Chapter 2

Methods for Short- and Long-Term Glycan Engineering at the Cell Surface to Control Biological Outcomes

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2.1 Abstract

Cell-surface GAGs are mediators of a variety of critical signaling events and can dictate the biological activity of cell populations through their interactions with cell-surface receptors and soluble ligands. Many approaches to connect structural determinants of the cellular glycocalyx with biological function employ reductive approaches by reducing or eliminating glycan structures from the extracellular matrix. However, a forward approach in which specific glycans are anchored onto plasma membranes would allow more control over the exact carbohydrate structures being displayed at the cell surface. Moreover, this tactic would provide direct evidence for biological activity as a result of a carbohydrate structure. Here, two methods are presented to modify cell surfaces with defined carbohydrate structures. The first method employs the functionalization of CS polysaccharides with an aminooxy group and conjugation to ketone-displaying liposomes to directly fuse with the cell membrane. The second approach describes the transgenic expression of a transmembrane HaloTag construct, which can covalently bond with chlorohexyl-functionalized HS polysaccharides. These two complementary methods provide both short- and long-term display of known carbohydrates, which allow for the direct control of cellular fate in both neuronal growth and stem cell differentiation.

2.2 Liposomal Delivery of CS GAGs to Control Neuronal Growth

2.2.1 Approach and Synthesis

Although some strategies for engineering cell-surface glycans have been reported,[1-8] the remodeling of cell membranes with complex polysaccharides such as GAGs has not been demonstrated. Moreover, the application of these methodologies prior to our work has been largely limited to imaging[2-5] or studying cell-surface phenomena, such as receptor clustering.[6] Here we developed a method to display specific sulfated GAG structures on cell surfaces using a liposomal fusion strategy. Tailoring membranes with the CS-E sulfation epitope activated growth factor-mediated signaling pathways and enabled the fine-tuned modulation of neuronal growth. These findings demonstrate that chemically controlling the presentation of exogenous glycans on cell surfaces can induce sustained effects on cellular signaling and function. Our studies also highlight the potential for glycan engineering to modulate complex cellular events, and they provide a powerful, new tool for remodeling cell membranes with a wide variety of important biomolecules.

We chose to utilize liposomes as glycan carriers due to their biocompatibility, ease of preparation, low cytotoxicity, and tunable biophysical properties.[9] Elegant studies have used glycan-presenting vesicles for intracellular antigen delivery[10, 11] or sugar-encapsulated vesicles with folate receptors for cell-specific metabolic labeling.[4] However, only two liposomal methods have been developed to our

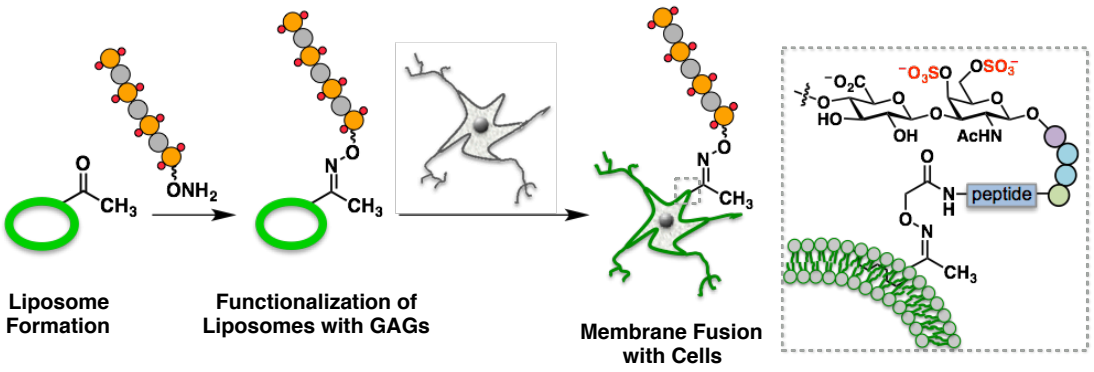


Figure 2-1. Schematic of liposomal delivery method for glycan engineering. Liposomes containing a ketone functional handle are produced and then functionalized with GAGs. Liposomes are then added to cells, where they fuse with the cell membrane to display carbohydrates on the cell surface.

knowledge for the cell-surface display of exogenous molecules, namely fluorophores.[12, 13] We expanded on these methods in an effort to incorporate large, sulfated GAGs into cell membranes (Figure 2-1). To promote membrane fusion and surface presentation of the glycans rather than intracellular uptake, we used cationic 1,2-dioleoyl-3-trimethylammoniumpropane chloride (DOTAP) and neutral 1,2-dioleoyl-*sn*-glycero-3-phosphatidylethanolamine (DOPE) as our primary lipids. Phosphatidylethanolamine lipids are an abundant, natural component of the neuronal cell membrane, and DOPE-based liposomes containing a lipid-functionalized fluorophore have been used to label a variety of cell types, including primary neurons.[12] We also incorporated 2-dodecanone into the liposomes to add a ketone handle for appending the glycans via oxime chemistry. CS polysaccharides containing a peptide fragment with an *N*-terminal amine were readily derivatized with an aminooxy group by coupling them to

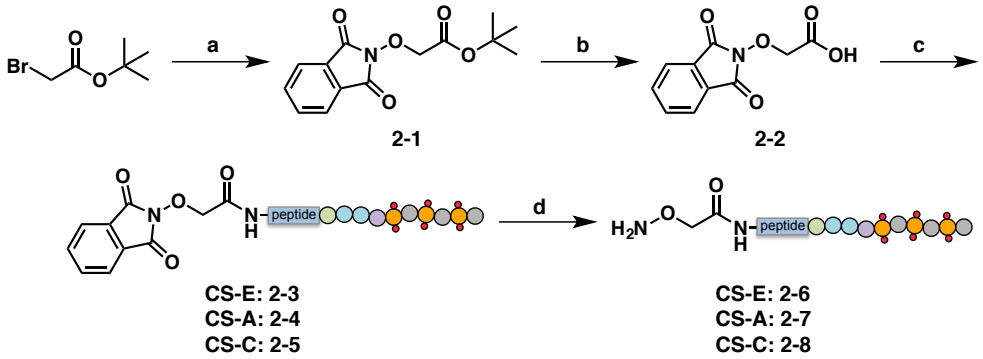


Figure 2-2. Synthesis of aminooxy-functionalized CS GAGs (2-6, 2-7, and 2-8).(a) *N*-hydroxyphthalimide (1.2 eq), $NaHCO_3$ (1.2 eq), DMF, 60 °C, 2 h, 88%; (b) TFA (3 eq), DCM, 1 h, 99%; (c) EDC (1.1 eq), sulfo-NHS (1.1 eq), 100 mM MES pH 6.0, 300 mM NaCl, RT, 30 min, then polysaccharide-NH_2, 50 mM $NaHCO_3$, RT, 12, 60%. (d) hydrazine monohydrate (3 eq), ddH_2O, RT, 3 h, 92%.

phthaloyl-protected aminooxyacetic acid followed by cleavage of the phthaloyl group with hydrazine monohydrate (Figure 2-2). The polysaccharides were then incubated with dodecanone-containing liposomes at 25 °C for 3 h to produce GAG-displaying vesicles. The synthetic ease and versatility of this approach represent advantages compared to existing methods, which require the radical-mediated synthesis of polymers end-functionalized with lipids.[5-8] With our approach, the lipid reagents are commercially available, and many biomolecules can be derivatized with aminooxy groups, including various glycans, peptides, lipids, nucleic acids, and proteins,[14-16] thus providing a general strategy for displaying a diverse range of bioactive molecules.

2.2.2 Validation

Preliminary optimization of liposomal membrane fusion was performed on rat pheochromocytoma (PC12) cells using liposomes functionalized with a

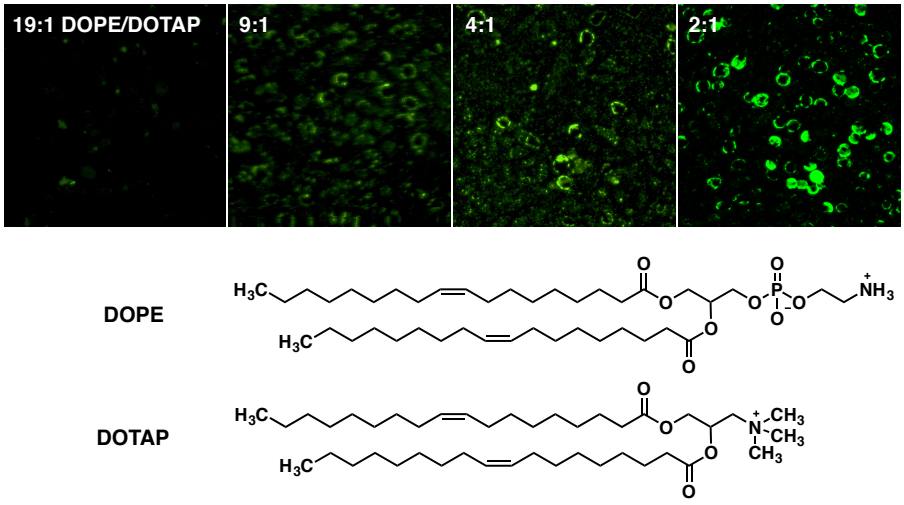


Figure 2-3. Optimization of lipid composition of liposomes for membrane fusion. Liposomes were produced with different w/w ratios of DOPE and DOTAP and 10% w/w dodecanone. AF488-hyd was incubated with the liposomes (ddH_2O, RT, 3h) to spontaneously react with the presented ketone moiety. PC12 cells were then incubated with the fluorescent liposomes (37 °C, 30 min) and imaged to visualize fusion efficiency.

hydrazide-conjugated fluorophore (AF488-hyd). We found that a 2:1 w/w ratio of DOPE:DOTAP was optimal for membrane fusion, as visualized by fluorescence microscopy (Figure 2-3). To approximate the relative levels of fluorophore incorporation at the cell surface, we incubated liposomes containing varying concentrations of AF488-hyd with PC12 cells on ice for 30 min. Cells labeled with liposomes containing 10 mol % AF488-hyd displayed similar fluorescence signal profiles by fluorescence-assisted cell sorting (FACS) analysis as cells labeled with an anti-CS-E monoclonal antibody[17] that detected endogenous CS-E levels (Figure 2-4). These results suggest that this liposomal strategy can incorporate exogenous molecules into cell membranes at levels roughly similar to those of endogenous CS polysaccharides. We next examined whether this approach could be used to

