Supporting Information

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SI Materials and Methods

Cell Culture, Transfection, and Viral Infection. HEK293T, U2OS, and MRC-5 cells were grown in DMEM containing 10% (vol/vol) FBS with 100 μg/mL penicillin/streptomycin (GIBCO) at 37 °C with 5% (vol/vol) CO_2. For small-scale transfection, FuGENE-6 (Roche) was used as recommended by the manufacturer. To establish Fam20B stable knockdown MRC-5 cell lines, lentiviral constructst (pLKO.1-TRC system; Sigma) encoding ShRNAs targeted against the human *FAM20B* ORF [target sequence 1 (ShRNA1), 5′-ATGCCTCTCTCACACTTGTA-3′ and target sequence 2 (ShRNA2): 5′-CTTGTGCTGATGGAGACATA-3′], and a control (Ctrl) ShRNA encoding scrambled *FAM20B* RNA, were cotransfected with viral packaging plasmids (psPAX2 and pMD2.G) into 293LTV cells, respectively. The resulting viral supernatants were applied to the cell lines used in these studies and stable cell pools encoding Ctrl and Fam20B knockdown shRNAs were obtained by puromycin (Sigma) selection. Stable HEK293T cell lines expressing FLAG-tagged Fam20B were generated using retroviral-based pQCXIP constructs (Clontech) essentially as described previously (1).

Large-Scale Protein Expression and Purification in Mammalian Cells. HEK293T cells (~1 × 10^8 cells) were seeded in five-layer CeLLSTACKS (Corning) 1 d before transfection. Approximately 450 μg of plasmid was mixed with 1.5 mL of 1 mg/mL polyethylenimine (Polysciences) and incubated at room temperature for 20 min before adding it to the cells (2). Conditioned medium was collected 3 d posttransfection and concentrated using a tangential flow filtration device (Vivaflow 200; Sartorius). FLAG-tagged proteins were immunopurified using anti-FLAG agarose and eluted by competition with 3×-FLAG peptide (Sigma). Strep-tagged proteins were purified using Tactin resin (Qiagen) and eluted by desthiobiotin (Sigma). Where indicated, proteoglycans were treated with alkaline phosphatase (New England Biolabs) while attached to the resin and washed extensively with Tris-buffered saline (TBS) before elution.

MBP-Fam20B(42-409) and MBP-GalT-II(31-329) Expression and Purification in Insect Cells. Bacmid was generated and recombinant baculovirus amplified using standard procedures as described (3). Hi5 cells were infected at a density of 2 × 10^6 cells/mL. After 48 h, the conditioned medium was collected, cleared of cell debris, and concentrated (Vivaflow 200; Sartorius). The fusion proteins were purified by nickel-agarose (Ni-NTA) affinity chromatography, and tobacco etch virus (TEV) protease was used to remove the N-terminal fusion tag when necessary.

GAG Treatment. Purified proteoglycans were digested at 37 °C for 15–30 min with 10 milliunit/mL (mU/mL) of heparinase I, II, and III (*Flavobacterium heparinum*; Seikagaku) and/or 10 mU/mL of chondroitinase ABC (Chon-ABC; Sigma) to specifically digest HS and/or CS. Where indicated, 0.01 mg/mL of pronase (type XIV protease from *Streptomyces griseus*; Sigma) was added to the reaction mixture to digest the proteins at 37 °C for 1 h.

Immunoblotting. M2-FLAG antibody was purchased from Sigma. DCN, SDC1, GPC1, and SDC4 antibodies were purchased from R&D Systems. The 3G10 antibody was purchased from Seikagaku. Fam20B polyclonal antibodies were purified from rabbit immune serum with recombinant human Fam20B covalently linked to NHS-HiTrap cloumns (GE Healthcare). To prepare samples for 3G10, SDC1, and GPC1 immunoblotting, cell monolayers were washed with Dulbecco's PBS (DPBS) and incubated with/without 2 mU/mL of heparinase I/II and 5 mU/mL of heparinase III/Chon-ABC in serum-free medium at 37 °C for 15 min. The cell monolayers were detached from the plates by enzyme-free and EDTA-based dissociation buffer (Invitrogen) for 15 min at 37 °C.

In Vitro Kinase Reactions Using Proteoglycans as Substrates. Aggrecan from bovine articular cartilage was purchased from Sigma. Aggrecan and other purified proteoglycans were incubated with 2.5–10 μg/mL of Fam20B or Fam20B (D309A) in kinase reaction buffer [50 mM Hepes pH 7.4, 10 mM $MnCl_2$, [γ-^{32}P]ATP (specific activity = 100–500 cpm/pmol)] at 30 °C. The reactions were terminated at the indicated time points by addition of 30 mM EDTA and 2 mM ATP and boiling in SDS sample buffer for 10 min. Reaction products were separated by SDS/PAGE and incorporated radioactivity was visualized by autoradiography.

