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Drosophila heparan sulfate 6-*O* endosulfatase regulates Wingless morphogen gradient formation

Adam Kleinschmit, Takashi Koyama, Katsufumi Dejima, Yoshiki Hayashi, Keisuke Kamimura, Hiroshi Nakato *

Department of Genetics, Cell Biology and Development, The University of Minnesota, Minneapolis, MN 55455, USA

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ABSTRACT

Heparan sulfate proteoglycans (HSPGs) play critical roles in the distribution and signaling of growth factors, but the molecular mechanisms regulating HSPG function are poorly understood. Here, we characterized Sulf1, which is a *Drosophila* member of the HS 6-*O* endosulfatase class of HS modifying enzymes. Our genetic and biochemical analyses show that Sulf1 acts as a novel regulator of the Wg morphogen gradient by modulating the sulfation status of HS on the cell surface in the developing wing. Sulf1 affects gradient formation by influencing the stability and distribution of Wg. We also demonstrate that expression of *Sulf1* is induced by Wg signaling itself. Thus, *Sulf1* participates in a feedback loop, potentially stabilizing the shape of the Wg gradient. Our study shows that the modification of HS fine structure provides a novel mechanism for the regulation of morphogen gradients.

Introduction

Morphogens, such as Wingless/Wnt, Hedgehog, and BMPs, form concentration gradients within tissues and dictate cellular positional cues to organize tissue growth and patterning during animal development. The extracellular environment in which signaling molecules are secreted heavily influences the localization and stability of a morphogen (Wang et al., 2008). Among the molecules that have a major impact on morphogen gradients are heparan sulfate proteoglycans (HSPGs). HSPGs are carbohydrate-modified proteins that play important roles in a variety of biological processes, such as growth factor signaling and cell adhesion (reviewed in Kirkpatrick and Selleck, 2007). Genetic studies have shown that mutations affecting HSPG core-proteins or HS biosynthetic enzymes cause defects in morphogen signaling in *Drosophila* (reviewed in Tabata and Takei, 2004).

Wg protein is secreted by several rows of cells along the dorsoventral (DV) border of the *Drosophila* larval wing disc and forms a gradient that helps to orchestrate adult wing development. Wg signaling in this tissue is regulated by *dally* and *dally-like* (*dlp*), the *Drosophila* members of the Glypican family of HSPGs that are attached to the cell surface via a glycosylphosphatidylinositol (GPI)-anchor. *dally* is known to be a positive regulator of the Wg pathway (Franch-Marro et al., 2005; Fujise et al., 2001; Han et al., 2005), while mutations in *dlp* have differential effects on Wg signaling depending on the region of the wing disc. *dlp* down-regulates Wg signaling near the wing margin whereas it has a positive effect on it in more peripheral regions (Kirkpatrick et al., 2004; Kreuger et al., 2004). It has been proposed that Dlp plays a role in long-range Wg diffusion by enhancing apical–basal trafficking of Wg (Gallet et al., 2008) or by acting as an exchange factor to regulate the amount of Wg available to the receptor (Yan et al., 2009).

The biological function of HSPGs is not only dependent on their core protein structure but also relies on the heterogeneous fine structure of their sugar chains. During HS biosynthesis, HS chains are polymerized by EXT proteins in the Golgi. The nascent polysaccharide subsequently undergoes a series of modification events including *O* sulfation at different positions. Since only a fraction of potential target units are modified in each biosynthetic step, the resulting HS chains have remarkable levels of structural heterogeneity. Several lines of evidence suggest that these HS fine structures regulate discrete signaling events at the cell surface (reviewed in Gorsi and Stringer, 2007). Recently, a novel family of HS modifying enzymes, the extracellular HS 6-*O* endosulfatases (Sulfs), were identified (Dhoot et al., 2001). Unlike other HS modifying enzymes that function in the Golgi during HS biosynthesis, Sulfs are believed to be secreted proteins that remove 6-*O* sulfate groups from internal sulfated domains of extracellular HS. The first identified Sulf molecule, QSulf1, was shown to increase Wnt signaling in avian embryonic somites (Dhoot et al., 2001). It was proposed that the activity of QSulf1 as well as other vertebrate homologues decreases the binding affinity between HS and the Wnt ligand, in turn promoting the access of Wnt to its receptor for signaling (Ai et al., 2003; Freeman et al., 2008; Nawroth et al., 2007; Tang and Rosen, 2009). In contrast, Sulfs

* Corresponding author. Fax: +1 612 626 5652.
E-mail address: nakat003@umn.edu (H. Nakato).

