The Role of *Drosophila* Heparan Sulfate 6-O-Endosulfatase in Sulfation Compensation

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Background: HS sulfation compensation provides robustness to cellular signaling and animal development, but its mechanism is unknown.

**Results:** Sulf1 is required for sulfation compensation in *Hs2st* mutants.

**Conclusion:** Sulfation compensation depends on the coordinated activities of *Hs2st*, *Hs6st*, and Sulf1.

**Significance:** The finding that Sulf1 is a novel component of HS sulfation compensation machinery provides novel mechanistic insight into this poorly understood phenomenon.

The biosynthesis of heparan sulfate proteoglycans is tightly regulated by multiple feedback mechanisms, which support robust developmental systems. One of the regulatory network systems controlling heparan sulfate (HS) biosynthesis is sulfation compensation. A previous study using *Drosophila* HS 2-O- and 6-O-sulfotransferase (*Hs2st* and *Hs6st*) mutants showed that loss of sulfation at one position is compensated by increased sulfation at other positions, supporting normal FGF signaling. Here, we show that HS sulfation compensation rescues both Decapentaplegic and Wingless signaling, suggesting a universal role of this regulatory system in multiple pathways in *Drosophila*. Furthermore, we identified Sulfl, extracellular HS 6-O-endsulfatase, as a novel component of HS sulfation compensation. Simultaneous loss of *Hs2st* and Sulfl led to 6-O-oversulfation, leading to patterning defects, overgrowth, and lethality. These phenotypes are caused at least partly by abnormal up-regulation of Hedgehog signaling. Thus, sulfation compensation depends on the coordinated activities of *Hs2st*, *Hs6st*, and Sulfl.

HSPGs serve as co-receptors for a number of signaling ligands such as FGFs, bone morphogenetic proteins, Wnt, and Hh family members, playing critical roles in development and tumorigenesis (1, 2). Genetic studies have shown that mutations affecting both core proteins and HS biosynthetic/modifying enzymes have a major impact on a number of developmental systems (3–6). However, the mechanism by which HSPG biosynthesis is regulated *in vivo* is poorly understood.

Control of HS structures can occur at two major steps as follows: 1) HS biosynthesis and modification in the Golgi, and 2) post-synthetic remodeling in the extracellular space. After the formation of a tetrasaccharide linkage attached to the serine residues of the core protein, EXT proteins in the Golgi apparatus polymerize HS chains as repeating disaccharides composed of N-acetylgalactosamine (GlcNAc) and glucuronic acid. The first HS-modifying enzyme, N-deacetylase/N-sulfotransferase, removes the acetyl groups from some of the GlcNAc residues and replaces them with sulfate groups. Subsequently, glucuronic acid can be converted to iduronic acid by the activity of HS C5-epimerase (Hsepi), and sulfate groups are added onto different ring positions of the HS chain. Such sulfation events include 2-O-sulfation of uronic acid and 6-O-sulfation of glucosamine residues catalyzed by HS 2-O- and 6-O-sulfotransferases (*Hs2st* and *Hs6st*), respectively. After HSPGs are transported to the cell surface, HS structure can be further modulated by extracellular HS 6-O-endsulfatas (Sulfs), which remove a specific subset of 6-O-sulfate groups within the highly sulfated domains on HS (7–12). In humans, mutations in Sulfs are associated with tumorigenesis (13, 14) and with Mesomelia-Synostoses syndrome, an autosomal dominant human disorder characterized by limb shortening, acral synostoses, and multiple congenital malformations (15). These Golgi and extracellular enzymes regulate the amount and pattern of sulfate groups on HS, which play critical roles in creating binding sites on HS for a variety of ligand proteins (16, 17).

The biosynthesis of HSPGs is controlled by multiple feedback mechanisms. For example, expression of Daily, a *Drosophila* glycan that functions as a co-receptor for Decapentaplegic (Dpp) morphogen (a *Drosophila* homologue of bone morphogenetic proteins), is negatively regulated by Dpp signaling (18). A computational study revealed that this type of feedback system can be a contributing factor to the robustness of morphogen gradient formation (19, 20). Similar negative feedback systems also work through HS-modifying enzymes. Sulfl, the only *Drosophila* homologue of vertebrate Sulfs, negatively regulates Wingless (Wg) signaling in the developing wing. Because Sulfl expression is induced by the Wg pathway (12, 21), this negative...
feedback loop appears to stabilize the Wg morphogen gradient. Thus, HSPG biosynthesis provides additional layers of fine regulation of cell-cell communication, supporting robust developmental systems.

Another feedback regulatory network controlling HS biosynthesis involves compensatory increases in sulfation at other positions in response to mutation of a HS sulfotransferase gene. This remarkable phenomenon, which is now known as “HS sulfation compensation,” was first discovered in a mammalian system (22, 23). HS purified from Hs2st−/− mouse embryonic fibroblasts did not have 2-O sulfate groups, but this loss was compensated for by increased N- and 6-O-sulfation. This compensation of HS sulfation is not unique to mammalian systems but appears to be a general feature of the HS biosynthetic machinery, because similar compensation events were also recognized in invertebrate models (24, 25). Drosophila Hs2st and Hs6st mutants show a remarkable increase in the levels of 6-O- and 2-O-sulfate groups, respectively, restoring a wild-type net charge on HS in both genotypes (24). As a result, a significant fraction of these mutants survive to adult stages, whereas Hs2st; Hs6st double mutants are completely lethal. Detailed analyses of FGF signaling during tracheal system formation in the mutants demonstrated that FGF signaling can occur in vivo in the absence of either 2-O- or 6-O-sulfate groups on HS. These data suggest that the lack of a specific sulfation sequence can be overcome when HS has a sufficient level of sulfate groups. Thus, HS sulfation compensation provides flexibility and stability to growth factor signaling.