Oligosaccharide Synthesis, Enzyme Kinetics, and Mass Spectrometry Analysis. The Tetra-Ben, Gal-Xyl-Ben, and Xyl-Ben were synthesized and characterized as previously described (4). Xyl-Xyl-Glc-EGF was a generous gift from Robert Haltiwanger, Stony Brook University, Stony Brook, NY. Kinase assays were typically performed in 50 mM Hepes, pH 7.4, 10 mM $MnCl_2$, [γ-^{32}P] ATP (specific activity = 100–500 cpm/pmol), and 2.5–10 μg/mL Fam20B-FLAG or Fam20B (D309A) at 30 °C for 10 min. For the kinetic studies, initial experiments were performed using these conditions to confirm the reactions were linear with respect to time. Reactions were terminated by 30 mM EDTA and loaded to Sep-Pack C_{18} cartridge (Waters) preequilibrated with 0.2 M $(NH_4)_2SO_4$. The columns were washed with 5 × 2 mL of 0.2 M $(NH_4)_2SO_4$ and the oligosaccharides were eluted by 1 mL methanol. Incorporated radioactivity was quantified by liquid scintillation counting (Beckman LS 6000IC). Analysis of the kinase reaction products of Tetra-Ben by mass spectrometry was done using an LTQ Orbitrap Discovery electrospray ionization mass spectrometer (Thermo Scientific) equipped with quaternary HPLC pump (Finnigan Surveyor MS pump) and a reverse-phase capillary column as previously described (5).

[^{32}P]Orthophosphate Metabolic Labeling. Control and Fam20B stable knockdown MRC-5 cells were grown to confluency in six-well plates and the cell monolayers were washed three times with DPBS. The cells were then radiolabeled for 6 h with 1 mCi/mL [^{32}P] orthophosphate in phosphate-free DMEM containing 10% (vol/vol) dialyzed FBS. Endogenous DCN was immunoprecipitated from the conditioned medium using anti-DCN antibody (R&D Systems) and treated with Chon-ABC to eliminate the CS chains before gel electrophoresis and immunoblotting. ^{32}P incorporation on DCN was visualized by autoradiography. The amount of DCN loading was normalized by anti-DCN immunoblotting.

^{35}S-GAG Labeling, Purification, and Treatment. Subconfluent cell cultures in six-well plates were incubated with 50 μCi/mL [^{35}S]sulfate for 20 h in DMEM containing 10% (vol/vol) dialyzed FBS. The medium was collected and centrifuged to remove cell debris. The cell monolayers were washed with DPBS, treated with trypsin for 10 min at 37 °C, and the cell suspensions were centrifuged. The cell pellets were lysed in radioimmunoprecipitation buffer (RIPA, 50 mM Tris, pH 8.0, 150 mM NaCl, 1.0% Nonidet P-40, 0.25% sodium deoxycholate, 1 mM EDTA, 0.1% SDS) containing protease inhibitor mixture (Roche). Total protein in the samples was quantified by BCA protein assay (Thermo Scientific Pierce) and used to normalize the amount of GAG. The

trypsin released soluble cell extracts (containing cell surface proteoglycans) and the conditioned medium (containing secreted proteoglycans) were exhaustively digested with 0.1 mg/mL pronase (Sigma) at 37 °C for 24 h followed by anion-exchange chromatography [diethylaminoethyl (DEAE)-Sephacel; GE Healthcare]. The columns were washed with 0.25 M NaCl and GAGs were eluted with 1 M NaCl. One-tenth volume of each of the elutes was analyzed by scintillation counting to calculate the amount of ^{35}S incorporated into the total GAG. The remainder of each eluate was diluted fivefold and digested overnight with 5 mU/mL of Chon-ABC or a mixture of 2 mU/mL of heparinase I/II and 5 mU/mL of heparinase III at 37 °C to specifically digest CS or HS, respectively. Released HS or CS was further purified by DEAE chromatography, quantified by scintillation counting, and normalized to total cellular protein.

Flow Cytometry. U2OS wild-type (WT) and *FAM20B* knockout (KO) cells were dissociated from the plates using EDTA-based dissociation buffer. Approximately 5×10^5 cells per clone were incubated with anti-SDC1 (1 μg/mL, R&D Systems), anti-SDC4 (1 μg/mL, antibody 5G9; Santa Cruz), and antibody 8G3 from Guido David, University of Leuven, Belgium, anti-GPC1 (1 μg/mL, R&D Systems), anti-GPC3 (1 μg/mL, a gift from Jorge Filmus, University of Toronto, Toronto), biotinylated Sambucus Nigra Lectin (SNA, 4 μg/mL; Vector Laboratories), or biotinylated FGF2 (2 μg/mL) in 100 μL of PBS for 1 h at 4 °C. After rinsing, the cells were stained with phycoerythrin (PE)-labeled secondary antibody or PE-streptavidin (eBioscience) at a dilution of 1:1,000 for 15 min and analyzed by flow cytometry (BD Biosciences) (6). In some experiments, cells were treated with heparinase I/II (2 mU/mL), III (5 mU/mL), and Chon-ABC (5 mU/mL) for 15 min at 37 °C before binding experiments.

