Heparan Sulfate 6-O-Sulfotransferase Is Essential for Muscle Development in Zebrafish*

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Heparan sulfate proteoglycans function in development and disease. They consist of a core protein with attached heparan sulfate chains that are altered by a series of carbohydrate-modifying enzymes and sulfotransferases. Here, we report on the identification and characterization of a gene encoding zebrafish heparan sulfate 6-O-sulfotransferase (hs6st) that shows high homology to other heparan sulfate 6-O-sulfotransferases. When expressed as a fusion protein in cultured cells, the protein shows specific 6-O-sulfotransferase activity and preferentially acts on the iduronosyl N-sulfoglucosamine. In the developing embryo, hs6st is expressed in the brain, the somites, and the fins; the same structures that were affected upon morpholino-mediated functional knockdown. Morpholino injections significantly inhibited 6-O- but not 2-O-sulfation as assessed by HPLC. Morphants display disturbed somite specification independent of the somite oscillator mechanism and have impaired muscle differentiation. In conclusion, our results show that transfer of sulfate to specific positions on glycosaminoglycans is essential for muscle development.

Heparan sulfate proteoglycans (HSPGs)1 are macromolecules with divergent structures and functions. They are located on the cell surface and in the extracellular matrix and are composed of a core protein to which highly sulfated glycosaminoglycan (GAG) sugar chains are attached. Heparan sulfate (HS) is a GAG of repeating disaccharide subunits that consists of glucosamine and glucuronic/iduronic acid and is modified in a complex series of steps involving deacetylation, sulfation, and epimerization (1). In each of these modification steps only part of the substrate is modified resulting in a high sequence diversity, which is thought to give HSPGs their functional specificity and versatility. HSs specifically bind to various extracellular molecules including growth factors, adhesion molecules, proteases, and receptors to regulate cell proliferation and differentiation during various developmental processes (2).

In Xenopus ectodermal explants, removal of GAGs from proteoglycans by heparanase results in inhibition of elongation and mesodermal differentiation in response to activin, FGF, and Wnt (3). Similar experiments in cockroach embryos perturbed the directed growth of axon pioneer fibers (4). In vertebrates, this function is conserved as it has been shown that exogenous addition of heparan sulfate causes misrouting of retinal axons at the tectal border (5). Recently, this misrouting of axon targeting was attributed to inhibition of sulfation of heparan sulfate (6). The zebrafish mutant knypek (kny) displays impaired convergent extension movements and the gene responsible for the mutant phenotype encodes a member of the glypican family of HSPGs. Kny is involved in the non-canonical Wnt/planar cell polarity pathway enhancing Wnt11 signaling in overexpression experiments and the kny mutation exacerbates the convergent extension defect of silverblicktsilb/until11 mutants (7). Genetic screens in Drosophila have yielded the sugarless (sgl) and sulfateless (sfl) mutants that are involved in HSPG biosynthesis as they encode UDP-glucose dehydrogenase and heparan sulfate N-deacetylase/N-sulfotransferase, respectively (8, 9). Their pleiotropic mutant phenotypes suggest that they have diverse functions in fibroblast growth factor

*mide-2-deoxy-4-O-(4-deoxy-a-L-threo-4-enepyranosyluronic acid)-6-O-sulfo-D-glucose; ΔDi-NS, 2-deoxy-2-sulfamino-4-O-(4-deoxy-a-L-threo-4-enepyranosyluronic acid)-D-glucose; ΔDi-5,6diS, 2-deoxy-2-sulfamino-4-O-(4-deoxy-a-L-threo-4-enepyranosyluronic acid)-D-glucose; ΔDi-5,6,9triS, 2-deoxy-2-sulfamino-4-O-(4-deoxy-a-L-threo-4-enepyranosyluronic acid)-D-glucose; PAPS, 3′-phosphoadenosine 5′-phosphosulfate; CDSNHS-heparin, completely desulfated and N-resulfated heparin; NS-heparan, desacylated, and N-sulfated heparan; CDSNHA-heparin, completely desulfated N-acetylated heparin; EHS, Engelbreith-Holm-Swarm; GAG, glycosaminoglycan; HPLC, high-performance liquid chromatography; hpf, hours post fertilization.

