 1 U/mL heparinase II (HepII, Sigma-Aldrich) for 2 h at 37 °C with gentle mixing every 15 min. HepII was quenched with 6 mL of complete DMEM, and the cells were pelleted and resuspended in complete DMEM. mESCs were then plated on six-well plates coated with 0.2% gelatin and incubated with HS-CL, de-HS-CL, deO-HS-CL, 6-deO-HS-CL, or 2-deO-HS-CL (5 μg/mL) overnight. Cells were serum-starved the following day with DMEM containing 0.5% FBS for 8 h and then stimulated for 15 m with DMEM containing 0.5% FBS and 10 ng/mL of FGF2. Cells were then quickly washed with 1 mL of ice-cold PBS, scraped, and pelleted. The pellet was lysed and homogenized as described above. Samples were boiled for 5 min with SDS-PAGE loading buffer, resolved by SDS-PAGE on 4-12% Bis-Tris gels, transferred to PVDF membrane, and blocked for 1 h at RT with Odyssey Blocking Buffer (LI-COR Biosciences). Membranes were immunoblotted with a rabbit anti-phospho-ERK monoclonal antibody (1:2000) overnight at 4 °C and then a mouse anti-ERK monoclonal antibody (1:2000) for 1 h at RT. Blots were then incubated with an AF680-conjugated goat anti-mouse IgG antibody (1:10000) and IRDye800-conjugated goat anti-rabbit IgG antibody (1:10000) for 1 h at RT. Membranes were washed three times with TBST for 5 min and then imaged and quantified using an Odyssey scanner (LI-COR Biosciences). Ratios of phospho-ERK to total ERK were calculated for each condition and normalized to an untreated control. Statistical analysis was performed using a one-way ANOVA with post-hoc analysis by Dunnett's test against the untreated control. Data represent the mean ± S.E.M. for three independent experiments ($n = 3$).

Neuronal differentiation assays. Pluripotent mESCs were transfected with the HTP plasmid and HepII treated as described above. The reaction was quenched with 6 mL of complete DMEM, pelleted, and resuspended in 1 mL of neural induction medium (1 part DMEM:F12 (1:1) 1% N-2 supplement, 1% P/S and 1 part Neurobasal, 1% GlutaMAX, 1% B-27, 1% P/S). HepII-treated cells were then added to 0.2% gelatin-coated plates in 2 mL of neural induction medium containing FGF2 (10 ng/mL) and HS-CL derivatives (untreated, HS-CL, or de-HS-CL; 5 μg/mL). The medium was replaced after 48 h with fresh neural induction medium containing FGF8b (10 ng/mL). At 3, 6, and 9 days after HepII treatment, the cells were subjected to RNA isolation for qRT-PCR and immunocytochemistry analyses.