Sialyltransferase Inhibitor Treatment. Approximately 5×10^5 WT and *FAM20B* KO U2OS cells were seeded in six-well plates. A total of 200 μM sialyltransferase inhibitor (3Fax-Peracetyl Neu5Ac) was added [0.1% (vol/vol) final DMSO concentration] to the KO cells. DMSO (0.1% vol/vol) was added to WT and KO cells as experimental controls (without inhibitor treatment). The cells were grown and subcultured routinely for 8 d in the presence of DMSO or 200 μM sialyltransferase inhibitor. On day 9, the cells were collected, washed, and subjected to flow cytometry analysis with SNA lectins and 3G10 immunoblotting as described above.

Identification of the Sugar Linkage by GC-MS/MS. The permethylated glycans were hydrolyzed to monosaccharides using 2NTFA at 100 °C for 4 h, followed by reduction with $NaBD_4$ and acetylation to form partially methylated alditol acetate (PMAA) conjugates. The PMAA samples were then analyzed by GC-MS/MS as described (7).

Generation of Gal-Xyl(P)-Ben and GalT-II Transferase Assay. Gal-Xyl-Ben was incubated with 10 μg/mL of Fam20B or Fam20B-D/A in the presence of kinase reaction buffer at 30 °C for 2 h to generate Gal-Xyl(P)-Ben. The reactions were stopped by heating for 10 min at 100 °C. After the solutions were chilled on ice, 5× galactosyltransferase buffer was added to each reaction to a final composition of 50 mM Mes, pH 6.0, 200 μM UDP-Gal with UDP-[6-^{3}H]Gal (specific activity: 100 cpm/pmole), 10 mM $MnCl_2$, 100 mM NaCl. Approximatey 20 μg/mL of MBP–GalT-II was used in each reaction. The reactions were performed at 30 °C for the indicated times, quenched by addition of 50 mM EDTA, and loaded onto Sep-Pak C_{18} columns. Nonreacted radioactive UDP-Gal was washed away from the column and the product was eluted from the column by methanol. Gal incorporation was quantified by scintillation counting.

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2. Lee JE, Fusco ML, Saphire EO (2009) An efficient platform for screening expression and crystallization of glycoproteins produced in human cells. *Nat Protoc* 4(4):592–604.
3. Xiao J, Tagliabracci VS, Wen J, Kim SA, Dixon JE (2013) Crystal structure of the Golgi casein kinase. *Proc Natl Acad Sci USA* 110(26):10574–10579.
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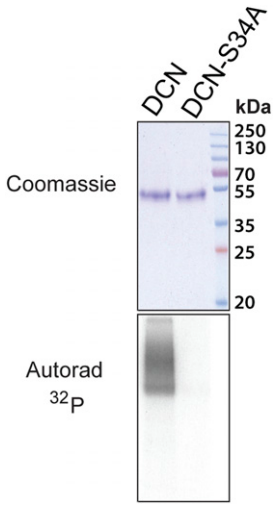

Fig. S1. In vitro Fam20B kinase reaction using DCN and DCN-S34A (GAG-deficient mutant) as substrates in the presence of [γ-^{32}P]ATP. The reaction products were separated by SDS/PAGE and proteins were visualized by Coomassie staining (*Top*). ^{32}P incorporation was visualized by autoradiography (autorad) (*Bottom*).