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PAPS was prepared as described previously (20).

becco and antibiotics for 72 h, the cells were washed with PBS.

CMV2 alone using LipofectAMINE according to the manufacturer's protocol, and the activities of Hs6sts in the supernatants and FLAG-bound fractions were measured as described below.

Assay for Sulfotransferase Activities—Sulfotransferase activities were determined as described previously (23). Briefly, the standard reaction mixture (50 μl) contained 2.5 μmol of imidazole-HCl, pH 6.8, 3.75 μg of protamine chloride, 25 nmol (as hexosamine; 500 μM) of acceptor glycosaminoglycans, 50 pmol of [35S]PAPS (about 5 × 10⁶ cpm, 1 μM), and enzyme. After incubation for 20 min at 37 °C, the reaction was stopped by heating at 100 °C for 1 min. Carrier chondroitin sulfate A (0.1 μM as glucuronic acid) was added to the reaction mixture and the 35S-labeled polysaccharides were isolated by precipitation with ethanol and containing 1% potassium acetate and 0.5 mM EDTA, followed by gel chromatography on a Fast Desalting column to remove [35S]PAPS and its degradation products. The amounts of enzymes added to the reaction mixture were chosen so as to obtain a linear incorporation of [35S]sulfate. One unit of enzyme activity was defined as the amount required to transfer 1 pmol of sulfate/min to CDSSN-heparin. Analysis of the reaction products for the specificity and sulfation position was performed by HPLC as described previously (23) with some modifications. Briefly, 35S-labeled products were digested with a mixture of 10 milliunits of heparitinase I, 5 milliunits of heparitinase II, and 10 milliunits of heparitinase III in 40 μl of 50 mM Tris-HCl, pH 7.2, 1 mM CaCl₂, and 4 μg of bovine serum albumin at 37 °C for 2 h. The digests were subjected to gel chromatography on Superdex 30 pg equilibrated with 0.2 mM NH₄HCO₃. The 35S-labeled disaccharide fractions were injected into a PAM column together with standard unsaturated disaccharides of the kit. Fractions of 0.6 ml were collected and radioactivity was measured.

Chromatographic Analysis of Isolated Glycosaminoglycans of Zebrafish Embryos—Embryos at the one-cell stage were injected with 1.0 ng of Hs6st-MO (1) and 1.5 ng Hs6st-MO (2) simultaneously. Embryos were collected at the 48 hpf stage and fixed with ice-cold 70% ethanol. The dried samples were treated with 0.2 M NaOH for 16 h at room temperature. Subsequently, samples were neutralized with 4 M HAc, DNase, and RNase were added and incubated for 2 h at 37 °C. Then proteinase K was added, and the reaction mixture was incubated for 1 h at 37 °C. The reaction was stopped by heating at 100 °C for 5 min, and samples were centrifuged at 13,000 rpm for 10 min to remove insoluble material. The supernatants were diluted with an equal volume of Tris-HCl buffer (50 mM, pH 7.2) and loaded on a DEA-Septahexyl column equilibrated with Tris-HCl buffer containing 0.2 M NaCl. The columns were washed with 10 column volumes of Tris-HCl buffer and then eluted with 4 column volumes of 2 M NaCl in Tris-HCl buffer. The eluates were precipitated with 2.5 volumes of cold 95% ethanol containing 1% potassium acetate and the glycosaminoglycans were recovered by centrifugation. The GAGs were digested with a mixture of 10 milliunits of heparitinase I, 5 milliunits of heparitinase II, and 10 milliunits of heparitinase III in 40 μl of 50 mM Tris-HCl buffer, 1 mM CaCl₂, and 4 μg of bovine serum albumin at 37 °C for 2 h. After filtration of the digests with Ultrafree-UC, unsaturated disaccharides in the filtrates were analyzed by a reversed-phase ion-pair chromatography using Senshu Pak column Docosil and determined with a fluorescence detector.

Bodipy- ceramide Staining—Bodipy-ceramide labeling was performed as described (21).