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have been shown to act as negative regulators of FGF signaling (Kamimura et al., 2006; Wang et al., 2004). Aberrant levels of Sulf expression are also associated with tumorigenesis (Li et al., 2005; Nawroth et al., 2007). Thus, post-synthetic remodeling of HS structures affects HSPG function and therefore provides a novel mechanism by which the activity and the distribution of growth factors can be regulated. However, the molecular basis for the in vivo function of Sulfs is poorly understood.

To elucidate the mechanism by which post-synthetic modulation of HS sulfation controls the activity and the distribution of secreted signaling molecules, we studied the role of *Drosophila* HS 6-*O* endosulfatase (Sulf1) in development. We show that *Sulf1* affects the Wg gradient by controlling the stability and distribution of Wg protein. Structural analysis of HS from *Sulf1* mutants showed that the mutant HS bears abnormally high levels of tri-sulfated (tri-S) disaccharide units. Collectively, these results suggest that specific desulfation of HS by Sulf1 is required for Wg morphogen gradient formation. We also demonstrate that *Sulf1* expression is under the control of Wg signaling, indicating that *Sulf1* is involved in a feedback system of this pathway. Our findings add a novel layer to the molecular network regulating the formation and stabilization of morphogen gradients.

Materials and methods

Fly strains

Detailed information for the fly strains used is described in Flybase (http://flybase.bio.indiana.edu/) except where noted. The wild-type strain used was Oregon R. Other strains used were: *P{GT1}GT000656*, a P-element insertion line in the *Sulf1* locus; *Df(3R)sdb26* (breakpoints, 89B9;89C7), a chromosomal deficiency line; *dally*^{gem}, a loss-of-function allele of *dally*; *Hs6st*^{d770}, a null allele of *Hs6st*; *neuralized*^{A101}; *wingless*^{en11} (*wg-lacZ*); *engrailed (en)-Gal4*; *apterous (ap)-Gal4*; *hedgehog (hh)-Gal4*; *decapentaplegic (dpp)-Gal4*; *C96-Gal4*; *A9-Gal4*; *UAS-FLP*; *UAS-Sulf1* (Kamimura et al., 2006); *UAS-dally* (Takeo et al., 2005; Tsuda et al., 1999), *UAS-dlp39.2* (Kirkpatrick et al., 2004), *UAS-GPI-Dfz2* (Cadigan et al., 1998; Rulifson et al., 2000), and *UAS-Arm*^{S10} (Pai et al., 1997). *Sulf1* homozygous mutant clones were generated by FLP-mediated mitotic recombination using FRT82B as previously described (Fujise et al., 2003). FLP was induced by heat-shock using *hs-FLP* (for mutant clones shown in Figs. 4D–F‴) or by *hh-Gal4 UAS-FLP* (for mutant clones shown in Figs. 2E–E″). FLP-OUT clones overexpressing *Sulf1* were induced as previously described (Struhl and Basler, 1993; Takeo et al., 2005) in wing disc bearing a *Act5C>CD2>Gal4* transgene cassette, *hsp70-flp*, *UAS-GFP*, and *UAS-Sulf1*.

To generate *Sulf1* mutations, a P-element in *P{GT1}GT000656* was excised by P-element transposase from *P{ry*$^{+}$*, Δ2–3} (99B)*. The resulting progeny were screened for loss of marker gene expression. Excision chromosomes were analyzed by PCR using flanking primers to identify deletions, and the extent of each deletion was determined by sequencing PCR products that spanned the junction.

Sulf1-ER was constructed by adding a KDEL coding sequence at the C-terminus of *Sulf1* cDNA (Munro and Pelham, 1987). *Sulf1-Golgi* consists of amino acids 1–122 from GalNAc-T3 (GenBank accession number X92689, a gift from S. Cohen) cloned in-frame with amino acids 27–1115 of Sulf1. Wild-type *Sulf1*, *Sulf1-ER*, and *Sulf1-Golgi* were cloned into vector pUASg.attB (a gift from K. Basler), and transgenic strains bearing these constructs were made by BestGene Inc. using ϕC31-mediated integration of respective plasmid DNA into Basler ZH line 68E (Bischof et al., 2007).

Immuno-staining and in situ RNA hybridization

Antibody staining was performed according to standard procedures (Fujise et al., 2001). The following antibodies were used: Mouse anti-Distal-less (Dll) (1:500, a gift from D. Duncan), mouse anti-Achaete (Ac) (1:5, Developmental Studies Hybridoma Bank), guinea pig anti-Senseless (Sens) (1:1000, a gift from H. Bellen), rabbit anti-Spalt (Sal) (1:30, a gift from S. Selleck), rabbit anti-pSMAD3 [pS423/425] (1:1000, Epitomics), mouse anti-Wg (1:100, 4D4, DSHB), mouse anti-β-galactosidase (1:50, DSHB), guinea pig anti-β-galactosidase (1:2000, a gift from M. Kanai, Kanai et al., 2005). Extracellular labelling of Wg protein was performed according to Strigini and Cohen (2000) (Strigini and Cohen, 2000) using the anti-Wg antibody (4D4) at 1:3 dilution. Secondary antibodies were from the AlexaFluor series (1:500; Molecular Probes).