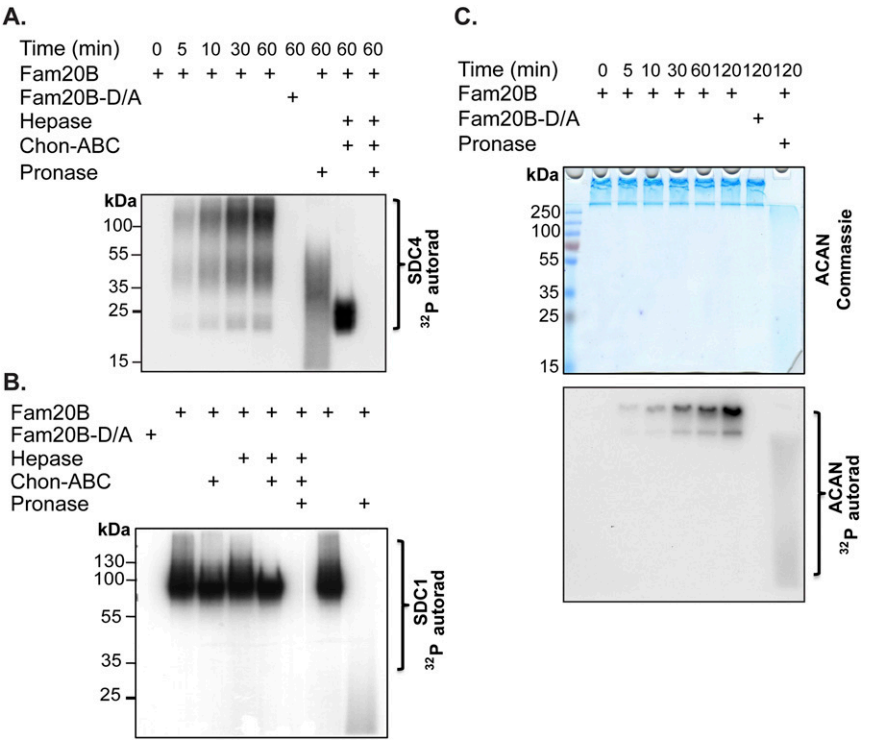

Fig. S2. Fam20B phosphorylates cell surface Syndecan 4 (SDC4), Syndecan 1 (SDC1), and cartilage aggrecan (ACAN). (*A*) In vitro kinase assays were carried out to demonstrate time-dependent incorporation of phosphate from [γ-^{32}P]ATP into recombinant SDC4 from HEK293T cells as catalyzed by Fam20B (1 μg/mL) or Fam20B D309A (D/A, 1 μg/mL). After the kinase reaction, the proteoglycans were treated with heparin lyases I, II, III (hepase), and/or chondroitinase ABC (Chon-ABC) to depolymerize the glycosaminoglycan (GAG) chains, or with pronase to digest the protein cores to demonstrate the incorporated ^{32}P was contained within the linkage region of GAG chains. (*B*) After in vitro kinase reactions with Fam20B WT or D309A, recombinant SDC1 from HEK293T cells was treated with hepase and/or Chon-ABC, and pronase. These results demonstrate the incorporated ^{32}P was contained within the linkage region of both chondroitin sulfate and heparan sulfate GAG chains. (*C*) Time-dependent phosphorylation of ACAN by Fam20B. Pronase treatment of the reaction products demonstrates that the incorporated ^{32}P was present on the GAG chains. Due to the high molecular mass of ACAN, it exhibited low electrophoretic mobility by SDS/PAGE. In *A*–*C*, reaction products were separated by SDS/PAGE and radioactivity was detected by autoradiography.

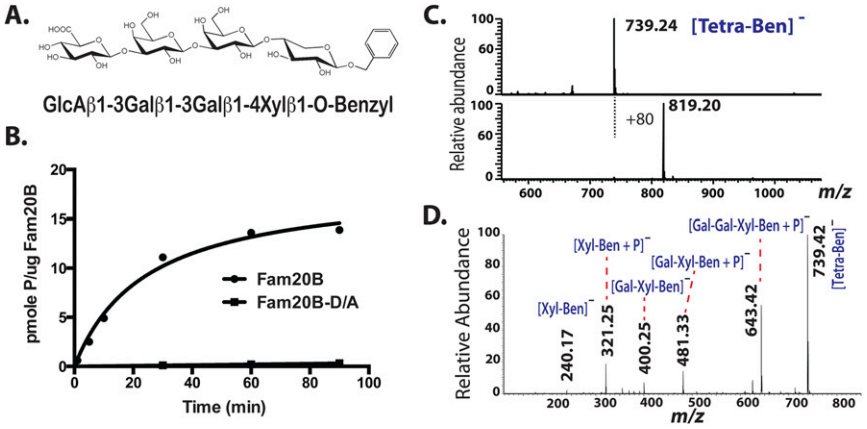

Fig. S3. Characterization of Fam20B xylose kinase activity using a model tetrasaccharide. (*A*) Structure of the synthesized tetrasaccharide linker analog (Tetra-Ben) used as a model Fam20B substrate. (*B*) Time-dependent incorporation of phosphate from [γ-^{32}P]ATP into Tetra-Ben (1 mM) by Fam20B (2 μg/mL) or Fam20B D309A (D/A, 2 μg/mL). The kinase assay reaction products were loaded onto preequilibrated Sep-Pak C_{18} cartridges. Tetra-Ben was eluted from the resin by methanol after extensive washing, and the incorporated ^{32}P was quantified by scintillation counting. (*C*) Negative mode mass spectrometry analysis of Tetra-Ben before and after Fam20B kinase reaction in the presence of nonradiolabeled ATP. After the kinase reaction with Fam20B WT, the molecular weight of Tetra-Ben shifted from 739.24 Da to 819.20 Da. The increase of 80 Da corresponds to phosphate incorporation. (*D*) The assignment of fragment ions after collision induced dissociation of the phosphorylated Tetra-Ben ion (*m/z* = 819.20) and the identification of phosphoxylose.

