

QSulf1 remodels the 6-O sulfation states of cell surface heparan sulfate proteoglycans to promote Wnt signaling

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The 6-O sulfation states of cell surface heparan sulfate proteoglycans (HSPGs) are dynamically regulated to control the growth and specification of embryonic progenitor lineages. However, mechanisms for regulation of HSPG sulfation have been unknown. Here, we report on the biochemical and Wnt signaling activities of QSulf1, a novel cell surface sulfatase. Biochemical studies establish that QSulf1 is a heparan sulfate (HS) 6-O endosulfatase with preference, in particular, toward trisulfated IdoA2S-GlcNS6S disaccharide units within HS chains. In cells, QSulf1 can function cell autonomously to remodel the sulfation of cell surface HS and promote Wnt signaling when localized

either on the cell surface or in the Golgi apparatus. QSulf1 6-O desulfation reduces XWnt binding to heparin and HS chains of Glypican1, whereas heparin binds with high affinity to XWnt8 and inhibits Wnt signaling. CHO cells mutant for HS biosynthesis are defective in Wnt-dependent Frizzled receptor activation, establishing that HS is required for Frizzled receptor function. Together, these findings suggest a two-state “catch or present” model for QSulf1 regulation of Wnt signaling in which QSulf1 removes 6-O sulfates from HS chains to promote the formation of low affinity HS–Wnt complexes that can functionally interact with Frizzled receptors to initiate Wnt signal transduction.

Introduction

Heparan sulfate proteoglycans (HSPGs)* are major components of the extracellular matrix that regulate transmission of developmental signals and also are implicated in the pathophysiology of diseases, including cancer, in which signals and tissue interactions malfunction (Selva and Perrimon, 2001; Nybakken and Perrimon, 2002). HSPGs are classified as soluble and membrane-intercalated subtypes such as Glypicans and Syndicans, which are composed of a core protein decorated with covalently linked heparan sulfate (HS) chains (Bernfield et al., 1999). HS chains are polysaccharides that are synthesized in the Golgi apparatus and contain repeating disaccharide units of uronic acid linked to glucosamine (Bernfield et al., 1999; Prydz and Dalen, 2000). The

disaccharide units are selectively sulfated at the N, 3-O, and 6-O positions of glucosamine and the 2-O position of uronic acid residues by actions of sulfotransferases in the Golgi apparatus. After biosynthesis, HSPGs are secreted to the cell surface or the extracellular matrix, where they have signaling and matrix functions (Bernfield et al., 1999; Nybakken and Perrimon, 2002). Cell surface HSPGs are also shredded and/or internalized by an endocytosis pathway involving HS degradation by catabolic enzymes, including exosulfatases for removal of terminal sulfates on sugar residues (Yanagishita and Hascall, 1992; Bai et al., 1997).

The extracellular signaling activities of HSPGs are mediated by their HS chains, which bind a diversity of developmental signaling ligands (Nybakken and Perrimon, 2002; Rapraeger, 2002). The sulfation states of HS chains influence their interactions with signaling molecules. FGF signal transduction is dependent on the sulfation of 2-O and 6-O positions on HS chains. The 2-O sulfation is required for bFGF binding to heparin and 6-O sulfation for bFGF-dependent dimerization and activation of the FGFR1 receptor, as revealed by both biochemical (Pye et al., 2000; Jemth et al., 2002) and the crystal structure studies of FGF–FGFR1–heparin ternary complexes (Schlessinger et al., 2000). Wnt (Wingless [Wg])

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*Abbreviations used in this paper: AP, alkaline phosphatase; CS, chondroitin sulfate; GAG, glycosaminoglycan; GlcNR6ase, GlcNR 6-O sulfatase; HS, heparan sulfate; HSPG, heparan sulfate proteoglycan; IR, immunoreactivity; Wingless, Wg.

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signaling also is controlled by HS sulfation. The *Drosophila sulfateless* gene encodes an HS N-deacetylase/N-sulfotransferase, and *sulfateless* mutants are completely deficient in HS sulfation and have disrupted Wg signaling (Lin and Perrimon, 1999; Toyoda et al., 2000). Furthermore, chlorate, which is a metabolic inhibitor of HS sulfation, blocks Wnt (Wg) signaling in *Drosophila* and mammalian cultured cells (Reichsman et al., 1996; Dhoot et al., 2001). Therefore, the signaling activities of HSPGs in extracellular signaling are regulated by HSPG sulfation.

HS sulfation is dynamically regulated and tissue specific. In particular, the 6-O sulfates of HSPGs are precisely positioned, leading to microheterogeneity along the length of HS chains (Brickman et al., 1998; Merry et al., 1999; Saifayan et al., 2000). Changes in HSPG 6-O sulfation have been correlated with regulatory changes in FGF signaling during neural development and tumor transformation (Brickman et al., 1998; Jayson et al., 1999). How the heterogeneous sulfation patterns of HSPGs are generated and dynamically maintained during the development has not previously been known. Most enzymes involved in Golgi-based HS biosynthesis and lysosomal HS degradation appear to be constitutively expressed in different tissues (Prydz and Dalen, 2000), and it has so far been difficult to pinpoint their roles as HS sulfation regulators. Furthermore, previously characterized HS sulfatases are exosulfatases that remove terminal sulfates from HS chains (Kresse et al., 1980; Raman et al., 2003) and, therefore, cannot generate intramolecular microheterogeneity of HS sulfation. Therefore, although HS sulfation is dynamically regulated to create HS microheterogeneity on HSPGs (Lindahl et al., 1998; Esko and Lindahl, 2001), mechanisms for regulation of HSPG sulfation remain unknown.

In this paper, we report on the biochemical and Wnt signaling activities of a novel extracellular sulfatase, QSulf1, which is a candidate developmental regulator of HSPG sulfation in embryonic progenitor lineages (Dhoot et al., 2001). QSulf1 has an enzymatic domain homologous to lysosomal HS-specific GlcNR 6-O sulfatase (GlcNR6Sase), which functions in the lysosomal degradation of HS. Unlike GlcNR6Sase, QSulf1 has an NH₂-terminal secretion signal peptide and hydrophilic domain for secretion and docking the cell surface. Homologues of QSulf1 have been identified in both vertebrates and invertebrates (Dhoot et al., 2001; Morimoto-Tomita et al., 2002; Ohto et al., 2002), and a second related family member, Sulf2, has been identified in mammals (Morimoto-Tomita et al., 2002) and birds (unpublished data). QSulf1 is essential for activation of the myogenic regulator *MyoD* for specification of muscle progenitors in embryonic somites and promotes Wnt-dependent signaling in myoblasts (Dhoot et al., 2001). Mutations that disrupt an essential N-formylglycine modification in the catalytic site blocked QSulf1 function in the Wnt signaling pathway, suggesting that QSulf1 functions as an enzymatically active sulfatase. We now show that QSulf1 is an HS-specific 6-O endosulfatase with a high degree of substrate specificity for 6-O-sulfated disaccharides of HS chains of HSPGs, including Glypican1, which is required for Wnt signaling (Lin and Perrimon, 1999; Tsuda et al., 1999; Baeg et al., 2001). QSulf1 localized on the cell surface or targeted

in the Golgi apparatus is functionally active in remodeling the 6-O sulfation states of HSPGs on the cell surface and promotes Wnt signaling. Biochemical and cell biological studies of Wnt–HS binding and Frizzled receptor activity reveal that QSulf1 functions as part of a two-state “catch or present” mechanism to regulate Wnt signaling, specifically to modulate the binding affinity of Wnts to HS chains on HSPGs to promote the HS-mediated presentation of Wnt ligand to its Frizzled receptor to initiate signaling.

Results

QSulf1 is an HS-specific sulfatase

QSulf1 has sequence homology with the catalytic domain of GlcNR6Sase, a 6-O exosulfatase involved in the lysosomal catabolism of HS. Mutation of a critical Cys89 in the QSulf1 catalytic domain blocks its Wnt signaling activity, suggesting that QSulf1 is an enzymatically active sulfatase (Dhoot et al., 2001). To investigate the enzymatic activity of QSulf1 on sulfated glycosaminoglycan (GAG) substrates, 293 cells were metabolically labeled with [³⁵S]SO₄, and high molecular mass, ³⁵S-labeled GAGs were isolated for enzymatic analysis. Myc-tagged QSulf1 expressed by transient transfection of 293 cells was isolated and purified 15-fold by affinity purification on Myc beads, as determined by Western blot analysis (unpublished data). QSulf1 in cell lysates and Myc bead-purified preparations was incubated with [³⁵S]GAGs and then assayed for [³⁵S]SO₄ release using a spin column method to monitor enzyme activity. As controls, cell extracts were prepared from 293 cells transfected with either empty expression vector or a vector expressing mutant QSulf1(C-A), which has Ala substitutions at Cys89,90 to block N-formylglycine modification, which is required for catalytic activity of all sulfatases (Schmidt et al., 1995). Wild-type QSulf1 was enzymatically active and released ~5% of [³⁵S]SO₄ from the [³⁵S]GAG substrate (Fig. 1 A). QSulf1 activity is optimal at pH 7.5 (Fig. 1 F), requires Mg²⁺, is enhanced by Pb²⁺, and is inhibited by 25 mM phosphate or sulfate. Control cell extracts prepared from cultures transfected with pAG empty vector or mutant QSulf1(C-A) were inactive in [³⁵S]SO₄ release, confirming the requirement of that Cys89,90 and N-formylglycine modification for enzyme activity and showing that cell extracts do not have measurable endogenous sulfatase activities. As GAGs are a mixture of HS and chondroitin sulfate (CS), [³⁵S]GAGs were differentially treated with heparinases I and II and chondroitinase ABC to selectively deplete HS or CS, which accounted for 78 and 22% of radioactivity in [³⁵S]GAG preparations, respectively. QSulf1 was fully active in [³⁵S]SO₄ release from HS-enriched [³⁵S]GAGs (Fig. 1 B) and was inactive on CS-enriched [³⁵S]GAGs (Fig. 1 C), establishing that QSulf1 is an HS sulfatase. QSulf1 is also active in HS desulfation on metabolically labeled [³⁵S]Glypican1 (Fig. 1 D), a cell surface HSPG that mediates Wnt signaling (Lin and Perrimon, 1999; Tsuda et al., 1999; Baeg et al., 2001), and, therefore, is the likely cellular substrate for QSulf1.