RESULTS

Isolation of Zebrafish hst—To isolate genes that may participate in neural plate regionalization, a subtractive hybridization experiment was performed.② One of the resulting cDNA clones, R1, was sequenced and appeared to be a partial hst cDNA clone. We obtained the full-length clone by searching the zebrafish EST database. One clone (MPMPg609N1048) was 100% identical to the cDNA fragment so we sequenced it to completion. The longest open reading frame encoded a protein of 469 amino acids (Fig. 1A), which was homologous to HS6STs from various organisms (Table I). Highest homology was found to the human and murine HS6ST-1 and HS6ST-2 proteins. However, by amino acid comparison (Table I) and phylogenetic tree analysis (Fig. 1B) we could not assign zebrafish Hst to

By calculating a Kyte-Doolittle hydrophobicity plot of the Hs6st protein sequence, a hydrophobic segment was found in the amino-terminal part (data not shown) that is characteristic for heparan sulfotransferases (23). This hydrophobic segment suggests that Hs6st is a type II transmembrane protein located in the Golgi apparatus where HSPGs are synthesized initially (24).

The genetic map position of hs6st was determined by radiation hybrid mapping (19).

Determination of Heparan Sulfate 6-O-Sulfotransferase Activity—The hs6st cDNA was transfected into cultured cells, and the expressed protein was examined for the HS6ST activity. The HS6ST activity, defined as the amount required to transfer 1 pmol of sulfate/minute to CDSNS-heparin, in cells transfected with hs6st was more than 3-fold higher as compared with the control vector (Table II). The HS2ST activity was not changed upon transfection of hs6st. These results demonstrate that recombinant Hs6st transfers sulfate to position 6 of N-sulfoglucosamine residues in CDSNS-heparin.

To compare the sulfotransferase activity of zebrafish Hs6st to that of other organisms, we investigated its sulfotransferase activity for various heparin derivatives, heparan sulfates from different sources, and other GAGs (Table III) including:

- CDSNS-heparin
- CDSNAc-heparin
- NS-heparosan
- Heparan sulfate (mouse EHS tumor)
- Heparan sulfate (pig aorta)
- 6ODS-heparin
- Chondroitin

Further, the ratio of the activity between HS-heparan and CDSN-heparin was less than 0.1 at all the concentrations tested.
This indicates that Hs6st preferred iduronosyl N-sulfoglucosamine since NS-heparosan contains only glucuronic acid and not its epimer iduronic acid. These data are very similar to the sulfotransferase activity values found for mHS6ST-1 and not to values for mHS6ST-2 (23).

To further determine the specificity of Hs6st function, the structure of the 35S-labeled products was analyzed after incubation of purified Hs6st, 35S-labeled PAPS and CDSNS-heparin or heparan sulfate of pig aorta (Fig. 2, B and C). Most of the radioactivity in the disaccharide fractions derived from both acceptor substrates was recovered at the position of \( \text{Di}(\text{N,6)}_{\text{diS}} \), and a low level of radioactivity eluted in an unknown peak between \( \text{Di}(\text{N,6,2)}_{\text{triS}} \) and \( \text{Di}(\text{N,6)}_{\text{diS}} \). Furthermore, 35S-labeled \( \text{Di}(\text{N,6,2)}_{\text{triS}} \) was also produced using heparan sulfate as acceptor. These results show that Hs6st transfers sulfate groups to position 6 of GlcNSO3 residues and to position 6 of GlcNSO3(6SO4) residues.

Expression Pattern of hs6st during Zebrafish Embryonic Development—Northern blot analysis revealed that hs6st was not maternally expressed (data not shown). The spatio-temporal pattern of zygotic hs6st expression was analyzed by whole mount in situ hybridization. At the onset of gastrulation, hs6st is ubiquitously expressed in the entire blastoderm (Fig. 3 A). During early somitogenesis the staining seems to concentrate to the anterior CNS (Fig. 3 B). Consistently, at 24 hpf the brain and the eyes of the embryo show intense staining. Importantly, expression was also restricted to the somitic boundaries as well as to the ventral part of the tail (Fig. 3, C–E). The rostral expression persisted until at least the 48 hpf stage when the fin buds contained high levels of the hs6st transcripts as well (Fig. 3 F).