HS biosynthesis regulatory networks play essential roles in normal development and cancer formation, but the molecular mechanism for HS sulfation compensation is unknown. We do not understand how compensatory increases occur nor how the mutant HS chains thus compensated can mediate signaling. To fill this critical knowledge gap, we used the Drosophila model to evaluate the degree of signaling rescued by HS compensation and to identify a novel component of the system. First, to determine whether the HS compensation rescues specifically FGF signaling or further more generally for multiple HS-dependent ligands, we examined Dpp and Wg signaling, the two well-established pathways that function through HSPG co-receptors. We found that both pathways were basically intact in Hs2st or Hs6st single mutants although they were severely impaired in cells deficient for both Hsst genes. These results showed that the HS sulfation compensation rescues both Dpp and Wg signaling, suggesting a universal role for this regulatory system in multiple pathways in Drosophila. Second, to identify a novel component involved in the compensation system, we focused on Sulf1. In Hs2st homozygous mutants, the net HS sulfation charge is compensated by an increase in the level of 6-O-sulfation (24). Because Sulf1 modulates the levels of 6-O-sulfate groups, we asked if loss of Sulf1 affects this compensatory balance. We found that Hs2st;Sulf1 double mutants exhibit high levels of lethality, patterning defects, and an overgrowth phenotype, which are associated with abnormal up-regulation of Hh signaling. HS structural analysis showed that HS isolated from Hs2st;Sulf1 double mutants lacks 2-O-sulfation but has a higher level of 6-O-sulfation compared with Hs2st mutants, suggesting that this “oversulfation” of HS led to the severe phenotype and up-regulated Hh signaling of Hs2st;Sulf1 double mutants. These results show that Sulf1 is involved in fine-tuning of 6-O-sulfation levels, which is required for normal HS sulfation compensation.

**Experimental Procedures**

**Fly Stocks**—Detailed information for the fly strains used is described in Flybase except where noted. The wild-type strain used was Oregon R. Other strains used were as follows: Hs2st267, a null allele of Hs2st (24); Hs6st8770, a null allele of Hs6st (24); Sulf1Δp1, a null allele of Sulf1 (12); Sulf1Δp2, a hypomorphic allele of Sulf1 (12); apterous (ap)-Gal4; engrailed (en)-Gal4; hedgehog (hh)-Gal4; breathless (btl)-Gal4; UAS-IR-Hs6st (24); and UAS-Hs2st (26).

Because Hs2st;Hs6st double mutants are embryonic lethal, we used two genetic approaches to obtain cells in which both Hsst genes are simultaneously reduced at later stages. First, we overexpressed an Hs6st RNAi transgene (UAS-IR-Hs6st) in selected Gal4 expression domains in an Hs2st mutant background. We used ap-Gal4 (a dorsal compartment-specific driver) and en-Gal4 (a posterior compartment-specific driver) for this purpose. Genotypes used for each driver were as follows: Hs2st267 ap-Gal4/Hs2st267;UAS-IR-Hs6st/+ and Hs2st267 en-Gal4/Hs2st267;UAS-IR-Hs6st/+ . These animals were raised at 30 °C. The validity of UAS-IR-Hs6st was confirmed in supplementary Fig. S1. Briefly, UAS-IR-Hs6st driven by btl-Gal4 eliminated Hs6st mRNA specifically in the tracheoblast, in which Hs6st is normally expressed at high levels (6, 24). Second, we generated FLP/FRT-mediated Hs2st mutant clones in an Hs6st mutant background. Hs2st clones were induced by FLP-mediated somatic recombination using FRT40A as described previously (18). The genotype used was hs-FLP/+; FRT40A Hs2st267/FRT40A ubi-GFP;Hs6st8770/Hs6st8770. FLP was induced by heat shock at 37 °C for 1 h at 48 h after egg laying.

**In situ hybridization showing Sulf1 mRNA expression in Sulf1Δp1 and Sulf1Δp2 mutant alleles is presented in supplementary Fig. S2.** No Sulf1 mRNA expression was detected in the Sulf1Δp2 wing disc, whereas the levels and patterns of Sulf1 mRNA signals in Sulf1Δp1 wing disc were indistinguishable from those of wild type. Detailed molecular and genetic analyses of Sulf1Δp1 and Sulf1Δp2 were described in our previous report (12). Based on sequencing of Sulf1Δp1 genomic DNA, this mRNA encodes a truncated protein with the enzymatic active domain of Sulf1 protein. Genetic analyses showed that Sulf1Δp2 behaved as a hypomorphic allele.

**Immunostaining and in Situ RNA Hybridization**—Third instar larval wing discs were dissected and fixed with 3.7% formaldehyde in PBS for 20 min at room temperature. Antibody staining was performed according to standard procedures (27). The following antibodies were used: rat anti-Ci (1:2, a gift from R. Holmgren; mouse anti-Distal-less (Dll) (1:500, a gift from D. Duncan); guinea pig anti-Senseless (Sens) (1:100, a gift from H. Bellen); rabbit anti-Spalt (Sal) (1:30, a gift from S. Selleck); rabbit anti-pSMAD3 (1:1000, Epitomics); mouse anti- patched (Ptc) (1:50 Apa-1, Developmental Studies Hybridoma Bank); mouse anti-Wg (1:100, 4D4, Developmental Studies Hybridoma Bank); and anti-Hh (1:1000, a gift from T. Tabata).
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Extracellular labeling of Wg protein was performed according to Strigini and Cohen (28) using the anti-Wg antibody (4D4) at 1:3 dilution (26). Secondary antibodies were from the Alexa-Fluor series (1:500; Molecular Probes).