QSulf1 is a 6-O endosulfatase

To define the substrate specificity of QSulf1, we assayed radiolabeled disaccharide products generated by deaminative cleavage of [³⁵S]GAG preparations (Shively and Conrad,

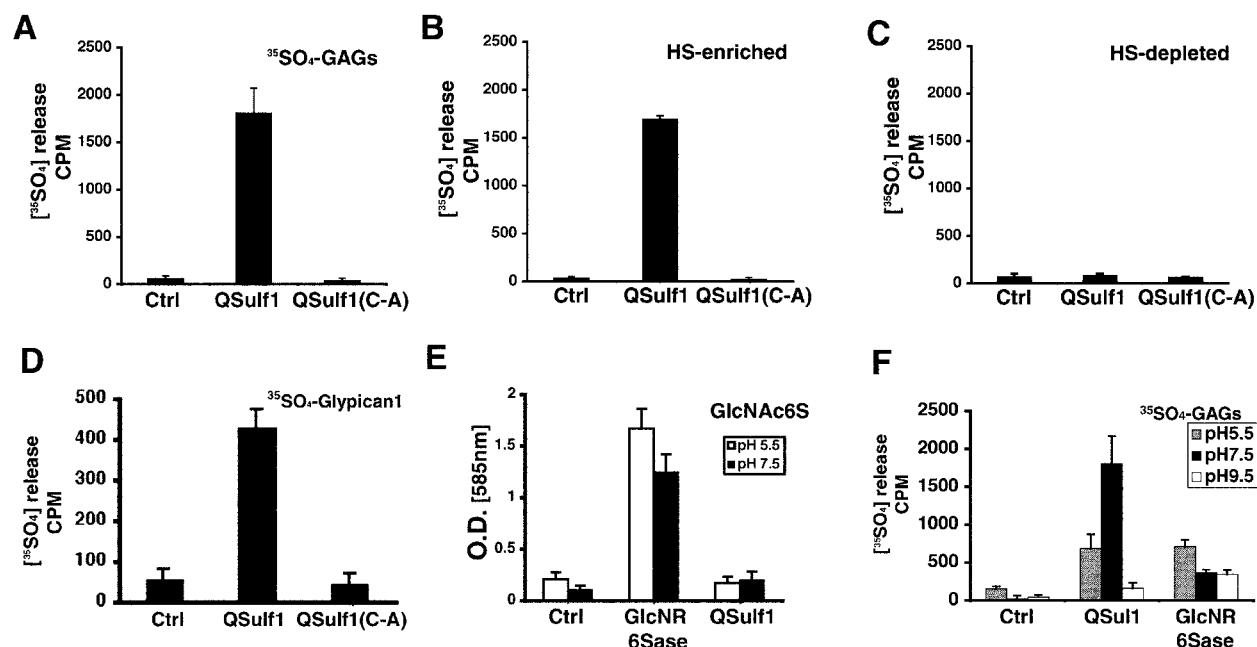


Figure 1. QSulf1 is an HS sulfatase with substrate specificities distinct from lysosomal GlcNR6Sase. (A) QSulf1 is enzymatically active in ^{35}S release from metabolically labeled ^{35}S GAGs. ^{35}S GAGs were reacted overnight (16 h) with QSulf1 in cell lysate. Myc bead-purified QSulf1 is similarly active. Control lysates were prepared from 293 cells transfected with either pAG empty vector (Ctrl) or a catalytic mutant (QSulf1[C-A]-MycHis). ^{35}S release was assayed by spin column fractionation of reaction products and quantitation by scintillation counting. QSulf1 released $\sim 5\%$ of total radioactivity from the ^{35}S GAGs, whereas control extracts had no activity. (B) QSulf1 desulfates HS-enriched GAGs. CS was depleted from ^{35}S GAG preparations by enzymatic digestion with chondroitinase ABC, which eliminated $\sim 22\%$ of the labeled GAGs. QSulf1 released $\sim 7\%$ of the ^{35}S from HS-enriched GAGs, equivalent to the loss from total ^{35}S GAG substrates. (C) QSulf1 does not desulfate CS-enriched GAGs. HS was depleted from ^{35}S GAG preparations by enzymatic digestion with heparinases I and II. QSulf1 is inactive in ^{35}S release from CS-enriched GAG substrate. (D) Glypican1 is a substrate for QSulf1. ^{35}S Glypican1-Myc was prepared by metabolically labeling Glypican1-Myc-transfected cells with ^{35}S , followed by Myc bead purification, and subsequently used as the substrate in the enzymatic assay. QSulf1 released ^{35}S from ^{35}S Glypican1, whereas control extracts from 293 cells transfected with pAG empty vector or catalytic mutant QSulf1(C-A)-MycHis plasmids had no ^{35}S release activity. (E) QSulf1 is inactive in 6-O sulfate release on monosaccharide GlcNAc6S substrates. GlcNAc6S was reacted with control, QSulf1, or lysosomal GlcNR6Sase at pH 5.5 and 7.5, in the presence of PbCl_2 . QSulf1 did not desulfate GlcNAc6S, whereas lysosomal GlcNR6Sase was highly active at both pHs. (F) QSulf1 showed pH optima at pH 7.5. Metabolically labeled ^{35}S GAGs were reacted overnight (16 h) with Ctrl, QSulf1, or lysosomal GlcNR6Sase at pH 5.5, 7.5, and 9.5. QSulf1 was most active in ^{35}S release at pH 7.5, whereas GlcNR6Sase showed low or little activity toward ^{35}S GAGs under these pHs.

1976; Rong et al., 2001) after incubation with wild type and (C-A) mutant forms of QSulf1 and with control cell extracts. Four major ^{35}S disaccharide products were resolved by HPLC chromatography: GlcA-GlcNS6S, IdoA-GlcNS6S, IdoA2S-GlcNS, and IdoA2S-GlcNS6S (Table I). QSulf1 is preferentially active in the 6-O desulfation of IdoA2S-GlcNS6S units, with less pronounced effect on GlcA-GlcNS6S and no detectable effect on IdoA-GlcNS6S (Table I). In these assays, GlcA-GlcNS6S and IdoA2S-GlcNS6S are significantly decreased as a percentage of total radioactivity by ~ 20 and $\sim 32\%$, respectively, with a parallel $\sim 50\%$ increase of the reaction product IdoA2S-GlcNS. QSulf1 did not desulfate IdoA-GlcNS6S, even when enzyme reactions were performed for extended times. These findings show that QSulf1 is an HS-specific 6-O sulfatase with substrate specificity for a subset of 6-O-sulfated disaccharide substrates in HS. The disaccharide analyses also establish that QSulf1 is an endosulfatase, based on the quantitative extent of its activity on HS chains and its specificity for selected 6-O disaccharide substrates located at internal sites along HS chains. In contrast to the lysosomal GlcNR6Sase, which functions exclusively as an exoenzyme on nonreducing-terminal

GlcNR6S residues (Kresse et al., 1980), QSulf1 desulfates GlcNS6S residues located on the reducing side of GlcA units, hence functioning in an endolytic mode (Table I). Furthermore, QSulf1 does not desulfate the monosaccharide substrate N-acetylglucosamine 6-O sulfate, consistent with its activity as an endosulfatase, in contrast to the lysosomal exosulfatase GlcNR6Sase, which actively hydrolyzes this monosaccharide substrate both at pH 5.5 and 7.5 (Reissig et al., 1955; He et al., 1993) (Fig. 1 E). QSulf1 is also significantly more active in ^{35}S release from ^{35}S GAGs than is lysosomal GlcNR6Sase at both pH 5.5 and 7.5 (Fig. 1 F), further distinguishing their activities.

QSulf1 remodels the 6-O sulfation state of HS cell surface in vivo

To investigate whether QSulf1 is enzymatically active in vivo, we tested the effects of QSulf1 expression on 10E4 antibody immunoreactivity (IR) of cell surface HSPGs. 10E4 antibody specifically recognizes sulfated N-acetylglucosamine residues (David et al., 1992; Yip et al., 2002), and 10E4 IR to cell surface HSPGs is sensitive to chlorate treatment at concentrations that preferentially block 6-O sulfa-

tion (Safaiyan et al., 1999; Yip et al., 2002). 80% of 3T3 cells have cell surface 10E4 IR, as determined using a live cell staining assay, and chlorate treatment reduces 10E4 IR to <20% of cells (Fig. 2, A and B). Transfected QSulf1 also reduces 10E4 IR to a similar extent, whereas enzymatically inactive QSulf1(C-A) has no effect. Significantly, QSulf1 does not disrupt 10E4 IR on immediately adjacent cells (Fig. 2 A), even though QSulf1 is abundant on the surface of expressing cells. These observations indicate that QSulf1 functions cell autonomously to remodel the sulfation states of HSPGs on expressing cells. QSulf1 expression does not disrupt the sulfation state of CS on the cell surface, as as-

sayed by live cell staining of QSulf1-transfected cells with a CS-specific antibody, CS56 (Avnur and Geiger, 1984), which recognizes sulfated epitopes also removed by chlorate treatment (Yip et al., 2002) (Fig. 2 C). These findings support the specificity of QSulf1 for HS, as observed in biochemical studies (Figs. 1, B and C).