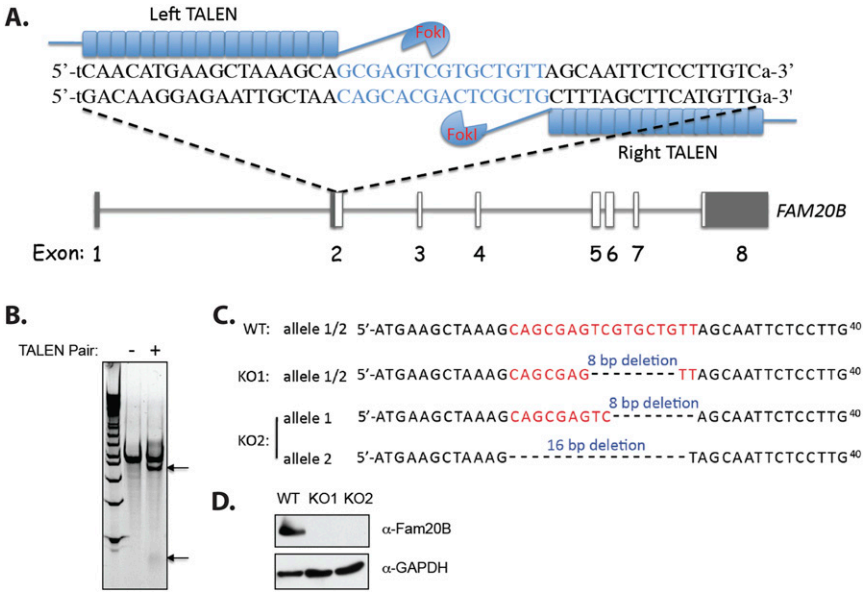

Fig. S4. Design of TALEN constructs targeting *FAM20B* and the identification of *FAM20B* knockout (KO) clones. (*A*) Exon structure of *FAM20B* and the targeting sequences of left and right *FAM20B* TALENs are illustrated. (*B*) Surveyor assay gel shows the cutting efficiency of the designed *FAM20B* TALENs. (*C*) *FAM20B* KO clones mutations were detected by sequencing analysis. The wild-type (WT) *FAM20B* sequence is shown on *Top* with the spacer region between the left and right TALENs highlighted in red. DNA sequences of two *FAM20B* KO clones that were identified show that one clone has a homozygous 8-bp deletion and the other clone has an 8-bp/16-bp deletion in the two alleles, respectively. (*D*) Fam20B immunoblotting demonstrating the lack of detectable Fam20B expression in the KO clones.