In situ RNA hybridization was performed as described previously (Kamimura et al., 2001). Digoxigenin (Dig)-labelled *Sulf1* RNA probes were synthesized using a DIG RNA Labelling Kit (Roche). For colorimetric staining, anti-Dig antibody conjugated with alkaline phosphatase was used as a secondary antibody. The signal was developed by a standard protocol using 3,3′-diaminobenzidine as a substrate. For fluorescent staining, wing discs from *hh-Gal4 UAS-GFP/UAS-GPI-Dfz2* or *dpp-GaL4 UAS-GFP/UAS-Arm*^{S10} animals were incubated with the *Sulf1* probe. The hybridized probes were reacted with anti-Dig-peroxidase (Roche), and the signal was detected using the TSA Biotin System (Perkin Elmer).

To confirm subcellular localization of Sulf1-ER and Sulf1-Golgi, we made HA-tagged forms of each respective Sulf1 construct. A single HA-tag was added on the C-terminal end of both wild-type Sulf1 and Sulf1-Golgi and between the C-terminus of Sulf1 and the KDEL sequence for Sulf1-ER. Subcellular localization of these modified forms of Sulf1 was examined using GFP-KDEL (a gift from K. Irvine) and Hs2st-Myc (Kamimura et al., 2006) as an ER and Golgi marker, respectively. Extracellular Sulf1 was detected by staining with rat anti-HA (1:100, Roche) using the extracellular staining methods described above (Strigini and Cohen, 2000).

Wg intensity plot analysis

Plot analyses of extracellular Wg staining were performed using ImageJ software and statistical analysis was completed with Microsoft Excel software. Raw data were obtained using a static rectangular box of arbitrary units in ImageJ placed in the central region of each wing disc. Extracted raw data from the plot profile menu in ImageJ were exported to an Excel spread sheet, where maximum peak intensity points were aligned for both wild-type and mutant discs. Averaged values for each genotype were then plotted in a Microsoft Excel graph together. For more direct comparison of gradient shape, wild-type and *Sulf1* graphs were transposed directly on top of each other using Adobe Illustrator.

Preparation and HPLC analysis of HS disaccharides

HS isolation and disaccharide composition analysis were carried out as previously described (Kamimura et al., 2006; Toyoda et al., 2000). Briefly, approximately 200 mg of adult flies was 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.

Results

Sulf1 regulates sensory bristle formation on the anterior wing margin

To study the roles of *Sulf1* in development, we isolated mutations in the *Sulf1* locus by imprecise excision of a P-element transposon. After inducing excision of *P{GT1}GT000656*, which is inserted in the third intron of the *Sulf1* locus, we screened for deletions in the genomic DNA neighboring the original P-element insertion site. Two

imprecise excision alleles were isolated by PCR-based screening and named *Sulf1*$^{\Delta P1}$ and *Sulf1*$^{\Delta P2}$. Breakpoints of both deletion alleles were mapped by sequencing. The molecular analysis of *Sulf1*$^{\Delta P1}$ showed that this mutant allele lacks exons 4–10, resulting in the loss of most of the protein coding sequence, including part of the catalytic domain of Sulf1 (Fig. 1A). *Sulf1*$^{\Delta P2}$ allele bears a smaller deletion in exon 3, which causes a frame shift at the amino acid residue Ser_{59}. This results in an early termination of translation at the amino acid 63. Residual nucleotides in the non-deleted region of *Sulf1*$^{\Delta P2}$ include sequences for the enzymatic active domain of the Sulf1 protein.

The isolated *Sulf1* mutants were viable and fertile. However, we found that both mutants show several specific adult phenotypes pertaining to the wing margin bristles. *Sulf1* mutations led to an increase in the number of chemosensory and mechanosensory bristles on the anterior wing margin (Figs. 1B and C). *Sulf1* mutants also have ectopic mechanosensory bristles which have shifted more posteriorly than their normal position. All these phenotypes are consistent with abnormally elevated Wg signaling near the DV boundary of the mutant wing (Cadigan et al., 1998; Gerlitz and Basler, 2002; Giraldez et al., 2002; Kirkpatrick et al., 2004).

