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Dimerized Glycosaminoglycan Chains Increase FGF Signaling during Zebrafish Development

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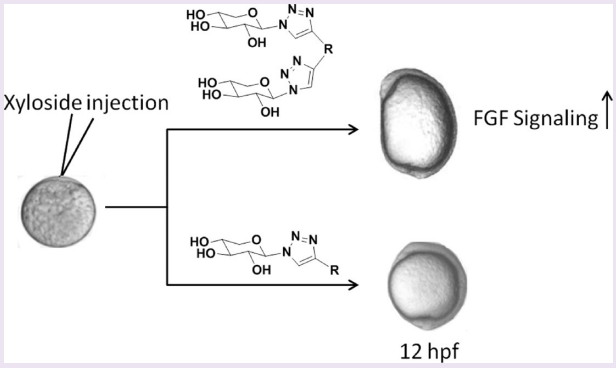
S *Supporting Information*

ABSTRACT: Proteoglycans (PGs) modulate numerous signaling pathways during development through binding of their glycosaminoglycan (GAG) side chains to various signaling molecules, including fibroblast growth factors (FGFs). A majority of PGs possess two or more GAG side chains, suggesting that GAG multivalency is imperative for biological functions *in vivo*. However, only a few studies have examined the biological significance of GAG multivalency. In this report, we utilized a library of bis- and tris-xylosides that produce two and three GAG chains on the same scaffold, respectively, thus mimicking PGs, to examine the importance of GAG valency and chain type in regulating FGF/FGFR interactions *in vivo* in zebrafish. A number of bis- and tris-xylosides, but not mono-xylosides, caused an elongation phenotype upon their injection into embryos. *In situ* hybridization showed that elongated embryos have elevated expression of the FGF target gene *mkp3* but unchanged expression of reporters for other pathways, indicating that FGF/FGFR signaling was specifically hyperactivated. In support of this observation, elongation can be reversed by the tyrosine kinase inhibitor SU5402, mRNA for the FGFR antagonist *sprouty4*, or FGF8 morpholino. Endogenous GAGs seem to be unaffected after xyloside treatment, suggesting that this is a gain-of-function phenotype. Furthermore, expression of a multivalent but not a monovalent GAG containing syndecan-1 proteoglycan recapitulates the elongation phenotype observed with the bivalent xylosides. On the basis of these *in vivo* findings, we propose a new model for GAG/FGF/FGFR interactions in which dimerized GAG chains can activate FGF-mediated signal transduction pathways.



The regulation of cell−cell and cell−matrix interactions is critical for various developmental processes. Secreted molecules such as FGFs, Wnts, and BMPs provide necessary informational cues through binding to their cognate cell surface receptors and subsequent activation of intracellular signaling pathways. It has been shown that proteoglycans (PGs) are essential to facilitate these interactions.[1−4]

PGs consist of a core protein to which multiple glycosaminoglycan (GAG) chains are attached at specific amino acids.[5,6] Several types of GAGs exist, including heparan sulfate (HS), chondroitin sulfate (CS), and dermatan sulfate (DS). Some PGs such as syndecans carry more than one type of GAG chain. Mutations altering GAG attachment sites on core proteins or disruptions of GAG biosynthetic enzymes affect signaling and lead to a variety of disorders in humans.[7−9] It is important to note that a majority of naturally occurring PGs possess two or more GAG side chains (Figure 1). This implies that multiple GAG chains are imperative for biological functions *in vivo*. However, only a few studies have examined the importance of GAG multivalency using cellular systems.[10−12]

A large body of *in vitro* data indicates that PGs are essential for FGF/FGFR signaling.[13,14] HS chains facilitate FGF-mediated dimerization of FGFRs and subsequently regulate intracellular signal transduction pathways during development.[15] Numerous models have been proposed to explain FGFR dimerization at the molecular level based on biochemical and biophysical studies.[16−18] However, these models have not

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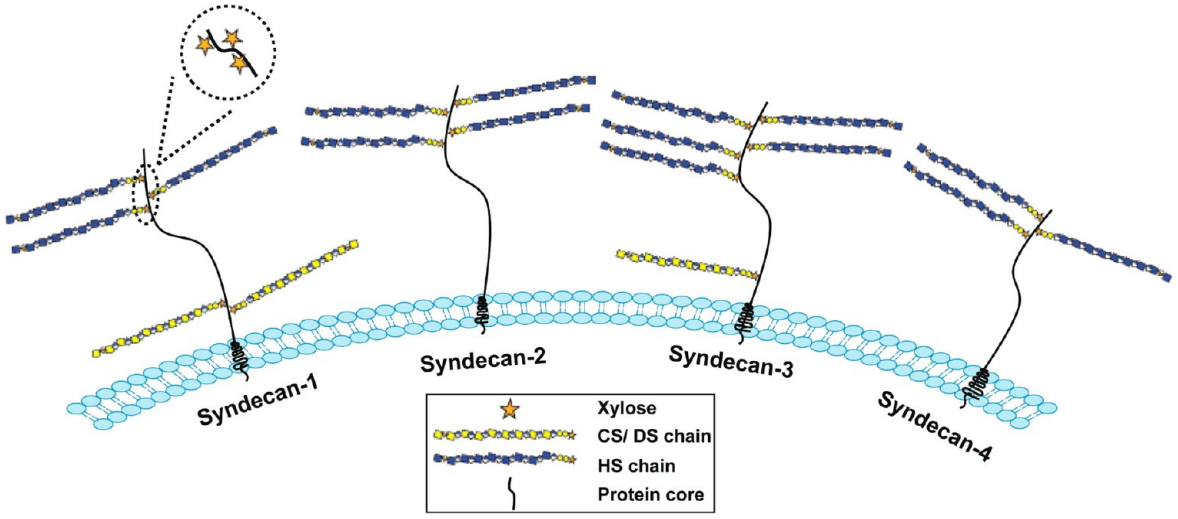
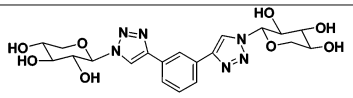
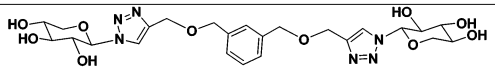
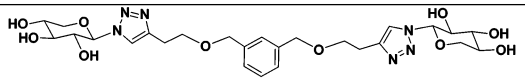
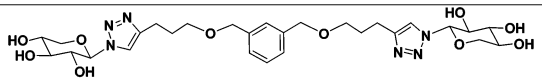
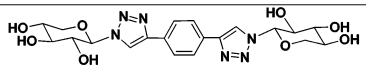
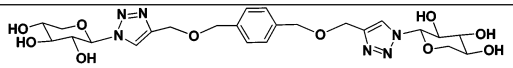
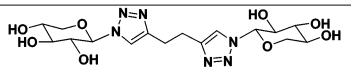
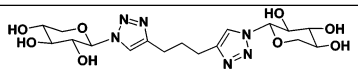
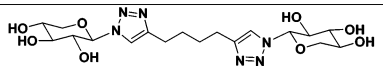
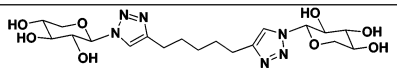
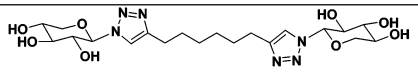
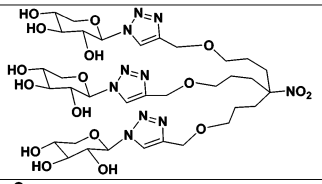
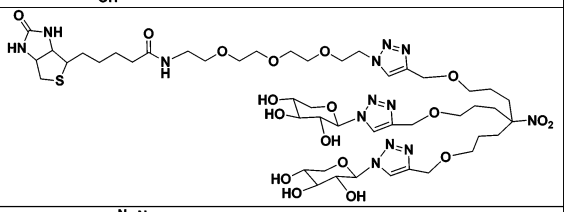
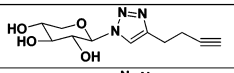
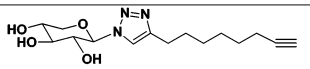
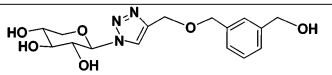
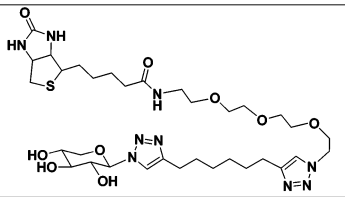
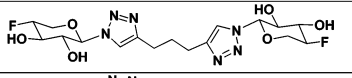
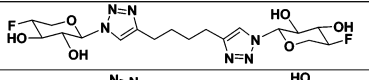
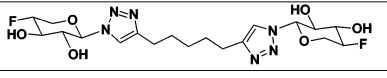