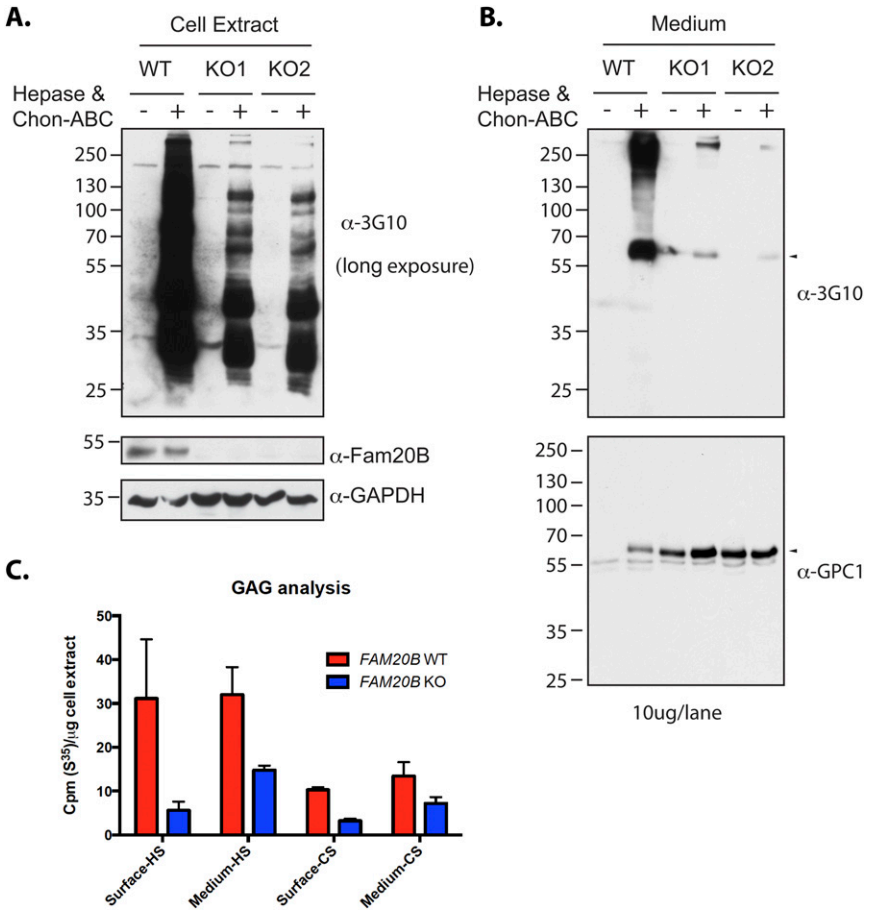

Fig. S5. *FAM20B* knockout (KO) in cells causes a dramatic decrease of CS and HS GAG chains on both cell surface and secreted proteoglycans. (*A*) Long exposure of a 3G10 immunoblot shows the neoepitope sugar stubs generated by hepase digestion of cell surface HS proteoglycans from *FAM20B* WT and KO U2OS cells. GAPDH was used as a loading control in each lane. (*B*) The 3G10 immunoblot showing the neoepitope sugar stubs on secreted proteoglycans following hepase digestion of secreted proteins from *FAM20B* WT and KO U2OS cells (*Top*). A total of 10 μg of protein derived from serum-free conditioned medium was loaded in each lane. (*Bottom*) An immunoblot of secreted glypican 1 (GPC1) in conditioned medium of WT and KO cells with/without hepase and Chon-ABC treatment. (*C*) Quantification of cell surface and secreted HS and CS GAGs from *FAM20B* WT and KO cells by [^{35}S]sulfate cell labeling. Samples were treated with pronase, Chon-ABC, and/or hepase before GAG purification using ion exchange chromatography.

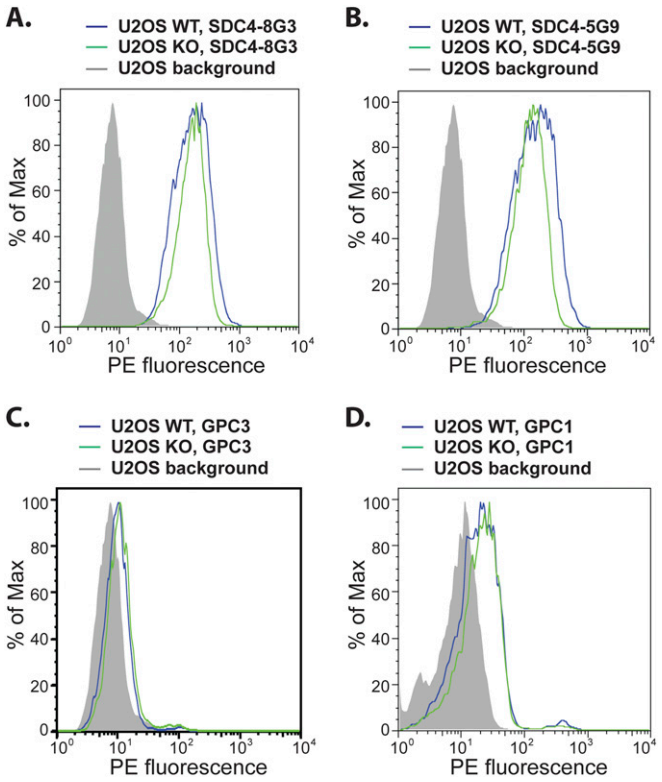

Fig. S6. *FAM20B* knockout (KO) has no effect on proteoglycan transit/secretion. Cell surface SDC4 in *FAM20B* WT and KO U2OS cells was quantified by flow cytometry using two different antibodies: 8G3 (*A*) and 5G9 (*B*). Cell surface GPC3 (*C*) and GPC1 (*D*) were quantified in *FAM20B* WT and KO U2OS cells using a single antibody specific to each protein.