In situ RNA hybridization was performed as described previously (6, 12, 29). Digoxigenin (Dig)-labeled Sulf1 or Hs6st RNA probes were synthesized using a DIG RNA labeling kit (Roche Applied Science). Anti-Dig antibody conjugated with alkaline phosphatase was used to detect Dig-labeled probes. For colorimetric staining, the signal was developed using 3,3’-diaminobenzidine as a substrate. For fluorescent staining, the secondary antibody was detected using Fast Red (Roche Applied Science).

Preparation and HPLC Analysis of HS Disaccharides—HS isolation and disaccharide composition analysis were carried out as described previously (24, 30). Briefly, 20 adult flies (~4 mg dry weight) were homogenized to isolate HS. The HS sample was digested with a heparitinase mixture (Seikagaku), and the resulting disaccharides were separated using reversed-phase ion pair chromatography. The effluent was monitored fluorometrically for post-column detection of HS disaccharides. The ratio of HS disaccharides was calculated based on the average of three independent experiments.

RESULTS

Adult Wing Development in Hs2st and Hs6st Mutants—We have previously shown that FGF signaling and FGF-dependent tracheogenesis can occur normally in a significant fraction of Hs2st or Hs6st single mutants (24). Disaccharide analyses of HS isolated from these mutants revealed a compensatory increase in sulfation by the predominant remaining sulfotransferase, thus keeping the same overall net sulfation charge as wild type. We asked whether or not this HS sulfation compensation mechanism in the Hsst mutants rescues additional signaling pathways other than FGF signaling during development. To address this question, we first analyzed adult wing phenotypes of Hs2st and Hs6st mutants. Adult survivors of both Hs2st and Hs6st null mutants exhibited a wild-type wing phenotype in terms of size and patterning (Fig. 1, A–C). There was no obvious abnormality in wing margin and vein structures.

We next analyzed tissues in which both Hsst genes are simultaneously reduced to block the compensation. Because Hs2st; Hs6st double mutants are embryonic lethal, we induced an Hs6st RNAi transgene in selected GAL4 expression domains in an Hs2st mutant background. Driving this construct with en-GAL4 in the posterior compartment or ap-GAL4 in the dorsal compartment, Hs2st mutant animals yielded wings with defects in cell proliferation and wing vein formation (Fig. 1, D–F). For example, en-GAL4-driven adult wings (en>Hs6st RNAi in Hs2st) showed a reduced wing area in the posterior compartment. These wings also exhibited loss of both anterior and posterior cross-veins and a reduction of longitudinal wing vein L5 (Fig. 1D). ap-GAL4 driver produced blistered wings (Fig. 1E), which trend to curl up toward the dorsal side (Fig. 1F). This phenotype is a hallmark of a size difference in the dorsal and ventral cell layers, suggestive of reduced cell proliferation in the dorsal compartment. Both the cell proliferation defects and wing vein phenotypes are suggestive of disrupted Dpp signaling (31, 32).

Heparan Sulfate Sulfation Compensation Rescues Dpp Signaling—Because adult wing phenotypes were consistent with abrogated Dpp signaling, we next assayed downstream markers of Dpp signaling in the developing wing. First, we examined expression of Spalt (Sal), a downstream Dpp transcriptional target, which is expressed in the central region of the wing pouch (Fig. 2A). Hs2st and Hs6st single mutants showed normal expression of Sal protein (Fig. 2, B and C). To obtain Hs2st; Hs6st double mutant cells, we generated FLP/FRT-mediated Hs6st mutant clones in an Hs2st mutant background. We found that Sal expression is severely disrupted in Hs2st; Hs6st mutant clones (Fig. 2, D–D’). Thus, expression of Sal is normal in Hs2st single mutants but disrupted in the double mutant cells.

We also examined phosphorylation of Mad protein, a direct readout of Dpp signaling, using an antibody specific to the phosphorylated form of Mad (pMad) (18, 33–35). In discs singly mutant for Hs2st or Hs6st, levels and patterns of pMad were indistinguishable from wild type (Fig. 2, E–G). We next examined discs with en-GAL4-driven Hs6st RNAi in an Hs2st mutant background (en>Hs6st RNAi in Hs2st). These discs showed a drastic reduction of the area of the posterior compartment (Hs6st knockdown in Hs2st homozygous background) compared with the anterior compartment (singly mutant for Hs2st), indicating that Dpp-dependent cell proliferation is impaired when both Hssts are disrupted (Fig. 2H). pMAD levels were also affected in the posterior compartment of en>Hs6st RNAi in Hs2st discs. The signal intensity of pMad staining dropped sharply a few cell diameters posterior to the AP compartment boundary (Fig. 2H), in contrast to the gradual reduction of pMad levels observed in wild-type or in single Hsst mutants (Fig. 2, E–G).
Thus, analyses of both adult wings and wing discs revealed that Dpp signaling is severely impaired in cells lacking normal levels of both Hssts but is relatively normal in single mutant cells. This observation strongly supports the idea that HS sulfation compensation rescues Dpp signaling as it does for the FGF pathway.

**Heparan Sulfate Sulfation Compensation Rescues Wg Signaling**—Does the sulfation compensation also affect another major HS-dependent pathway, Wg signaling? To address this question, we first observed the formation of sensory organs at the wing margin, a well established Wg-directed event, in Hsst mutants. We observed no significant change in the number and positions of wing margin chemosensory and mechanosensory bristles for Hs2st or Hs6st mutants (data not shown, see Table 1 for Hs2st), suggesting that Wg signaling in the wing margin formation is unaffected. We next examined the expression pattern of Sens, a downstream transcriptional target of Wg signaling. When Hs6st RNAi was driven by en-GAL4, Sens expression was disrupted in the dorsal compartment (Fig. 3A). Similarly, posterior compartment-specific knockdown of Hs6st by en-GAL4 also altered the Sens staining pattern (Fig. 3E); the space between the dorsal and ventral Sens expressing domains was reduced, and the two stripes of Sens-positive cells fused at the DV compartment border. Thus, cells impaired for both Hssts exhibited reduced levels of Sens staining, whereas cells singly mutant for either Hs2st or Hs6st receive Wg signaling normally. This result suggests that compensatory increases in 6-O- and 2-O-sulfation restore wild-type Wg signaling in the Hs2st and Hs6st mutants, respectively.