To further characterize the *Sulf1* alleles, we used a deficiency line, *Df(3R)sdb26*, which lacks the cytological region 89B9–89C7 including the entire *Sulf1* locus. The phenotypes of increased chemosensory and mechanosensory bristles, and ectopic mechanosensory bristles of *Sulf1*$^{\Delta P1}$ homozygous animals were as severe as those of transheterozygotes bearing *Sulf1*$^{\Delta P1}$ over *Df(3R)sdb26* (Fig. 1C). These results, together with the molecular mapping of the deletion, defined *Sulf1*$^{\Delta P1}$ as a null allele of *Sulf1*. The phenotypes of *Sulf1*$^{\Delta P2}$ homozygous adults showed slightly less severe phenotypes compared to those of *Sulf1*$^{\Delta P2}$/*Df(3R)sdb26* heterozygotes and *Sulf1*$^{\Delta P1}$ homozygotes, implying that this allele is hypomorphic. The *Sulf1*$^{\Delta P1}$ allele was thus used for all further analyses described below.

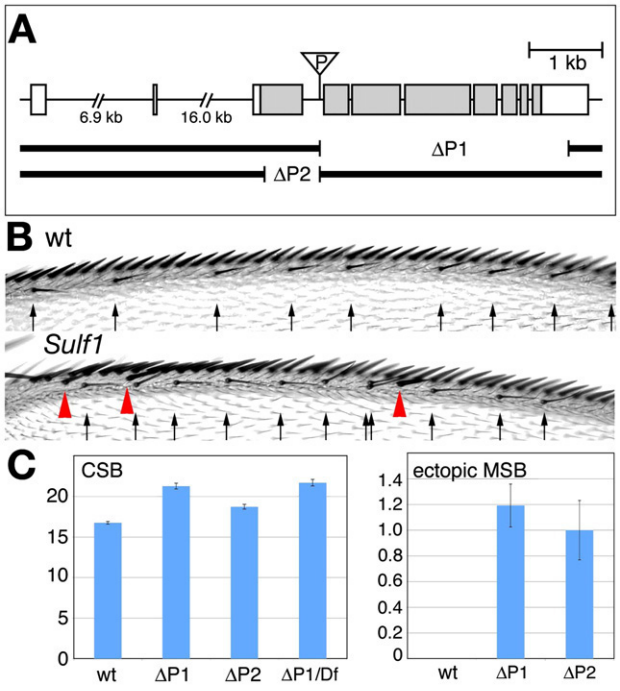

Fig. 1. Phenotypes of *Sulf1* mutants. (A) Genomic organization of the *Sulf1* locus. The shaded area indicates the protein coding region. Two *sulf1* deletion alleles, *Sulf1*$^{\Delta P1}$ (ΔP1) and *Sulf1*$^{\Delta P2}$ (ΔP2), were generated by P-element imprecise excision. *Sulf1ΔP1* lacks exons 4–10, which includes most of the protein coding region. *Sulf1ΔP2* has a smaller deletion located in exon 3, which corresponds to the N-terminal domain of Sulf1 protein. This deletion causes a frame shift at amino acid residue Ser_{59}. (B) Dorsal view of the anterior wing margin of wild-type (wt) and *Sulf1* mutant adult wings. The arrows and red arrowheads indicate positions of chemosensory bristles and ectopic mechanosensory bristles, respectively. These phenotypes are consistent with an expanded region of high-level Wg signaling. (C) Bar graphs showing the number of chemosensory bristles (CSB; left) and ectopic mechanosensory bristles (MSB; right) in *Sulf1* mutants. Values are shown for wild-type (wt), *Sulf1*$^{\Delta P1}$ (ΔP1), *Sulf1*$^{\Delta P2}$ (ΔP2), and *Sulf1*$^{\Delta P1}$/*Df(3R)sdb26* (ΔP1/Df). Both *Sulf1*$^{\Delta P1}$ and *Sulf1*$^{\Delta P2}$ alleles had a significant increase in chemosensory ($p<0.001$), mechanosensory ($p<0.001$), and ectopic mechanosensory ($p<0.001$) bristles compared to wild-type. *Sulf1*$^{\Delta P1}$/*Df(3R)sdb26* had a similar bristle number as *Sulf1*$^{\Delta P1}$ ($p=0.2$), which along with molecular data indicate that it is a null allele. Wing margin bristles were counted for more than 20 wings for each genotype. Error bars for each genotype were calculated using standard error (STDEV/SQRT(n)). P-values for the wing margin bristles were obtained using a one tailed, unpaired, Student's t-test with equal variance in Microsoft Excel.