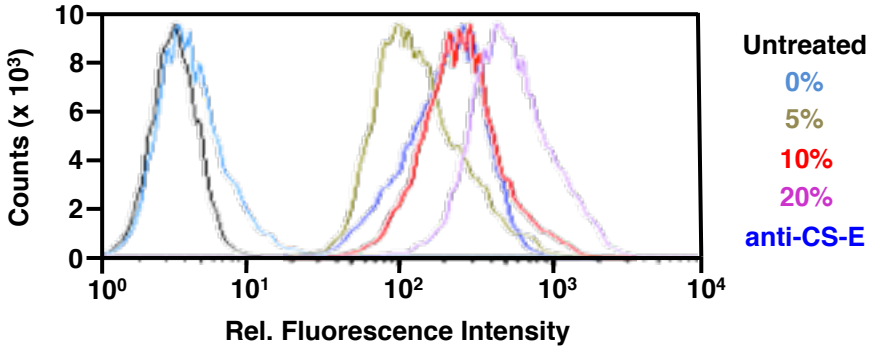
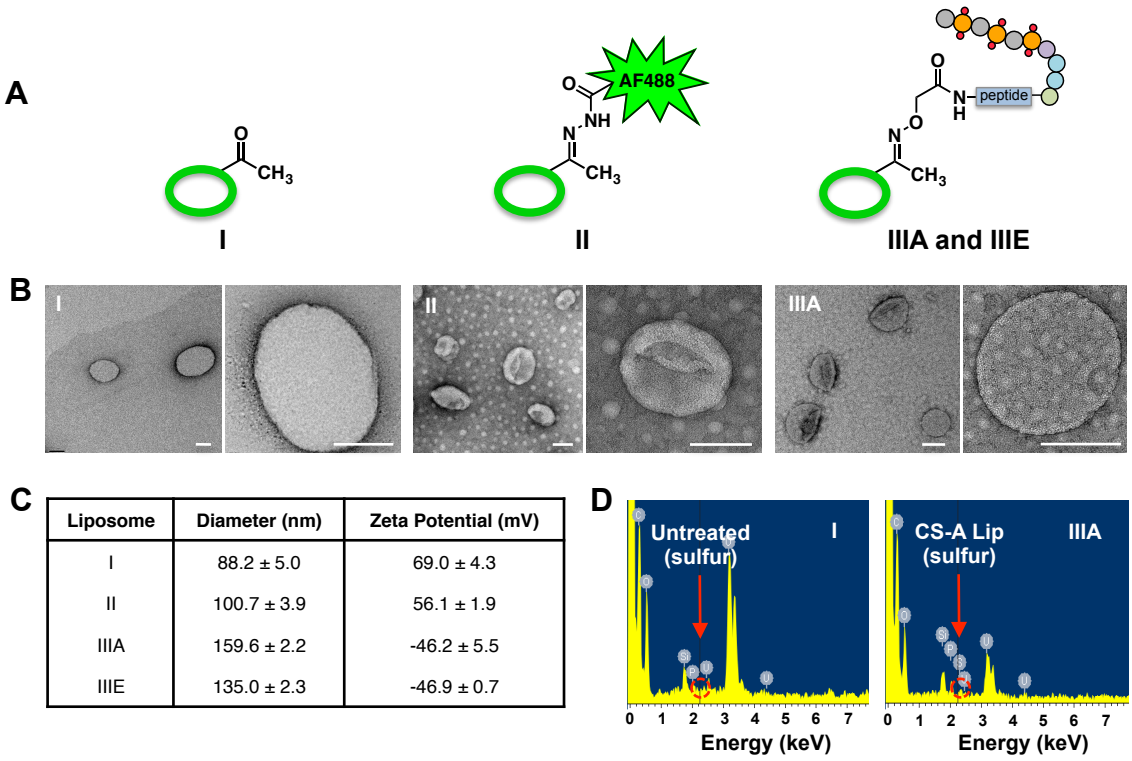


Figure 2-4. Quantifying cell surface labeling by liposomal delivery. PC12 cells were functionalized with liposomes containing different amounts of 2-dodecanone (w/w) that had been reacted with AF-488 and analyzed by flow cytometry. As an approximation, these data were compared to PC12 cells labeled with a CS-E monoclonal antibody.



Liposome	Diameter (nm)	Zeta Potential (mV)
I	88.2 ± 5.0	69.0 ± 4.3
II	100.7 ± 3.9	56.1 ± 1.9
IIIA	159.6 ± 2.2	-46.2 ± 5.5
IIIE	135.0 ± 2.3	-46.9 ± 0.7

Figure 2-5. Biophysical characterization of conjugated liposomes. (A) Schematic of different liposomes used for characterization. (B) Liposomes were negatively stained with uranyl acetate and imaged using TEM (scale bar = 50 nm). (c) DLS was used to measure average liposome hydrodynamic diameters, which showed increases after functionalization. Zeta potential analysis showed a negative shift in potential after functionalization with sulfated GAGs. (d) Energy dispersion spectroscopy showed the incorporation of sulfur after functionalization with sulfated GAGs.

display large GAG polysaccharides on cell surfaces. Liposomes containing 2:1 w/w DOPE:DOTAP and 10% w/w dodecanone were functionalized with CS-E-enriched polysaccharides (~70 kDa). To characterize their biophysical properties, we used transmission electron microscopy (TEM), dynamic light scattering (DLS), and zeta potential measurements. The liposomes exhibited parameters predicted to favor membrane fusion, including spherical morphologies, average diameters ranging between 132.6 and 159.6 nm, and good stabilities (zeta potentials of ±46–69 mV; Figure 2-5b). The change from positive to negative electrokinetic potential (69 to −46 mV; Figure 2-5c) confirmed successful conjugation of the sulfated polysaccharides. Furthermore, energy dispersive spectroscopy (EDS) verified the presence of sulfur on CS-functionalized liposomes after CS conjugation (Figure 2-5d). To test for membrane fusion, PC12 cells were treated with chondroitinase to remove endogenous CS, incubated with CS-E-modified liposomes, and immunostained with an anti-CS-E antibody. Importantly, strong immunostaining for CS-E was observed on the surfaces of cells treated with CS-E-functionalized liposomes compared to chondroitinase-treated cells without liposome addition, indicating efficient incorporation of the polysaccharides (Figure 2-6).

2.2.3 Controlling Intracellular Signaling

Having validated the method, we investigated whether the approach could be used to control cellular signaling pathways. Previous studies from our laboratory

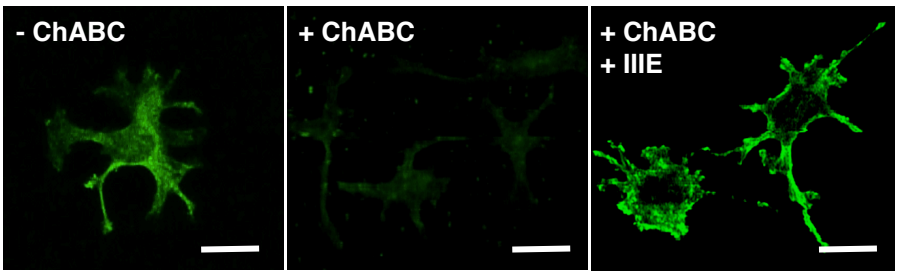


Figure 2-6. Validation of cell functionalization with CS-E via liposomal delivery. PC12 cells were left untreated, treated with chondroitinase ABC (ChABC), or treated with ChABC and then CS-E displaying liposomes (**IIIE**) and then immunostained with a CS-E monoclonal antibody.

have demonstrated that CS-E polysaccharides can recruit nerve growth factor (NGF) to the cell surface and promote the assembly of NGF-tropomyosin receptor kinase (Trk) complexes.[18] Complex formation, in turn, activates protein kinase B (Akt) and phosphatidylinositol 3-kinase (PI3K) signaling pathways and thereby enhances neurite outgrowth.[19] Thus, we reasoned that cell-surface presentation of exogenous CS-E polysaccharides might recruit NGF to the membrane and assist in the formation of activated signaling complexes to induce these biological responses. Embryonic day 18 (E18) rat cortical neurons were cultured *in vitro* for 7 days and then treated with liposomes displaying CS-E- or CS-C-enriched polysaccharides (**IIIE** or **IIIC**, 30 min, 37 °C). Neurons were stimulated with the neurotrophin NGF for 0, 10, 30, or 60 min, and Akt activation was monitored using a phospho-Ser473 Akt antibody. Remarkably, neurons remodeled with CS-E polysaccharides showed approximately a 3-fold increase in Akt activation relative to untreated neurons at each time point (Figure 2-7), consistent with increased recruitment of NGF to the cell surface and robust

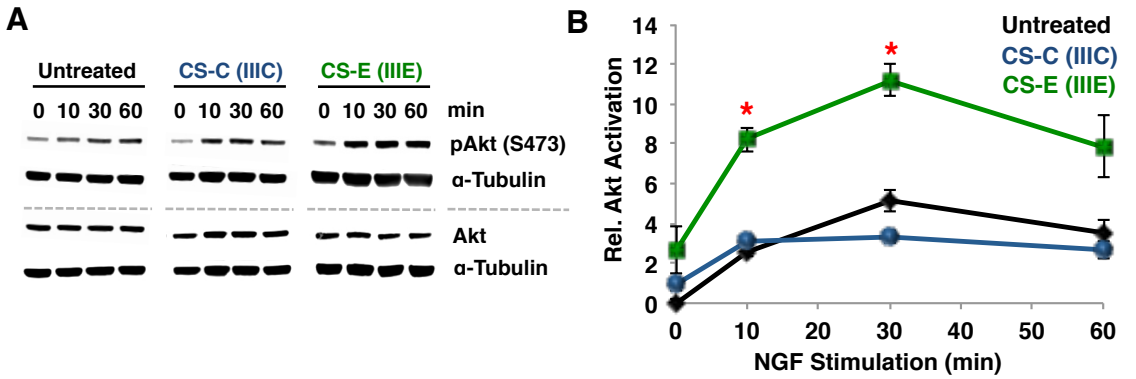


Figure 2-7. Potentiation of Akt signaling via cell surface glycan engineering. Embryonic cortical neurons were left untreated or functionalized with CS-C- (**IIIC**) or CS-E- (**IIIE**) displaying liposomes. Cells were then stimulated with NGF, and Akt activation was monitored by (A) Western blotting with (B) quantification. * $P < 0.05$.

activation of Trk receptors. In contrast, neurons remodeled with CS-C polysaccharides showed phospho-Akt levels similar to those of untreated neurons. These results support the importance of the CS-E motif in NGF-stimulated Trk activation.[18] Moreover, they show that liposomal-mediated presentation of specific, sulfated CS polysaccharides on cell surfaces can activate important neuronal signaling pathways.