Figure 1. Structural feature of syndecans carrying multiple glycosaminoglycan side chains. A majority of proteoglycans including syndecans carry two or more glycosaminoglycan side chains such as heparan sulfate, chondroitin sulfate, and dermatan sulfate.

Table 1. Structures of Xylosides Studied in Zebrafish Embryos

Number	Structure
I	
II	
III	
IV	
V	
VI	
VII	
VIII	
IX	
X	
XI	

Number	Structure
XII	
XIII	
XIV	
XV	
XVI	
XVII	
XVIII	
XIX	
XX	

considered the impact that GAG valency may have on FGFR dimerization. In particular, researchers have not yet elucidated the effects of bivalent GAGs on FGF-mediated signaling pathways *in vivo*.

We hypothesized that the presence of multiple GAG chains on endogenous PGs is essential for biological functions *in vivo*. In this study, we investigated whether GAG multivalency, as found in endogenous PGs, can regulate FGF/FGFR-mediated

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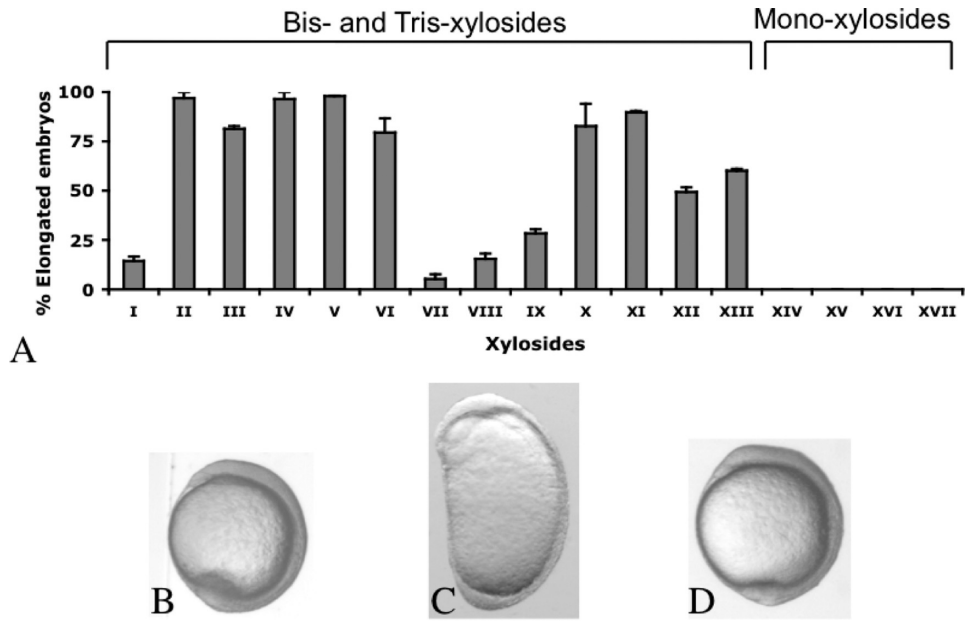

Figure 2. Xylosides cause elongation of developing zebrafish embryos at 12 hpf. (A) Treatment with bis- and tris-xylosides causes elongation. Mean ± SEM from 3 experiments (n = 145−246 embryos) are presented. (B) Control embryo (WT). (C) Elongated embryo treated with bis-xyloside **II**. (D) Nonelongated embryo treated with mono-xyloside **XIV**. Lateral views, dorsal right, anterior up. Xyloside structures are shown in Table 1. Xylosides (33 pmol) were injected into the blastomere of one-cell stage embryos.

signaling pathways during development. It has been shown that mono-xylosides, which prime GAG chains free of core proteins, modulate development in various systems.[19,20] A mono-xyloside that inhibits endogenous CS PG biosynthesis at very high concentration has been shown to affect heart development in zebrafish embryos.[21] Our laboratory has recently utilized click chemistry to generate mono-xylosides and their fluorinated analogues that stimulate or inhibit GAG biosynthesis, respectively.[22,23] In our companion studies, we have utilized a similar chemical approach to synthesize a library of cluster-xylosides and have probed their priming activity and the mechanism of GAG biosynthesis in a cellular system.[24] Here, we use the embryonic zebrafish as an *in vivo* system to examine whether these xylosides produce proteoglycan mimetics *in vivo* and then further define their roles in modulating FGF/FGFR-mediated signaling pathways. On the basis of our findings, we propose that GAG multivalency plays a major role in the regulation of FGF-mediated signal transduction pathways during development.