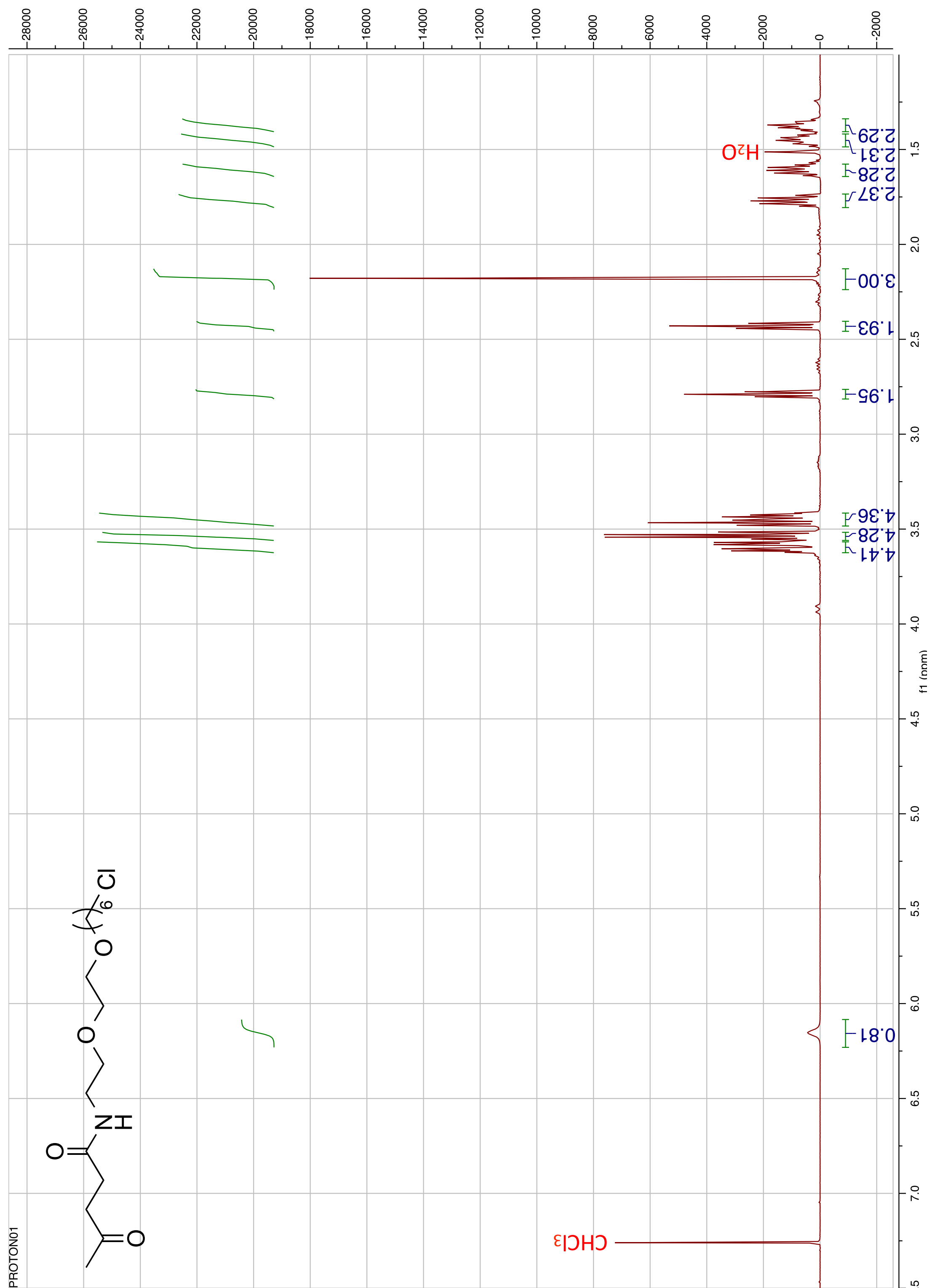
Real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR). mESCs were cultured and subjected to neuronal differentiation as described above. At different time points (Day 3, 6, and 9), RNA was extracted from untreated cells and cells treated with HS-CL or de-HS-CL using the RNeasy Mini Kit (Qiagen) per the manufacturer's instructions. RNA concentrations were obtained with a NanoDrop 2000 UV-Vis spectrophotometer (Thermo Scientific), and then the RNA samples were converted to cDNA using the iScript Advanced cDNA Synthesis Kit (Bio-Rad). qRT-PCR was performed using an Applied Biosciences 7300 Real-Time PCR System with primers for GAPDH, SDHA, NANOG, SOX1, and TUJ1 (Table S1). The following conditions were used for each 20-μL qRT-PCR reaction: 5 μL of cDNA (10 ng/μL), 1 μL of primer mixture (10 μM forward and 10 μM reverse), 10 μL of 2x SYBR master mix, and 4 μL of ddH_2O. The cycle threshold (Ct) values for NANOG, SOX1, and TUJ1 were first normalized against the geometric mean of GAPDH and SDHA at each condition and time point. Each data point was then normalized to the untreated value at day 3 to report relative changes. Statistical analysis was performed for each gene at each time point using a one-way ANOVA with post-hoc analysis by Dunnett's test against the untreated control. Data represent the mean ± S.E.M. from two samples run in triplicate (n = 2).

Immunocytochemistry. Untreated mESCs and mESCs treated with HS-CL or de-HS-CL were subjected to neuronal differentiation as described above. At 3, 6, and 9 days after HepII treatment, mESCs were fixed with 4% paraformaldehyde in PBS (15 min), rinsed with PBS (2 x 1 mL), permeabilized with a solution containing 1% BSA and 0.1% Triton X-100 (20 min), and incubated with a rabbit anti-NANOG monoclonal antibody (1:1000), a goat

anti-SOX1 polyclonal antibody (1:250), or a rabbit anti-TUJ1 monoclonal antibody (1:1000) for 3 h at RT. Cells were washed with PBS (2 x 1 mL) and then incubated with an AF488-conjugated goat anti-rabbit antibody (1:5000), AF568-conjugated donkey anti-goat antibody (1:5000), or AF568-conjugated goat anti-mouse antibody (1:5000), respectively, for 1 h at RT. Cells were imaged using a Zeiss LSM 700 inverted confocal microscope.