Morpholino-mediated Knockdown of Hs6st Results in Specific 6-O-Sulfation Inhibition—To assess the developmental function of Hs6st, we have selectively blocked the translation of the hs6st mRNA using morpholino antisense oligonucleotides, which have been proven to be effective and specific translational inhibitors in zebrafish (18) and were shown to phenocopy a wide variety of earlier described mutations (25). For knockdown experiments we used two morpholinos of non-overlapping sequence as this method yielded a higher frequency of knockdown phenotypes whereas possible nonspecific undesired mistargeting effects were reduced (Ref. 26, data not shown).
This technique allowed us to specifically inhibit 6-O-sulfation, which is a great advantage when compared with the more general inhibition of proteoglycan/protein sulfation by for example chlorate (27–29). As controls, inverted morpholinos were used (see “Experimental Procedures”) that resulted in wild-type embryos upon injection.

Specificity of the injected morpholinos was assessed by determining the sulfation profile of disaccharide components of wild-type versus morpholino-injected embryos that showed the morphant phenotype. In 48 hpf embryos, highest decrease of sulfation (40.3%) in morphants was found for 6-O-sulfation of N-sulfoglucosamine residues (production of ΔDi(N, 6)diS (Table IV)). As compared with the disaccharide components of wild-type embryos, N-sulfation increased, and 2-O-sulfation decreased slightly (Table IV). Analysis of heparan sulfate compositions was performed in duplicate with a similar outcome. From these results, we conclude that Hs6st morpholino injection inhibits 6-O-sulfation specifically albeit not completely.

**Phenotypic Characteristics of Hs6st Morphants**—The morpholino-mediated knockdown of Hs6st activity resulted in dramatic phenotypic abnormalities that were already visible during somitogenesis (Fig. 4). Control experiments in which embryos were injected with inverted Hs6st-morpholinos did not alter the wild-type phenotype throughout development indicating the specificity of the morpholinos (Fig. 4, A and B). Embryos injected with Hs6st morpholinos demonstrated convergent-extension defects, reminiscent of knypek (knyp)glypican zebrafish mutants (7), that became more obvious as development proceeded (Fig. 4C, see also Fig. 6, G–J). Often the tail was curled and to a certain extent, morphants displayed transient and variable brain apoptosis between 24 and 48 hpf (Fig. 4C). As a consequence, the size of the head in morphants was reduced later in development (Fig. 4, D and E). In contrast, the brain ventricles had an inflated and oedemic appearance possibly due to the overproduction of cerebrospinal fluid. Transverse sections of the brain of 48 hpf embryos show that both the size of the pectoral fins at 96 hpf (Fig. 4, D and E), a structure that showed expression of hS6ST (Fig. 3F).

**Knockdown of Hs6st Disrupts Muscle Development in Zebrafish**—The most obvious defects in Hs6st knockdown embryos were seen in somite development. Nomarski images of somites of 48 hpf morphant embryos reveal abnormal muscle fiber structure with undifferentiated cells present (Fig. 5, A–C). To investigate somite abnormalities further, we utilized Bodipy-ceramide, a lipophilic, vital dye to reveal cell morphology. In 24 hpf wild-type embryos, somites have a chevron shape with clear boundaries. The adaxial cells at the horizontal myoseptum can be clearly distinguished by their morphology (Fig. 5D). Morpholino injections resulted in a range of phenotypes that could qualitatively be categorized as weak, intermediate, or strong. In weakly affected Hs6st morphants, muscle cell morphology is normal but there are local interruptions in the vertical myoseptae, which result in cells spanning across the somitic boundary (Fig. 5E). In embryos with qualitatively more severe defects, however, muscle differentiation seems to be affected at an earlier step. Some somitic cells are smaller, do not show the classic elongated shape observed in wild-type embryos and breakdown of intersomitic boundaries is even more pronounced (Fig. 5F). By 72 hpf, somite defects become very severe in morphant embryos (Fig. 5, G–I). In less strongly affected embryos, somites have large rounded cells, which appear to be only empty shells with intact membrane surrounding them as shown by their exclusion of Bodipy-ceramide (Fig. 5H). In more severe cases, muscle degeneration is almost complete, the empty cells observed in weaker affected embryos are completely reabsorbed leaving large intercellular spaces with barely a few muscle cells remaining connected to each other and to the remnants of the intersomitic boundaries (Fig. 5I). The fact that we observed slight qualitative differences in severity of morphant phenotypes within the same batches of injection experiments as indicated by weak (Fig. 5, B, E, H) and strong (Fig. 5, C, F, I) phenotypes may be due to unequal spreading of injected morpholino solution resulting in slightly different active concentrations in the early embryo. This could