RESULTS AND DISCUSSION

Bis- and Tris-xylosides Affect the Early Development of Zebrafish Embryos. Monoxylosides have been used in various systems to define the role of GAG chains.[14,19−21] We have also previously reported the synthesis of *click*-xylosides and their ability to prime distinct GAG chains in the absence of a core protein in a cellular system.[22,25] However, mono-xylosides can prime only a single GAG chain per xyloside. On the other hand, cluster-xylosides can prime multiple GAG chains that are covalently attached on the same scaffold, better mimicking naturally occurring PGs.[26] Various xylosides were synthesized using click chemistry in such a way that some scaffolds carry single xylose units and others carry two or three xylose units, allowing us to determine the importance of GAG multivalency as found in endogenous PGs (Table 1). Furthermore, a number of bis-xylosides were prepared with variable distances between two xylose residues. All of these xylosides were tested by injection into the blastomere of one-cell stage zebrafish embryos at a series of concentrations. Such injections provide convenient access to the cytoplasmic compartment without the need for plasma membrane permeability; as embryonic cells divide, injected material is partitioned into daughter cells. Bis- and tris-xylosides (xylosides **I**−**XIII**) at dosages of 33 pmol/embryo effectively caused elongation at 12 hpf (Figure 2A,C), whereas mono-xylosides (xylosides **XIV**−**XVII**) failed to cause any elongation at 33 pmol/embryo (Figure 2A,D) or even at higher dosages (up to 160 pmol/embryo). Xylosides **I**−**VI**, in which two xylose residues are linked by an aromatic ring, all effectively induced elongation in a majority of embryos, with the exception of xyloside **I**, which induced elongation in only 14.3% of treated embryos (n = 152). It is interesting to note that xyloside **I** has the shortest distance between the two xylose residues. In the case of xylosides **VII**−**XI**, in which two xylose residues are linked by a linear alkyl chain, a longer spacer between the two xylose residues induced elongation in a higher percentage of embryos (Figure 2A). These results suggest that both the dimeric nature and the conformational flexibility of the newly primed bivalent GAG chains are critical to have an effect on signal transduction during developmental processes.

Elongated Embryos Show Specific Increases in FGF Signaling. Elongation of an embryo can result from activation of WNT, FGF, or Nodal signaling or from inhibition of BMP signaling.[27−30] To distinguish between these possibilities, we analyzed patterns of early gene expression in embryos treated with bis- or tris-xylosides and control embryos using *in situ* hybridization and immunostaining. When embryos were treated with bis-xyloside **II**, the expression of the FGF pathway target gene *mkp3* at 8 hpf[31] was expanded (Figure 3A). The *mkp3* pattern, a broadened ring around the margin, is just what would be expected from enhanced FGF signaling, given the expression of Fgfs at this stage in a marginal ring.[31] A similar effect on

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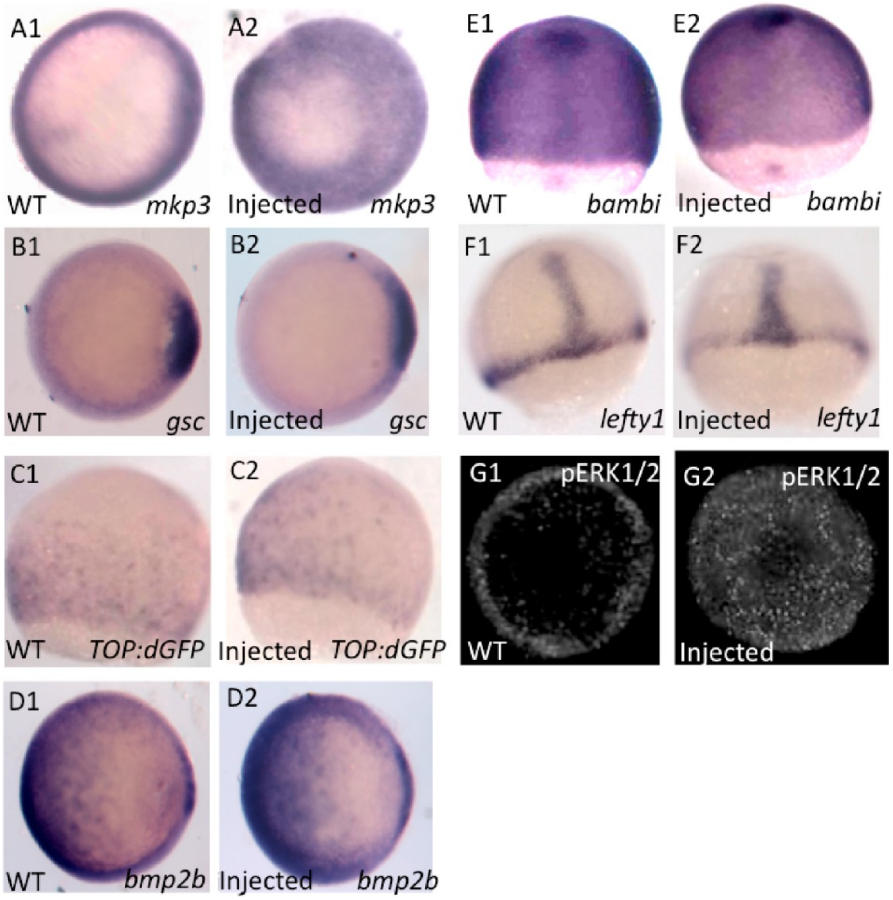

Figure 3. Analysis of developmental patterning in WT and bis-xyloside II treated embryos. (A–F) *In situ* hybridization of control (A1–F1) and bis-xyloside **II** treated (A2–F2) embryos at 8 hpf with probes for the FGF signaling target gene *mkp3*, dorsal marker *gsc*, WNT signaling reporter *TOP:dGFP*, BMP signaling ligand *bmp2b*, BMP signaling target gene *nma/bambi*, and Nodal signaling target gene *lefty1*, respectively. A, B, D: animal pole view, dorsal right; C: lateral view, dorsal right; E, F: dorsal view. Only the expression of *mkp3* was expanded suggesting that FGF signaling is predominantly affected at 8 hpf. (G) Immunostaining with phospho-Erk 1/2 antibody in control (G1) and bis-xyloside **II** treated (G2) embryos at 6 hpf. Animal pole view, dorsal right. Phospho-Erk 1/2 was expanded toward the animal pole in bis-xyloside **II** treated embryos compared to control.