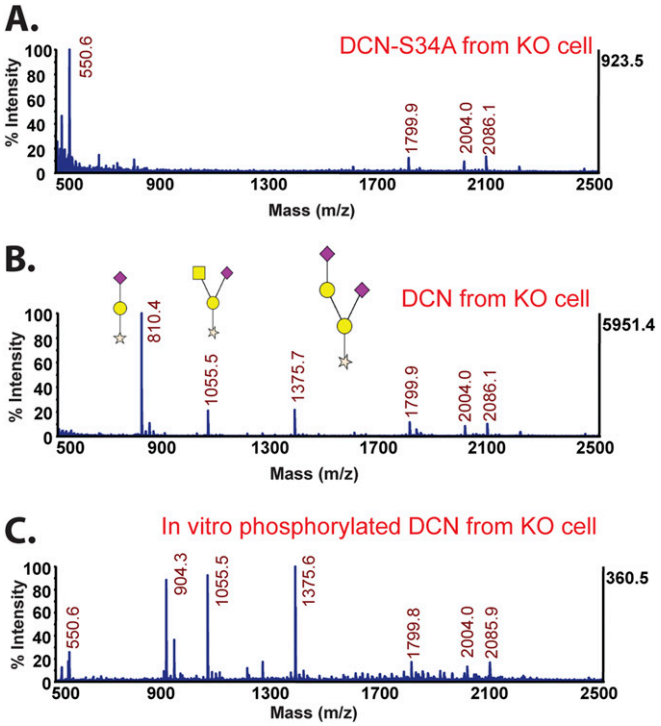

Fig. S7. Generation of Sia-Gal-Xyl on the GAG attachment site of decorin (DCN) from *FAM20B* KO cells. (*A*) Mass spectrum of permethylated glycans released from a DCN-S34A GAG-deficient mutant purified from *FAM20B* KO cells was generated by MALDI-TOF. (*B*) Mass spectrum of permethylated glycans released from DCN from *FAM20B* KO cells. The *m/z* = 810.4 ion species (Sia-Gal-Xylitol) was the dominant peak in the spectrum, which was not present in the DCN-S34A sample. The *m/z* = 1055.5 and *m/z* = 1375.5 were identified based on their molecular weight and collision induced dissociation (CID) fragments as branched glycans with a core of Gal-Xyl. Diamond, sialic acid; circle, hexose; square, *N*-acetylhexosamine. (*C*) DCN purified from *FAM20B* KO cells was first phosphorylated by Fam20B in vitro, then the O-linked glycans were released, permethylated, and analyzed by mass spectrometry. The *m/z* = 810.4 ion species was shifted to *m/z* = 904.3, which corresponds to Sia-Gal-Xyl with the addition of a permethylated phosphate. Notably, the glycans corresponding to *m/z* = 1055.5 and *m/z* = 1375.5 were not phosphorylated by Fam20B, even though they have xylose as the first sugar.

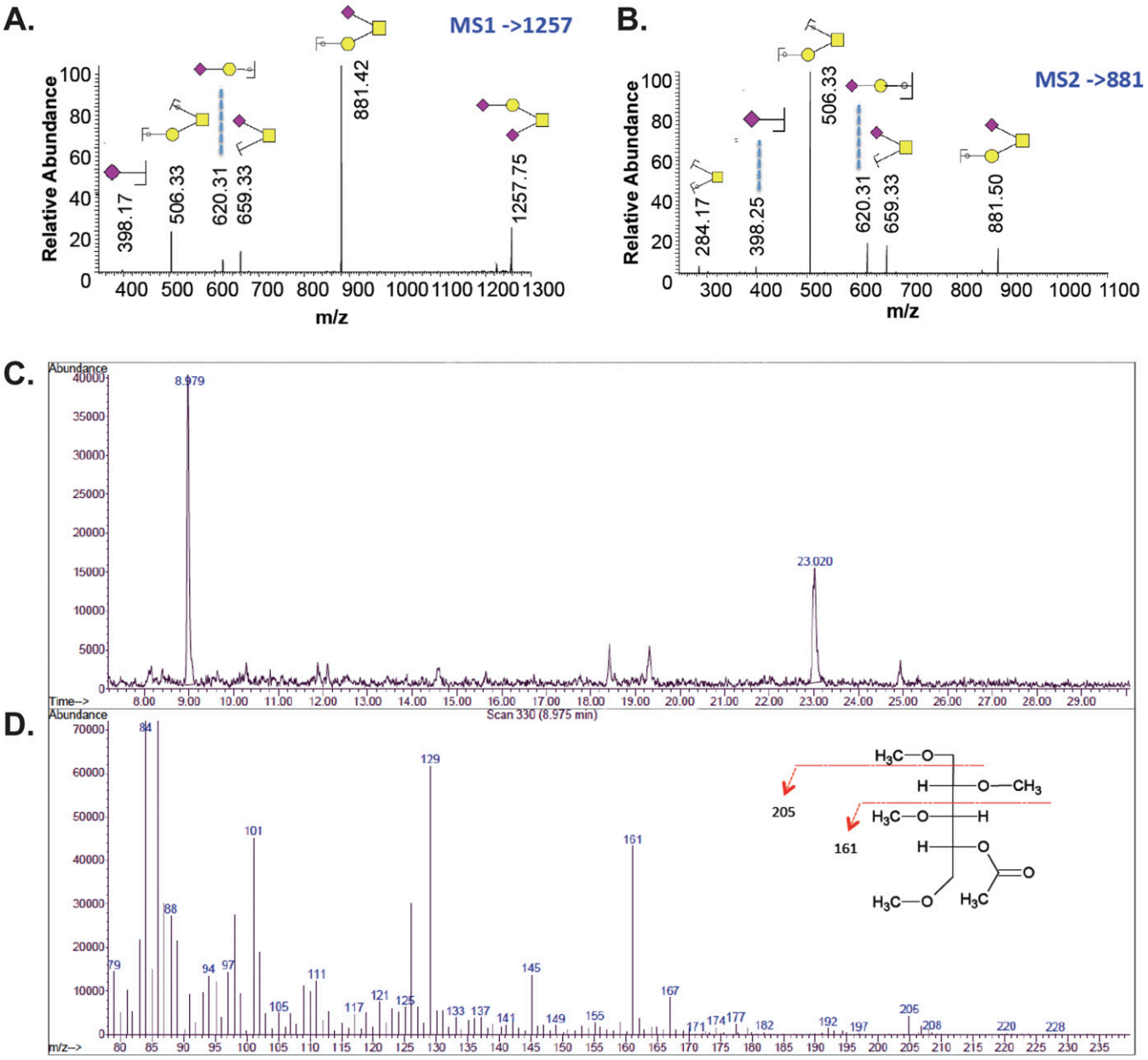

Fig. S8. Assignment of *m/z* = 1257 ion species as a branched O-glycan and GC-MS/MS identification of C4-linked xylitol on Sia-Gal-Xyl. (*A*) Fragmentation of *m/z* = 1257 ion by collision induced dissociation (CID) and the assignment of daughter ions (MS^2). (*B*) Further fragmentation of *m/z* = 881.5 ion by CID and the assignment of MS^3 ions. Diamond, sialic acid; circle, hexose; square, *N*-acetylhexosamine. (*C*) Gas chromatography profile of alditol acetates derived from hydrolyzed Sia-Gal-Xylitol and the identification of xylitol and galactose retention peaks. (*D*) Fragmentation of xylitol acetate (retention time: 8.98 min) and the identification of signature fragments of C4-linked xylitol (*m/z* = 161 and *m/z* = 205).