QSulf1 also is an active 6-O endosulfatase *in vivo* with similar disaccharide substrate specificities as observed in biochemical assays (Table I; Fig. 3). [³⁵S]GAGs were prepared from 293 cells that were stably transfected with wild-type and C-A mutant QSulf1 and metabolically labeled with [³⁵S]SO₄. Disaccharide analysis shows that labeled GlcA-

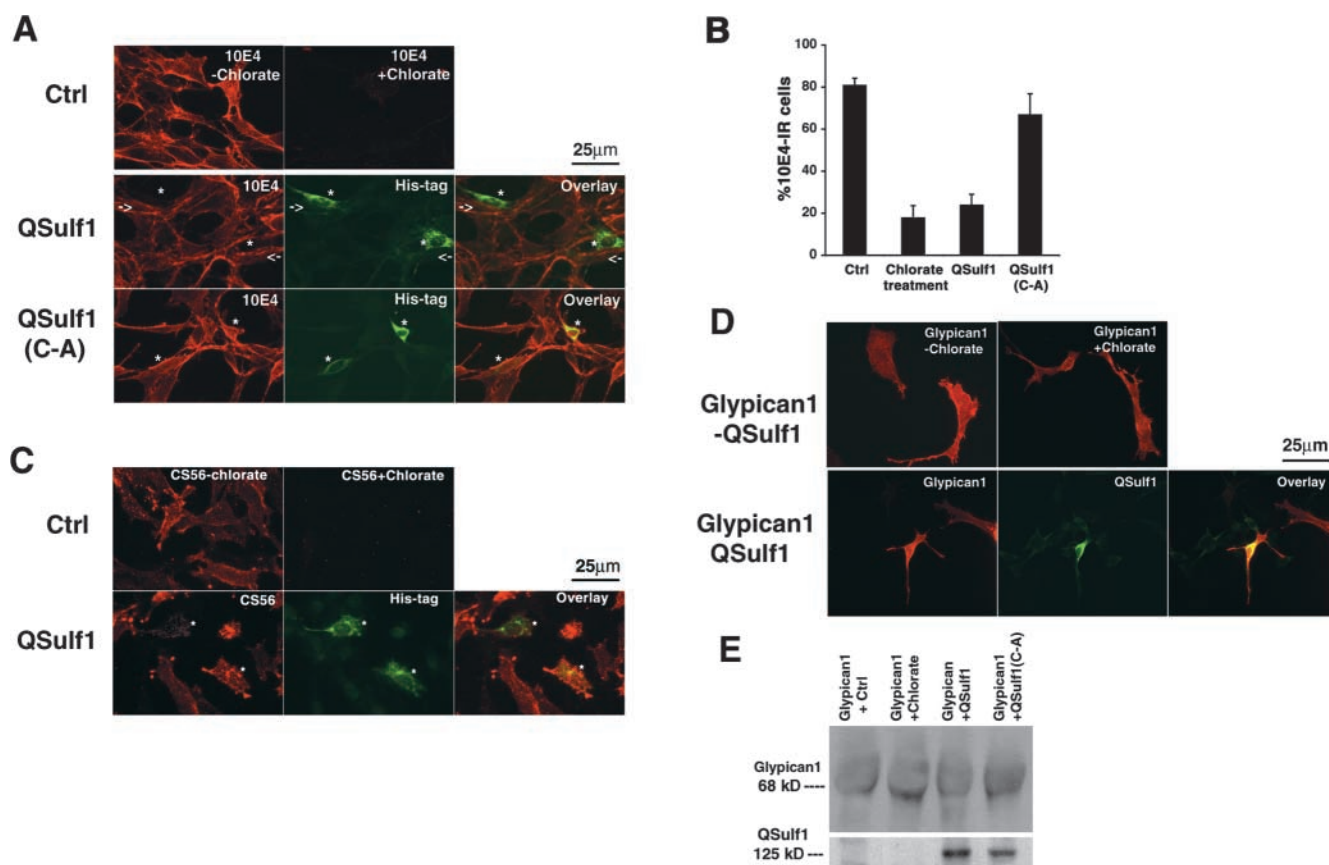


Figure 2. QSulf1 desulfates cell surface HSPGs in living cells without affecting the stability of HSPG core proteins. (A) QSulf1 and chlorate desulfate cell surface HS, as assayed by IR to 10E4 antibody. Control 3T3 cells were transfected with empty vector, cultured with or without 25 mM chlorate to block sulfation, and then live cell stained with 10E4 antibody, and antibody reactivity was assayed by fluorescence microscopy. A majority of untransfected cells (Ctrl) express 10E4 IR on the cell surface, and chlorate treatment removes 10E4 IR. Cells transfected with QSulf1–MycHis or catalytic mutant QSulf1(C-A)–MycHis were live stained for extracellular 10E4 IR and then permeabilized to assay QSulf1 or QSulf1(C-A) with a His antibody. QSulf1-expressing cells (QSulf1) lose cell surface 10E4 IR, whereas QSulf1(C-A)-expressing cells remain immunoreactive, as shown in the overlay. Note that QSulf1 expression does not alter 10E4 IR on adjacent cells. An asterisk marks transfected cells and an arrow marks cells adjacent to QSulf1-expressing cells. (B) Quantitative analysis of the effects of QSulf1 expression and chlorate treatment on 10E4 IR. Cells stained with 10E4 were counted, and the percentage of 10E4 IR cells was calculated as the percent of total cells that were 10E4 stained in the control assay (Ctrl), or as the percent of transfected cells that expressed either QSulf1 or QSulf1(C-A). (C) QSulf1 expression does not alter the sulfation of cell surface CS. Extracellular CS was visualized with CS56 antibody in untransfected control (Ctrl) cultures treated with or without chlorate or in QSulf1-transfected cultures. An asterisk marks the cells transfected with QSulf1. Assays were conducted in duplicate in three independent experiments, counting >100 cells in each assay. (D) The protein core of Glypican1 remains on the cell surface of QSulf1-expressing cells and chlorate-treated cells. 3T3 cells cotransfected with Glypican1–Myc and untagged QSulf1 were live cell stained with Myc antibody to detect cell surface Glypican1–Myc. Cells were then permeabilized and immunostained for QSulf1 expression with QSulf1 antibody. Control cells were cotransfected with Glypican1–Myc and pAG empty vector plasmids, with or without chlorate treatment, followed by live cell staining to assay extracellular Glypican1–Myc. Similar Glypican1 staining was detected in control and QSulf1-transfected cells. (E) QSulf1 expression and chlorate treatment do not alter the stability or gel mobility of Glypican1. Western blot assays of cell extracts from 293 cells cotransfected with Glypican1–Myc, with pAG empty vector (Ctrl), QSulf1, or QSulf1(C-A) plasmids. Western blots were probed with anti-Myc and anti-QSulf1 antibodies.

Table I. Summary of disaccharide analysis

Heparan sulfate	O-[³⁵ S]-sulfated disaccharides (% of total O-[³⁵ S]-sulfated disaccharides)			
	GlcA-GlcNS6S (GMS)	IdoA-GlcNS6S (IMS)	IdoA2S-GlcNS (ISM)	IdoA2S-GlcNS6S (ISMS)
QSulf1 treatment	13.0 ± 1.0 ^a	6.3 ± 1.2	50.7 ± 3.1 ^a	30.0 ± 4.6 ^a
QSulf1(C-A) treatment	16.0 ± 2.0	5.0 ± 1.0	35.3 ± 4.5	43.7 ± 2.3
Untreated control	19.0 ± 3.5	4.8 ± 1.8	33.0 ± 4.2	43.5 ± 0.7
QSulf1-expressing cells	14.0 ± 1.0 ^b	11.0 ± 3.6	58.0 ± 3.0 ^a	17.0 ± 3.6 ^a
QSulf1(C-A)-expressing cells	16.3 ± 2.0	7.7 ± 2.5	38.0 ± 8.6	38.0 ± 10.1

[³⁵S]HS prepared from metabolically labeled 293T cells was reacted with Myc bead-purified QSulf1 or catalytic mutant QSulf1(C-A). Untreated control was [³⁵S]HS without treatment. To test whether QSulf1 functions in vivo, [³⁵S]HS was prepared from stable 293T cell lines expressing QSulf1 or QSulf1(C-A) by metabolic labeling with [³⁵S]SO₄. ³⁵S-labeled disaccharides were generated by deaminative cleavage of HS, and reaction products were resolved by HPLC anion exchange chromatography. The radioactivity in each disaccharide product was quantified by scintillation counting. Results are presented as mol-percent of specific disaccharide products in three independent experiments. M in disaccharide abbreviations stands for the 2,5-anhydromannitol deamination products of GlcNS residues (see also Fig. 3).

^aP < 0.05 (t test).

^bP < 0.1.

GlcNS6S and IdoA2S-GlcNS6S disaccharide units were quantitatively reduced in QSulf1-expressing cells compared with C-A mutant controls, and this decrease is accompanied by a parallel increase in IdoA2S-GlcNS. Disaccharide IdoA-

GlcNS6S was unchanged, establishing that QSulf1 has the same substrate specificity in vivo as in vitro. Compared with biochemical assays, QSulf1 in vivo generated a greater decrease of trisulfated IdoA2S-GlcNS6S disaccharide component (55% in vivo vs. 30% in vitro), suggesting that QSulf1 functions more efficiently in cells.

QSulf1 activity does not disrupt the accumulation of Glypican1 HSPG core protein, which is attached on the cell surface by a GPI linker. Immunological localization analysis shows that QSulf1-expressing 3T3 cells and chlorate-treated 3T3 cells have abundant levels of myc-tagged Glypican1 on these cell surfaces (Fig. 2 D). The electrophoretic mobility and levels of Glypican1 core protein also are unchanged in QSulf1-expressing cells, indicating that QSulf1 activity does not lead to processing or degradation of Glypican1 core proteins or its HS chains (Fig. 2 E). These findings support the conclusion that QSulf1 activity remodels the 6-O sulfation states of GlcA-GlcNS6S and IdoA2S-GlcNS6S disaccharide components of HSPGs, which are retained on the cell surface in their desulfated states.

Golgi-targeted QSulf1 is active in cell surface remodeling and Wnt signaling

During biosynthesis, QSulf1 transits through the ER and Golgi apparatus, where HSPGs are assembled (Prydz and Dalen, 2000), and transports together with HSPGs to the surface of expressing cells (Dhoot et al., 2001; Ohto et al., 2002; unpublished data). To investigate whether QSulf1 localization on the cell surface is required for its activity, we expressed Golgi-tethered (QSulf1-Golgi) and ER-tethered (QSulf1-ER) forms of QSulf1 in 3T3 cells and C2C12 myoblasts and then assayed for their biochemical and cell biological activities in remodeling the sulfation states of HS and for Wnt induction activities. Neither QSulf1-Golgi nor QSulf1-ER is detected on the cell surfaces of expressing cells (unpublished data), but they colocalize with Golgi and ER markers, respectively (Fig. 4 A). ER- and Golgi-tethered QSulf1 isolated from expressing cells are enzymatically active in biochemical assays for sulfate release using [³⁵S]GAG substrates (Fig. 4 B). QSulf1-ER protein is inactive in remodeling 10E4 IR and has little or no activity in Wnt signaling, whereas the QSulf1-Golgi protein is fully active in remodeling 10E4 IR as well as inducing Wnt signaling (Figs.