mkp3 was seen at 6 hpf. When treating with bis-xylosides **VII**–**XI**, the percentage of embryos with expanded *mkp3* expression varied with linker length in concordance with the percentage of elongated embryos (Supplementary Table S2). On the other hand, the expression of *mkp3* remained normal in embryos treated with mono-xylosides (Supplementary Table S2 and Figure S1). By contrast, the expression of the dorsal marker *gsc*[32] was unaffected by the injection of bis-xylosides (Figure 3B and Supplementary Figure S1). Thus at 8 hpf, bis-xylosides enhanced FGF signaling without a detectable effect on dorsoventral patterning.

To investigate whether the Wnt, BMP, or Nodal signaling pathways were also affected, the expression of corresponding pathway readouts was examined at 8 hpf. The expression of the transgenic Wnt reporter *TOP:dGFP*,[33] which carries 4 TCF/LEF binding sites driving destabilized GFP, appeared unchanged following bis-xyloside **II** injection (Figure 3C). Similar observations were made for the BMP ligand *bmp2b*,[34] the BMP pathway target *nma/bambi*[35] and the Nodal pathway target *lefty1*[36] (Figure 3D–F). The expression of these genes was also unchanged following mono-xyloside injections (Supplementary Figure S1). Furthermore, immunostaining revealed that bis-xyloside-treated embryos have higher phospho-Erk 1/2 expression than controls, again with the greatest effect near the margin, indicating increased FGF activity through the FGFR/Ras/Mapk signaling pathway (Figure 3G). These results suggest that the FGF pathway is the only, or at least the primary, signaling pathway affected by bis-xyloside at 8 hpf. Once FGF signaling is affected, we would expect secondary effects on other pathways. For example, Furthauer *et al.* have shown that FGF signaling inhibits BMP signaling during early development.[28] Hence, expression patterns of the FGF target *mkp3* and BMP target *nma/bambi* were analyzed at 12 hpf. The expression of *mkp3* expanded in the ventral ectoderm, while the expression of *bambi* decreased in bis-xyloside **II**-injected embryos compared to WT (Supplementary Figure S2). These results indicate that the primary effect of multivalent GAG chains primed by bis-xylosides is increased FGF signaling, with decreased BMP signaling as a later, secondary consequence.

Inhibition of FGFR-Mediated Signaling Rescues Elongation Phenotype. If bis-xylosides primarily activate the FGF/FGFR-mediated intracellular signaling pathway, we predicted that its inhibition would prevent the elongation phenotype. We used a well-known FGFR tyrosine kinase inhibitor, SU5402,[37] and applied a concentration (25 μM) at which the kinase inhibitor has no significant effect on the normal morphology of the embryos through 12 hpf. After injection with bis-xyloside **II**, the embryos were immediately treated with SU5402. After continuous treatment through 12

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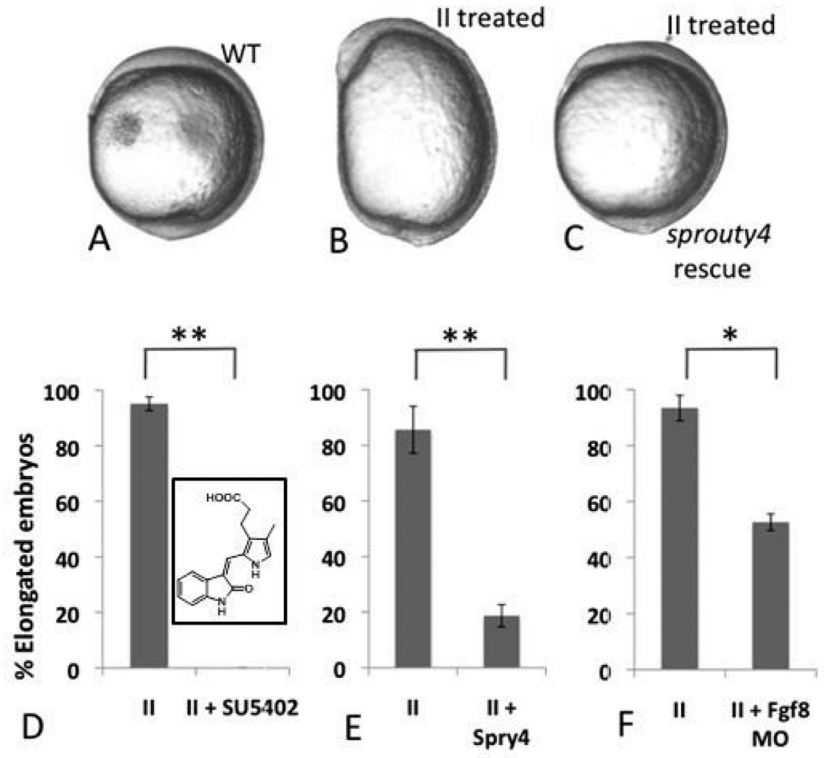

Figure 4. Rescue of elongation phenotype by inhibition of FGF signaling. (A–C) Examples of control, elongated and rescued embryos at 12 hpf. Lateral views, dorsal right, anterior up. (A) Control embryo. (B) Embryo treated with bis-xyloside **II**. (C) Embryo treated with bis-xyloside **II** and rescued by *sprouty4* mRNA injection. (D–F) Percentage of elongated embryos following treatment with bis-xyloside **II** alone or with bis-xyloside **II** as well as 25 μM SU5402 (D, n = 111), 70 pg of *sprouty4* mRNA (E, n = 149), or 6 ng of *fgf 8* morpholino (F, n = 98). All three treatments show significant rescue. *, $p < 0.05$; **, $p < 0.01$ using Student's t test.