Sulf1 mutation up-regulates Wg signaling in the developing wing

Since *Sulf1* mutant alleles affect the formation of wing margin bristles, a Wg-dependent process, we assayed downstream markers of Wg signaling in the developing wing. First, we visualized sensory organ precursors (SOPs) using *neuralized*^{A101} enhancer-trap. SOP formation is induced by high levels of Wg signaling in the anterior part of the DV boundary of the wing disc in the late third instar larval stage (Huang et al., 1991; Phillips and Whittle, 1993). Anti-β-gal antibody staining revealed a regular pattern of SOPs in wild-type discs (Fig. 2A). *Sulf1* mutants exhibited an increase in SOP number (average number = 20.5, n = 27) when compared to wild-type (average number = 18.4, n = 21, $p<0.005$) (Fig. 2B). We often observed an irregular, broader distribution of the SOPs, suggesting that Wg signaling is enhanced at the presumptive wing margin. This phenotype is consistent with the increased number and ectopic distribution of wing margin bristles observed in *Sulf1* mutant adults.

We next analyzed expression of Distal-less (Dll), a low-threshold target gene of Wg signaling. We found that Dll levels were consistently elevated in *Sulf1* mutant wing discs compared to the wild-type counterparts (Figs. 2C and D). To confirm this result, *Sulf1* mutant clones were generated by somatic recombination and their effects on Dll expression was examined. Figs. 2E–E″ show a wing disc that has a large *Sulf1* mutant clone covering almost the entire posterior compartment. The area of cells positive for anti-Dll antibody staining was remarkably expanded in the mutant clone compared to wild-type control region. Thus, Wg signaling is up-regulated throughout *Sulf1* mutant wing discs, indicating that *Sulf1* is a novel negative regulator of Wg signaling.

Overexpression of Sulf1 compromises Wg signaling

Based on the observation that Wg signaling is elevated in *Sulf1* mutant discs, we hypothesized that *Sulf1* overexpression would disrupt Wg signaling. To test this idea, a *UAS-Sulf1* transgene was driven in the wing disc by various Gal4 drivers. We found that *Sulf1*-overexpressing adult wings exhibited a wide range of phenotypes. *Sulf1* expression at a moderate level using *C96-Gal4*, a driver for the DV border of the wing disc, or *A9-Gal4*, a driver for the entire wing disc, resulted in a significantly reduced number of wing margin bristles (Figs. 3A and B). *Sulf1* expression at higher levels, driven by *en-Gal4* or *hh-Gal4*, led to notched wings (penetrance: 96%, n = 27; Fig. 3C). These phenotypes are consistent with compromised Wg signaling (Phillips and Whittle, 1993). To determine if *Sulf1* overexpression indeed affects Wg signaling, larval wing discs were stained for Dll. *Sulf1* misexpression in the D compartment driven by *ap-Gal4* abrogated Dll expression (Fig. 3D), confirming that *Sulf1* inhibits Wg signaling. On the other hand, the effect of *Sulf1* overexpression on Senseless (Sens), a high-threshold target for Wg signaling, was less evident (Fig. 3D′). Similarly, Sens expression was not obviously affected by *Sulf1* overexpression by *hh-Gal4* (Figs. 3E and E′). Thus, low- and high-threshold markers exhibited different sensitivities to alterations of *Sulf1* activity. Collectively, these results are consistent with the idea that Sulf1 is a novel negative regulator of Wg signaling at the DV boundary of the developing wing.