To further confirm the results obtained with Hs6st RNAi, we created FLP/FRT-mediated Hs6st mutant clones in an Hs2st mutant background. Discs bearing the double mutant clones were stained for Distal-less (Dll), a low threshold downstream Wg transcriptional target. We found that Dll expression was reduced within the double mutant clones (Fig. 3, F–F”). These results demonstrated that sulfation compensation creates a functional environment for normal Wg signaling.

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**TABLE 1**

| Genotype   | Lethality | Malformed wing discs | Ectopic MSB |
|------------|-----------|----------------------|------------|
| Wild type  | ND        | 0.0                  | 0.0 ± 0.0  |
| Sulf1      | 8.0       | 3.6                  | 1.2 ± 0.2  |
| Hs2st      | 27.6      | 0.0                  | 0.6 ± 0.1  |
| Hs2st/Sulf1| >99.9     | 41.3                 | 5.9 ± 0.7  |

**FIGURE 2.** Dpp signaling is aberrant in Hs2st and Hs6st double mutant cells. A–C, Sal immunostaining of wild-type (A), Hs2st/+ (B), and Hs6st/+ (C) third instar larval wing discs. D–D”, anti-Sal antibody staining of a wing disc bearing Hs2st/+ homozygous clones in Hs6st background. Position of mutant clones is marked by loss of GFP signal (D) and Sal expression is shown in D’. D’ is a merged image of D and D”. Sal signal intensity was decreased in the double mutant clones (red arrows). E–H, pMad immunostaining of wild-type (E), Hs2st/+ (F), Hs6st/+ (G), and Hs2st/+ en-Gal4/Hs2st/+ UAS-IR-Hs6st/UAS-GFP (H–H”) third instar larval wing discs. When Hs6st RNAi was driven by en-GAL4 in an Hs2st/+ background, area of the posterior compartment (p, shown by GFP expression in H” and H”) was significantly reduced, and pMad gradient was disrupted in the posterior cells.

**FIGURE 3.** Wg signaling is aberrant in Hs2st and Hs6st double mutant cells. A–E, anti-Sens antibody staining of wild-type (A), Hs2st/+ (B), Hs6st/+ (C), Hs2st/267 ap-Gal4/Hs2st/+;UAS-IR-Hs6st/+ (D), and Hs2st/267 en-Gal4/Hs2st/+;UAS-IR-Hs6st/+ (E). In a disc for ap-GAL4-driven Hs6st RNAi in an Hs2st/+ background, Sens signal in the dorsal side (d) was disrupted, whereas the one in the ventral side was not affected (v). F–F”, a wing disc bearing Hs2st/267 mutant clones in an Hs6st/+ mutant background. Positions of Hs2st/267 mutant cells are shown by loss of GFP signal (F). Dll expression was reduced in Hs2st/267 mutant clones (arrowheads in F”). F” shows a merged image of F and F”.

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The lethality, the percentage of larval wing discs with abnormal overall structure, and the numbers of ectopic mechanosensory bristles (ectopic MSB) at the anterior dorsal wing margin are shown for the indicated genotypes. Hs2st/267 and Sulf1/267 were used as null alleles for each gene. For ectopic mechanosensory bristles, values represent means ± S.E. for bristle numbers counted for 20 or more wings for each genotype. ND means not determined.
Sulfation Compensation Regulates Extracellular Wg Gradient—To further analyze the mechanism by which Hs2st and Hs6st regulate Wg signaling, we examined the level of extracellular Wg protein in Hsst mutants. Wing discs were stained using a protocol that is designed to specifically detect Wg protein in the extracellular space (Fig. 4) (28). We first assayed extracellular Wg protein in Hs2st and Hs6st single mutant discs. In both genotypes, Wg forms a normal extracellular gradient (Fig. 4, B and C). In contrast, ap-GAL4-driven Hs6st RNAi in an Hs2st null background virtually eliminated signals for Wg in the dorsal compartment (Fig. 4D). In a similar experiment using en-GAL4, overall levels of Wg were markedly reduced in the posterior cells defective for both Hs2st and Hs6st (Fig. 4E). To further confirm these results, Hs6st homozygous wing discs bearing Hs2st mutant clones were stained for extracellular Wg (Fig. 4, F–F”). We found that Wg protein levels were drastically reduced on Hs2st/Hs6st mutant cells. These observations demonstrated that sulfation compensation regulated by Hssts rescues Wg morphogen gradient formation.

Altogether, we conclude that sulfation compensation not only rescues FGF-dependent tracheal system formation, but it also functions more generally for multiple HS-dependent pathways.

Sulfation Compensation Is Sensitive to Loss of Sulf1—Our observations described above show that the sulfation compensation mechanism in Hs2st and Hs6st single mutants supports normal morphogenesis by sustaining multiple signaling pathways. One key component of the compensation system is 6-O-sulfation, which is regulated by Hs6st. Another molecule that can affect the 6-O-sulfation status of HS is extracellular 6-O-endosulfatase, Sulf1 (12, 21). To better understand the mechanisms of HS sulfation compensation, we asked if Sulf1 is involved in this regulatory system. To this end, we examined genetic interactions between the Hs2st and Sulf1 genes.

Previous studies have shown that both Hs2st and Sulf1 single mutants are relatively healthy and show only low levels of lethality (Table 1) (12, 24). To our surprise, Hs2st animals show nearly 100% lethality when Sulf1 is eliminated (Table 1); Hs2st; Sulf1 double mutants exhibited a high rate of embryonic or early larval mortality. In addition, Sulf1 heterozygotes in an Hs2st null background were observed at a much lower than expected genetic frequency (data not shown).