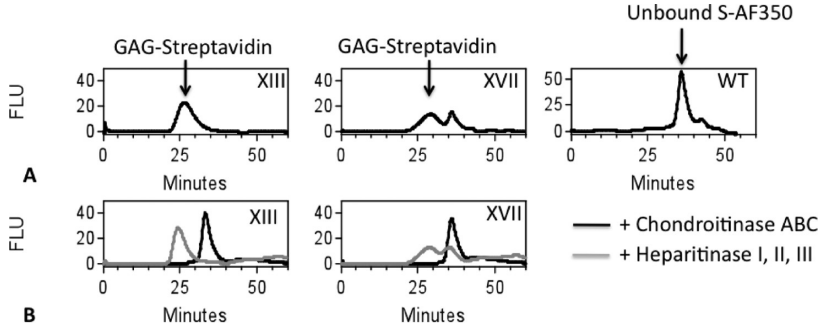

Figure 5. Structural analysis of GAG chains primed by xylosides. (A) Fluorescent detection of GAG chains primed in embryos by biotinylated bis-xyloside **XIII** or biotinylated mono-xyloside **XVII** or untreated embryos, after purification, complexing with streptavidin-Alexa Fluor 350, and elution from an HPLC-size exclusion column. (B) Fluorescent detection of GAG chains primed by biotinylated xylosides (**XIII** and **XVII**) after treatment with chondroitinase ABC or heparitinases I, II, and III to determine the relative proportion of HS and CS/DS, respectively.

hpf, there was a complete rescue: no elongation was observed (n = 111; Figure 4D). When embryos were treated with SU5402 starting at 8 hpf instead of immediately after injection with bis-xyloside **II**, only 24% (n = 115) of embryos developed normally, suggesting that bis-xyloside **II** exerts its activity by augmenting FGF signaling before or during gastrulation. We also tested whether expression of *sprouty4*, an FGF signaling antagonist, could rescue the phenotype. When injected with bis-xyloside **II** and 70 pg of *sprouty4* mRNA, 18.7% (n = 149) of the embryos were elongated at 12 hpf. In comparison, 85% of the embryos (n = 119) were elongated when injected with bis-xyloside **II** alone (Figure 4C,E). These results confirm that the bis-xyloside-mediated elongation phenotype depends on FGF/FGFR signaling.

Suppression of FGF8 Rescues Elongation Phenotype. FGF signaling is essential for many developmental processes,[38] and FGFs 3, 8, 17b, and 24 are the main FGFs expressed during zebrafish gastrulation.[39] Among these, FGF8 is thought to have the greatest role in early developmental events. Therefore, we investigated whether FGF8 is involved in causing elongation of embryos when treated with bis-xylosides. Bis-xyloside **II** and *fgf 8* translation blocking morpholino were sequentially injected into one-cell embryos. After 12 hpf, 52.6% of these embryos were elongated (n = 98), whereas 93% of those that were injected with bis-xyloside **II** only were elongated (n = 125; Figure 4F). These results suggest that bis-xylosides activate FGFR signaling pathways at least partially via FGF8 and

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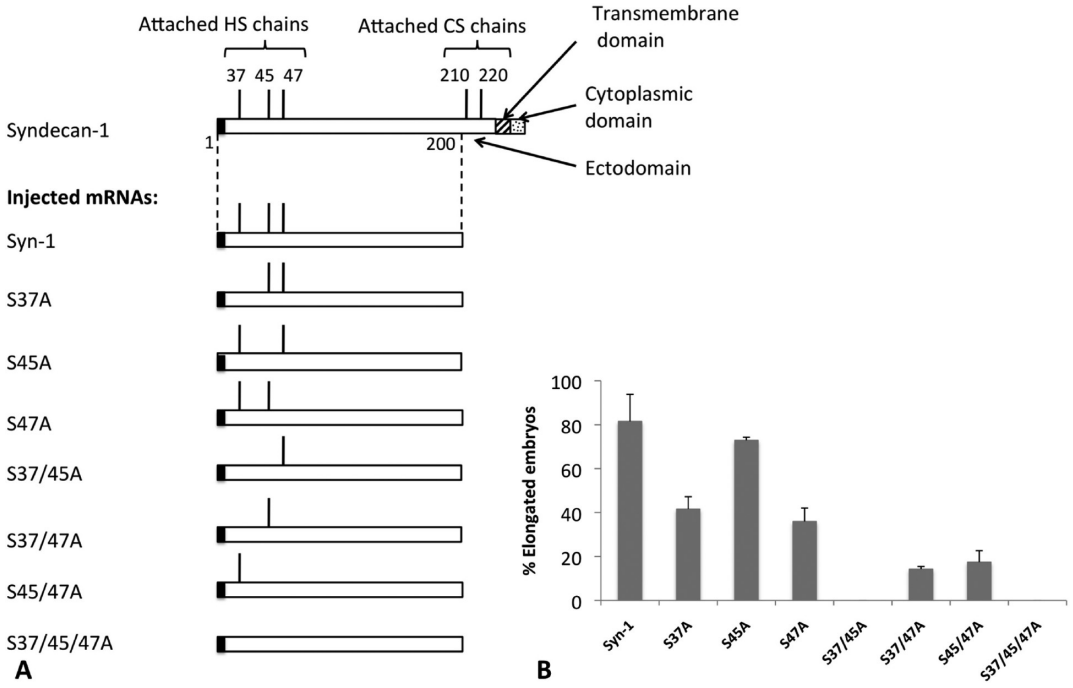

Figure 6. Injection of mRNAs encoding mutated syndecan-1 carrying two and three HS chains significantly caused the elongation phenotype. (A) Structures of syndecan-1 and mutated syndecan-1 with none, one, two, or three HS initiation sites. (B) Percentage of elongated embryos after the treatment with 45 pg of each mRNA at one-cell stage. Total numbers of embryos injected with Syn-1, S37A, S45A, S47A, S37/45A, S37/47A, and S45/47A are 188, 181, 126, 163, 199, 144, 160, and 159, respectively. Syn-1 carries three HS chains; S37A, S45A, and S47A carry two HS chains; S37/45A, S37/47A, and S45/47A carry one HS chain; and S37/45/47A does not carry any HS chain. Using the Bonferroni adjustment, the difference between none/one HS chain and two/three HS chains is statistically significant ($p < 0.01$).

furthermore show that the effect of bis-xyloside **II** is FGF8-dependent.