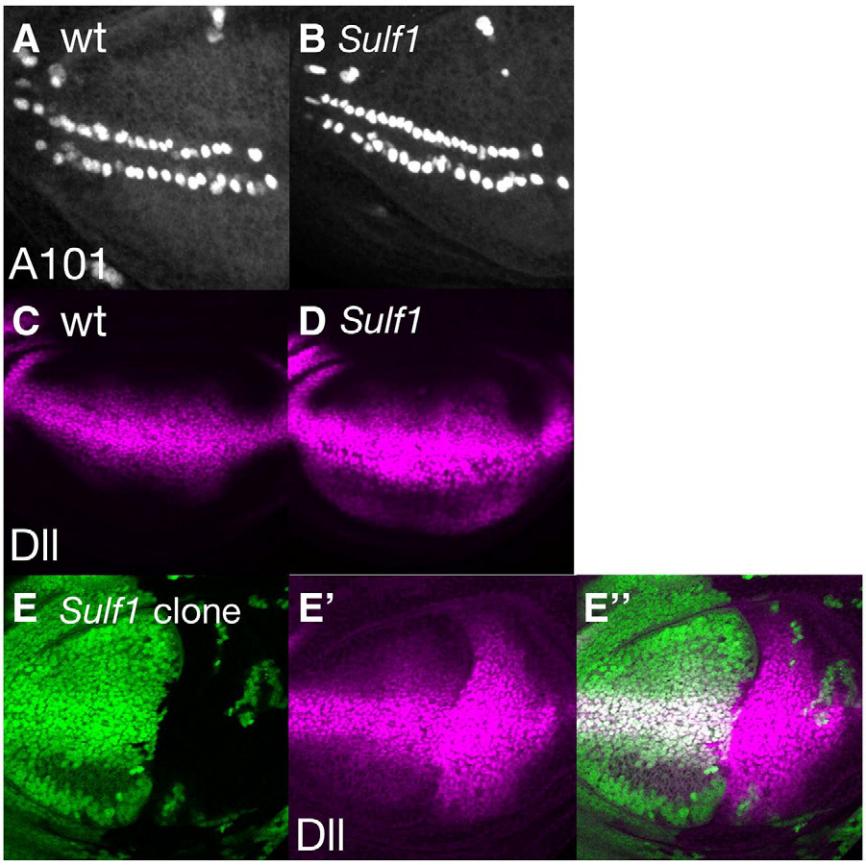

Fig. 2. Wg signaling is up-regulated in *Sulf1* mutants. (A and B) neu^{A101} expression was visualized by anti-β-gal antibody staining of wild-type (A) and *Sulf1* (B) mutant wing discs. For this and all other wing disc images, anterior is to the left and dorsal is to the top. (C and D) Anti-Dll antibody staining of wild-type (C) and *Sulf1* (D) mutant wing discs. (E–E″) A wing disc bearing a large *Sulf1* mutant clone was stained with anti-Dll antibody. Posterior clones were induced by expression of *UAS-FLP* by *hh-Gal4*. Positions of *Sulf1* mutant cells are shown by loss of GFP signal (green in E) and Dll staining is shown in magenta (E′). E″ shows a merged image of E and E′.

In addition to the Wg-dependent processes described above, *Sulf1* overexpression also induced other phenotypes characteristic of reduced Dpp signaling. For example, regions overexpressing *Sulf1* were substantially reduced in size, suggesting that cell proliferation is affected (Figs. 3D–E′). Since Dpp signaling is required for the normal proliferation of wing cells (Burke and Basler, 1996), this observation supports the idea that *Sulf1* overexpression disrupts this pathway. The adult phenotypes of these animals are also consistent with this hypothesis: wings from a large portion of *en>Sulf1* adult flies lacked crossveins or had incomplete anterior and posterior crossveins, and exhibited defects in longitudinal veins L4 and L5 (Fig. 3C). As would be expected for reduced Dpp signaling, we observed that *ap>Sulf1* wing discs showed a reduced area expressing Spalt (Sal), a downstream Dpp transcriptional target (Figs. S1A and A′). In addition, phosphorylation of Mad protein, a direct readout of Dpp signaling, is also significantly decreased by *Sulf1* overexpression (Figs. S1B and B′). The pMad staining was lost in the receiving cells, but was higher in the Dpp-expressing domain. Interestingly, this pattern in the dorsal compartment of *ap>Sulf1* wing discs resembles that of *dally* mutant wing discs (Fujise et al., 2003), suggesting that *Sulf1* overexpression may disrupt the co-receptor function of Dally. Thus, the adult phenotypes and analysis of Dpp targets indicated that excess *Sulf1* activity compromises Dpp signaling as well as the Wg pathway.

Sulf1 affects extracellular levels of Wg protein

To analyze the mechanism for the regulation of Wg signaling by Sulf1, we examined the level of Wg protein in *Sulf1* homozygotes. Wing discs were stained with anti-Wg antibody using a protocol designed to specifically detect Wg protein in the extracellular space (Strigini and Cohen, 2000). We found that overall levels of Wg protein in the mutant discs were higher than those of wild-type (Figs. 4A–B′). In addition to the change in the overall levels of the Wg gradient, we noticed a moderate but consistent difference in the staining pattern between wild-type and *Sulf1* discs. In the mutant discs, the signal for extracellular Wg was more intense and concentrated at the DV boundary, when compared to wild-type discs, which show a broader distribution of Wg protein near the Wg-expressing cells. The differential distribution of Wg was further confirmed by a quantitative analysis on multiple discs. Average Wg signal intensity plots generated from both wild-type and *Sulf1* homozygous discs confirmed elevated Wg protein levels throughout the mutant wing disc (Fig. 4C). Furthermore, the intensity plots indicated that the shape of the gradients is remarkably different. Directly overlaying the intensity plots for wild-type and *Sulf1* mutants revealed that the extracellular level of Wg protein near the DV boundary forms a sharp peak in the mutants whereas the wild-type curve is more gradual (dashed red line in Fig. 4C), which suggests that *Sulf1* affects Wg distribution near the DV boundary. These results suggest that Sulf1 has two effects on the Wg gradient: It reduces extracellular levels of Wg protein throughout the wing disc and facilitates the lateral diffusion of Wg near the DV boundary.