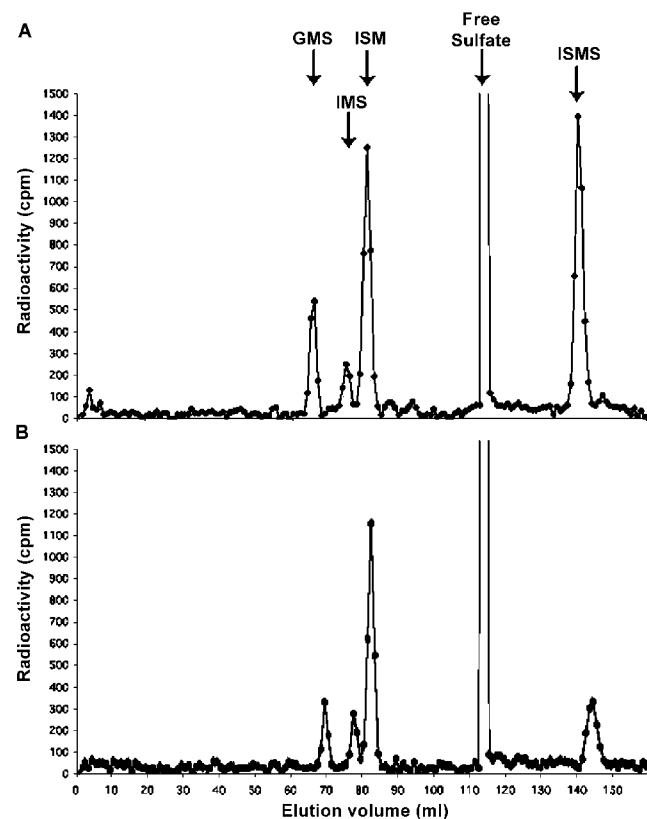
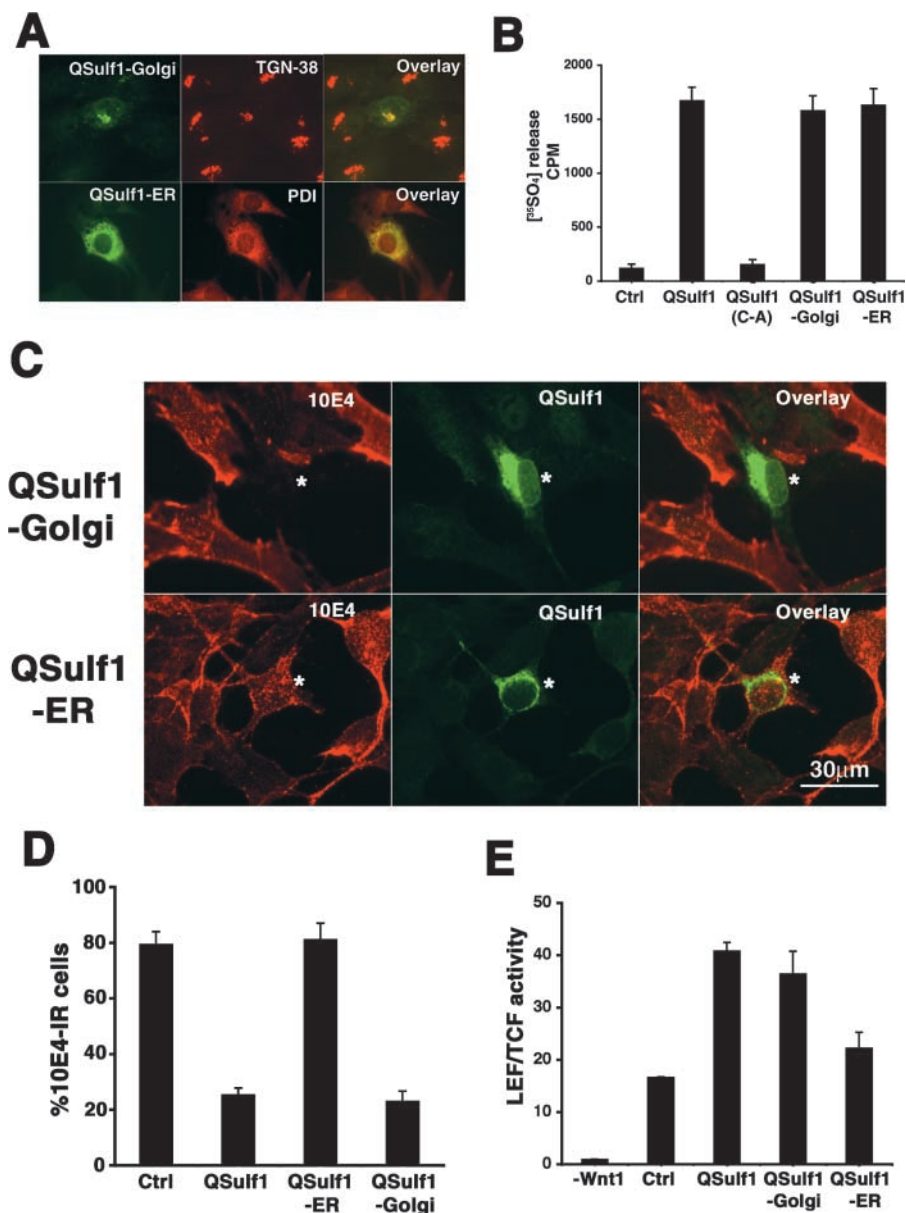


Figure 3. QSulf1 selectively removes 6-O sulfates from HS in vivo. [³⁵S]HS prepared from metabolically labeled stable 293T cell lines that expressed wild-type QSulf1 and enzymatically inactive QSulf1(C-A) mutant was prepared for disaccharide analysis. The resulting disaccharide fractions were resolved by HPLC anion exchange chromatography and analyzed as described previously. The arrows correspond to the elution positions of disaccharide components. (A) The disaccharide components of 293T cells that expressed inactive QSulf1(C-A) mutant protein. (B) The disaccharide components of QSulf1-expressing stable 293T cell lines. GMS, GlcA-GlcNS6S; IMS, IdoA-GlcNS6S; ISM, IdoA2S-GlcNS; ISMS, IdoA2S-GlcNS6S.

Figure 4. Golgi-targeted QSulf1 desulfates cell surface HSPGs and enhances Wnt1 signaling.

(A) QSulf1 targeted to the Golgi apparatus or ER colocalizes with Golgi or ER markers. 3T3 cells transfected with QSulf1-Golgi or QSulf1-ER were permeabilized and double stained with QSulf1 antibody and antibody against TGN 38 or ER resident protein PDI. QSulf1-Golgi and QSulf1-ER were localized in the Golgi apparatus and ER, respectively, as shown in overlay. (B) QSulf1 targeted to the Golgi apparatus or ER is enzymatically active on cellular HS substrate. QSulf1-Golgi and QSulf1-ER expressed in 293T cells by transient transfection were incubated with [³⁵S]GAGs overnight (16 h) and released similar amounts of radioactivity as QSulf1. (C) Golgi-targeted, but not ER-targeted, QSulf1 decreases cell surface 10E4 IR. 3T3 cells transfected with QSulf1-Golgi or QSulf1-ER were live cell stained with 10E4 antibody to assay cell surface 10E4 IR and then permeabilized and double stained with QSulf1 antibody. Expression of QSulf1-Golgi resulted in loss of 10E4 IR, whereas the expression of QSulf1-ER had no effect on 10E4 IR, as shown in overlay. An asterisk marks QSulf1-transfected cells. (D) QSulf1-Golgi expression decreases the percentage of cells with extracellular 10E4 IR. Cells stained with 10E4 were counted, and the percentage of 10E4 IR cells was calculated as the percent of total cells that were 10E4 stained in the control assay (Ctrl), or as the percent of transfected cells that expressed either QSulf1, QSulf1-Golgi, or QSulf1-ER. (E) QSulf1-Golgi enhances Wnt1 signaling activity. *Lef/TCF* luciferase reporter activity in C2C12 cells transfected with empty vector as control (Ctrl), QSulf1, QSulf1-Golgi, or QSulf1-ER. Luciferase activity was normalized to activities of extracts from control cells not induced by Wnt1.



4, C–E). These findings establish that QSulf1 plays an indirect role in promoting Wnt signal transduction by remodeling the 6-O sulfation states of extracellular HSPGs, either through its activity on the cell surface or its activity in the Golgi apparatus during HS biosynthesis.

QSulf1 regulates binding affinities of XWnt8 for HS chains and promotes an HS-dependent presentation of Wnt ligand to its Frizzled receptor

To investigate the mechanisms by which QSulf1 6-O desulfation activity promotes Wnt signaling, we first tested the activity of soluble heparin and 6-O and 2-O chemically desulfated heparin on Wnt signal transduction in cells (Fig. 5 A). For these studies, Wnt1-expressing cells were cocultured with Wnt1-responsive C2C12 cells transfected with a TCF luciferase reporter to monitor Wnt signaling activity. At a concentration of 10 μ g/ml, both soluble heparin and 2-O-desulfated heparin completely

inhibit Wnt signaling activity, whereas 6-O-desulfated heparin has no inhibitory effect (Fig. 5 A). Therefore, soluble 6-O-sulfated HS could inhibit Wnt signaling by blocking the Frizzled receptor from binding the Wnt ligand. Alternatively, 6-O-sulfated HS could bind to Wnts and prevent Wnt presentation to its Frizzled receptor. To investigate whether HS blocks Frizzled receptor activity, we compared the Wnt1 signaling activity in wild-type CHO cells that express HSPGs and in *pgsd677* mutant CHO cells that are defective in HS biosynthesis (Bai et al., 1999). Wnt1 signaling in CHO cells is dependent on expression of a transfected Frizzled 3 (mFZ3) receptor, which increases the response of these cells to Wnt1 by 12–15-fold in a coculture system with Wnt1-expressing cells, as assayed using a LEF/TCF luciferase reporter (unpublished data). CHO cells are dependent on transfected mFZ3 receptor for Wnt1 signal transduction, which makes it possible to directly monitor the requirement for

HS in the response of Frizzled receptor to Wnt signal. In these mFZ3-dependent Wnt signaling assays, wild-type and *pgsd677* mutant CHO cells were found to be inactive in Wnt1 signal transduction in the absence of Wnt1 ligand (Fig. 5 B), whereas in response to Wnt1 ligand, mFZ3-expressing wild-type CHO cells more actively respond to Wnt1 than do HS-deficient *pgsd677* cells (which have only a slightly elevated response to Wnt1 compared with unstimulated control cells) (Fig. 5 B). These findings show that HS does not act as a general repressor of ligand-independent mFZ3 receptor activity. On the contrary, they provide evidence that HS is required for efficient mFZ3 receptor activation in response to Wnt1 ligand. Therefore, soluble sulfated heparin inhibits Wnt signaling probably by binding to Wnt ligand and subsequently preventing the release of the Wnt ligand to the receptor.