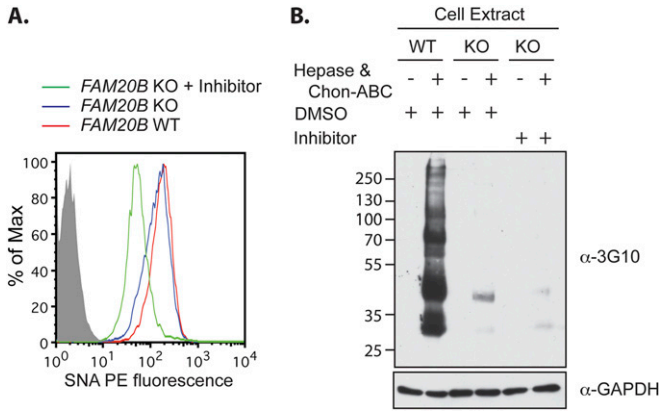

Fig. S9. Global sialyltransferase inhibition does not restore GAG elongation in *FAM20B* KO cells. (*A*) *FAM20B* KO cells were grown in DMEM with 200 mM sialyltransferase inhibitor ($3F_{ax}$-peracetyl *N*-acetylneuraminic acid) for 8 d. The efficacy of the treatment was assayed by quantifying cell surface exposed Sia-Gal using biotinylated *Sambucus nigra* lectin (SNA). SNA binds preferentially to sialic acid attached to terminal galactose in α2,6 and to a lesser degree, α2,3 linkage. Cell surface bound SNA was recognized by phycoerythrin (PE)-labeled streptavidin and the relative abundance of Sia-Gal is indicated by the intensity of PE fluorescence. (*B*) The 3G10 blot of *FAM20B* KO cells treated with/without sialyltransferase inhibitor.

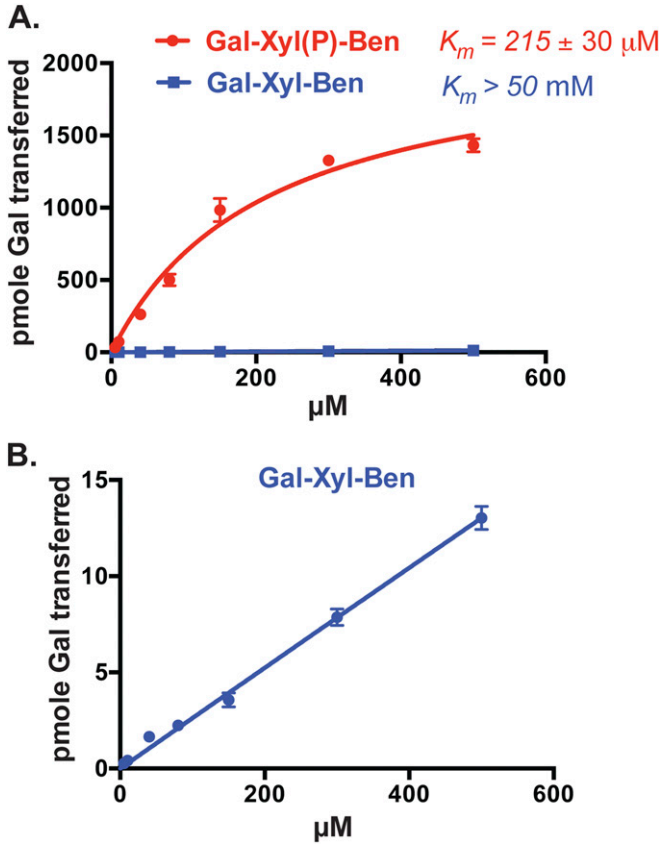

Fig. S10. Determination of kinetic parameters for recombinant MBP–GalT-II against Gal-Xyl-Ben and Gal-Xyl(P)-Ben substrates. (*A*) In vitro galactosyltransferase assays were carried out with MBP–GalT-II using unphosphorylated and Gal-Xyl-Ben and Fam20B-phosphorylated Gal-Xyl(P)-Ben substrates. The data are represented graphically as pmol Gal transferred vs. substrate concentration. The respective K_m values determined for each substrate are indicated. (*B*) Galactosyltransferase activity of MBP–GalT-II against unphosphorylated Gal-Xyl-Ben substrate with expanded *y* axis.