2.2.4 Controlling Neuronal Growth

We next examined whether the exogenous CS-E-mediated activation of Trk pathways could direct neuronal growth. Rat E18 hippocampal neurons were cultured *in vitro* for 2 days and subsequently treated for 30 min with liposomes prefunctionalized with CS-A-, CS-C-, or CS-E-enriched polysaccharides. Neurons were then incubated in media lacking liposomes for an additional 24 h and immunostained with an anti-α-tubulin antibody to image the processes using

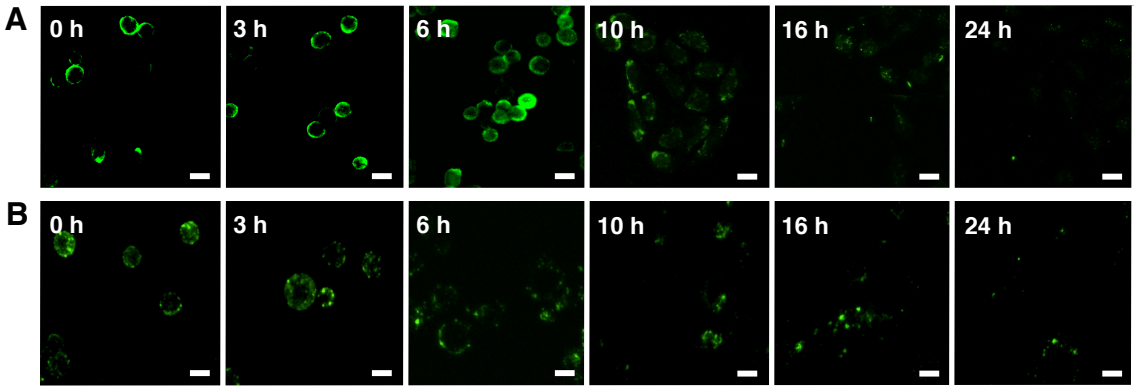


Figure 2-8. Surface lifetime of lipid-anchored molecules. PC12 cells were functionalized with (A) AF488- (**II**) or (B) fluorescent CS-E- (**IIIE**) functionalized liposomes and monitored by microscopy. The membrane lifetime of both molecules lasts only a few hours, with the signal from the small molecule dye being slightly longer lived. Scale bar = 50 μm for (A) and 30 μm for (B).

confocal fluorescence microscopy. Initial studies revealed no appreciable difference in neurite outgrowth between neurons displaying the different sulfation motifs. We postulated that the membrane lifetime of the exogenous CS GAGs might be too short to elicit functional responses such as neurite outgrowth, which requires *de novo* protein and lipid biosynthesis. Therefore, we assayed the membrane lifetime of the exogenous lipids by treating PC12 cells with liposomes bearing fluorophore-conjugated CS-E polysaccharides and monitoring the fluorescence signal over 24 h (Figure 2-8). A decrease in signal was observed within 6 h, and loss of the signal progressed over the course of 10 h. After 16 h, weak fluorescence was detected around the cell periphery, suggesting that the CS-conjugated lipids had been internalized or had diffused into the medium. To circumvent this problem, we repeated the outgrowth assays with multiple additions of liposomes every 8 h over a 24-h period. Under these conditions, we

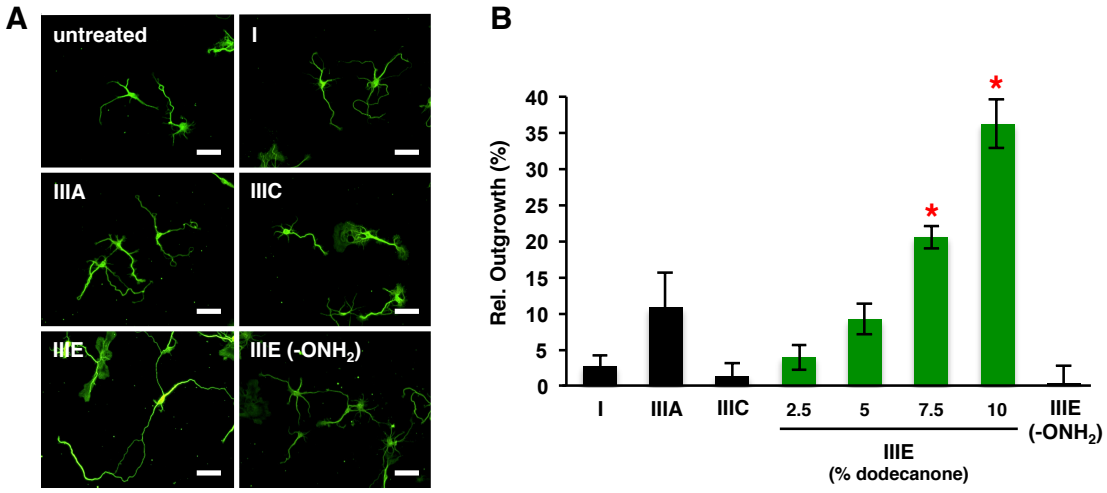


Figure 2-9. Controlling neurite outgrowth via cell surface glycan engineering. Embryonic hippocampal neurons were left untreated or treated with CS-A- (**IIIA**), CS-C- (**IIIC**), or CS-E- (**IIIE**) functionalized liposomes once every 8 hours for 24 h. Samples were (A) visualized by immunofluorescence and (B) quantified. For all samples, liposomes were produced with 10% w/w 2-dodecanone except for the panel of **IIIE** liposomes. Neurite outgrowth could be finely tuned by the amount of CS-E added onto the surface. Treatment with liposomes that had been incubated with CS-E without the aminooxy group showed no difference in outgrowth compared to control samples. Scale bar = 50 μm. * $P < 0.05$.

found that cell-surface presentation of CS-E polysaccharides significantly enhanced neurite outgrowth by 36.3 ± 3.3% relative to untreated neurons (Figure 2-9). As expected, neurons displaying CS-A or CS-C polysaccharides showed minimal neurite outgrowth when compared to untreated neurons (10.8 ± 4.8% and 1.3 ± 1.8%, respectively). As a further control, dodecanone-containing liposomes were reacted with CS-E polysaccharides lacking the aminooxy functionality and then incubated with the cells. No difference in neurite outgrowth was observed relative to untreated neurons. Remarkably, the extent of neurite outgrowth could be finely tuned by controlling the concentration of CS-E

polysaccharides at the cell surface. Liposomes containing 0% to 10% dodecanone were conjugated with aminooxy-functionalized CS-E and then incubated with E18 rat hippocampal neurons as above. Notably, we observed a dose-dependent increase in neurite outgrowth from 4.0% to 36.3% as the dodecanone concentration was increased from 2.5% to 10% (Figure 2-9). Together, these studies demonstrate that this approach for engineering glycans on cell surfaces can be used to finely modulate both the signaling and functional responses of neurons.

2.3 HaloTag Anchoring of HS GAGs to Control Stem Cell Differentiation

2.3.1 Approach and Synthesis

Heparan sulfate (HS) glycosaminoglycans (GAGs) have recently been identified as important regulators of stem cell differentiation.[20-24] HS GAGs exert this control by selectively interacting with proteins involved in the differentiation process, such as fibroblast growth factors (FGFs), bone morphogenic proteins (BMPs), and wingless-type MMTV integration site family members (Wnts).[22, 25] Furthermore, specific sulfation patterns of HS have been implicated in the progression of ESCs from self-renewal to a differentiated state. For example, undersulfated HS is found on pluripotent cells,[23] whereas highly sulfated HS is associated with differentiated cells and has been proposed to promote interactions between soluble FGF and BMP factors and their receptors.[22] However, the precise sulfated epitopes and mechanisms involved in the generation of specific cell lineages remain unclear. We postulated that the presentation of particular HS GAG structures on ESC surfaces might enable the selective activation of signaling pathways and thereby induce desirable cell fates. Such an approach would also provide novel insights into the structure-function relationships of HS GAGs and their roles in stem cell biology.

Elegant studies have recently shown that the short-term display of synthetic HS glycopolymers can promote stem cell specification to form intermediate neural rosettes.[26] However, directing the generation of fully differentiated,

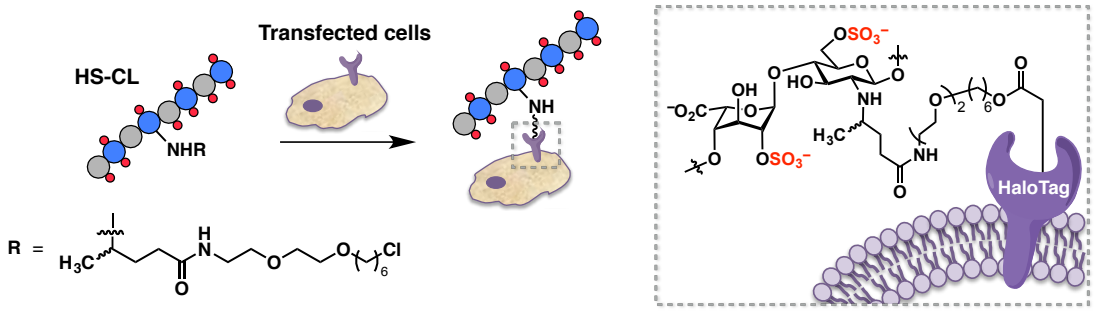


Figure 2-10. Schematic of HTP glycan engineering approach. HS GAGs modified with the CL linker are added directly to cells expressing the transmembrane HTP construct, which covalently bonds to the CL linker.

mature cell types will likely require the development of new methods to enable the long-term, stable presentation of defined HS GAGs. Although our liposomal method as well as the passive lipid insertion technique can elicit short-term cellular responses,[8, 27] the lipid tail anchor limits the membrane lifetime of the exogenous glycans to several hours. To address these shortcomings, we developed a method to tailor cell surfaces with specific HS derivatives using membrane-bound HaloTag proteins (HTPs) as anchors (Figure 2-10). Molecules covalently attached to HTPs displayed prolonged cell-surface lifetimes of more than one week, circumventing the temporal limitation of lipid anchors. Moreover, mouse ESCs remodeled with heparin/trisulfated HS underwent accelerated exit from selfrenewal and commitment to a neural lineage through early activation of extracellular signal-regulated kinase (ERK)/mitogen-activated protein kinase (MAPK) signaling pathways. These results highlight the potential to elucidate the functional roles of HS GAGs and direct cell differentiation by remodeling the glycocalyx of stem cells.

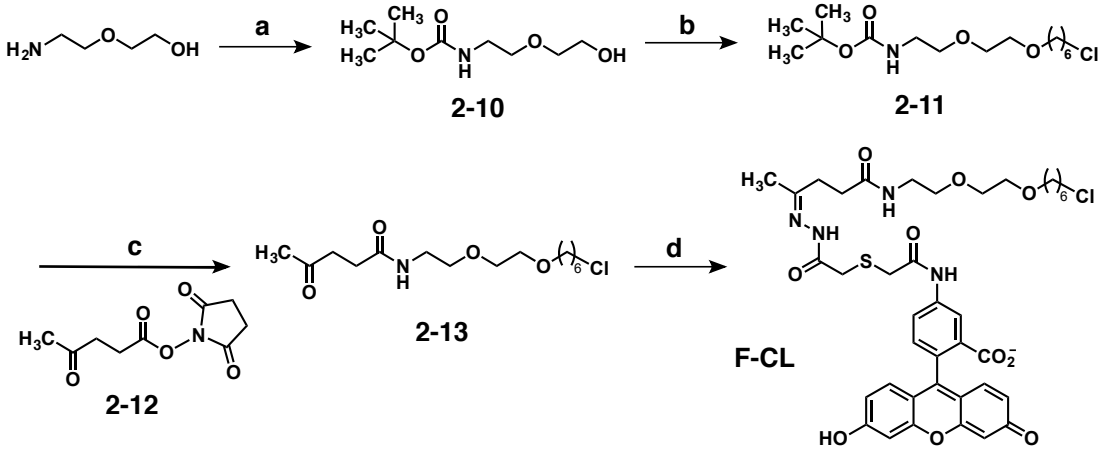


Figure 2-11. Synthesis of 2-13 and F-CL. (a) Boc_2O (2 eq), MeOH, 0 °C, 2 h, 91%; b) 60% NaH (1.5 eq), DMF, 0 °C, 1 h, then 1-chloro-6-iodohexane (1.2 eq), 24 h, 37%; c) TFA (6 eq), DCM, 4 h, then **2-12** (1 eq), DCM, 24 h, 67%; d) 5-(((2-(carbohydrazino)methyl) thio)acetyl)-aminofluorescein (**F**) (0.5 eq), MeOH, 16 h, 75%.