To investigate whether 6-O desulfation of heparin affects Wnt binding, we developed a competition assay with heparin-agarose beads to compare the binding affinity of XWnt8 to heparin or 6-O-desulfated heparin. XWnt8 tagged with HA is soluble and biologically active in the canonical Wnt signaling pathway (Hsieh et al., 1999; Piccolo et al., 1999). In this assay, HA-XWnt8 binds with high affinity to hepa-

rin-agarose beads (unpublished data), consistent with earlier studies of Wg binding to HS (Reichsman et al., 1996). We then tested the ability of increasing concentrations of heparin, pretreated either with QSulf1 or inactive QSulf1(C-A) mutant protein, to release bound HA-XWnt8 from heparin-agarose beads, as assayed by Western blot analysis of HA-XWnt8 released into the supernatant fraction (Fig. 5 C). We found that heparin treated with inactive QSulf1 (C-A) promoted the concentration-dependent release of HA-XWnt8 from heparin beads; by contrast, QSulf1-treated heparin released only small amounts of HA-XWnt8, and this release was not concentration dependent, reflecting low affinity binding of HA-XWnt8 to 6-O-desulfated heparin (Fig. 5 C).

The activity of QSulf1 in Wnt binding to HS was also tested in XWnt8 binding to Glypican1, a cell surface HSPG that mediates Wnt signaling (Lin and Perrimon, 1999; Tsuda et al., 1999; Baeg et al., 2001). A soluble form of Glypican1 tagged with alkaline phosphatase (AP) was used in the binding assay to allow Glypican1 purification from the media of transfected cells (Chen and Lander, 2001). HA-tagged XWnt8 was incubated with the soluble Glypican1-AP, and complexes formed were immuno-

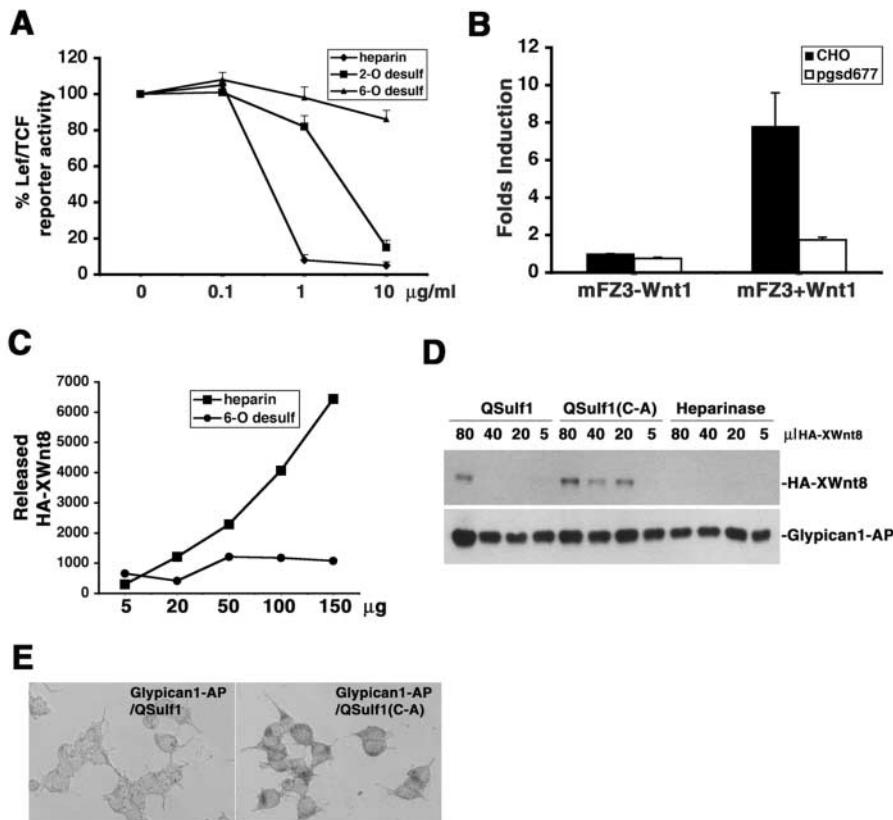


Figure 5. QSulf1 regulates the interaction between HS and Wnt ligand to promote Wnt signaling. (A) 6-O-desulfated heparin does not inhibit Wnt signaling. C2C12 cells transfected with Tcf/LEF luciferase reporter to monitor Wnt signaling activity were stimulated by Wnt1-secreting cells in the presence of heparin or chemically 2-O-desulfated or 6-O-desulfated heparin. Luciferase activity was normalized to activities from cells cultured without heparin. 6-O-desulfated heparin had no effect on Wnt signaling, whereas heparin or 2-O-desulfated heparin completely inhibited Wnt signaling activity at a concentration of 10 µg/ml. (B) HS is required for Frizzled 3 receptor activation by Wnt1. Wild-type CHO cells or HS-deficient *pgsd677* mutant CHO cells were transfected with a Frizzled 3 expression vector to initiate Wnt signaling. Expression of Frizzled 3 receptor alone did not activate Wnt signaling in wild-type CHO cells or mutant *pgsd677* cells. Mutant *pgsd677* cells are defective in Wnt1 signaling. (C) QSulf1 activity reduces the binding affinity between heparin and XWnt8. Heparin pretreated with QSulf1 or inactive QSulf1(C-A) mutant was incubated with HA-XWnt8 that was bound to heparin-agarose beads. The released HA-XWnt8 in the supernatant was

measured by Western blot. Heparin treated with inactive QSulf1(C-A) mutant released HA-XWnt8 in a concentration-dependent manner, whereas QSulf1-desulfated heparin released much less HA-XWnt8. QSulf1 treatment reduced the competitive activity of heparin for HA-XWnt8 release consistently in four experiments, although the quantitative extent of research varied. (D) QSulf1 decreased XWnt8 binding to Glypican1. Glypican1-AP was treated by QSulf1, inactive QSulf1(C-A) mutant, or heparinase and then incubated with HA-XWnt8 to allow the binding. Glypican1-AP-HA-XWnt8 complex was separated by immunoprecipitation with AP antibodies and then analyzed by Western blot. QSulf1(C-A)-treated Glypican1 bound more HA-XWnt8 than QSulf1-treated Glypican1, and the binding was abolished by heparinase treatment. (E) QSulf1 decreased the binding of Glypican1 to HA-XWnt8-expressing cells. Glypican1-AP pretreated with either QSulf1 or QSulf1(C-A) was incubated with XWnt8-expressing cells. The bound Glypican1-AP on the cell surface was visualized by AP substrate staining.

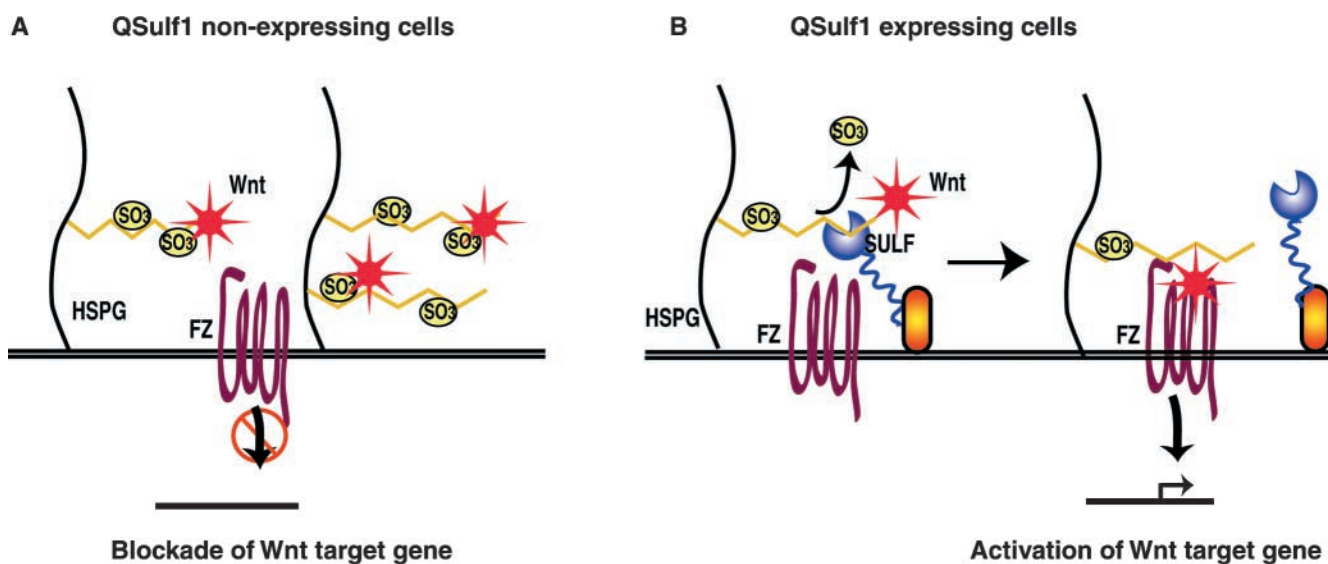


Figure 6. **A two-state catch or presentation model of QSulf1 regulation of Wnt signaling.** (A) In QSulf1-nonexpressing embryonic cells, HS chains on cell surface HSPGs are in a 6-O-sulfated state, which binds with high affinities to catch Wnt ligands, preventing functional interactions of bound Wnts with their Frizzled receptors. (B) In QSulf1-expressing cells, selective 6-O desulfatase activity of QSulf1 removes 6-O sulfates from HS chains on cell surface HSPGs to a low affinity binding state for Wnts. 6-O-desulfated HS then can present Wnt ligands to Frizzled receptor and can form functionally active Wnt-HS-Frizzled receptor complexes for initiation of Wnt signal transduction.