Although the lethality of Hs2st;Sulf1 double mutants was extremely high, we could obtain a few escapers from a large scale culture. The double mutants exhibited several morphological abnormalities. We first noticed that these larval, pupal, and adult survivors are remarkably larger in size. Fig. 5A shows a size comparison of pupae with indicated genotypes. Sulf1, but not Hs2st, homoyzogous pupae show a modest overgrowth. Hs2st;Sulf1 double mutants exhibit a much more significant overgrowth phenotype than Sulf1 null animals. This was Sulf1 dosage-dependent, because Sulf11 allele (a null allele) showed a stronger effect compared with hypomorphic Sulf11.

Imaginal discs were also overgrown and malformed in Hs2st; Sulf1 third instar larvae. Abnormally shaped wing discs were observed in Sulf1 mutants at a low frequency (3.6%), but the penetrance of this phenotype increased to 41.3% in Hs2st;Sulf1
double mutants (Table 1). The double mutant wing discs are often partially duplicated due to secondary axis formation (Fig. 5B).

Hs2st and Sulf1 also showed genetic interactions in an adult wing phenotype. In wild type, mechanosensory bristles are aligned in a stereotypic pattern at the anterior wing margin. These structures are highly sensitive to altered expression of HSPGs and HS-modifying enzymes (12, 27, 36). Both Hs2st and Sulf1 single mutants have ectopic mechanosensory bristles at a low penetrance (Table 1). We found that double mutant adult escapers had a significant increase in the number of ectopic mechanosensory bristles (Table 1). It is worth noting that the effect of Sulf1 mutations in Hs2st mutants matches with its expression patterns. Previous studies have shown that Sulf1 is expressed at high levels near the DV boundaries of the wing disc (12, 29). We found that Sulf1 expression was not affected by loss or gain of Hs2st (supplemental Fig. S3). Because the wing margin of the adult wing derives from the DV border of the wing disc, the wing margin defect observed in Hs2st;Sulf1 is consistent with high levels of Sulf1 expression at the DV border. Taken together, these remarkable synergistic enhancements of Hs2st mutant phenotypes by Sulf1 mutation suggest that Sulf1 activity is required for proper HS sulfation compensation and normal development of Hs2st mutants.

Hh Signaling Is Up-regulated in Hs2st;Sulf1 Double Mutants—We next asked if any of the known HS-dependent signaling pathways is altered in Hs2st;Sulf1 double mutants. Because both overgrowth and wing duplication phenotypes are caused by ectopic Hh signaling or overactivation of the Hh pathway (37–39), and Sulf1 has been shown to regulate Hh activity (29), we examined if this pathway is affected in Hs2st;Sulf1 discs. To this end, we analyzed the expression of two Hh signaling targets, which are high and low threshold markers of Hh signaling activity. Hh protein is exclusively expressed in the posterior compartment and secreted into the anterior compartment to regulate the activity of a key target molecule, Ci, a Drosophila member of Gli family of transcription factors. Hh signaling inhibits proteolytic digestion of the latent transcriptional activator Ci-155, thus stabilizing this form of Ci protein. Anti-Ci antibody staining detects high levels of Ci as far as 8–10 cells away from the AP compartment boundary in wild-type wing discs (Fig. 5, C–C′) (40, 41). Ci is directly involved in transcriptional activation of Hh target genes, including dpp and patched (ptc). Expression of Ptc, a high threshold Hh target gene, is detected in a thinner stripe of cells (five cells) at the AP compartment border (Fig. 5C′) (42).

Sulf1 has dual functions in Hh signaling; it acts as a positive and a negative regulator in the Hh producing and receiving cells, respectively (29). Together, as a sum of these effects, Hh signaling is modestly increased in Sulf1 homozygous wing discs. Consistent with this, in Sulf1 mutant discs, the overall level of Ci staining is higher, and the domain with high levels of Ci was extended in the anterior compartment (Fig. 5D), but Ptc staining was indistinguishable from that of the wild type (Fig. 5D′).

In Hs2st;Sulf1 wing discs, the domain with high levels of Ci was further expanded toward the anterior edge of the wing pouch (Fig. 5E). In addition, the Ptc stripe was significantly wider (Fig. 5E′), suggesting that more cells received high levels of Hh signaling at the AP border. These results suggest that Hh signaling is abnormally up-regulated in Hs2st;Sulf1 double mutant wing discs.

To address if the level of Hh protein contributes to enhanced Hh signaling in Hs2st;Sulf1 double mutants, we stained wild-type, Hs2st, and Hs2st;Sulf1 wing discs with anti-Hh antibody (supplemental Fig. S4). We detected no obvious change in the levels and patterns of Hh protein between these genotypes. Even in Hs2st;Sulf1 discs with abnormal morphology, we detected no sign of increased Hh protein levels (supplemental Fig. S4D). This result suggests that Sulf1 mutations mainly affect the sensitivity of the receiving cells to Hh rather than the ligand distribution.

HS Disaccharide Profiles of Hs2st;Sulf1 Double Mutants—To explore the mechanism of Sulf1 function in HS compensation, we analyzed HS disaccharide structure in Hs2st;Sulf1 double mutants. Briefly, HS purified from adult flies was completely digested into disaccharide units by heparin lyases. Differently sulfated disaccharide species were separated by reverse phase HPLC. As shown previously, HS isolated from Sulf1 mutants has substantially increased levels of the tri-S disaccharide unit (ΔUA2S-GlcNS6S) at the expense of the 2SNS unit (ΔUA2S-GlcNS) (Table 2 and Fig. 6A) (12). This profile reflects the fact that the major enzymatic target of Sulf1 is 6-O-sulfate groups at tri-S ΔUA2S-GlcNS6S disaccharide units of HS (8, 12, 43). In Hs2st mutants, the two disaccharide species containing 2-O-sulfated uronic acid (ΔUA2S-GlcNS and ΔUA2S-GlcNS6S) are not detectable (Table 2 and Fig. 6A) (24). This loss is compensated by a substantial increase of NS6S disaccharide unit (ΔUA-GlcNS6S).