Structural Analysis of Primed GAG Chains. Biotin-conjugated bis-xyloside (xyloside **XIII**) and mono-xyloside (xyloside **XVII**) were synthesized to identify primed GAG chains and to distinguish them from endogenous GAGs. Thirty-three pmoles of biotinylated xylosides **XIII** and **XVII** were injected into embryos at the one-cell stage. The GAG chains primed by these biotinylated xylosides were purified and captured with fluorescent-tagged streptavidin, S-AF350, as described in Methods. Size exclusion chromatography (SEC) analysis confirmed the priming activity of these two xylosides: in both cases, the unbound S-AF350 peak was reduced or absent and replaced by a GAG-streptavidin peak (Figure 5A). This result indicates that both mono- and bis-xylosides prime GAG chains in zebrafish embryos (Figure 5A). In the case of mono-xyloside, a small fraction of the unbound S-AF350 peak still remained at 37 min. This result suggests that mono-xyloside **XVII** may not be as good a primer as bis-xyloside **XIII**. This is contrary to our findings, reported in our companion paper, that mono-xylosides are better primer than bis-xylosides.[24] To exclude the possibility that differential priming activity may account for the elongation phenotype, we screened a number of additional mono-xylosides, which were found to be better primers than bis-xylosides (Supplementary Table S1). None of these mono-xylosides caused an elongation phenotype. In order to further demonstrate that the GAG chain valency plays a central role in causing the elongation phenotype, bis-xyloside XIII was injected at a lower concentration (15 pmol per embryo). At this concentration, bis-xyloside XIII was found to have a similar priming activity as mono-xyloside **XVII** (Supplementary Figure S3A) but still caused the elongation phenotype in 33% of treated embryos ($n = 240$). Thus these experimental outcomes suggest that the bivalency of the newly primed GAG chains is essential in causing the elongation phenotype. Since streptavidin is a globular tetrameric protein, we could not determine the exact molecular weights of the GAG chains. We estimated the HS/CS/DS composition of primed GAG chains by determining the extent to which GAG-streptavidin conjugates were susceptible to heparitinase I, II, and III or chondroitinase ABC treatment. The results indicated that both bis-xyloside **XIII** and mono-xyloside **XVII** primed mostly CS/DS (>95%) and very little HS (<5%) (Figure 5B). Similar results were obtained with GAG chains from 6 hpf embryos (Supplementary Figure S3C).

Kinetics of *in Vivo* Xyloside Priming. To characterize the kinetics of priming, GAG chains primed by bis-xyloside **XIII** at different time points (2, 4, 6, 8, 10, and 12 hpf) were isolated, and their size exclusion profiles were compared (Supplementary Figure S3B). Primed GAGs were detectable at 2 hpf; nearly all xyloside appeared to have primed by 4 hpf. The results suggest that the amount of primed GAGs increased throughout early development and furthermore that the structure of primed GAGs continued to change, as reflected in differential SEC elution times (Supplementary Figure S3B). The sulfation density of bis-xyloside primed GAG chains at 10 hpf was compared with the sulfation density of heparin using anion-exchange chromatography. The bis-xyloside primed GAG chains eluted at 33 min, while heparin eluted at 55 min (Supplementary Figure S3D), suggesting that the extent of sulfation of GAG chains synthesized during early development was much lower than that of heparin. Finally, disaccharide profiles of HS and CS/DS in WT embryos, bis-xyloside **VI**-injected embryos and mono-xyloside **XVI**-injected embryos

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were analyzed using HPLC coupled to a postcolumn reactor (see the Supporting Information). The disaccharide composition of HS and CS chains were largely identical for WT-, mono-xyloside-, and bis-xyloside-injected embryos, suggesting that these synthetic scaffolds do not overwhelm or disturb the GAG synthetic machinery *in vivo* and that xyloside-primed GAGs have relatively normal sulfation (Supplementary Figure S5).

Determination of GAG Chain Type Responsible for the Elongation Phenotype. Our data showed that both mono- and bis-xylosides primed mostly CS/DS and a small amount of HS (Figure 5B). Therefore, we investigated which GAG type, HS or CS/DS, could cause the elongation of zebrafish embryos when assembled on bis-xylosides. GAG chains were purified from 12 hpf xyloside **II**-treated embryos as described in the Supporting Information. The GAG chains were then subjected to heparitinase I, II, and III or chondroitinase ABC treatment to obtain CS/DS or HS, respectively. Both samples were injected back into zebrafish embryos at 4 hpf. At 12 hpf, 22% of embryos (n = 102) were elongated after injection with HS, and 16% of embryos (n = 100) were elongated after injection with CS/DS. These results imply that both HS and CS/DS primed by xylosides can cause the elongation phenotype. In order to further define the roles of different GAG types, we injected commercially available monomeric GAG chains including heparin, CS-A, CS-C, and DS (1 ng/embryo) into zebrafish embryos. Injection of heparin, DS, CS-A, and CS-C caused, respectively, 95%, 90%, 5%, and 17% (n > 300) elongation phenotype at 12 hpf. Most notably, heparin caused 90% of the embryos to exhibit an elongation phenotype even at a lower dose (0.2 ng/embryo), whereas other GAG types failed to cause the effect at this dose. Expression of the FGF target *mkp3* was analyzed after injection with heparin, DS, CS-A, or CS-C. Also, 77% (n = 35) of embryos injected with heparin and 72.5% (n = 40) of embryos injected with DS overexpressed *mkp3*, compared to CS-A (11%, n = 45) and CS-C (16.3%, n = 43) injected embryos (Supplementary Figure S4). HS and DS, but not CS, injection results in the elongation phenotype as that observed with the bis- and tris-xylosides injection. This result is consistent with a model in which the bis- and tris-xylosides are acting through HS- or DS-dependent FGF signaling pathways. These novel findings further corroborate recent studies that suggest the roles for GAG in FGF signaling.[40,41]

Syndecan-1 Carrying Multivalent HS Chains Causes Elongation Phenotype. One of the limitations of xyloside treatment in zebrafish embryos is our inability to ascertain the *in vivo* priming activity of each xyloside except in the case of biotinylated xylosides. Therefore, in order to confirm that GAG valency, rather than xyloside priming activity, is important in causing the elongation phenotype, ectodomains of mutant syndecan-1 mRNAs carrying one, two, three, or no HS initiation sites were injected (Figure 6). Injection of mRNA carrying all three HS initiation sites caused the elongation phenotype at 12 hpf in 81.5% of treated embryos. Injection of mRNAs carrying two HS initiation sites at positions 45 and 47, 37 and 47, or 37 and 45 caused the elongation phenotype at 12 hpf in 41.5%, 72.9%, or 35.9% of treated embryos, respectively. Injection of mRNAs carrying only one HS initiation site at position 47, 45, or 37 caused the elongation phenotype in 0%, 14.2%, or 17.4% of the treated embryos at 12 hpf. On the other hand, injection of mRNA carrying no HS initiation site did not cause any elongation phenotype at 12 hpf. This result unequivocally indicates that proteoglycans with two or three HS side chains are more effective than proteoglycans with one HS side chain in hyperactivating FGF signaling in zebrafish embryos.