**TABLE IV**

Disaccharide composition analysis in wild-type and Hs6st morphants

<table>
<thead>
<tr>
<th></th>
<th>Wild type</th>
<th>Morpholino injected</th>
<th>Relative in/decrease</th>
</tr>
</thead>
<tbody>
<tr>
<td>Di-OS</td>
<td>53.0</td>
<td>57.3</td>
<td>+8.1</td>
</tr>
<tr>
<td>Di-NS</td>
<td>15.4</td>
<td>17.2</td>
<td>+11.7</td>
</tr>
<tr>
<td>Di-6S</td>
<td>5.5</td>
<td>4.4</td>
<td>−20.0</td>
</tr>
<tr>
<td>Di-(N,6)diS</td>
<td>6.2</td>
<td>3.7</td>
<td>−40.3</td>
</tr>
<tr>
<td>Di-(N,UDiS)</td>
<td>9.7</td>
<td>8.7</td>
<td>−10.3</td>
</tr>
<tr>
<td>Di-(N,6,UtriS)</td>
<td>10.2</td>
<td>8.8</td>
<td>−13.7</td>
</tr>
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</table>

**Fig. 4. Phenotypical defects of Hs6st morphants.**

A, lateral view of a 48 hpf wild-type embryo. B, lateral view of a 48 hpf embryo injected with 3.75 ng Hs6st inverted morpholino. C, lateral view of a 48 hpf Hs6st morphant of reduced size displaying a curvy tail. D and E, dorsal view of a 96 hpf wild-type embryo (D) and a 96 hpf morphant (E). The size of the mid- and hindbrain as can be seen by the distance from the middle of the lens to the posterior end of the ear (arrow) as well as the size of the fin (arrow) in a representative morphant (G) as compared with the wild-type (F). Injections were done with 1.0 ng of Hs6st-MO (1) and 1.5 ng of Hs6st-MO (2). Wt, wild type; inv. MO-inj., inverted Hs6st morpholino-injected; MO-inj., Hs6st morpholino-injected.
result in mild and strong phenotypes at later stages of development.

Knockdown of Hs6st Does Not Affect the Somite Oscillator Mechanism but Disturbs Expression of Anterior Somite Markers—Next we set out to investigate what mechanism leads to the abnormal somitic boundaries observed in morphant embryos. To this end expression of different marker genes was assessed in qualitative terms. In 10-somite stage wild-type embryos myoD, a bHLH transcription factor involved in muscle differentiation is expressed in adaxial cells and in the posterior part of the newly formed somites (Refs. 30 and 31, Fig. 6A). In 15-somite stage Hs6st morphants the striped pattern in the mature somites is severely disturbed (Fig. 6B). We hypothesized that the oscillator mechanism regulating zebrafish somitogenesis might be affected. To this end, we analyzed the expression pattern of her1 and deltaD, two effectors of the zebrafish somitic clock (32, 33, 34). In embryos with perturbed oscillator function, her1 and deltaD are expressed throughout the presomatic mesoderm instead of the normal stripe pattern (35). Even severe Hs6st morphants, however, displayed a wild-type-like her1 and deltaD stripes of expression in the most recent forming somites (Fig. 6, C–F) suggesting that the oscillator function is not affected by Hs6st knockdown. Interestingly however, the striped pattern shows left-right asymmetry in about one-third of the injected embryos, which suggests asynchrony during somite development.

To examine antero-posterior specification within the somites, notch5 and notch6, two genes encoding Delta receptors and expressed in the posterior and anterior part of the somites, respectively, were analyzed (36). At the 10-somite stage, somites were expanded laterally as judged from notch5 and notch6 expression patterns most likely due to early aberrant convergent extension movements (Fig. 6, G–J). In contrast to the defects observed with later myoD distribution, Hs6st morphants did not display obvious change in the expression pattern of notch5 indicating that posterior somite identity was unaffected (Fig. 6, G and H). Expression of notch6 in the last 4–5 somites however was somewhat reduced, which suggests that anterior somite specification may be impaired (Fig. 6, I and J). Mesp-b is a functional homologue of mammalian mesp2 (37), a bHLH transcription factor, which is segmentally expressed in two or three stripes in the anterior regions of somite primordia and is responsible for anterior somite specification upstream of notch5 and notch6 in zebrafish (Refs. 38 and 39, Fig. 6K). Knockdown of Hs6st results in reduced expression of mesp-b at the 5-somite stage as compared with wild-type expression but the segmental pattern of the transcript is still detectable (Fig. 6, K and L). These results suggest that partial loss-of-function of Hs6st does not impair the somitic clock mechanism but affects later mesp-b-dependent steps during somite patterning.