To further confirm that *Sulf1* affects Wg protein levels, wing discs bearing *Sulf1* mutant clones were stained for extracellular Wg. In small mutant clones, typically one or a few cell diameter in size, we observed two separate phenotypic classes. The first class exhibited accumulation of extremely high levels of Wg protein in a cell autonomous fashion (penetrance: 60%, n = 103, arrowhead, Figs. 4D–E″). The second class displayed more moderately elevated levels of Wg (penetrance: 40%, data not shown). The penetrance of

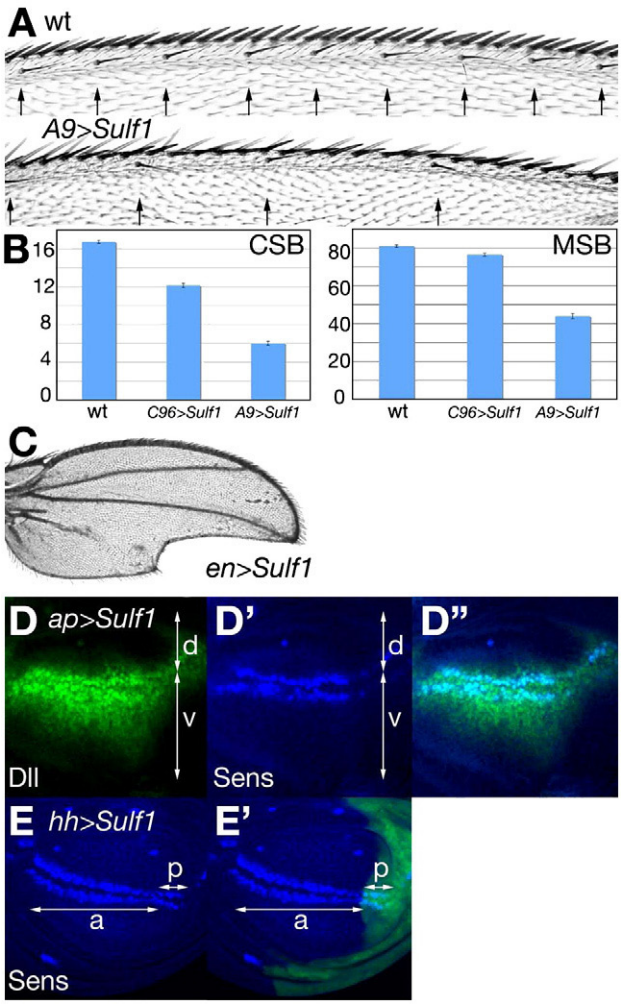

Fig. 3. Wg signaling is abrogated in *Sulf1* overexpressing cells. (A) Anterior wing margins of wild-type (upper) and *A9>Sulf1* (lower) adult wings. Arrows indicate positions of chemosensory bristles. (B) Bar graphs showing the number of chemosensory bristles (CSB; left) and mechanosensory bristles (MSB; right) in wild-type (wt), *C96>Sulf1* and *A9>Sulf1* animals. A significant decrease in chemosensory (*C96>Sulf1* and *A9>Sulf1*: $p<0.001$) and mechanosensory (*C96>Sulf1*: $p=0.03$; *A9>Sulf1*: $p<0.001$) bristles was observed. Wing margin bristles were counted for more than 20 wings for each genotype. Error bars and p-values were calculated as described in Fig. 1. (C) Wing notching phenotype observed in *en>Sulf1* adult flies. (D–D″) Expression of Dll (green, D and D″) and Sens (blue, D′ and D″) in an *ap>Sulf1* larval wing disc. Arrows mark the dorsal (d) and ventral (v) compartments. (E and E′) Expression of Sens in an *hh>Sulf1* larval wing disc (blue). GFP in E′ marks the posterior compartment expressing *Sulf1* (green). Arrows indicate the anterior (a) and posterior (p) compartments.

either of the two Wg accumulation phenotypes was independent of clone position relative to the Wg-expressing cells. To determine if the accumulation of high levels of Wg protein in small *Sulf1* clones is associated with the synthesis of Wg, *wg* expression was monitored by wg^{en11} (*wg-lacZ*). We observed that Wg accumulation occurs without the induction of *wg* transcription (Figs. 4F′–F‴). Interestingly, all larger clones showed the phenotypes of the second class, which is similar to homozygous null mutant discs. It is possible that during the growth of clones, cells with extremely high levels of Wg undergo apoptosis (Adachi-Yamada and O'Connor, 2002), allowing only more phenotypically mild clones to grow. Together, these results indicate that the up-regulation of Wg signaling in *Sulf1* mutant tissue is due to the abnormal accumulation of Wg protein. Some HSPGs have been shown to regulate morphogen gradient formation by stabilizing ligand on the cell surface (Akiyama et al., 2008). Therefore, Sulf1-mediated modification of HS fine structure appears to modulate the activity of HSPGs to decrease extracellular levels of Wg protein.