Molecular Mechanism for the Activation of GAG-Mediated FGF Signaling. In earlier studies, mono-xyloside primers have been shown to compete with endogenous core proteins for GAG priming and function as "decoys" by preventing HS from being attached to its acceptor sites on endogenous core proteins.[19,20] In this case, xylosides with higher priming activity should function as better decoys. However, in our companion studies we have shown that mono-xylosides have priming activity higher than that of bis-xylosides, and thus bis-xylosides would be expected to function as worse decoys and therefore cause less elongation than mono-xylosides do.[24] Contrary to the above argument, bis-xylosides are actually much more effective at causing elongation, implying that elongation is not caused by decoy activity. The similarity in the types of sulfation of GAG chains obtained from WT and xyloside-injected embryos suggests that xyloside primers do not disturb the endogenous GAG biosynthesis (Supplementary Figure S5). In our companion studies as well, we have shown that cluster-xylosides do not affect biosynthesis of endogeneous PGs.[24] Moreover, bis-xyloside primed GAG chains purified from zebrafish embryo, irrespective of whether these GAGs are susceptible to heparin lyases or chondroitinases, can cause the FGFR hyperactivation and the elongation phenotype. Under these conditions, endogenous GAGs are largely unaffected. The Kiessling group has shown in an elegant study that FGF8s can oligomerize on a non-heparin polymer scaffold and activate FGFR-mediated signaling.[42] Therefore, one may argue that cluster-xylosides themselves may likewise facilitate FGF dimerization, but not mono-xylosides, leading to signal activation irrespective of their GAG priming ability. To exclude these possibilities, a number of bis-fluoroxylosides, **XVIII**, **XIX**, or **XX** (Table 1), which are bis-xyloside analogues containing a fluorine instead of a hydroxyl group at the C4 position of corresponding bis-xylosides **VIII**, **IX**, or **X**, are synthesized and injected into embryos to determine if they could activate FGF8 signaling without priming dimeric GAG chains. These bis-fluoroxylosides did not cause the elongation phenotype. Taken together, these results confirm that bis-xylosides do not act as "decoys" but rather act through their primed GAG chains. The findings that elongation is seen after injecting either exogenous GAGs or *in vivo*-primed GAGs back into the embryos demonstrate that these GAGs act extracellularly, thus further suggesting that *in vivo*-primed GAGs are secreted and act outside the cell. It is important to note that GAG chains primed by xylosides *in vivo* have a sulfation density lower than that of commercial GAG chains (Supplementary Figure S3D). Therefore, we argue that the high sulfation of heparin and DS allows them to activate FGF-mediated signaling, while under *in vivo* conditions, primed GAG chains with less sulfation must be at least dimeric to facilitate ternary complex formation with FGF and FGFR, subsequently leading to FGFR dimerization and signal transduction. Thus, the elongation phenotype is best explained by a *gain of function* mechanism in which xyloside-primed dimeric GAG chains facilitate the formation of a GAG/FGF/FGFR signaling complex and lead to FGFR hyperactivation. This explanation is further corroborated by the fact that up to 160 pmol of mono-xyloside, 5 times the injected dose for bis-xylosides, did not cause any elongation phenotype. Thus, primed GAG chains need to be at least dimeric to affect FGF signaling.

On the basis of our current *in vivo* study, we propose a molecular model in which two covalently linked GAG chains induce FGF8-dependent FGFR dimerization that leads to the elongation phenotype (Figure 7). Each GAG chain from the

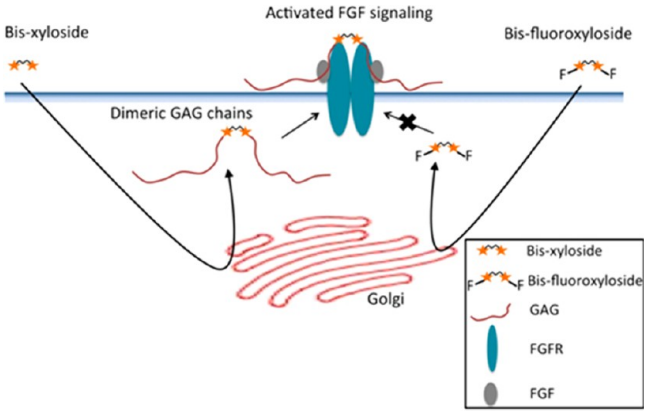

Figure 7. Mechanistic roles of bis-xylosides and bis-fluoroxylosides in the activation of FGF signaling in zebrafish embryos. Bis-xyloside enters the Golgi apparatus and primes dimeric GAG chains. The dimeric GAG chains are secreted to the extracellular matrix and form the ternary complexes with FGFs and FGFRs. Two complexes are then brought closer for active FGFR dimerization leading to elongation phenotype in zebrafish embryos. On the other hand, bis-fluoroxylosides enter the Golgi but cannot prime GAG chains. Therefore, bis-fluoroxylosides cannot activate FGF signaling in zebrafish embryos and fail to cause elongation phenotype.

bis-xyloside scaffold interacts with both an FGF8 molecule and its cognate FGFR simultaneously, forming two independent stable 1:1:1 FGF8/FGFR/GAG ternary complexes. Two complexes are then brought into close proximity for active FGFR dimerization only if the two xylose units are separated by at least three -CH_2- units, providing sufficient conformational flexibility. This model is also strengthened by the fact that a majority of naturally occurring PGs bear multiple GAG chains. One may alternatively argue that FGF8 could be sequestered in specific regions under normal condition through its interaction with endogenous proteoglycans. However, when bis- or tris-xylosides are injected into embryos, the primed GAG chains effectively compete with endogenous PGs, increase FGF8-mediated FGFR dimerization, and lead to the elongation of embryos. Examining such an alternative model is beyond the scope of the current work. Biological importance of GAG multivalency is also further strengthened from observations made in other studies: multivalent CS chains have much more potentiating effect on neurite outgrowth than monovalent CS chains do; dendrimeric heparin conjugates potentiate FGF2-mediated signaling.[43−45]

In summary, the results presented here, for the first time, provide a structural basis for the ability of certain synthetic bis- and tris-xylosides to promote FGF8-mediated FGFR activation *in vivo*. It is, however, noteworthy that GAG multivalency may not be necessary for FGF/FGFR pairs that can form ternary complexes on a single GAG chain. Thus, a large GAG chain may allow FGFRs to slide along the chain to form an active signaling complex. Efforts are currently underway to elucidate the requirement of specific sulfate groups for the activation of FGF8 signaling and the nature of interactions among GAG, FGF8, and FGFR in the formation of signaling complex.