Hs6st Is Required for Proper Muscle Differentiation—To investigate the molecular basis of somitic defects we analyzed marker genes involved in muscle development. In 24 hpf wild-type embryos, myoD is expressed in the individual somites in which expression of myoD in the posterior-most somites is slightly stronger (Fig. 7A). In morphant embryos, myoD is clearly expressed more intensely in all somites along the antero-posterior axis (Fig. 7B). As development proceeds, myoD is down-regulated in wild-type embryos with only remnants of myoD mRNA left in the somites (Fig. 7, C and E). Interestingly, myoD expression is maintained at high level in the somites of 48 hpf morphant embryos (Fig. 7, D and F) suggesting that differentiation is perturbed. As it is possible that lingering activity of myoD interferes with the process of normal differentiation, we next examined a-tropomyosin expression, one of the first structural protein genes to be activated during this process (40). However, no changes were detected in its expression indicating that the initial steps of differentiation proceeded correctly (Fig. 7, G and H). Slow muscle in zebrafish develops from a subset of somitic cells, which can be found closest to the notochord and therefore referred to as adaxial cells (41, 42). Muscle pioneer (MP) cells differentiate from the adaxial cells and express eng2 (43, 44). As early myoD expression indicated that adaxial cell specification is normal, we examined whether separation of MP cells from the adaxial cell lineage occurred properly. We found that eng2 mRNA levels were significantly higher along the A-P axis but no lateral or
dorsoventral expansion was detected (Fig. 7, I–L). These results suggest that somitic muscle differentiation is arrested at an early stage, which might lead to its later degeneration.

As signals from axial tissues are known to regulate gene expression in paraxial mesoderm, we next investigated structural integrity and signaling from the axial mesoderm. In zebrafish, the gene coding for the α chain of type II collagen (col2a1), is expressed in a number of chondrogenic and non-chondrogenic tissues including the floorplate, the notochord and the hypochord (Refs. 45 and 46, Fig. 8A). In 24 hpf morphants, expression is present in all three tissues but mRNA levels remained elevated in the notochord whereas floorplate and hypochord expression decreased (Fig. 8B). Also, the cellular morphology of the vacuolar cells in the notochord is affected, which indicates that cellular proliferation or differentiation might be perturbed by Hs6st morphants.

Shh and other members of the Hedgehog family have been shown to regulate somite differentiation in zebrafish as well as...
in higher vertebrates (47–49). In 24 hpf zebrafish embryos, shh is expressed in the brain, the floorplate and in the posterior end of the notochord (Ref. 50, Fig. 8, C, D, G, H). In 24 hpf morphants, notochordal, and floor plate expression was normal, but the dorsal extension of the zona limitans interthalamica, was absent (Fig. 8, E and F). To investigate if Hedgehog signaling from axial tissues is normal in morphant embryos we used the patched1 (ptc1) marker gene. Ptc1 is a shh receptor, expressed in a pattern complementary to that of shh in the central nervous system and in the paraxial mesoderm of zebrafish embryos and has been shown to be regulated by Hedgehog signaling (51). In Hs6st morphants, somitic ptc1 expression surrounding the notochord is not affected indicating that enhancement of eng2 expression is unlikely to be a result of altered Hedgehog signaling by Hs6st (Fig. 8, I–N).

In summary, the above in vivo experiments have shown that Hs6st is essential during somite specification and differentiation of muscle cells albeit Hs6st acts independent of the somite oscillator mechanism.

**DISCUSSION**

Enzymes that modify HSPGs play essential roles during development. The fact that an enormous structural diversity of GAGs is created by deacetylation, sulfation, and epimerization indicates that a complex scaffold of proteoglycans is attached to the cells. Heparan sulfate 6-O-sulfotransferase is one of these modifying enzymes and here we describe its expression, biochemical activity, and examine its functions during zebrafish muscle development.