precipitated by agarose beads coupled with monoclonal AP antibodies, followed by probing Western blots of protein complexes with HA-specific antibodies (Fig. 5 D). Pretreatment of Glypican1 with heparinase completely blocked its binding to XWnt8, establishing that XWnt8 binding to Glypican1 is mediated through the HS chains and not the protein core. QSulf1 treatment of Glypican1-AP significantly diminished, but did not completely block, formation of HA-XWnt8 binding, compared with treatment with C-A mutant QSulf1, which did not diminish complex formation (Fig. 5 D). QSulf1 treatment also blocked the binding of Glypican1-AP to XWnt8 expressed on the surface of living transfected 293T cells, as detected by immunohistochemical detection in a cell binding assay (Hsieh et al., 1999; Wu and Nusse, 2002), whereas QSulf1(C-A) treatment did not prevent binding (Fig. 5 E). Control and C-A mutant QSulf1-treated Glypican1-AP abundantly bound to the surfaces of HA-XWnt8-expressing cells, as detected by double immunostaining with HA antibodies (unpublished data). Together, these Wnt binding studies reveal that QSulf1 6-O sulfatase activity reduces the binding of Wnt to HS on Glypican1. The results of these binding studies are consistent with a two-state catch or presentation model (Fig. 6) in which 6-O-sulfated HS on cell surface HSPGs binds Wnts in a high affinity state to “catch” Wnt ligands and compete with the binding of Wnts to Frizzled receptor (Fig. 6 A). The HS 6-O desulfatase activity of QSulf1 would convert the cell surface HS to a low affinity binding state for Wnts, which allows their HS-dependent “presentation” to the Frizzled receptors to initiate Wnt signal transduction (Fig. 6 B). By this mechanism, QSulf1, which is expressed in localized populations of somite and neural progenitor cells, would pattern Wnt signaling responses to localized populations of progenitors responding to widely dispersed Wnt signals.

Discussion

Our biochemical and cell expression studies identify QSulf1 as an HS-specific 6-O endosulfatase with substrate specificity for disulfated GlcA-GlcNS6S and trisulfated IdoA2S-GlcNS6S disaccharide components of cellular HS chains. QSulf1 does not appear to desulfate -IdoA-GlcNS6S- sequences, providing evidence for functional selectivity of QSulf1 activities. Also, QSulf1, both in vitro and in vivo, desulfates only a small fraction of GlcA-GlcNS6S disaccharide residues, suggesting that QSulf1 is active with only a subset of GlcA-GlcNS6S residues in HS chains. Compared with its low activity on GlcA-GlcNS6S residues, QSulf1 induces appreciable 6-O desulfation of trisulfated IdoA2S-GlcNS6S units, which are major sulfated constituents of cellular HS chains. These findings are consistent with immunochemical studies with 10E4 antibody in which we show that QSulf1 expression remodels the 10E4 IR on the surface of expressing cells. HSulf1 and HSulf2, human orthologues of QSulf1, also desulfate -IdoA2S-GlcNS6S- units on intestinal heparin (Morimoto-Tomita et al., 2002). Heparin is enriched in IdoA and O-sulfation, in contrast to cell-derived HS, which contains more GlcA and has less 2-O-sulfated sugars (Vives et al., 1999), which accounts for our identification of GlcA-GlcNS6S as a QSulf1 substrate. The substrate specificity and high level activity of QSulf1 on cell-derived HS establish QSulf1 as a unique 6-O endosulfatase that removes 6-O sulfates from sulfated residues located within HS chains, in contrast to lysosomal GlcNR6Sase, which is an exosulfatase involved in the sequential degradation of HS chains (Kresse et al., 1980).

QSulf1 6-O endosulfatase activity for disulfated GlcA-GlcNS6S and trisulfated IdoA2S-GlcNS6S disaccharide components of cellular HS chains attenuates the binding affinity of XWnt8 ligand to HS chains on Glypican1, which plays a central role in developmental signaling (Lin and Perri-

mon, 1999; Tsuda et al., 1999; Baeg et al., 2001; Nybakken and Perrimon, 2002), leading to stimulation of Wnt signaling. Although the specific structural interactions between Wnt ligand and HS residues are not yet known, it is notable that the trisulfated IdoA2S-GlcNS6S disaccharide contributes directly to the high affinity binding of bFGF to HS, and the 6-O sulfate of this trisulfated disaccharide is required for FGF-dependent receptor dimerization and activation (Schlessinger et al., 2000; Pye et al., 2000). These observations predict that QSulf1 activity will block HS-dependent FGF signaling, in contrast to Wnt signaling, which is stimulated by QSulf1 (Dhoot et al., 2001). QSulf1, therefore, may have a dual developmental function as a positive regulator of Wnt signaling and a negative regulator of FGF signaling.

Our findings provide evidence that QSulf1 plays a positive role in the regulation of Wnt signaling. QSulf1 is tethered to the cell surface and cell autonomously remodels the 6-O sulfation of extracellular HSPGs of Wnt-responsive somite and neural progenitors in embryos (Dhoot et al., 2001). Notum is another HSPG-modifying enzyme that regulates Wnt signaling in embryos (Giraldez et al., 2002). However, in contrast to QSulf1, Notum is released from Wg-producing cells to establish a morphogen gradient of Wg ligand presumably through its activity to degrade HS on HSPGs. *Drosophila Notum* mutants show much expanded Wg distribution and enhanced Wg activity in embryonic patterning, suggesting that Notum negatively regulates Wg signaling in Wg-responsive cells. By contrast, QSulf1 positively regulates Wnt signaling when localized either on the cell surface or targeted to the Golgi apparatus by actively remodeling the 6-O sulfation states of HS chains, which does not degrade HS or promote the turnover of HSPG core protein.

Our study of QSulf1 activity on Wnt binding to HS sheds light on the mechanism of QSulf1 function in Wnt signaling. We show that soluble 6-O-sulfated heparin binds to Wnt ligand with high affinity and strongly inhibits Wnt signaling. QSulf1 significantly reduces, but does not eliminate, the binding of XWnt8 to HS through its 6-O endosulfatase activity to promote Wnt signaling. Selective 6-O desulfation by QSulf1 does not remove all sulfates from HS. The residual sulfated residues on QSulf1-desulfated HS are essential for HS-mediated transduction of Wnt signals and Frizzled activation, based on previous findings that complete loss of HS sulfation in *sulfateless* mutant *Drosophila* or by chlorate treatment blocks Wnt signaling (Reichsman et al., 1996; Lin and Perrimon, 1999; Dhoot et al., 2001; Toyoda et al., 2000). CHO cells deficient in HS biosynthesis are also defective in Frizzled-dependent Wnt signaling, further establishing that HS is required for Frizzled function. Our findings also reveal that QSulf1 targeted to the cell surface or the Golgi apparatus actively remodels the sulfation states of cell surface HSPGs and promotes Wnt signaling. Therefore, QSulf1 itself does not function dynamically in the presentation of Wnt ligands to Frizzled receptors, but rather selectively removes 6-O sulfates from HS on the cell surface to convert the HS from a high affinity binding state to a low affinity binding state to allow the formation of functional Wnt–Frizzled complexes. Together, these findings support a static two-state catch or presentation model for QSulf1 regulation of Wnt signaling (Fig. 6). According to this model,

embryonic cells that do not express QSulf1 would have sulfated HSPGs on the cell surface in a high affinity state that catch Wnts and compete for binding of Wnts to their Frizzled receptors. QSulf1 expression in localized populations of embryonic progenitors would reduce 6-O HS sulfation to lower the binding affinity between Wnts and HSPGs, and 6-O-desulfated HS in the low affinity binding state would present Wnt ligand to promote the formation of Wnt–Frizzled receptor complexes to initiate Wnt signaling. In this regard, it is interesting to note that Frizzled-related proteins, such as sFRP-1, have heparin-binding domains that promote the formation of sFRP-1 and Wg complexes (Chong et al., 2002), suggesting that Wnts interacting with QSulf1-desulfated HS chains form a ternary complex with the Frizzled receptor, similar to the FGF–HS–FGFR complex that is required to initiate FGF signaling (Schlessinger et al., 2000). Therefore, the presentation of Wnt ligand by 6-O-desulfated HS in the low affinity binding state would provide a static mechanism to promote Wnt–Frizzled interaction in QSulf1-expressing cells. However, we also show that soluble heparin blocks Wnt signaling, suggesting that trisulfated HS may immobilize Wnt ligand on the cell surface, and QSulf1 activity reduces Wnt binding affinity of HS to enhance the access of Wnt ligand to Frizzled receptor, as shown by biochemical binding assays. We also show that chemically 6-O-desulfated soluble heparin does not promote Wnt signaling, suggesting that the residual 6-O sulfate groups on QSulf1-desulfated HS may be involved in Wnt binding to its Frizzled receptor. Structural studies of the interactions of QSulf1-modified HS substrates with Wnt ligands and their Frizzled receptors will provide insights into the specific mechanisms by which QSulf1 activity regulates Wnt signaling and will provide a basis for the development of therapeutic reagents to attenuate pathological HSPG-dependent signaling responses through control of angiogenesis and cell migration in diseases such as cancer (Sasaki et al., 1999; Lundin et al., 2000; Selva and Perrimon, 2001).

Materials and methods

Expression plasmids

QSulf1 and Glypican1 were cloned in pAG expression vector with a Myc-His COOH-terminal tag. The expression vector for a soluble form of Glypican1–AP lacking GPI linkage groups was provided by A. Lander (University of California, Irvine, CA) (Chen and Lander, 2001). HA–XWnt8 was cloned into pCS2 expression vector (Piccolo et al., 1999). QSulf1-ER expression vector was constructed by replacing the COOH-terminal Myc-His tag with a KDEL coding sequence (Munro and Pelham, 1987). QSulf1-Golgi expression plasmid was constructed by replacing the NH₂-terminal signal peptide of QSulf1 (amino acids 1–21) with NH₂-terminal amino acids 1–81 of the human β 1,4-galactosyltransferase (Gleeson et al., 1994). pECFP-Golgi and pECFP-ER were used as transfection markers to assay Golgi apparatus and ER expression (CLONTECH Laboratories, Inc.).