HTP is a modified alkane dehalogenase that forms a covalent adduct with chloroalkane substrates.[28] Strategies based on HTP have been adapted for diverse applications, ranging from cancer diagnostics to chemical proteomics.[29-31] However, most reported applications have used HTP methods to append molecules that serve as detection or capture agents. We chose to exploit the HTP platform to modulate biological processes in living cells. We first investigated the membrane lifetime of molecules conjugated to HTPs. *N*-*tert*-Butyloxycarbonyl (Boc)-protected 1-(2-(2-amino-ethoxy)ethoxy)-6-chlorohexane **2-11**[31] was deprotected with trifluoroacetic acid and reacted with *N*-hydroxysuccinimidyl levulinate **2-12** to obtain chloroalkane linker (CL) **2-13** (Figure 2-11). Condensation of **2-13** with a fluorescein-hydrazide derivative gave CL-conjugated fluorescein (**F-CL**).

2.3.2 Validation

To test the approach, hemagglutinin (HA)-tagged HTP was stably expressed in Chinese hamster ovary (CHO) cell membranes by fusing it to the platelet-derived growth factor receptor (PDGFR) transmembrane domain. Cells were then incubated with F-CL for one hour at 37 °C, and individual wells were fixed and imaged every twelve hours for eight days. Remarkably, we observed a strong fluorescence signal that persisted for at least eight days after only a single F-CL treatment (Figure 2-12). In contrast, no fluorescence signal was observed when cells were treated with the fluorescein-hydrazide derivative alone or with cells lacking HTP. These results indicate that the display of HTP conjugates is specific and long-lived despite membrane turnover, highlighting the potential of this approach to exert long-lasting effects on cellular function. We next examined whether this method could be used to present HS GAGs on cell surfaces. Although the glucosamine sugars in heparin/HS are mostly *N*-acetylated or *N*-sulfated, the free amine is also present in low abundance (1–3%), which provides a convenient functional handle for attaching the chloroalkane linker in a single step. We biotinylated HS (B-HS) and conjugated it to **1** by reductive amination chemistry (**B-HS-CL**; Figure 2-13). CHO cells stably expressing HTP were incubated with **B-HS** or **B-HS-CL** at 37 °C for six hours. Cells were lysed and subjected to blotting analysis using an anti-HA antibody and streptavidin IRDye800. Importantly, biotinylated HS was detected only from HTP-expressing

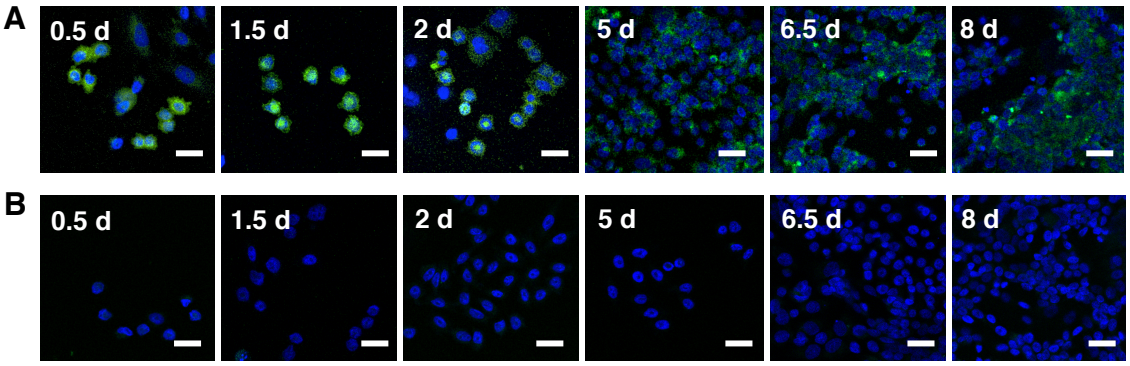


Figure 2-12. Surface lifetime of HTP-anchored molecules. CHO cells expressing HTP were treated with (A) **F-CL** or (B) **F** and monitored by microscopy for over one week. Surface labeling was observed at 8 days with no nonspecific labeling from unlabeled fluorescein (**F**). Scale bar = 50 μm.

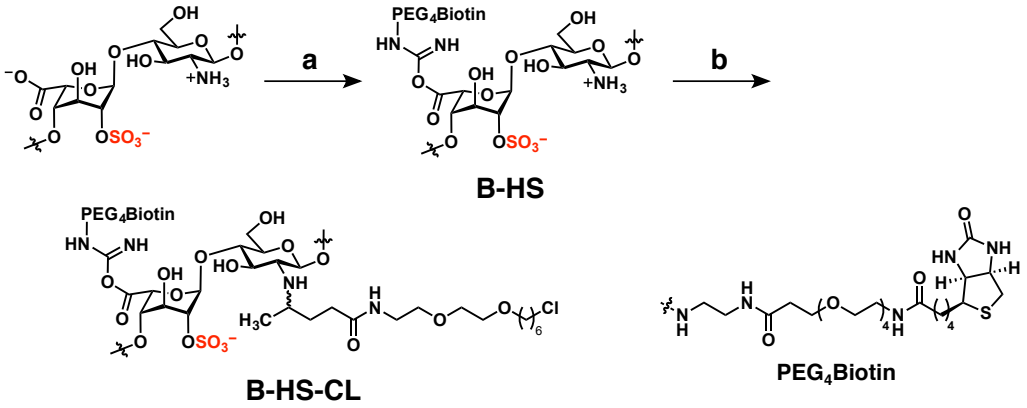


Figure 2-13. Synthesis of B-HS and B-HS-CL. (a) BrCN (excess), 0.2 M NaOH pH 11, RT, 10 min, then BiotinPEG_4NH_4 (excess), 0.2 M sodium borate pH 8, RT, 12 h; (b) **2-13** (excess), $NaBH_3CN$ (excess), MeOH, ddH_2O, RT, 16 h.

cells treated with **B-HS-CL**, but not **B-HS**, which lacks the chloroalkane moiety (Figure 2-14). Immunocytochemical analysis of cells further confirmed the presence of biotinylated HS on HTP-expressing cells incubated with **B-HS-CL**, but not **B-HS**. Together, these studies demonstrate the selective display of HS GAGs on cell surfaces using HTP anchors.

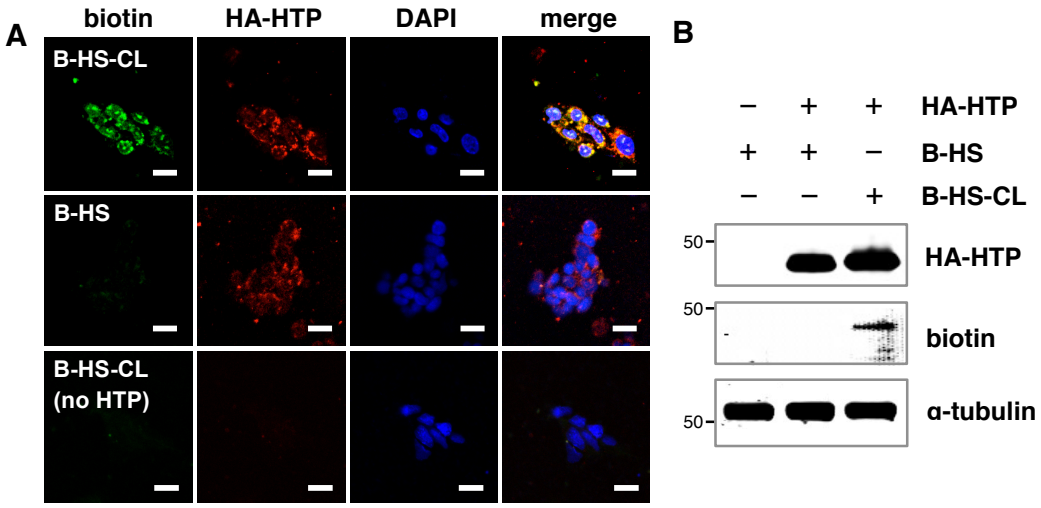
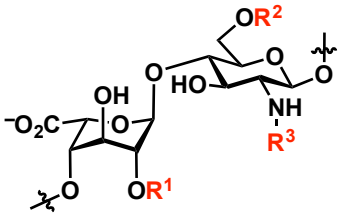


Figure 2-14. Glycan engineering with HS GAGs. CHO cells expressing HTP or not were incubated with **B-HS-CL** or **B-HS**. Incorporation of HS onto the cell surface was validated by (A) immunofluorescence or (B) Western blotting. Scale bar = 50 μm.

