Supplementary Results

Systemic inactivation of Hs2st in mice. The construction of the conditional loxP-flanked allele of *Hs2st* is described in Experimental Procedures. The gene was inactivated in oocytes by crossbreeding *Hs2st*$^{f/+}$ mice with transgenic mice bearing *Zp3Cre*, in which the expression of Cre recombinase is under control of the regulatory sequences of the mouse zona pellucida 3 gene (1). *Zp3Cre* is exclusively expressed in the growing oocyte prior to the completion of the first meiotic division. Thus, Cre expression resulted in recombination and inactivation of the single copy of *Hs2st*^{f} allele, generating the *Hs2st*$^{-}$ mutant allele, and heterozygous offspring (*zp3Hs2st*$^{+/-}$*)*. *zpHs2st*$^{+/-}$ mice thrived, reproduced normally, and were indistinguishable in health and behavior from wild-type littermates. Interbreeding of *zp3Hs2st1*$^{+/-}$ mice yielded pups in the expected Mendelian ratio (24% +/+, 53% f/+, and 23% f/f, n= 53) at birth, but *zp3Hs2st*$^{-/-}$ neonates died within a day after birth. Gross anatomical evaluation of the mice showed complete kidney agenesis (n = 10). The developmental defect and neonatal lethality of *zp3Hs2st*$^{-/-}$ mice was indistinguishable to that reported for *Hs2st*$^{gt/gt}$ mice (2), indicating that the *Hs2st*$^{f/f}$ allele can be efficiently inactivated by Cre-recombinase in vivo.

Zp3Hs2st$^{-/-}$ *mice exhibit reduced 2-O-sulfotransferase activity* - To establish the consequence of inactivating Hs2st on heparan sulfate 2-O-sulfotransferase activity, a cell free homogenate was prepared from whole neonate mouse bodies and assayed for Hs2st activity using [^{35}S]PAPS as sulfate donor and 2-O-desulfated heparin as acceptor. The assay was proportional to time up to 60 min and to the amount of tissue protein up to 300 μg (3,4). Enzymatic activity was reduced from 3700 ± 650 cpm in the wild-type (mean ± SD, n= 3) to 700 ± 300 cpm, which was just above the background determined from assays performed in the absence of extract (**Fig. S1**). Because the extent of gene inactivation was complete and only one gene encoding Hs2st exists in mice, the minor residual transfer activity arose presumably from transfer to sulfate to other positions in the substrate as observed previously (3).

Hs2st$^{-/-}$ *mice exhibit nearly complete absence of 2-O-sulfation* – To confirm that inactivation of Hst2st led to loss of 2-O-sulfation of heparan sulfate, glycosaminoglycans were extracted from neonates and digested to disaccharides using a blend of heparin lyases I, II, and III. The disaccharide composition was analyzed using anion exchange chromatography with post-column derivatization as described (5). The results demonstrated all four 2-*O*-sulfated disaccharides were reduced in heparan sulfate from *zp3Hs2st*$^{-/-}$ neonates as observed in *Hs2st*$^{gt/gt}$ mice (6). A small amount of disaccharide with a retention time consistent with D2S6 was detected, but this method does not distinguish other trisulfated disaccharides such as D0S9 (ΔUA-GlcNS3S6S) that might be present in whole animals (**Fig. S2**). LC/MS analysis of a sample demonstrated molecular ions characteristic of D2S6 (data not shown, (7)) As only one gene encoding Hs2st is thought to occur in vertebrates (8), these findings suggest the possibility of a small contribution of heparan sulfate from a maternal source. Quantitation of the individual disaccharides showed that 2-O-sulfate groups were reduced from 21/100 disaccharides in the wild-type (*zp3HS2st*$^{+/+}$) to 1.8/100 disaccharides in the *zp3Hs2st*$^{-/-}$ mutant, showing >90% reduction of 2-O-sulfate in *zpHS2st*$^{-/-}$ HS (**Fig. S2**).