Cell culture and DNA transfection

All cell lines were cultured in DME with 10% FBS (Invitrogen). Cells were transfected with Eugene 6 (Roche) in 24-well plates for immunohistological analysis and Wnt1 assays, as described previously (Dhoot et al., 2001). QSulf1, Glypican1, Glypican1–AP, and XWnt8 proteins were expressed in 293T cells cultured in 100-mm dishes using a standard lipofectamine transfection protocol (Invitrogen). Stable QSulf1-expressing 293T cell lines were generated by selecting transfected 293T cells with 250 μ g/ml hygromycin (Sigma-Aldrich) 48 h after transfection. Expressed proteins, including QSulf1, Glypican1, and HA–XWnt8, were extracted from cells 24 h after transfection. To produce Glypican1–AP, transfected cells were

switched to serum-free DME/F12 (Invitrogen) 24 h after transfection, and conditioned medium was collected 16 h later. Protein expression was monitored by Western blot electrophoresis, followed by enzymatic or binding assays, as described below. As noted, chlorate-treated cultures were incubated with 25 mM chlorate for 18 h before being processed for immunocytochemistry and Western blot analysis.

Preparation of [³⁵S]GAG substrates

Confluent 293T cell cultures in 100-mm dishes, either untransfected or stable lines expressing QSulf1 or control proteins, were transferred to low sulfate F12 medium with 1% FBS and metabolically labeled for 5 h in 100 μ Ci/ml of carrier-free [³⁵S]SO₄ (NEN Life Science Products). After rinsing with TBS, cells were lysed in 0.25% Triton X-100, followed by overnight digestion with proteinase K (10 μ g/ml; Sigma-Aldrich) at 55°C. Proteinase K was then heat inactivated, and [³⁵S]GAGs were precipitated in three volumes of 95% ethanol, 1 μ g/ml dermatan sulfate (Sigma-Aldrich), and 1/10 volume of 5 M NaAc overnight at -20°C. After centrifugation, the [³⁵S]GAG pellet was air dried, dissolved in water, and then centrifuged in a 5-kD filter unit (UFC3BCC00; Millipore) to remove molecules with molecular masses <5 kD. [³⁵S]GAGs were reconstituted in water at a concentration of 10⁷ cpm/ml for use as substrates in QSulf1 enzyme assays or directly for disaccharide assays. To differentially deplete [³⁵S]GAGs of HS or CS, samples were digested for 4 h at 37°C with either heparinases I and II (0.1 U/ml; Sigma-Aldrich) or chondroitinase ABC (0.1 U/ml; Sigma-Aldrich), followed by heat inactivation. Prior to enzyme assays, low molecular mass digestion products were removed from heparinase- and chondroitinase-treated [³⁵S]GAG preparations by centrifugation in 5-kD filter units.

QSulf1 enzyme assays

293T cell cultures transfected with Myc-tagged QSulf1 and control plasmids were lysed in 500 μ l of 0.25% Triton X-100. Freshly prepared cell lysates were dialyzed in TBS to remove phosphate and sulfate and other small molecules or incubated overnight with Myc beads (CLONTECH Laboratories, Inc.) at 4°C. Myc beads were then washed and resuspended in TBS. QSulf1-Myc was purified 15–20-fold by incubation with Myc beads, as assayed by Western blot analysis. QSulf1 enzyme assays with [³⁵S]GAGs were conducted in 200- μ l reaction mixtures, including 30 μ l of cell lysate (10 μ g) or Myc bead-purified enzyme (0.5 μ g), 10 μ l of [³⁵S]GAG substrate in 50 mM Tris buffer (pH 7.5), and 50 mM MgCl₂. Samples were digested overnight at 37°C. Enzyme reactions were 95% complete after 4 h. After incubation, the reaction mixture was centrifuged to remove Myc beads and denatured proteins, and the released, low molecular mass [³⁵S]SO₄ was assayed by centrifugation of samples at 14,000 rpm for 30 min using the 5-kD filter unit, followed by scintillation counting for quantization of [³⁵S]SO₄ release. Assays with *N*-acetylglucosamine 6-O sulfate monosaccharide substrate (Sigma-Aldrich) were modified from previously described assays by the addition of 50 mM MgCl₂ and 3 mM PbCl₂ to reactions (He et al., 1993). *N*-acetylglucosamine formation was measured by the Reissig method (Reissig et al., 1955). Control extracts for enzymatic assays with [³⁵S]GAG and GlcNAc6S substrates were prepared from 293 cells transfected with empty vector or mutant QSulf1 (C-A) expression plasmids.

Structural analysis of ³⁵S-labeled HS

HS chains were cleaved at *N*-sulfated glucosamine residues by treatment with nitrous acid at pH 1.5 (Shively and Conrad, 1976), followed by reduction with NaBH₄. Labeled deamination products were fractionated by gel chromatography on a column (1 \times 180 cm) of Sephadex G-25 eluted with 0.2 M NH₄HCO₃. Samples of isolated disaccharides were analyzed on a Partisil-10 SAX column eluted at a rate of 1 ml/min with stepwise increasing concentrations of KH₂PO₄ (Rong et al., 2001).

Immunocytochemistry and Western blots

Cells for immunochemical analysis were cultured on acid-treated coverslips. To assay cell surface markers, cells were live cell stained by incubating cells with the primary antibody in culture medium for 2 h at 37°C, rinsed with PBS, and then permeabilized by fixation with 4% paraformaldehyde/PBS at room temperature for 15 min for immunostaining to identify QSulf1-MycHis-expressing cells. After washing, cells were incubated for 2 h with the secondary antibody or double stained in antibody dilution buffer (0.1% Triton X-100 in PBS, 20% goat serum). Cells were subsequently washed with PBS, and coverslips were mounted and analyzed by fluorescence microscopy. Western blots were prepared as described previously (Dhoot, et al., 2001). The primary antibodies used included mouse anti-Myc (9E10; 1:300 for immunocytochemistry and 1:2,000 for Western

blots), rabbit anti-His (1:200; Santa Cruz Biotechnology, Inc.), mouse anti-HS (10E4; 1:150; Seikagaku), mouse anti-CS (CS56; 1:100; Sigma-Aldrich), rabbit anti-QSulf1 hydrophilic domain (1:200 for immunostaining and 1:2,000 for Western blotting; Calico), mouse anti-TGN 38 (Ab-1; 1:1,000; Oncogene); mouse anti-PDI (1:100; Affinity BioReagents, Inc.), mouse anti-alkaline phosphatase (8B6; 1:4,000; Sigma-Aldrich), and mouse anti-HA (1:1,000; CLONTECH Laboratories, Inc.). Secondary antibodies included goat anti-mouse Cy3 (1:1,000; Jackson ImmunoResearch Laboratories), goat anti-rabbit Cy2 (1:1,000; Jackson ImmunoResearch Laboratories), peroxidase-conjugated goat anti-mouse (diluted 1:4,000; Vector Laboratories), peroxidase-conjugated goat anti-rabbit (diluted 1:4,000; Vector Laboratories).

Binding assay for HA-XWnt8- or HA-XWnt8-expressing cells

To produce HA-XWnt8, 293T cell cultures transfected with HA-XWnt8 expression plasmid were lysed in 500 μ l of 0.25% Triton X-100. The cell lysate was spun for 5 min to separate the nuclei, and the supernatant was collected. NaCl was added to the supernatant to a final concentration of 2 M to salt off any membrane-bound HA-XWnt8, and extra NaCl in the cell lysate was diluted by dialysis overnight at 4°C in PBS. To generate soluble Glypican1-AP, conditioned medium collected from transfected cells was concentrated 100-fold through 10-kD Centriprep (Amicon) columns, and the amount of concentrated proteins was quantified using a colorimetric dye concentrate reagent (Bio-Rad Laboratories). The concentrated proteins containing Glypican1-AP were treated with QSulf1 or control overnight. For binding to HA-XWnt8, 50 μ g concentrated protein was immunoprecipitated after enzymatic treatment using 50 μ l AP beads (CLONTECH Laboratories, Inc.). The beads were then separated by centrifugation and washed with HBSS with 20 mM Hepes (pH 7.0). An aliquot of 10 μ l beads with bound Glypican1-AP was incubated with HA-XWnt8 for 20 min at RT. After centrifugation and washes, the beads were collected, and HA-XWnt8 bound to Glypican1-AP was quantified by Western blot. For binding to HA-XWnt8-expressing cells, Glypican1-AP treated with either QSulf1 or control was added to 293T cells transfected with HA-XWnt8 expression vector at a concentration of ~50 μ g/ml. The protocol for the binding was performed as previously described (Hsieh et al., 1999).

Competition assay for HA-XWnt8 binding to heparin

Heparin immobilized on 100 μ l agarose beads (Sigma-Aldrich) was blocked with 0.5 mg/ml BSA in HBSS with 20 mM Hepes (pH 7.0) at RT for 1 h, followed by incubation with 400 μ l HA-XWnt8 at RT for 30 min. Heparin beads were separated by centrifugation and washed three times with HBSS with 20 mM Hepes (pH 7.0), and equal quantities were then aliquoted into 10 tubes. Heparin was treated overnight either with QSulf1 or control QSulf1 (C-A) mutant proteins and then added to heparin beads to compete for HA-XWnt8 binding. After 30 min at RT, heparin beads were separated by centrifugation, and HA-XWnt8 in the supernatant was quantified by Western blot. The intensity of protein corresponding to HA-XWnt8 protein on Western blots was quantified by IQant.