The disaccharide profile of Hs2st;Sulf1 mutant HS revealed that the NS6S unit was further increased (Table 2 and Fig. 6A).

Based on the disaccharide composition, we calculated the rela-
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**FIGURE 6. HS disaccharide profiling of Hs2st;Sulf1 mutants.** A, graphical depiction of disaccharide composition of HS from each respective genotype. Bar graphs show percentage of the following disaccharides: ΔUA-GlcNAc (gray), ΔUA-GlcNS (blue); ΔUA-GlcNAc6S (light blue); ΔUA-GlcNS6S (green); ΔUA2S-GlcNS (yellow); and ΔUA2S-GlcNS6S (red). The values were obtained from three independent experiments, and each bar represents the mean ± S.D. *p < 0.05; ***, p < 0.001. B, relative levels of total sulfation (blue) and 6-O-sulfation (red) are shown for each respective genotype compared with wild-type levels (1.0).

A study showed an unexpected level of flexibility in biosynthesis and function in vivo. The level of total sulfate groups on HS is very similar in all genotypes we tested (wild-type, Sulf1, Hs2st, and Hs2st;Sulf1) (Fig. 6B). In contrast, the level of 6-O-sulfation was elevated in Sulf1 and Hs2st and further elevated in the Hs2st; Sulf1 double mutant HS. Thus, the severe phenotypes and up-regulated Hh signaling observed in Hs2st;Sulf1 double mutants are associated with 6-O-oversulfation. This observation demonstrates that Sulf1 plays a role in regulating the levels of 6-O-sulfation in Hs2st mutants. In Hs2st mutant cells, Sulf1 functions as a brake to fine-tune 6-O-sulfation to an appropriate level. It is worth noting that in the Hs2st mutant background (in the absence of tri-S unit), the main target substrate of Sulf1 is the NS6S unit (ΔUA-GlcNS6S) (Table 2 and Fig. 6A).

**DISCUSSION**

A previous study showed that a fraction of Hs2st (9%) and Hs6st (39%) null embryos exhibited defects in Breathless (FGF receptor)-dependent tracheal branching (24), demonstrating that specific sulfation events are important for FGF signaling in vivo. At the same time, tracheal morphology of a large fraction of these single mutants was indistinguishable from that of wild-type embryos (24). Intact FGF signaling was observed in these mutant animals. These observations indicated that living cells show an unexpected level of flexibility in biosynthesis and function of HS. This in vivo evidence suggests correct positioning of sulfate groups on HS can be bypassed if the overall level of sulfation is maintained. One model that explains this phenomenon is that HS chains are flexible enough to twist and bend to place sulfate groups at appropriate three-dimensional (spatial) positions to form or stabilize the FGF-FGF receptor signaling complexes, even without specific sulfation sequences. Thus, although clearly there are ideal (most efficient) binding sites specific to each ligand protein, such HS fine structures should be regarded as the distribution of charge in three-dimensional space rather than a linear sulfation sequence. It is worth emphasizing that the concept of the sulfation compensation does not conflict with the importance of specific sulfation of HS for signaling events.

To understand HS biosynthesis and function in vivo, it is critical to elucidate the molecular mechanisms of the HS sulfation compensation system, which maintains proper overall sulfation even when particular modifications are disrupted. In this study, we asked two fundamental questions as follows. 1) To what extent does sulfation compensation rescue cellular signaling? 2) What component other than Hs2st and Hs6st is involved in compensation? We first demonstrated that sulfation compensation, which buffers fluctuating levels of Hs2st or Hs6st, rescues not only FGF signaling but also the Wg and Dpp pathways. This finding suggests that this regulatory mechanism plays general roles in multiple HS-dependent pathways. However, this result does not exclude the possibility that some pathway(s) cannot be rescued by the compensation. Although surviving adult Hs2st or Hs6st null mutants do not show obvious morphological defects, they are both male and female sterile (24). This fact suggests that germ line development depends on a signaling molecule that requires more strictly defined HS fine structures and thus cannot be rescued by the compensation. Alternatively, HS sulfation may not be fully compensated in the testis and ovary. Additional studies will be required to distinguish between these possibilities.

We also demonstrated that proper compensation in Hs2st mutants requires Sulf1 activity. In Hs2st mutants, 6-O-sulfation is increased by sulfation compensation, whose mechanism is unknown. The elevation of 6-O-sulfate groups in Hs2st mutants, however, has to be repressed by Sulf1, presumably at a post-synthetic step. The morphological defects, lethality, and abnormally up-regulated Hh signaling observed in Hs2st;Sulf1 mutants were associated with 6-O-oversulfation. The increase in the levels of the NS6S unit in Hs2st;Sulf1 mutant HS compared with Hs2st appeared to be relatively modest. However, because we used HS samples from rare Hs2st;Sulf1 adult survivors (<0.1%), the results of the HS structural analysis may represent ones of healthier animals for this genotype and therefore underestimate the increase in NS6S unit levels. In either case, there seems to be a critical threshold of 6-O-sulfation to support normal Hh signaling. This idea is consistent with recent observations that relatively small changes in 6-O-sulfation level showed a dramatic impact on FGF2-induced cell signaling (44) and angiogenic growth factor signaling (45). It is possible that other HS-dependent signaling pathways, in addition to Hh signaling, may be also affected in the double mutants. In fact, the ectopic mechanosensory bristle phenotype observed in Hs2st; Sulf1 animals is also seen in animals with increased Wg signal-
ing, suggesting that this pathway also contributes to the lethality and morphological phenotypes of the double mutants. Interestingly, we observed an alteration in Sulf1 substrate specificity during the compensation; Sulf1 can remove 6-O-sulfate groups from the NS6S unit in the absence of its natural target HS structure, the tri-S unit. Together, our findings provide evidence that sulfation compensation is achieved by coordinated activities of Hs2st, Hs6st, and Sulf1. Moreover, the requirement for Drosophila Sulf1 in sulfation compensation, which contributes to robust cell signaling systems through a number of HS-dependent factors, may provide a novel insight into future development of new diagnostic and therapeutic strategies for Mesomelia-Synostoses syndrome.