2.3.3 Deconvoluting the Role of HS GAG Sulfation Epitopes in FGF Activation

With a method for the long-lived presentation of GAGs in hand, we examined the ability of our non-natural presentation of HS GAGs to stimulate stem cell signaling pathways. HS GAGs regulate FGF-FGF receptor (FGFR) signaling events involved in stem cell differentiation by assisting in the assembly of active growth factor/receptor complexes.[32, 33] Activation of FGF2-FGFR1, in turn, initiates several intracellular signaling pathways, including the ERK/MAPK cascade.[34] A distinct period of increased ERK1/2 phosphorylation through FGF signaling triggers ESCs to exit self-renewal and the transition to a neural cell fate.[35, 36] To display particular sulfated HS structures on ESCs and stimulate the FGF2-FGFR1 pathway, we conjugated **2-13** to various heparin/HS polysaccharides. The commercially available polysaccharides (12–13 kDa) were derived from a single



HS-CL: $R^1 = SO_3^-$, $R^2 = SO_3^-$, $R^3 = SO_3^-$, Ac, or CL
de-HS-CL: R^1 = H, R^2 = H, R^3 = H, Ac, or CL
deO-HS-CL: R^1 = H, R^2 = H, $R^3 = SO_3^-$, Ac, or CL
6-deO-HS-CL: $R^1 = SO_3^-$, R^2 = H, $R^3 = SO_3^-$, Ac, or CL
2-deO-HS-CL: R^1 = H, $R^2 = SO_3^-$, $R^3 = SO_3^-$, Ac, or CL

Figure 2-15. Chemical structures of HS GAGs.

natural source and chemically desulfated to produce heparin/HS with defined sulfation motifs. Specifically, CL-functionalized heparin/trisulfated HS (**HS-CL**), fully desulfated heparin/HS (**de-HS-CL**), *O*-desulfated heparin/HS (**deO-HS-CL**), 6-*O*-desulfated heparin/HS (**6-deO-HS-CL**), and 2-*O*-desulfated heparin/HS (**2-deO-HS-CL**) were readily generated in one step under standard reductive amination conditions (Figure 2-15). A homogeneous population of pluripotent mouse ESCs were obtained commercially and transiently transfected with the HTP construct. The ESCs were treated with heparinase II to remove endogenous HS GAGs and incubated overnight in serum-depleted medium containing the various HS-CL derivatives. Cells were then stimulated with FGF2 and assayed for ERK1/2 activation by immunoblotting with antibodies against phosphorylated (pThr202/pTyr204) and total ERK1/2. To our delight, ESCs engineered to display heparin/trisulfated HS exhibited a 1.5-fold increase in ERK1/2 phosphorylation compared to untreated cells (Figure 2-16a). **HS-CL** had no effect on ESCs lacking HTP, confirming that ERK1/2 activation required the HTP anchors (Figure 2-16b). Moreover, ERK1/2 activation was dependent on the sulfation pattern, as

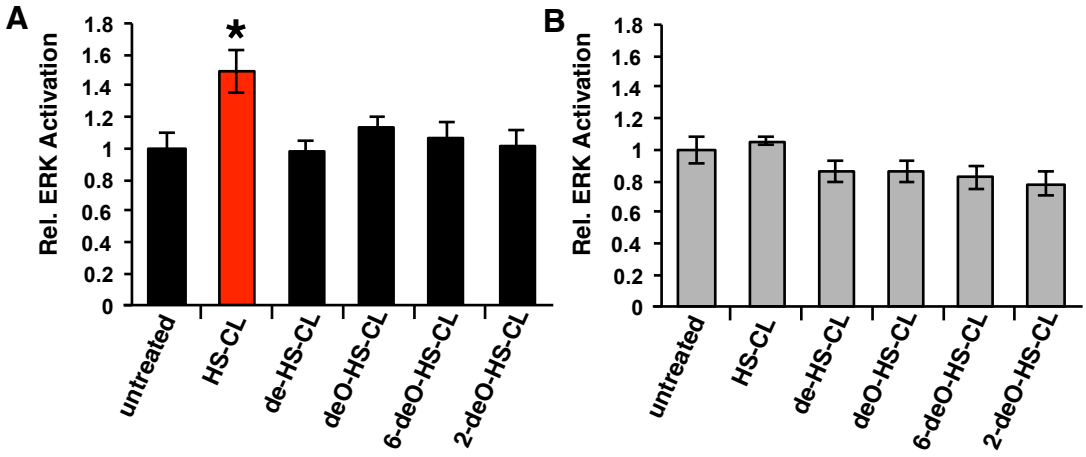


Figure 2-16. ERK1/2 activation by cell surface engineering. (A) mESCs were transfected with the HTP construct, functionalized with different **HS-CL** structures and then stimulated with FGF2. ERK1/2 phosphorylation was measured by Western blotting and quantified. (B) The same experiment was conducted with untransfected mESCs. * $P < 0.05$.

cells engineered with other selectively desulfated heparin/HS variants showed no significant increase in phospho-ERK1/2 levels.

Structural and biochemical studies have led to conflicting reports on the pivotal determinants of HS-FGF2-FGFR1 complexation and ERK1/2 activation.[33, 37, 38] For example, crystallographic studies suggested that 6-*O*-sulfation is critical for both ligand and receptor binding,[33] whereas certain biochemical studies indicated that downstream ERK1/2 activation elicited by the complex is not significantly attenuated by loss of the 6-*O*-sulfate group.[38] Interestingly, the same crystallographic studies showed that the other sulfate groups are also important for interactions with FGF2, suggesting that all three of the sulfate groups may be required for formation of the ternary HS-FGF2-FGFR1 complex.[33] To clarify the role of sulfation, we used GAG microarrays[18] to probe the ternary interaction

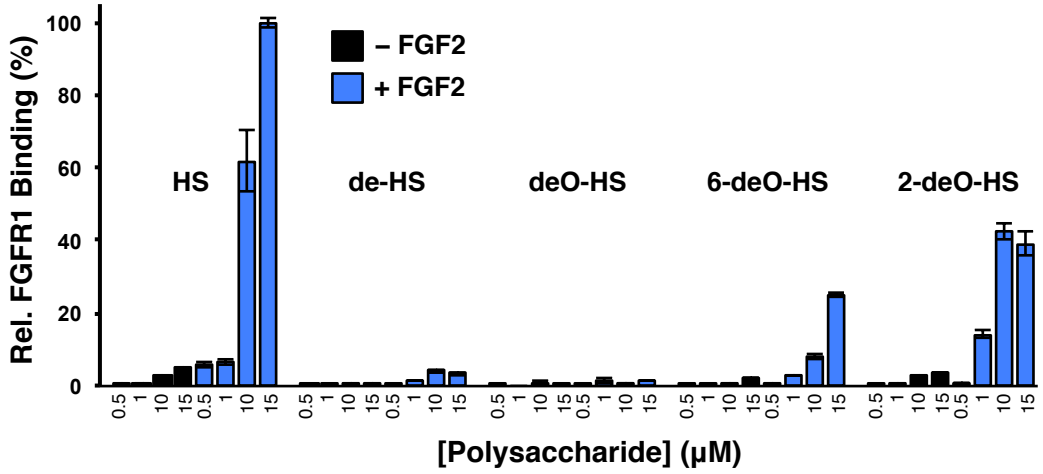


Figure 2-17. Ternary complex formation between FGF2 and FGFR1 with HS. Microarrays were incubated with FGFR1-Fc with and without FGF2. Bound FGFR1-Fc was visualized by an anti-human Fc antibody.

more closely. Microarrays printed with various concentrations of the HS derivatives were incubated with an FGFR1-Fc fusion protein in the presence or absence of FGF2. Binding of FGFR1-Fc was visualized using an anti-Fc antibody conjugated to AlexaFluor 647. We found that FGFR1 bound preferentially to heparin/trisulfated HS only in the presence of FGF2, suggesting the formation of a ternary complex (Figure 2-17). Consistent with our ERK1/2 activation studies, binding of FGFR1 was significantly attenuated by either 2-*O*- or 6-*O*-desulfation of HS. Taken together, our studies suggest that both 2-*O*- and 6-*O*-sulfation of HS are critical for FGF2-FGFR1-mediated ERK1/2 activation in ESCs. Thus, an HTP-based approach can be used to hijack endogenous signaling pathways and deconvolute the sulfation requirements of complex GAG-mediated processes.

2.3.4 Accelerating Stem Cell Differentiation

Finally, we investigated whether the HTP-dependent presentation of specific heparin/HS epitopes could promote stem cell differentiation into mature cell types. Dopaminergic neurons from fetal sources have shown long-term cell survival and preliminary clinical benefits when implanted into patients with Parkinson's disease.[39, 40] As such, the development and refinement of *in vitro* systems to generate neuronal cell populations could have widespread biomedical implications for the treatment of neurological diseases. The differentiation of ESCs into dopaminergic neurons is promoted by FGF8b.[41] Interestingly, our microarray studies indicated that FGF8b and its receptor FGFR3c are capable of forming ternary complexes with heparin/trisulfated HS, whereas complex assembly was attenuated with 2-*O*-desulfated or 6-*O*-desulfated heparin/HS and was not observed with fully *O*-desulfated heparin/HS (Figure 2-18). Pluripotent mouse ESCs transiently expressing HTP were treated with heparinase II and then grown in neural induction medium supplemented with FGF2 and **HS-CL**. For comparison, ESCs were treated identically, but in the absence of **HS-CL** (untreated) or replacing **HS-CL** with **de-HS-CL**. After two days, the FGF2-containing medium was substituted with a neural induction medium containing FGF8b. We monitored the differentiation process at specific time points by profiling the gene expression levels of specific markers using real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR) analyses

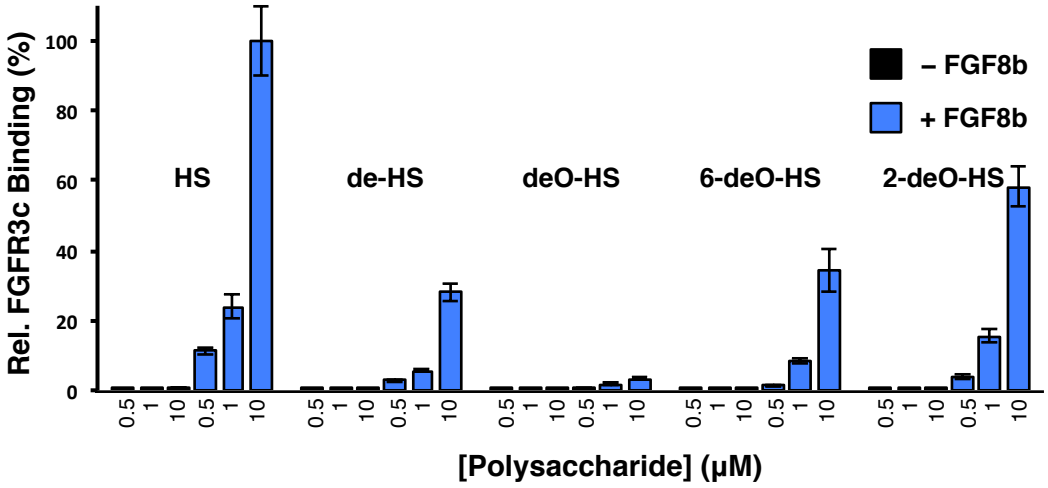


Figure 2-18. Ternary complex formation between FGF8b and FGFR3c with HS. Microarrays were incubated with FGFR3c-Fc with and without FGF8b. Bound FGFR3c-Fc was visualized by an anti-human Fc antibody.