METHODS

Animals and Materials. Zebrafish were raised and bred according to standard procedures. Strains used were Tübingen wildtypes and *Tg(TOP:dGFP)*^{w}.[25] Zebrafish experiments were approved by the University of Utah Institutional Animal Care and Use Committee. Xylosides (Table 1) were synthesized using click chemistry as described in our companion paper and in earlier studies.[22−24] Xylosides were dissolved in distilled water to a final concentration of 100 mM for injection into embryos. *lefty1* probe, SU5402, *sprouty4*, and syndecan constructs were generously provided by Drs. H. J. Yost, T. Piotrowski, L. Maves, and R. D. Sanderson, respectively. Commercial sources: rabbit anti-phospho ERK1/2, Cy-3 anti-rabbit secondary antibody and disaccharide standards, Sigma-Aldrich; SU5402, Pfizer; DEAE-sepharose and sephacel gel, NAP-10, Amersham Biosciences; streptavidin-Alexa Fluor 350 conjugate (S-AF350), Invitrogen; G3000SWxl columns, Tosoh Bioscience LLC; Sep-Pak Plus column, Waters; heparin (18−25 kDa), chondroitin sulfate A (~50 kDa), chondroitin sulfate C (~50 kDa), and dermatan sulfate (~40 kDa), Seikagaku Corporation.

Screening of Xylosides in Zebrafish Embryos. Embryos were obtained by natural mating and raised at 28.5 °C in E3 buffer. Xylosides were diluted with 1% phenol red, and each xyloside (33 pmoles) was microinjected in a 1 nL bolus into the blastomere of one-cell stage embryos. Embryos were staged, and their phenotypes were compared to those of wild type (WT) embryos.[46]

RNA *in Situ* Hybridization. Antisense digoxigenin-labeled probes were generated from cDNA clones using a SP6 or T7 RNA polymerase-based labeling kit. The following probes were used: *mkp3*,[31] *gsc*,[32] *gfp*,[33] *bmp2b*,[34] *bambi*,[47] and *lefty1*.[36] Embryos were fixed overnight in 4% PFA, and then washed with PBST (0.1% Tween-20 phosphate buffered saline). Whole mount *in situ* hybridization experiments were performed as previously described.[48] Embryos were then stained with BM Purple for imaging.

Whole Mount Antibody Staining. Embryos were fixed with 4% PFA for 2.5 h at RT, washed with PBST, blocked with NCST (10% heat-inactivated newborn calf serum, 0.1% Tween-20, 1% DMSO, in PBS) overnight at 4 °C, incubated with rabbit anti-phospho ERK1/2 antibody at 1:300 for 12 h at 4 °C, washed with PBST, incubated with Cy-3 conjugated anti-rabbit secondary antibody at 1:300 overnight at 4 °C, washed with PBST, mounted, and finally imaged on an Olympus confocal microscope.

FGFR Tyrosine Kinase Inhibitor SU5402 Treatment. Twenty-five pmoles of xyloside **II** was injected per embryo at the one-cell stage, and the embryos were subsequently dechorionated. Two-thirds of the embryos were placed in 3 mL of E2 buffer with 25 μM SU5402, and the remaining one-third were placed in 3 mL of E2 buffer as controls. SU5402 treated embryos were then compared to untreated embryos at 12 hpf.

mRNA and Morpholino Injection. *Sprouty4* mRNA was synthesized using a SP6 mMessage mMachine transcription kit. An antisense morpholino against *fgf8* [49] was obtained from Gene Tools (5′- GAGTCTCATGTTTATAGCCTCAGTA -3′; start codon is underlined). To ensure uniform xyloside dosing, we used a double-injection protocol. First, xyloside **II** (25 pmol/embryo) was injected into all embryos at the one-cell stage. Then, two-thirds of xyloside treated embryos were injected with 70 pg of *sprouty4* mRNA or 6 ng of *fgf8* morpholino. These embryos were then compared at 12 hpf with the remaining one-third embryos.

Injection of Purified GAGs into Zebrafish Embryos. GAGs were isolated (see the Supporting Information) from approximately 1,000 embryos (12 hpf) that were injected with xyloside **II**. The isolated GAG chains were then digested with heparitinases I, II, and III or chondroitinase ABC overnight at 37 °C. The undigested CS/DS or HS, respectively, was then purified and concentrated to a final volume of 20 μL using 3000 MWCO Amicon columns. One nanoliter of each sample was injected into the animal pole of dome-stage embryos at 4 hpf. The phenotypes of these embryos were compared to those of WT embryos that had been injected with 1% phenol red. Commercially available heparin, chondroitin sulfate A (CS-A), chondroitin sulfate C

(CS-C), and dermatan sulfate (DS) were also injected into the animal pole of 4 hpf embryos (0.2 or 1 ng per embryo), and their phenotypes were compared to that of WT.

Injection of Mutated Syndecan-1 mRNAs into Zebrafish Embryos. Seven mutant constructs of syndecan-1, in which serine residues at positions 37, 45, and 47 were replaced by alanine residues in all possible combinations, were generously provided by Dr. R.D. Sanderson (University of Alabama at Birmingham). The DNA fragments encoding first 200 amino acids of each of the seven mutant constructs and wild type were subcloned into pCS2+ at *Bam*HI and XhoI sites using forward primer 5′- GATC**ATGGAT**CCATGAGAC-GCGCGGCGCT -3′ and reverse primer 5′- CAT**CTCGAG**TTA-GTGATGGTGATGGTGATGTTCCTTCCTGTCCAAAA -3′. mRNAs of these DNA fragments carrying none, one, two or three HS initiation sites were synthesized using a SP6 mMessage mMachine transcription kit. Each mRNA was injected into the blastomere of one-cell stage embryos at the concentration of 45 pg per embryo. These treated embryos were then compared at 12 hpf with control embryos.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

This material is available free of charge *via* the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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