Supplementary Experimental Procedures

Heparan sulfate 2-O-Sulfotransferase Assay - Two embryos from each mouse genotype (zp3Cre*Hs2st*$^{+/+}$, zp3Cre*Hs2st*$^{+/-}$ *and zp3CreHs2st*$^{-/-}$) were homogenized (Polytron tissue homogenizer) in 50 mM MES buffer (pH 6) containing protease inhibitors (Sigma). Each sample was centrifuged and the supernatant was frozen at -80 ^{o}C. Hs2st enzymatic activity was measured as reported previously (3). Briefly, the assay (25 µl) contained 50 mM MES (pH 6.5), 1% TX-100, 10 mM $MgCl_2$, 10 mM $MnCl_2$, 5 mM $CaCl_2$, 87.5 mM NaF, [^{35}S]PAPS (~2.7 x 10^5 cpm/nmol), 1 µg of 2-O-desulfated heparin as an acceptor and 150 µg cell protein. The reaction was incubated for 1 h at 37 ^{o}C and stopped by quenching with 0.1 M EDTA. Shark cartilage chondroitin sulfate was added (2 mg) as carrier and the volume was increased to 500 µl with water. The sample was applied to 0.25-ml column of DEAE-Sephacel in a disposable polypropylene tips as described (9). The column was washed with 10 ml of 0.25 M NaCl, 20 mM sodium acetate (pH 6.0) and eluted with 2.5 ml of a 1 M NaCl in 20 mM sodium acetate buffer (pH 6.0). An aliquot (1 ml) was counted by liquid scintillation (Ultima Gold X-R, Packard BioScience). *P. Chrysogenum* APS Kinase was a kind gift from Dr. Irwin Segel, University of California, and was used to prepare [^{35}S]PAPS as described (10).

Heparan sulfate analysis. Heparan sulfate was isolated from neonatal mice (Experimental Procedures) and subjected to exhaustive enzymatic digestion with 2.5 mU heparin lyases I, II, and III for 16 h at 37°C in 100 mM sodium acetate (pH 7.0) containing 0.1 mM $CaCl_2$. The reaction was stopped by heat inactivation. Samples were filtered through Microcon centrifugal filters (Microcon YM-10, Millipore), dried, resuspended in 100 µl water and analyzed by HPLC using an anion-exchange ProPac PA1 column (CarboPacTM PA1, DIONEX) with post-column derivatization with 2-cyanoacetamide (5). For LC/MS analysis, the digests were lyophilized and reconstituted in water, ion pairing reagent dibutylamine and then analyzed by LC/MS as reported earlier (7).

Supplementary Figure Legends

Supplementary Figure S1. Heparan sulfate 2-O-sulfotransferase enzyme activity in embryos. Cell-free extracts were prepared from whole neonates of the indicate genotype and assayed for heparan sulfate 2-O-sulfotransferase activity using 2-O-desulfated heparin as substrate. Inactivation of *Hs2st* results in dramatically reduced enzyme activity.

Supplementary Figure S2. Heparan sulfate disaccharide analyses in embryos. Heparan sulfate was extracted from neonates and depolymerized with heparin lyases. The individual disaccharides were analyzed by GRIL-LC/MS (Experimental Procedures) and designated as: D0H0, ΔUA-GlcNH$_2$; D0A0, ΔUA-GlcNAc; D0H6, ΔUA-GlcNH$_2$-6S; D0S0, ΔUA-GlcNS; D0A6, ΔUA-GlcNAc6S; D2H6, ΔUA2S-GlcNH$_2$6S; D0S6, ΔUA-GlcNS6S; D2S0, ΔUA2S-GlcNS; D2A6, ΔUA2S-GlcNAc6S; D2S6, ΔUA2S-GlcNS6S, where ΔUA = 4,5-unsaturated uronic acid (11).

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