(Figure 2-19). The transcription factors NANOG and SOX1 are well-established markers for pluripotency and self-renewal exit/neural lineage commitment, respectively, whereas TUJ1 is widely used as a specific marker for mature, fully differentiated neurons.[42] In parallel, the presence of NANOG, SOX1, and TUJ1 in cells was imaged by immunocytochemistry (Figure 2-20). We found that mRNA NANOG levels declined most rapidly in ESCs treated with **HS-CL**, which is consistent with an accelerated loss of pluripotency. In contrast, the levels remained high until day nine in untreated cells and cells treated with **de-HS-CL**. The decrease in NANOG levels at day nine for untreated and **de-HS-CL** treated cells is most likely due to *de novo* HS biosynthesis following the single heparinase treatment. Consistent with an accelerated loss of pluripotency, the decline in NANOG levels in **HS-CL**-treated cells was accompanied by a corresponding

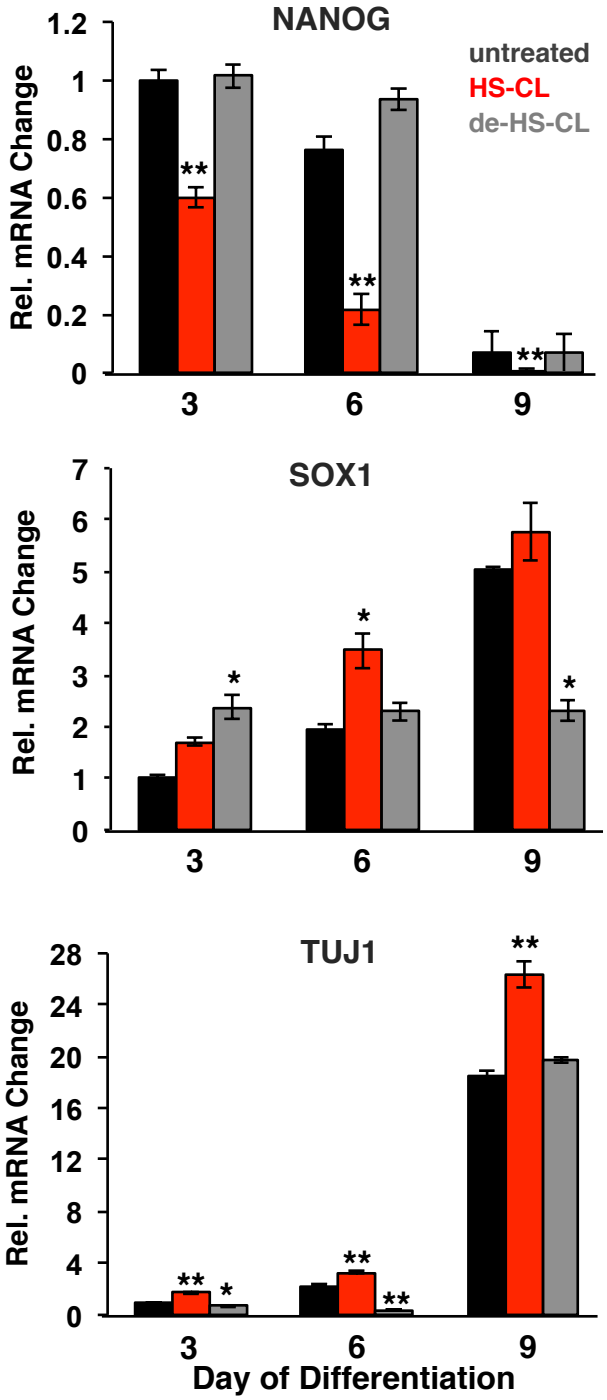


Figure 2-19. Accelerated stem cell differentiation with glycan engineering (qRT-PCR). mESCs expressing HTP were incubated with nothing, **HS-CL**, or **de-HS-CL** and cultured in differentiation medium as described. Differentation was monitored by qRT-PCR of NANOG, SOX1, and TUJ1. * $P < 0.05$, ** $P < 0.01$.

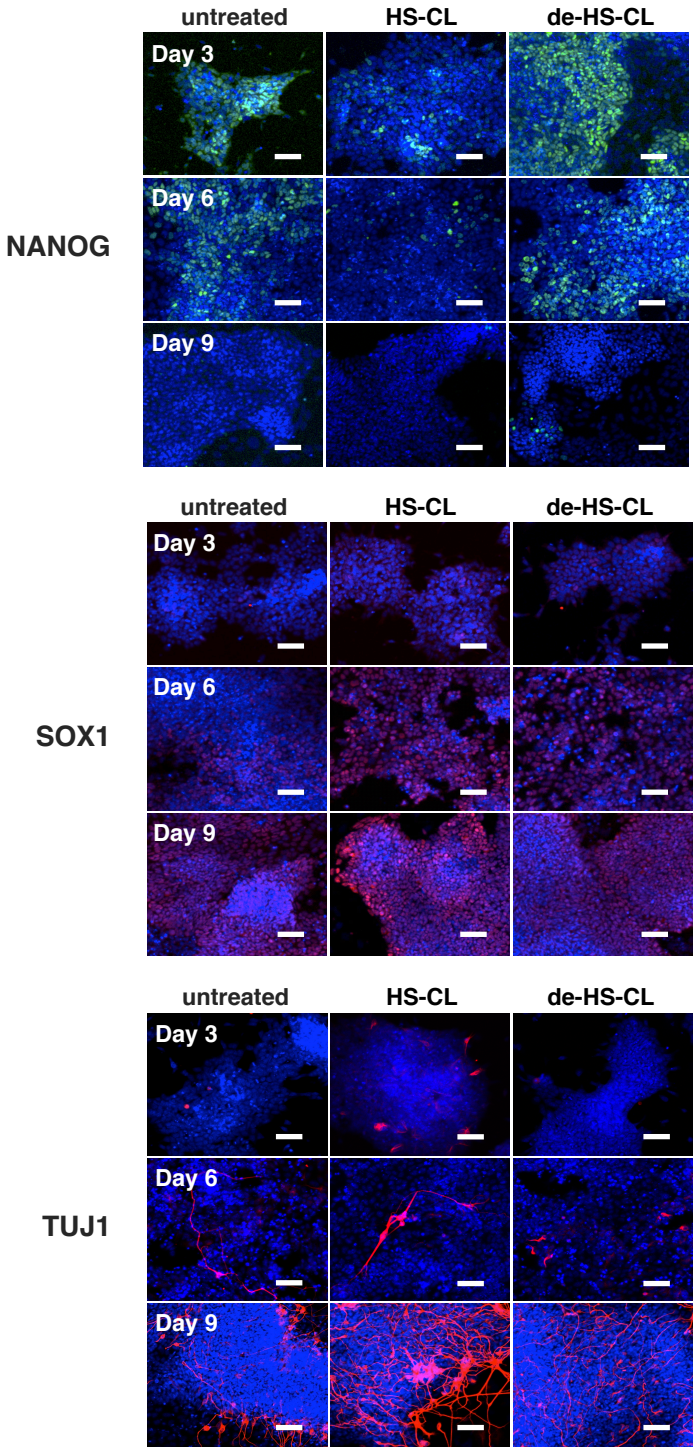


Figure 2-20. Accelerated stem cell differentiation with glycan engineering (immunofluorescence). mESCs from the experiment described in Figure 2-18 were stained for NANOG (green), SOX1 (red), or TUJ1 (red) and DAPI (blue). Scale bar = 50 μm.

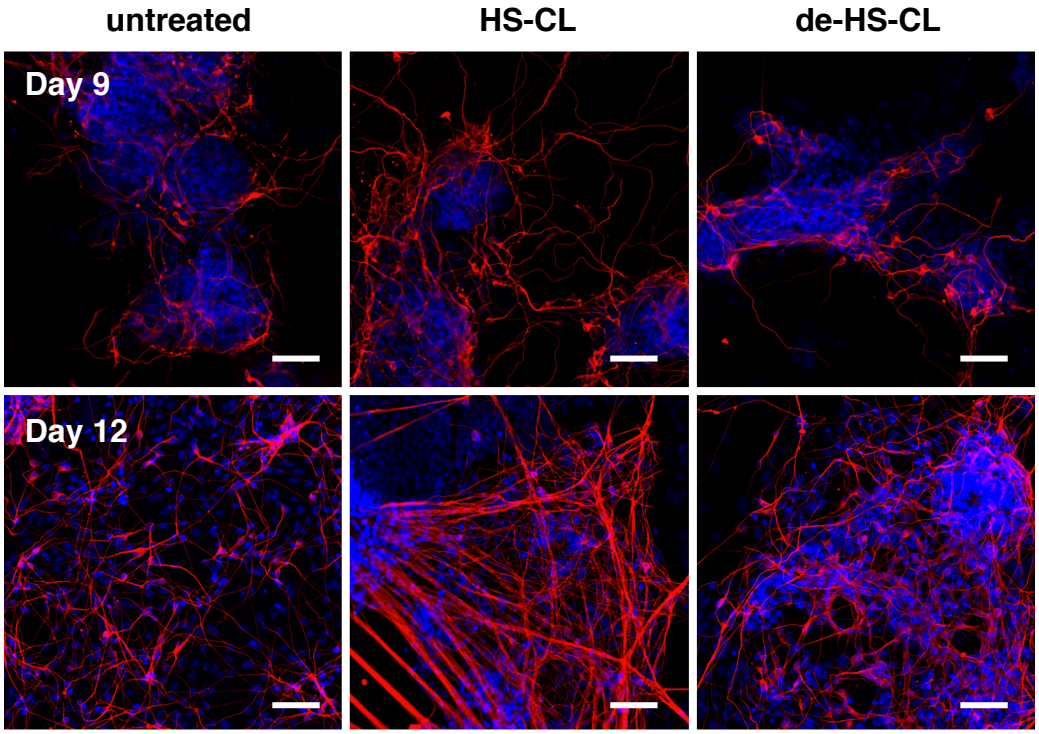


Figure 2-21. Mature neuronal processes on differentiated mESCs. mESCs from the experiment described in Figure 2-18 were allowed to grow to 9 or 12 days and were stained for TUJ1 (red) and DAPI (blue). Scale bar = 50 μm.

increase in the neuroectoderm-specific marker SOX1. At day six, SOX1 levels were significantly higher in **HS-CL**-treated cells than in untreated or **de-HS-CL**-treated cells, suggesting that **HS-CL**-treated cells had undergone accelerated exit from self-renewal and commitment to an intermediate, neuroectoderm state. Importantly, cells remodeled with **HS-CL** also showed significantly higher levels of TUJ1 than untreated or **de-HS-CL**-treated cells at all time points, which is indicative of faster progression to a mature, differentiated neuronal phenotype. As expected, the cells also developed more elaborate neurite processes than the untreated cells and **de-HS-CL** treated cells (Figure 2-21). Interestingly, cells

remodeled with **de-HS-CL** showed significantly lower amounts of SOX1 at day nine and TUJ1 at days three and six compared to untreated cells, which is consistent with studies suggesting that undersulfated HS may help to maintain pluripotency and restrict differentiation.[23] Together, our results illustrate the power of using long-term cell-surface displays of HS GAGs to activate specific signaling events and drive the differentiation of stem cells into mature neuronal populations.