In contrast to the mouse and human that have multiple HS6ST homologues (23, 52, 53), *Xenopus*, *Drosophila* and zebrafish have only one described gene coding for HS6ST so far (Refs. 6 and 54, this study). The 3-fold increase in HS6ST activity as found for zebrafish Hs6st was comparable to the values found in *Drosophila* for HS6ST activity (54). The phylogenetic analysis using simple homology search does not indicate clearly which is the true mouse orthologue of zebrafish hs6st. Comparison of expression data is not very informative in this regard as the study describing the expression patterns of putative homologues in mouse and *Xenopus* have been limited to later stages of development (6, 23). Biochemical evidence however, demonstrates that based on the ratio of HS6ST activity between CDSNS-heparin and NS-heparin (23), the zebrafish gene is acting in a fashion most similar to murine HS6ST-1. In biological assays to examine its cellular function we observed a specific HS6ST activity as sulfate groups were predominantly transferred to position 6 of N-sulfoglucosamine. Surprisingly, injection of two non-overlapping morpholinos did not result in a 100% inhibition of sulfate transfer as assessed by the analysis of disaccharide components in 48 hpf morphant and wild-type embryos. Unfortunately, due to the lack of an antibody against Hs6st, we were not able to analyze whether morpholino injection abolished Hs6st protein levels completely in morphant embryos, which leaves two possible explanations for the observed specific but partial 6-O-sulfation in morphants. Zebrafish may possess more than one gene coding for an enzyme with HS6ST activity similarly to the situation in the mouse and in humans (52, 53). Alternatively Hs6st activity may partially be recovered by 48 hpf of development, similarly to what was found for other proteins in transient knockdown studies (55–57).

In summary, our data suggest that zebrafish Hs6st is the homologue of mHS6ST-1. Whether zebrafish encodes multiple homologues of Hs6st can only be answered definitively upon completion of the zebrafish genome sequence.

The expression pattern of hs6st suggested a role in several developmental processes, which were confirmed in the functional knockdown experiments. Hs6st mRNA is present in three main areas in the zebrafish: the brain, the pectoral fins, and the somites. One characteristic of Hs6st morphants was the reduction of white matter in which axon bundles reside. HSPGs are highly abundant in the white matter of rat and chick embryonic brains and are involved in neurite outgrowth and axonal guidance (58, 59). Moreover, it has been shown that the composition of heparan sulfate with respect to 2-O and 6-O sulfation might be responsible for proper targeting of retinal ganglion cell axons in *Xenopus* (60). It is possible that in ze-
brafish other proteoglycans are involved primarily in this process as in our knockdown experiments optic nerve formation and the optic tract is not affected (data not shown). Supporting this idea is the recent data demonstrating that strong repellent guidance of regenerating optic axons in zebrafish embryos has mainly been ascribed to chondroitin sulfates but not to heparan sulfates (60). Hs6st expression was also found in the pectoral fins of 48 hpf zebrafish embryos. In accordance, morphant embryos display reduced pectoral fins at 5 dpf. The mechanism of Hs6st action in fin development is at present unclear and requires further study.

In vertebrates, almost all skeletal muscle is derived from the somites. Knockdown of Hs6st function appears to affect muscle development at several levels including: 1) perturbing mesp-b-dependent somite patterning demonstrating the impaired anterior somite specification, 2) maintaining high levels of myoD expression during somitogenesis in contrast to wild-type embryos that display a down-regulation of myoD labeling, and myoD expression at 5 dpf. The mechanism of Hs6st action in fin development is at present unclear and requires further study.

The zebrafish somitic clock, responsible for directing proper division of the unsegmented paraxial mesoderm, consists of her1 and components of the Delta-Notch signaling pathway although the exact details of its mechanism are still under dispute (35, 61, 62). Expression of notch6 that is localized to the anterior part of the somites is slightly reduced in Hs6st morphant embryos, particularly in the posteriorly, normally formed somites while expression of the posterior marker notch5 is unaffected. Mesp-b, a gene involved in promoting anterior somite fate (38) was also perturbed in morphants suggesting that zebrafish Hs6st is required in a step of the A-P patterning mechanism. As the striped expression pattern of both her1 and deltaD is present in Hs6st morphants, the observed mesp-b-dependent defects seem to occur without the direct involvement of the somite oscillator mechanism. These findings are in agreement with her1 being active during anterior somite specification independently of mesp-b (38).