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REFERENCES

1. Kirkpatrick, C. A., and Selleck, S. B. (2007) Heparan sulfate proteoglycans at a glance. J. Cell Sci. 120, 1829–1832
2. Bishop, J. R., Schuksz, M., and Esko, J. D. (2007) Heparan sulfate proteoglycans fine-tune mammalian physiology. Nature 446, 1030–1037
3. Nakato, H., Futch, T. A., and Selleck, S. B. (1995) The division abnormally delayed (daily) gene: a putative integral membrane proteoglycan required for cell division patterning during postembryonic development of the nervous system in Drosophila. Development 121, 3687–3702
4. Tsuda, M., Kamimura, K., Nakato, H., Archer, M., Staatz, W., Fox, B., Humphrey, M., Olson, S., Futch, T., Kaluz, V., Siegfried, E., Stam, L., and Selleck, S. B. (1999) The cell-surface proteoglycan Dally regulates Wingless signaling in Drosophila. Nature 400, 276–280
5. Lin, X., and Perrimon, N. (1999) Dally cooperates with Drosophila Frizzled 2 to transduce Wingless signaling. Nature 400, 281–284
6. Kamimura, K., Fujise, M., Villa, F., Isumi, H., Habuchi, H., Kimata, K., and Nakato, H. (2001) Drosophila heparan sulfate 6-O-sulfotransferase (dHS6ST) gene. Structure, expression, and function in the formation of the tracheal system. J. Biol. Chem. 276, 17014–17021
7. Dhoot, G. K., Gustafsson, M. K., Ai, X., Sun, W., Standiford, D. M., and Emerson, C. P., Jr. (2001) Regulation of Wnt signaling and embryo patterning by an extracellular sulfatase. Science 293, 1663–1666
8. Ai, X., Do, A. T., Lozynska, O., Kutsche-Gullberg, M., Lindahl, U., and Emerson, C. P., Jr. (2003) QSulf1 remodels the 6-O-sulfation states of cell surface heparan sulfate proteoglycans to promote Wnt signaling. J. Cell Biol. 162, 341–351
9. Ai, X., Kitazawa, T., Do, A. T., Kutsche-Gullberg, M., Labosky, P. A., and Emerson, C. P., Jr. (2007) SULF1 and SULF2 regulate heparan sulfate-mediated GDNF signaling for esophageal innervation. Development 134, 3327–3338
10. Morimoto-Tomita, M., Uchimura, K., Werb, Z., Hemmerich, S., and Rosen, D. S. (2002) Cloning and characterization of two extracellular heparin-degrading endosulfatases in mice and humans. J. Biol. Chem. 277, 49175–49185
11. Viviano, B. L., Paine-Saunders, S., Gasiunas, N., Gallagher, J., and Saunders, S. (2004) Domain-specific modification of heparan sulfate by Qsulf1 modulates the binding of the bone morphogenetic protein antagonist Noggin. J. Biol. Chem. 279, 5604–5611
12. Kleinschmit, A., Koyama, T., Dejima, K., Hayashi, Y., Kamimura, K., and Nakato, H. (2010) Drosophila heparan sulfate 6-O-endosulfatase regulates Wingless morphogen gradient formation. Dev. Biol. 345, 204–214
13. Lembjabar-Alaoui, H., van Zante, A., Singer, M. S., Xue, Q., Wang, Y. Q., Tsay, D., He, B., Jablons, D. M., and Rosen, S. D. (2010) Sulf-2, a heparan sulfate endosulfatase, promotes human lung carcinogenesis. Oncogene 29, 635–646
14. Rosen, S. D., and Lemjabbar-Alaoui, H. (2010) Sulf-2: an extracellular modulator of cell signaling and a cancer target candidate. Expert Opin. Ther. Targets 14, 935–949
15. Isidor, B., Pichon, O., Redon, R., Day-Salvatore, D., Hamel, A., Siwicka, K. A., Bitner-Glindzicz, M., Heymann, D., Kjellén, L., Kraus, C., Leroy, J. G., Mortier, G. R., Rauch, A., Verloes, A., David, A., and Le Caïgne, C. (2010) Mesomelia-synostoses syndrome results from deletion of SULF1 and SLCOS1 genes at 8q13. Am. J. Hum. Genet. 87, 95–100
16. Esko, J. D., and Selleck, S. B. (2002) Order out of chaos: assembly of ligand binding sites in heparan sulfate. Annu. Rev. Biochem. 71, 435–471
17. Nakato, H., and Kimata, K. (2002) Heparan sulfate fine structure and specificity of proteoglycan functions. Biochim. Biophys. Acta 1573, 312–318
18. Fujise, M., Takeo, S., Kamimura, K., Matsuo, T., Aigaki, T., Izumi, S., and Nakato, H. (2003) Daily regulates Dpp morphogen gradient formation in the Drosophila wing. Development 130, 1515–1522
19. Lander, A. D., Nie, Q., and Wan, F. Y. (2002) Do morphogen gradients arise by diffusion? Dev. Cell 2, 785–796
20. Lander, A. D., Nie, Q., and Wan, F. Y. (2007) Membrane-associated non-receptors and morphogen gradients. Bull. Math. Biol. 69, 33–54
21. You, J., Bellenkaya, T., and Lin, X. (2011) Sulfated is a negative feedback regulator of wingless in Drosophila. Dev. Dyn. 240, 640–648
22. Merry, C. L., Bullock, S. L., Swan, D. C., Backen, A. C., Lyon, M., Beddington, R. S., Wilson, V. A., and Gallagher, I. T. (2001) The molecular phenotype of heparan sulfate in the Hs2st−/− mutant mouse. J. Biol. Chem. 276, 35429–35434