^{1}H NMR spectra of representative compounds.

N-(2-(2-(6-Chlorohexyloxy)ethoxy)ethyl)-4-oxopentanamide (**1**)



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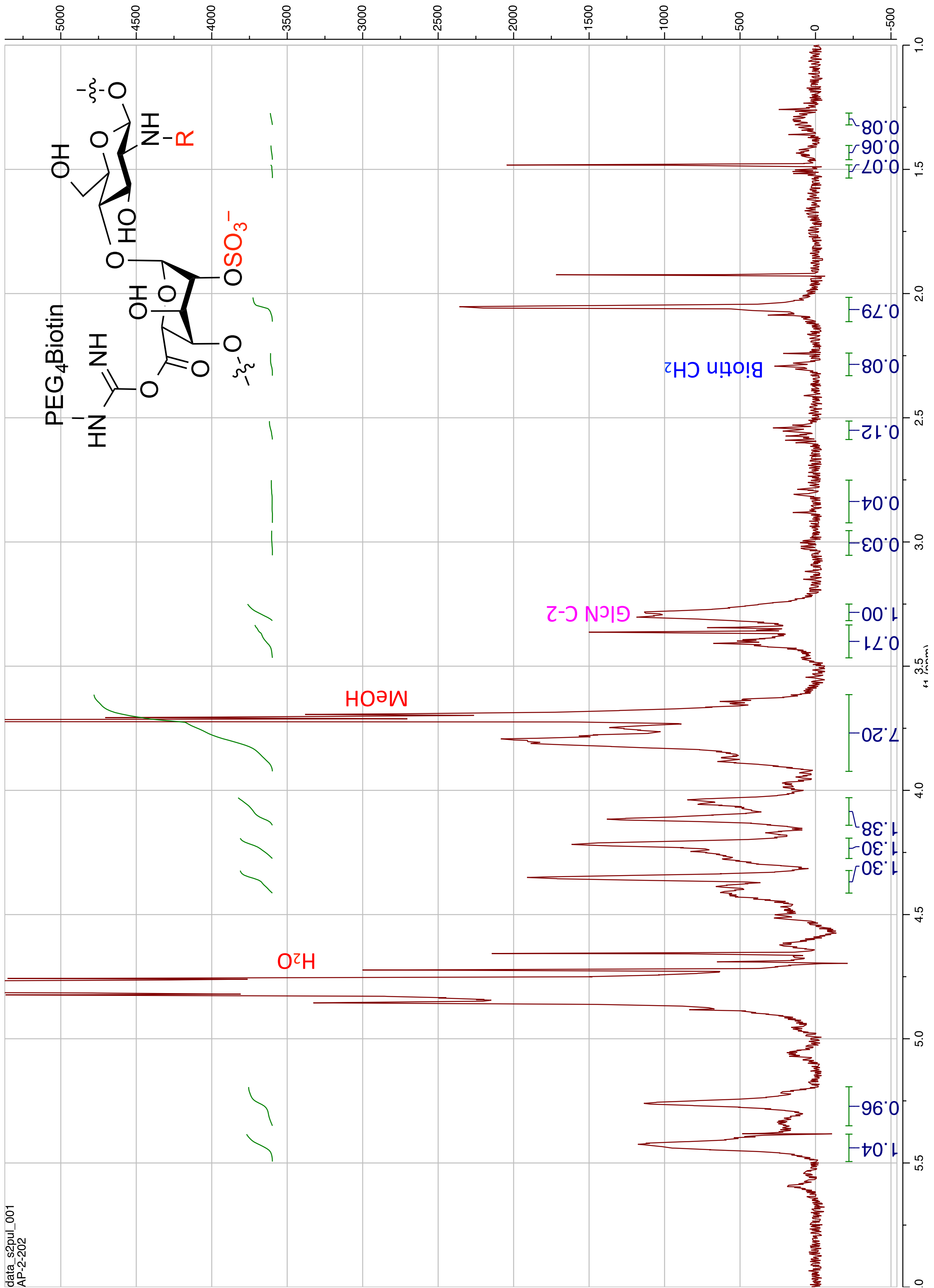

6-deO-HS-CL

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