For proper differentiation of muscle precursor cells in the somites, the correct spatio-temporal activation of myogenic genes is required and these genes are regulated by Wnts, Hhs BMPs, and FGFs (63, 64) secreted by cells of various surrounding tissues. Recently, it has been demonstrated that expression of myogenin in the mouse limb bud (65), is temporally and spatially coincident with expression of two proteoglycan core protein genes, decorin, and syndecan-3 (66). Furthermore, syndecan-3 is synthesized by myoblasts and inhibition of its expression results in acceleration of muscle differentiation in an FGF-2-dependent manner (67). In Hs6st morphant zebrafish embryos, myoD expression in the somites is maintained and increased as compared with wild-type embryos suggesting that proper sulfation might play a role in controlling proper spatio-temporal pattern of muscle differentiation. This is supported by the fact that we also observed an increase of eng2 expression, a homeodomain repressor gene that is expressed in muscle pioneer cells along the somitic myoseptum (41). At later stages, we observed severe degeneration of muscle cells in the somites of Hs6st morphants indicating that the activity of Hs6st is essential for proper muscle cell development. Whether this feature is a direct consequence of the perturbed differentiation is currently unclear but our observation that myoD expression is not reduced in morphant somites might indicate a connection between these events. As eng2 expression marks a subset of slow muscle forming adaxial cells in zebrafish that are not affected by knockdown of hs6st, further experiments are necessary.

HSPGs have a significant role in modifying various cellular signaling processes (2). One of the pathways, that has been shown in this context, is the Wnt pathway. Wnts have been shown to control myogenesis in mammalian systems (68, 69, 70). Wnts bind with high affinity to heparin and heparan sulfate and it is now generally thought that Wnt signaling is regulated through their binding to heparan sulfate moieties on cell surface HSPGs in the extracellular matrix (15, 71–75). Our data supports a genetic interaction between Hs6st and Wnt signaling as in Hs6st morphants we observed an up-regulation of myoD and eng2 expression, which are both described as Wnt target genes in mice (76, 77). Also, the strong Hs6st knockdown phenotype is reminiscent to kny, a glypicant mutant, which has severe convergent extension defects, that potentiates Wnt11 function in a dose-dependent manner (7). However, further experiments are needed to address the precise functional link between Hs6st and Wnt signaling in myogenesis.

Hedgehog signaling has also been linked to HSPG synthesis and sulfation. Toot-velu, a heparan-sulfate copolymerase gene related to the mammalian tumor suppressor genes EEXT1 and EEXT2, is required for proper diffusion of the long-range Hedgehog signal during wing disc patterning in Drosophila (10, 12). Mutants of the sulfatase gene have impaired N-deacytelysae N-sulfotransferase function and lack N-, 2-, O-, and 6-O-sulfation. The sulfatase gene is essential for proper Wg, FGF as well as Hh signaling in Drosophila (9, 78, 79). Our experiments have revealed an increase in eng2 expression in the somites; a feature that has been obtained previously by increased Hedgehog signaling (47). In contrast to our results however, these experiments also resulted in V-V as well as lateral expansion of eng2-expressing cells in the somites. Since this is not the case in Hs6st morphants it appears that Hs6st might not act by affecting midline Hedgehog signaling. This was confirmed by the unchanged expression of ptc1, a known Hh target gene (51).

In conclusion, this is the first report that demonstrates that reduced transfer of 6-O-sulfation by Hs6st to GAG chains causes developmental defects as shown in an in vivo situation during vertebrate development. Gross HSPG modifications are brought about by chemical compounds such as chlorate, a very potent, but nonspecific inhibitor of sulfation that reduces the PAPS concentration (27–29) and these modifications interfere with developmental processes such as neural tube closure (80). Our results demonstrate that even subtle changes in sulfate moieties of GAGs brought about by morpholino-mediated knockdown of Hs6st show pleiotropic developmental phenotypes with most obvious effects on proper muscle formation in an intact in vivo model.

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