23. Merry, C. L., and Wilson, V. A. (2002) Role of heparan sulfate-2-O-sulfotransferase in the mouse. Biochim. Biophys. Acta 1573, 319–327
24. Kamimura, K., Koyama, T., Habuchi, H., Ueda, R., Masu, M., Kimata, K., and Nakato, H. (2006) Specific and flexible roles of heparan sulfate modifications in Drosophila FGF signaling. J. Cell Biol. 174, 773–778
25. Townley, R. A., and Bülow, H. E. (2011) Genetic analysis of the heparan modification network in Caeonhabditis elegans. J. Biol. Chem. 286, 16824–16831
26. Kamimura, K., Maeda, N., and Nakato, H. (2011) In vivo manipulation of heparan sulfate structure and its effect on Drosophila development. Glycoconjugate J. 28, 6581–6592
27. Wojcinski, A., Nakato, H., Soula, C., and Glise, B. (2011) DSulfatase-1 fine-tunes Hedgehog patterning activity through a novel regulatory feedback loop. Dev. Biol. 358, 168–180
28. Toyoda, H., Kinoshita-Toyoda, A., and Selleck, S. B. (2000) Structural analysis of glycosaminoglycans in Drosophila and Caenhabditis elegans and demonstration that tout-velu, a Drosophila gene related to EXT tumor suppressors, affects heparan sulfate in vivo. J. Biol. Chem. 275, 2269–2275
29. Yu, K., Sturtevant, M. A., Biehs, B., François, V., Padgett, R. W., Blackman, R. K., and Bier, E. (1996) The Drosophila decapentaplegic and short gastrulation genes function antagonistically during adult wing vein development. Development 122, 4033–4044
30. Burke, R., and Basler, K. (1996) Dpp receptors are autonomously required for cell proliferation in the entire developing Drosophila wing. Development 122, 2261–2268
31. Tanimoto, H., Itoh, S., ten Dijke, P., and Tabata, T. (2000) Hedgehog creates a gradient of DPP activity in Drosophila wing imaginal discs. Mol. Cell 5, 59–71
32. Akiyama, T., Kamimura, K., Firkus, C., Takeo, S., Shimm, O., and Nakato, H. (2008) Daily regulates Dpp morphogen gradient formation by stabilizing Dpp on the cell surface. Dev. Biol. 313, 408–419
33. Dejima, K., Kanai, M. I., Akiyama, T., Levings, D. C., and Nakato, H. (2011) Novel contact-dependent bone morphogenetic protein (BMP) signaling mediated by heparan sulfate proteoglycans. J. Biol. Chem. 286, 17103–17111
34. Kirkpatrick, C. A., Dimitroff, B. D., Rawson, J. M., and Selleck, S. B. (2004)
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Spatial regulation of Wingless morphogen distribution and signaling by Dally-like protein. *Dev. Cell* 7, 513–523
37. Basler, K., and Struhl, G. (1994) Compartment boundaries and the control of *Drosophila* limb pattern by hedgehog protein. *Nature* 368, 208–214
38. Tabata, T., and Kornberg, T. B. (1994) Hedgehog is a signaling protein with a key role in patterning *Drosophila* imaginal discs. *Cell* 76, 89–102
39. Takeo, S., Akiyama, T., Firkus, C., Aigaki, T., and Nakato, H. (2005) Expression of a secreted form of Dally, a *Drosophila* glypican, induces overgrowth phenotype by affecting action range of Hedgehog. *Dev. Biol.* 284, 204–218
40. Aza-Blanc, P., Ramírez-Weber, F. A., Laget, M. P., Schwartz, C., and Kornberg, T. B. (1997) Proteolysis that is inhibited by hedgehog targets cubitus interruptus protein to the nucleus and converts it to a repressor. *Cell* 89, 1043–1053
41. Strigini, M., and Cohen, S. M. (1997) A Hedgehog activity gradient contributes to AP axial patterning of the *Drosophila* wing. *Development* 124, 4697–4705
42. Alexandre, C., Jacinto, A., and Ingham, P. W. (1996) Transcriptional activation of hedgehog target genes in *Drosophila* is mediated directly by the cubitus interruptus protein, a member of the GLI family of zinc finger DNA-binding proteins. *Genes Dev.* 10, 2003–2013
43. Ai, X., Do, A. T., Kusche-Gullberg, M., Lindahl, U., Lu, K., and Emerson, C. P., Jr. (2006) Substrate specificity and domain functions of extracellular heparan sulfate 6-O-endosulfatases, QSulf1 and QSulf2. *J. Biol. Chem.* 281, 4969–4976
44. Jastrebova, N., Vanwildemeersch, M., Lindahl, U., and Spillmann, D. (2010) Heparan sulfate domain organization and sulfation modulate FGF-induced cell signaling. *J. Biol. Chem.* 285, 26842–26851
45. Ferreras, C., Rushton, G., Cole, C. L., Babur, M., Telfer, B. A., van Kuppevelt, T. H., Gardiner, J. M., Williams, K. J., Jayson, G. C., and Avizienyte, E. (2012) Endothelial heparan sulfate 6-O-sulfation levels regulate angiogenic responses of endothelial cells to fibroblast growth factor 2 and vascular endothelial growth factor. *J. Biol. Chem.* 287, 36132–36146