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References

- Avnur, Z., and B. Geiger. 1984. Immunocytochemical localization of native chondroitin-sulfate in tissues and cultured cells using specific monoclonal antibody. *Cell*. 38:811–822.
- Baeg, G.H., X. Lin, N. Khare, S. Baumgartner, and N. Perrimon. 2001. Heparan sulfate proteoglycans are critical for the organization of the extracellular distribution of Wingless. *Development*. 128:87–94.
- Bai, X., K.J. Bame, H. Habuchi, K. Kimata, and J.D. Esko. 1997. Turnover of heparan sulfate depends on 2-O sulfation of uronic acids. *J. Biol. Chem.* 272: 23172–23179.
- Bai, X., G. Wei, A. Sinha, and J.D. Esko. 1999. Chinese hamster ovary cell mu-

- tants defective in glycosaminoglycan assembly and glucuronosyltransferase I. *J. Biol. Chem.* 274:13017–13024.
- Bernfield, M., M. Gotte, P.W. Park, O. Reizes, M.L. Fitzgerald, J. Lincecum, and M. Zako. 1999. Functions of cell surface heparan sulfate proteoglycans. *Annu. Rev. Biochem.* 68:729–777.
- Brickman, Y.G., M.D. Ford, J.T. Gallagher, V. Nurcombe, P.F. Bartlett, and J.E. Turnbull. 1998. Structural modification of fibroblast growth factor-binding heparan sulfate at a determinative stage of neural development. *J. Biol. Chem.* 273:4350–4359.
- Chen, R.L., and A.D. Lander. 2001. Mechanisms underlying preferential assembly of heparan sulfate on glypican-1. *J. Biol. Chem.* 276:7507–7517.
- Chong, J.M., A. Uren, J.S. Rubin, and D.W. Speicher. 2002. Disulfide bond assignments of secreted Frizzled-related protein-1 provide insights about Frizzled homology and netrin modules. *J. Biol. Chem.* 277:5134–5144.
- David, G., B. Van der Schueren, P. Marynen, J.J. Cassiman, and H. van den Berghe. 1992. Molecular cloning of amphiglycan, a novel integral membrane heparan sulfate proteoglycan expressed by epithelial and fibroblastic cells. *J. Cell Biol.* 118:961–969.
- Dhoot, G.K., M.K. Gustafsson, X. Ai, W. Sun, D.M. Standiford, and C.P. Emerson, Jr. 2001. Regulation of Wnt signaling and embryo patterning by an extracellular sulfatase. *Science.* 293:1663–1666.
- Esko, J.D., and U. Lindahl. 2001. Molecular diversity of heparan sulfate. *J. Clin. Invest.* 108:169–173.
- Giraldez, A.J., R.R. Copley, and S.M. Cohen. 2002. HSPG modification by the secreted enzyme Notum shapes the Wingless morphogen gradient. *Dev. Cell.* 2:667–676.
- Gleeson, P.A., R.D. Teasdale, and J. Burke. 1994. Targeting of proteins to the Golgi apparatus. *Glycoconj. J.* 11:381–394.
- He, W., Y.V. Voznyi, A.M. Boer, W.J. Kleijer, and O.P. van Diggelen. 1993. A fluorimetric enzyme assay for the diagnosis of Sanfilippo disease type D (MPS IIID). *J. Inher. Metab. Dis.* 16:935–941.
- Hsieh, J., A. Pattner, P.M. Smallwood, and J. Nathans. 1999. Biochemical characterization of Wnt-Frizzled interactions using a soluble, biologically active vertebrate Wnt protein. *Proc. Natl. Acad. Sci. USA.* 96:3546–3551.
- Jayson, G.C., C. Vives, C. Paraskeva, K. Schofield, J. Coutts, A. Fleetwood, and J.T. Gallagher. 1999. Coordinated modulation of the fibroblast growth factor dual receptor mechanism during transformation from human colon adenoma to carcinoma. *Int. J. Cancer.* 82:298–304.
- Jemth, P., J. Kreuger, M. Kusche-Gullberg, L. Sturiale, G. Gimenez-Gallego, and U. Lindahl. 2002. Biosynthetic oligosaccharide libraries for identification of protein-binding heparan sulfate motifs. *J. Biol. Chem.* 277:30567–30573.
- Kresse, H., E. Paschke, K. von Figura, W. Gilberg, and W. Fuchs. 1980. Sanfilippo disease type D: deficiency of N-acetylglucosamine-6-sulfate sulfatase required for heparan sulfate degradation. *Proc. Natl. Acad. Sci. USA.* 77:6822–6826.
- Lin, X., and N. Perrimon. 1999. Dally cooperates with *Drosophila* Frizzled to transduce Wingless signaling. *Nature.* 400:281–284.
- Lindahl, U., M. Kusche-Gullberg, and L. Kjellen. 1998. Regulated diversity of heparan sulfate. *J. Biol. Chem.* 273:24979–24982.
- Lundin, L., H. Larsson, J. Kreuger, S. Kanda, U. Lindahl, M. Salmivirta, and L. Claesson-Welsh. 2000. Selectively desulfated heparin inhibits fibroblast growth factor-induced mitogenicity and angiogenesis. *J. Biol. Chem.* 275:24653–24660.
- Merry, C.L., M. Lyon, J.A. Deakin, J.J. Hopwood, and J.T. Gallagher. 1999. Highly sensitive sequencing of the sulfated domains of heparan sulfate. *J. Biol. Chem.* 274:18455–18462.
- Morimoto-Tomita, M., K. Uchimura, Z. Werb, S. Hemmerich, and S.D. Rosen. 2002. Cloning and characterization of two extracellular heparin-degrading endosulfatases in mouse and human. *J. Biol. Chem.* 277:49175–49185.
- Munro, S., and H.R. Pelham. 1987. A C-terminal signal prevents secretion of luminal ER proteins. *Cell.* 48:899–907.
- Nybakken, K., and N. Perrimon. 2002. Heparan sulfate proteoglycan modulation of developmental signaling in *Drosophila*. *Biochim. Biophys. Acta.* 1573:280–291.
- Ohto, T., H. Uchida, H. Yamazaki, K. Keino-Masu, A. Matsui, and M. Masayuki. 2002. Identification of a novel nonlysosomal sulphatase expressed in the floor plate, choroid plexus and cartilage. *Genes Cells.* 7:173–185.
- Piccolo, S., E. Agius, L. Leyns, S. Bhattacharyya, H. Grunz, T. Bouwmeester, and E.M. De Robertis. 1999. The head inducer Cerberus is a multifunctional antagonist of nodal, BMP and Wnt signals. *Nature.* 397:707–710.
- Prydz, K., and K.T. Dalen. 2000. Synthesis and sorting of proteoglycans. *J. Cell Sci.* 113:193–205.
- Pye, D.A., R.R. Vives, P. Hyde, and J.T. Gallagher. 2000. Regulation of FGF-1 mitogenic activity by heparan sulfate oligosaccharides is dependent on specific structural features: differential requirement for the modulation of FGF-1 and FGF-2. *Glycobiology.* 10:1183–1192.
- Raman, R., J.R. Myette, Z. Shriver, K. Pojasek, G. Venkataraman, and R. Sasisekharan. 2003. The heparin/heparan sulfate 2-O sulfatase from *Flavobacterium heparinum*. *J. Biol. Chem.* 278:12167–12174.
- Rapraeger, A.C. 2002. Heparan sulfate-growth factor interactions. *Methods Cell Biol.* 69:83–109.
- Reichsman, F., L. Smith, and S. Cumberledge. 1996. Glycosaminoglycans can modulate extracellular localization of the wingless protein and promote signal transduction. *J. Cell Biol.* 135:819–827.
- Reissig, J.L., J.L. Strominger, and L.F. Leloir. 1955. A modified colorimetric method for the estimation of N-acetyl amino sugars. *J. Biol. Chem.* 217:959–966.
- Rong, J., H. Habuchi, K. Kimata, U. Lindahl, and M. Kusche-Gullberg. 2001. Substrate specificity of the heparan sulfate hexuronic acid 2-O-sulfotransferase. *Biochemistry.* 40:5548–5555.
- Safaiyan, F., S.O. Kolset, K. Prydz, E. Gottgridsson, U. Lindahl, and M. Salmivirta. 1999. Selective effects of sodium chlorate treatment on the sulfation of heparan sulfate. *J. Biol. Chem.* 274:36267–36273.
- Safaiyan, F., U. Lindahl, and M. Salmivirta. 2000. Structural diversity of N-sulfated heparan sulfate domains: distinct modes of glucuronyl C5 epimerization, iduronic acid 2-O-sulfation, and glucosamine 6-O-sulfation. *Biochemistry.* 39:10823–10830.
- Sasaki, T., H. Larsson, J. Kreuger, M. Salmivirta, L. Claesson-Welsh, U. Lindahl, E. Hohenester, and R. Timpl. 1999. Structural basis and potential role of heparin/heparan sulfate binding to the angiogenesis inhibitor endostatin. *EMBO J.* 18:6240–6248.
- Schlessinger, J., A.N. Plotnikov, O.A. Ibrahim, A.V. Eliseenkova, B.K. Yeh, A. Yayon, R. Linhardt, and M. Mohammadi. 2000. Crystal structure of a ternary FGF-FGFR-heparin complex reveals a dual role for heparin in FGFR binding and dimerization. *Mol. Cell.* 6:743–750.
- Schmidt, B., T. Selmer, A. Ingendoh, and K. von Figura. 1995. A novel amino acid modification in sulfatases that is defective in multiple sulfatase deficiency. *Cell.* 82:271–278.
- Selva, E.M., and N. Perrimon. 2001. Role of heparan sulfate proteoglycans in cell signaling and cancer. *Adv. Cancer Res.* 83:67–80.
- Shively, J.E., and H.E. Conrad. 1976. Formation of anhydrosugars in the chemical depolymerization of heparin. *Biochemistry.* 15:3932–3942.
- Toyoda, H., A. Kinoshita-Toyoda, B. Fox, and S.C. Selleck. 2000. Structural analysis of glycosaminoglycans in animals bearing mutations in sugarless, sulfataseless, and tout-velu. *J. Biol. Chem.* 275:21856–21861.
- Tsuda, M., K. Kamimura, H. Nakato, M. Archer, W. Staaz, B. Fox, M. Humphrey, S. Olson, T. Furch, V. Kaluza, et al. 1999. The cell-surface proteoglycan Dally regulates Wingless signaling in *Drosophila*. *Nature.* 400:276–280.
- Vives, R.R., D.A. Pye, M. Salmivirta, J.J. Hopwood, U. Lindahl, and J.T. Gallagher. 1999. Sequence analysis of heparan sulphate and heparin oligosaccharides. 1999. *Biochem. J.* 339:767–773.
- Wu, C., and R. Nusse. 2002. Ligand receptor interactions in the Wnt signaling pathway in *Drosophila*. *J. Biol. Chem.* 277:41762–41769.
- Yanagishita, M., and V.C. Hascall. 1992. Cell surface heparan sulfate proteoglycans. *J. Biol. Chem.* 267:9451–9454.
- Yip, G.W., P. Ferreti, and A.J. Copp. 2002. Heparan sulfate proteoglycans and spinal neurulation in the mouse embryo. *Development.* 129:2109–2119.