2.4 Materials and Methods for Liposomal Experiments

2.4.1 Chemicals, Biochemical Reagents, Cell Lines, and Animals

All chemicals and reagents were of analytical grade, obtained from Sigma Aldrich, and used without further purification unless specified. Chondroitin sulfate (CS)-A, -C, and -E polysaccharides and chondroitinase ABC (ChABC) were purchased from Seikagaku Corporation. 1,2-Dioleoyl-*sn*-glycero-3-phosphoethanolaime (DOPE) and 1,2-dioleoyl-3-trimethylammoniumpropane chloride (DOTAP), were purchased from Avanti Polar Lipids, Inc. All secondary antibodies (AlexaFluor (AF) 680 goat anti-rabbit, AF680 goat anti-mouse, and AF488 goat anti-mouse), AF488 hydrazide sodium salt (AF488-hydrazide), and cell culture media and reagents were obtained from Life Technologies Corporation. Adherent pheochromocytoma cells (PC12 cells; CRL-1721.1), horse serum (HS), and F-12K media (30-2004) were obtained from American Type Culture Collection. Accutase (AM105) was received from Innovative Cell Technologies, Inc. Nerve growth factor (NGF) was obtained from R&D Systems, Inc. Primary antibodies against phospho-Akt (Ser473) (4060) and total Akt (4691) were purchased from Cell Signaling Technology, Inc. Sprague-Dawley rats were obtained from Charles River Laboratories, Inc.

2.4.2 Synthetic Methods

tert-*Butyl 2-((1,3-dioxoisoindolin-2-yl)oxy)acetate* (**2-1**). To a solution of *N*-hydroxyphthalimide (3.03 g, 18.6 mmol, 1.20 eq) in DMF (30 mL) was added

$NaHCO_3$ (1.56 g, 18.6 mmol, 1.20 eq). The mixture was stirred at 60 °C for 1 h, after which *tert*-butyl 2-bromoacetate (2.77 mL, 15.5 mmol) was added. The reaction mixture was stirred for 16 h at 60 °C. The mixture was then diluted with DCM (30 mL), extracted with 1 M $NaHCO_3$ until the aqueous layer became colorless, washed with ddH_2O (2 x 25 mL) and brine (1 x 25 mL), dried over $MgSO_4$, and concentrated to afford a yellow solid **2-1**. The product was purified by silica flash chromatography (Hex:EtOAc, 85:15) to afford a white solid **2-1** (3.76 g, 88%). ^{1}H NMR (500 MHz, $CDCl_3$): δ 1.48 (s, 9H, CH_3), 4.71 (s, 2H, CH_2), 7.75-7.76 (m, 2H, J = 5 Hz, Ar-H), 7.84-7.85 (m, 2H, J = 5 Hz, Ar-H).

2-((1,3-Dioxoisoindolin-2-yl)oxy)acetic acid (**2-2**). Compound **2-2** was synthesized as previously described.[43] Briefly, TFA (2 mL) was slowly added to a solution of 4 (0.51 g, 1.8 mmol) in DCM (6.0 mL). The mixture was stirred at RT for 2 h under Ar. The reaction was then concentrated and azeotroped with DCM (3 x 5 mL) to afford a white solid 5 (0.40 g, 99%). ^{1}H NMR (500 MHz, $CDCl_3$): δ 4.88 (s, 2H, CH_2), 7.85-7.87 (m, 2H, J = 10 Hz, Ar-H), 7.93-7.94 (m, 2H, J = 10 Hz, Ar-H).

2-((1,3-Dioxoisoindolin-2-yl)oxy)-conjugated CS polysaccharides (**2-3, 2-4, 2-5**). To a buffered solution containing 0.1 M MES and 0.5 M NaCl (pH 6.0), **2-2** (5.0 mg, 0.023 mmol), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC; 4.2 mg, 0.027 mmol, 1.2 eq), sulfo-*N*-hydroxysuccinimide (sulfo-NHS; 7.5 mg, 0.035 mmol, 1.5 eq) were added, and the mixture was stirred at RT for 30 min. Concurrently, CS-E, CS-A, or CS-C (4.0 mg, ~70 μmol, ~0.5 eq) was stirred in

0.05 M $NaHCO_3$ for 30 min. The polysaccharide amine solution was then added to the small molecule acid mixture and allowed to react for 12 h at RT under Ar. The mixture was then purified via dialysis against ddH_2O for 48 h (6 x 8 h) using a molecular weight membrane of 35,000 Da, followed by gel filtration chromatography (G-25). Fractions containing the polysaccharide were lyophilized to afford white solids. For **2-3** CS-E: ^1H NMR (500 MHz, $CDCl_3$): δ 2.04 (s, 3H, CH_3), 3.28-3.49 (m, 2H, CH), 3.51-4.35 (m, 9H, CH, CH_2OSO_3-), 4.42-4.67 (m, 1H, CH), 7.48 (m, 2H, Ar-H), 7.58 (m, 2H, Ar-H). 64% yield. For **2-4** CS-A: 1H NMR (500 MHz, $CDCl_3$): δ 2.03 (s, 3H, CH_3), 3.40 (br s, 1H, CH), 3.64 (br s, 1H, CH), 3.72-3.95 (m, 6H, CH), 4.03 (br s, 2H, CH), 4.51-4.63 (m, 2H, CH), 7.62 (m, 1H, Ar-H), 7.77 (m, 1H, Ar-H). 60% yield. For **2-5** CS-C: 1H NMR (500 MHz, $CDCl_3$): δ 2.02 (s, 3H, CH_3), 3.39 (br s, 1H, CH), 3.64 (br s, 1H, CH), 3.72-4.06 (m, 6H, CH, CH_2OSO_3-), 4.12-4.27 (m, 3H, CH), 4.57 (br s, 1H, CH), 7.62 (m, 2H, Ar-H), 7.75 (m, 2H, Ar-H). 78% yield.

Aminooxy-conjugated CS polysaccharides (**2-6**, **2-7**, **2-8**). Hydrazine monohydrate (1 µL, 0.031 mmol, 4.3 eq) was added dropwise to a solution of **2-3**, **2-4**, or **2-5** (4.0 mg, ~70 µmol) in ddH_2O (4 mL), and the mixture was stirred for 3 h at RT under Ar. The solution was then purified via dialysis against ddH_2O for 48 h (6 x 8 h) using a molecular weight membrane of 35,000 Da, followed by gel filtration chromatography (G-25). Fractions containing the polysaccharide were lyophilized to afford white solids. For **2-6** CS-E: ^1H NMR (600 MHz, $CDCl_3$): δ

2.04 (s, 3H, CH_3), 3.38 (br s, 1H, CH), 3.56-3.85 (m, 5H, CH), 3.86-4.33 (m, 4H, CH, CH_2OSO_3-), 4.42-4.67 (m, 2H, CH). For **2-7** CS-A: ^{1}H NMR (500 MHz, $CDCl_3$): δ 2.04 (s, 3H, CH_3), 3.39 (br s, 1H, CH), 3.61 (br s, 1H, CH), 3.69-3.87 (m, 6H, CH), 4.08 (br s, 2H, CH), 4.46-4.64 (m, 2H, CH). For **2-8** CS-C: ^{1}H NMR (500 MHz, $CDCl_3$) δ 2.03 (s, 3H, CH_3), 3.38 (br s, 1H, CH), 3.61 (br s, 1H, CH), 3.69-4.06 (m, 5H, CH, CH_2OSO_3-), 4.15-4.27 (m, 3H, CH), 4.46-4.65 (m, 2H, CH).

Fluorophore-conjugated aminooxy-CS-E (**2-9**). The protocol for conjugating aminooxy-CS-E with fluoresceinamine was adapted from Glabe *et al.*[44] To a stirring solution of **2-6** (1.00 mg, 14.29 μmol) in ddH_2O (1 mL) was added cyanogen bromide (1.00 mg, 0.00094 mmol, excess) in ddH_2O (0.3 mL). The pH of the reaction was adjusted to 11.0 using 0.2 M NaOH, which was stirred for 10 min at RT. The mixture was then desalted on a disposable PD-10 Sephadex column (17-0851-01, GE Healthcare Biosciences) using 0.2 M sodium borate buffer (pH 8.0). The CS-E fractions were pooled (3 mL) and immediately stirred with fluoresceinamine (1.00 mg, 0.0029 mmol, excess) in the dark overnight (12 h). The mixture was concentrated and purified on a G-25 Sephadex column in the dark. The CS-E fractions were again pooled, flash frozen, and lyophilized to afford an orange solid 2-**9**.

2.4.3 Liposomal Formation and Characterization

Liposome formation. A solution of 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE, 120 μL, 10 mg/mL in $CHCl_3$), dodecanone (12 μL, 10 mg/mL in $CHCl_3$),

and 1,2-dioleoyl-3-trimethylammoniumpropane chloride salt (DOTAP, 60 µL, 10 mg/mL in $CHCl_3$) was thoroughly mixed and then dried with a stream of argon followed by high vacuum for 12 h. The dried lipids were reconstituted in ddH_2O (2 mg/mL total lipids) and hydrated for 30 min. The mixture was then sonicated for 2 min and extruded (11 passes through the membrane) to form large unilaminar vesicles using a mini-extruder (Avanti Polar Lipids, Inc.) according to the manufacturer's instructions.

Liposome conjugation. AF488-hydrazide-presenting liposomes: Liposomes (100 µL, 2.24 mM dodecanone) were incubated with AF488-hydrazide (12.8 µL, 1.75 mM, 10:1 ketone/hydrazide) for 3 h at RT in the dark overnight at a stoichiometric ratio to label approximately 10% of the available ketones. This ratio was chosen after method optimization to conserve carbohydrate material but maintain a significant amount of cell labeling. CS-aminooxy-tethered liposomes: Liposomes (100 µL, 2.24 mM dodecanone) were reacted with CS-A/C/E-aminooxy-polysaccharides **2-6**, **2-7**, or **2-8** (~20 µL, 33.3/31.7/25.6 µM) for 3 h at RT in the dark overnight. Mixed liposomes: Liposomes (100 µL, 2.24 mM dodecanone) were reacted with CS-A/C/E-aminooxy polysaccharides **2-6**, **2-7**, or **2-8** (~20 µL, 33.3/31.7/25.6 µM) for 4 h at RT in the dark, followed by AF488-hydrazide at a stoichiometric ratio to label approximately 10% of the available ketones.

Dynamic light scattering (DLS). Ketone-presenting (**I**) and AF488-hydrazide- (**II**), CS-A-ONH_2- (**IIIA**), CS-E-ONH_2- (**IIIE**), and mixed AF488/CS-A-ONH_2- (**IV**) conjugated liposomes were generated as described above and diluted (1:20) with 1.5 mM KCl. The size distributions of these liposomal samples were then determined using a DynaPro NanoStar (Wyatt Technologies) DLS instrument. The data are represented as the mean liposomal hydrodynamic diameter ± S.E.M. with eight to ten measurements conducted per experiment (n = 3).

Zeta potential analyses. Ketone-presenting (**I**) and AF488-hydrazide- (**II**), CS-A-ONH_2- (**IIIA**), and CS-E-ONH_2- (**IIIE**), mixed AF488/CS-A-ONH_2- (**IV**) conjugated liposomes were generated as described above, diluted (1:20) with 1.5 mM KCl, and subjected to zeta potential analyses using a ZetaPALS (Brookhaven Instruments Corp.) analyzer. The data are represented as the mean zeta potential ± S.E.M. with three to ten measurements conducted per experiment (n = 3).

Transmission electron microscopy (TEM) and energy dispersion spectroscopy (EDS). Dodecanone-containing liposomes were prepared and conjugated with AF488-hydrazide and CSA-aminooxy polysaccharide **2-7** as described above. A drop of each liposome sample was placed on a TEM copper/carbon grid for 30 s, which was then blotted using filter paper. A drop of 2% uranyl acetate was then added to each sample for 5 min followed by blotting with filter paper. The grid was then rinsed with ddH_2O and dried. TEM and elemental analyses of each liposome sample were performed using an FEI Tecnai F30UT (300 kV) equipped with a

high angle annular dark field detector, an Oxford energy dispersion X-ray detector, and an AMT CCD camera.

2.4.4 Biological Procedures

Cell culture. PC12 cells were cultured on collagen-coated (10 µg/mL) tissue culture plates in F-12K media supplemented with 6.5% HS, 6.5% FBS, and 1x penicillin/streptomycin (P/S) at 37 °C with 5% CO2. Hippocampal and cortical neurons were cultured in Neurobasal media supplemented with B27, 20 mM GlutaMAX, and 1x P/S at 37 °C with 10% CO_2.

Cell-surface remodeling. AF488-hydrazide-bearing liposomes were diluted 1:100 with the appropriate cell culture medium and incubated with PC12 cells or hippocampal neurons for 30-45 min. The medium was removed, and the cells were washed three times with 0.5 mL of sterile 1x phosphate buffered saline (PBS). Fresh medium (0.5 mL) was then added, and the cells were imaged live under a Zeiss LSM 5 Pascal inverted confocal microscope (Carl Zeiss Microscopy). For labeling differentiated PC12 cells with CS-E, PC12 cells were cultured on collagen-coated tissue culture plates and stimulated with NGF (100 µM; 256-GF, R&D Systems) to induce differentiation. After 4 d of NGF treatment, the cells were treated with 1 U/mL ChABC (1:1 activation buffer (0.4 M Tris/HCl pH 8.0, 0.4 M NaOAc, 0.1% BSA)/complete medium) for 2 h (37 °C, 5% CO2). The cells were then washed with PBS (4 x 0.5 mL), and medium that was supplemented with CS-E-functionalized liposomes (diluted 1:100) was added for

30-45 min. After fusion, the cells were fixed with 4% paraformaldehyde (PFA) in PBS for 20 min, stained with a monoclonal mouse anti-CS-E antibody (1:250 in 1% BSA/0.1% Triton X-100/PBS; 4 °C, 12 h), followed by an AF488-conjugated goat anti-mouse antibody (1:1000 in 1% BSA/0.1% Triton X-100/PBS; RT, 1 h), and imaged using a Zeiss LSM-5 Pascal inverted confocal microscope.

Immunochemistry. For imaging, cells were fixed with 4% PFA in PBS (15 min), rinsed with PBS twice, permeated, and blocked with a solution containing 1% BSA and 0.1% Triton X-100 (20 min), incubated with a monoclonal mouse anti-CS-E antibody (1:250; 4 °C, 12 h), washed with PBS (2 x 5 min), incubated with an anti-mouse AF488 antibody (1:1000; RT, 1 h), washed with PBS (2 x 5 min), and imaged under a Zeiss LSM 5 Pascal inverted confocal microscope.

Flow cytometry. Dodecanone-containing liposomes were prepared as mentioned above with the exception that the ratio of dodecanone to DOPE was varied (0, 5, 10, 15, and 20% w/w). PC12 cells were cultured until ~80% confluent and then detached from the tissue culture plate using TrypLE. The cells were twice washed with cold PBS (500 µL) and spun down (1,400 x *g*, 2.5 min). For AF488-hydrazide labeling, the cells with resuspended and incubated on ice in a buffered solution (45 mM BSA, 10 mM HEPES, and DNase I (0.5 U/mL) in HBSS at pH 7.4) containing 0, 5, 10, 20% w/w ketone-hydrazide conjugated liposomes for 30 min (diluted 1:100). The dye concentration was adjusted per condition according to the ketone concentration in the liposomes at a stoichiometric ratio to label

approximately 10% of the available ketones. After labeling, the cells were again twice washed with cold PBS (500 µL) and spun down (1,400 x *g*, 2.5 min). After resuspension in the buffer listed above, the cells were filtered through a 40 µm-filtered FACS tube, and subjected to flow cytometric analyses using a FACS-Calibur flow cytometer (BD Biosciences). For CS-E labeling, PC12 cells were incubated with a monoclonal mouse anti-CS-E antibody (1:250) for 1 h followed by two wash cycles, incubated with an AF488-conjugated goat anti-mouse antibody (1:1000) for 1 h on ice, washed again twice, and subjected to flow cytometric analyses. The labeled samples were referenced to untreated cells. As controls, PC12 cells were separately incubated with liposomes without the ketone and hydrazide-AF488 or the AF488-conjugated goat anti-mouse antibody. Each sample was run in duplicate, and each sample set was performed twice.

Cell internalization assay. Liposomes were reacted with 9 as mentioned above for 3 h at RT in the dark. The liposome stock solution was diluted 1:100 with HBSS (45 mM BSA, 10 mM HEPES, pH 7.4) and added (400 µL) to PC12 cells for 30 min on ice in the dark. After labeling, the cells were diluted with PBS (500 µL), washed (3 x 500 µL wash/centrifuge cycles), and added to RPMI medium without phenol red. The cells were imaged live at 0, 3, 6, 10, 16, and 24 h after labeling under a Zeiss 700 LSM confocal microscope.

Neurite outgrowth assays. Hippocampal neurons from E18 Sprague-Dawley rats were dissected as previously described[45] and plated at 2,000 cells/well on poly-

lysine-coated 96-well plates (BD Biosciences) in 50 µL of complete DMEM (10% FBS, 1% P/S) for 30 min, followed by 150 µL of complete Neurobasal (B27, 20 mM GlutaMAX, 1% P/S). After 48 h, CS-A, -C, and -E-conjugated liposomes (10% w/w dodecanone; 2.5-10% ketone for CS-E dose-dependent studies) were diluted 1:100 in 200 µL of complete Neurobasal and added. The medium was replaced after 30 min to remove the remaining liposomes and unreacted CS. Due to endocytosis of CS after approximately 6-10 h, cells were treated with polysaccharide-displaying liposomes again at 8 and 16 h, followed by medium replacement 30 min after addition. At each time point, the cells were fixed with 4% PFA (20 min), washed with PBS (2 x 5 min), permeabilized with 1% BSA and 0.1% Triton X-100 in PBS (15 min), washed with PBS (2 x 5 min), incubated with a mouse α-tubulin antibody (Sigma-Aldrich; 1:1000) at 4 °C for 12 h, washed with PBS (2 x 5 min), incubated with a AF488-conjugated goat anti-mouse antibody (1:1000) at RT for 1 h, washed with PBS (2 x 5 min), and imaged under a Zeiss LSM 5 Pascal inverted confocal microscope as previously described.[17] Each well was reconstructed in Adobe Illustrator after capturing a 9 x 9 grid of 2034-pixel images. Four to five wells were blindly subjected to MetaMorph (Molecular Devices) neurite outgrowth software analyses to calculate the mean outgrowth per neuron. As controls, untreated neurons, neurons subjected to ketone liposomes without polysaccharide, and neurons treated with ketone liposomes (+CS-E without an aminooxy group) were assayed for outgrowth. The data are

represented as the mean outgrowth percentage (± S.E.M.) with n = 7 (200 cells measured per assay).

Stimulation assays. Cortical neurons from E18 Sprague-Dawley rats were dissected as previously described[46] and plated at 5 x 10^6 cells per poly-ornithine-coated (Sigma Aldrich) 10-cm plate in complete DMEM (10% FBS, 1% P/S) for 30 min, followed by complete Neurobasal (B27, 20 mM GlutaMAX, 1% P/S). After 7 d, CS-C- and CS-E-conjugated ketone liposomes (10% w/w dodecanone) were added to the complete Neurobasal for 30 min. The medium was then replaced with fresh complete Neurobasal that contained 50 ng/mL NGF (256-GF; R&D Systems), and the neurons were stimulated for 0, 10, 30, and 60 min. At each time point, the medium was evacuated, the cells were scraped and collected in cold PBS (1 mL), centrifuged (4 °C, 3,000 x *g*, 2.5 min), and homogenized in 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 inhibitors (Roche) by 10 passages through a 26-gauge 3/8" needle on ice. The lysates were then cleared (4 °C, 16,000 x *g*, 15 min), and the total protein concentration was determined for each sample using a BCA assay kit (Thermo Scientific Pierce). Western blot analyses were performed as previously described,[17] blotting separately with a rabbit anti-phospho-Ser473 Akt antibody and a rabbit anti-Akt antibody at 1:1000 in 5% BSA. Akt activation was calculated by determining the ratio of phospho-Akt to total Akt for each time point and

condition normalized to α-tubulin staining. Relative Akt activation was reported with respect to the untreated values (+NGF, no liposomes).

2.5 Materials and Methods for HaloTag Experiments

2.5.1 Synthetic Methods

N-Boc-2-(2-hydroxyethoxy)ethylamine (**2-10**). Compounds **2-10** and **2-11** were synthesized as previously described.[31] 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 **2-10** (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 (**2-11**). A solution of **2-10** (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 **2-11** 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 (**2-12**). *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 **2-12** (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 (**2-13**). Trifluoroacetic acid (TFA) (0.14 mL, 1.8 mmol, 6 eq) was added dropwise to a stirring solution of **2-11** (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 **2-12** (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 **2-13** 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, $CH_2C(O)$), 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.

Fluorescein-chloroalkane linker conjugate (**F-CL**). To a solution of **2-13** (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). ^{1}H 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 **2-13** (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). ^{1}H 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.

General procedure for chloroalkane heparin/heparan sulfate derivatives. In a typical procedure, **2-13** (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).

2.5.2 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) to introduce a 5' SacII restriction site and a 3' SalI restriction site using the following primers:

```
HTP-upstream:   TTATCCGCGGTGGATCCGAAATCGGTACTGGCTTT
HTP-downstream: ACTAGTCGACACCGGAAATCTCCAGAGTAGACAG
```

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). 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 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 (pgsB-618, ATCC) 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 FACS-Aria 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.) 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) 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) 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.[18] 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. Primers for amplification of genes of interest are listed below.

```
GAPDH-F: AACAGAAACTCCCACTCTTC
GAPDH-R: CCTGTTGCTGTAGCCGTATT (111 bp amplicon)


SDHA-F:  GCTGGAGAAGAATCGGTTATGA
SDHA-R:  GCATCGACTTCTGCATGTTTAG (97 bp)

NANOG-F: TTTGGAAGCCACTAGGGAAAG
NANOG-R: CCAGATGTTGCGTAAGTCTCATA (115 bp)

SOX1-F:  ACACACACACACACACTCTC
SOX1-R:  CCTCAAGATCTGGTCAGGAATG (101 bp)

TUJ1-F:  CCATTCTGGTGGACTTGGAA
TUJ1-R:  GCACCACTCTGACCAAAGATA (103 bp)
```

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.

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