Sulf1 overexpression reduces the extracellular levels of Wg protein

To further examine the role of *Sulf1* in the Wg pathway, we directly visualized the distribution of Wg protein in wing discs overexpressing *Sulf1*. Staining for extracellular Wg protein revealed that its distribution was dramatically disrupted in the D compartment of *ap>Sulf1* wing discs (Fig. 5A). However, because *Sulf1* overexpression over a wide area decreases the compartment size, it was difficult to directly compare the shape of the Wg gradient between the D and V compartments. Therefore, we examined the effects of ectopically expressed *Sulf1* in randomly induced clones using the FLP-OUT system (Struhl and Basler, 1993). Extracellular Wg protein levels were significantly reduced in the *Sulf1* overexpressing FLP-OUT clones at the surface of both Wg-expressing and receiving cells (Figs. 5B–E'). Remarkably, the function of Sulf1 appears to be cell autonomous (Figs. 5B–E′).

To determine whether Sulf1 affects *wg* expression and/or secretion, we detected Wg protein using a conventional immunostaining protocol, which emphasizes intracellular Wg. *Sulf1* overexpression did not show a detectable change in Wg staining by this staining method, indicating that expression or secretion of Wg protein is not significantly affected (Figs. 5F and F′). We also monitored *wg* expression using *wg-lacZ*. No significant change was observed in *wg-lacZ* signals between inside and outside of *Sulf1* overexpressing clones (Figs. 5G and G′), also supporting that *wg* transcription was not altered. These results together suggest that *Sulf1* modulates extracellular levels of Wg protein in a cell autonomous fashion without affecting *wg* expression.

The cell autonomous nature of Sulf1 activity raised the question of whether this enzyme can act in intracellular compartments. To clarify this point, we generated transgenic strains bearing UAS constructs to express an ER-tethered form (*Sulf1-ER*) and a Golgi-tethered form (*Sulf1-Golgi*) of Sulf1. Sulf1-ER and Sulf1-Golgi both localized to the expected compartments when expressed in S2 cells (Fig. S2). We expressed wild-type and the modified forms of *Sulf1* in the posterior compartment of wing discs by *en-Gal4*, and assessed their ability to decrease extracellular Wg in vivo (Figs. 5H–J′). A significant decrease in extracellular Wg levels was observed in the posterior compartment of *en>Sulf1* but not *en>Sulf1-ER* discs (Figs. 5H–I′). Importantly, *Sulf1-Golgi* showed the activity to reduce extracellular Wg levels similar to wild-type form (Figs. 5J and J′). These results indicated that Sulf1 can function either in the Golgi or on the cell surface, or it may act in both locations. A similar observation was reported for QSulf1 (Ai et al., 2003).

Relationship between Hs6st and Sulf1

The discovery of Sulfs raises several questions. For example, what is the biological significance of having an enzyme that re-modifies HS structures? Why do only 6-*O* sulfate groups undergo the step of desulfation? To better understand the post-synthetic function and substrate specificity of Sulf1, we examined the genetic interactions between *Sulf1* and *Hs6st*, which catalyzes 6-*O* sulfation of the glucosamine residues of HS in the Golgi. *Sulf1* mutants display an increased number of wing margin bristles (Table 1). On the other hand, *Hs6st* mutants have a wild-type wing margin bristle phenotype. Our previous study on *Hs2st* and *Hs6st* showed that HS modifications can be adjusted in response to a defect in one type of sulfation: the loss of 6-*O* sulfation can be compensated by increased 2-*O* sulfation, and vice versa, thus maintaining growth factor signaling essential for normal development in both *Hs2st* and *Hs6st* mutants (Kamimura et al., 2006).

If Sulf1 acts in a post-synthetic manner as has been proposed in vertebrate models (Ai et al., 2003; Dhoot et al., 2001; Morimoto-Tomita et al., 2002; Ohto et al., 2002; Wang et al., 2004), one can predict that *Hs6st* would be epistatic to *Sulf1*. *Hs6st* mutants have no detectable 6-*O* sulfation, so Sulf1 would be expected to have little or no

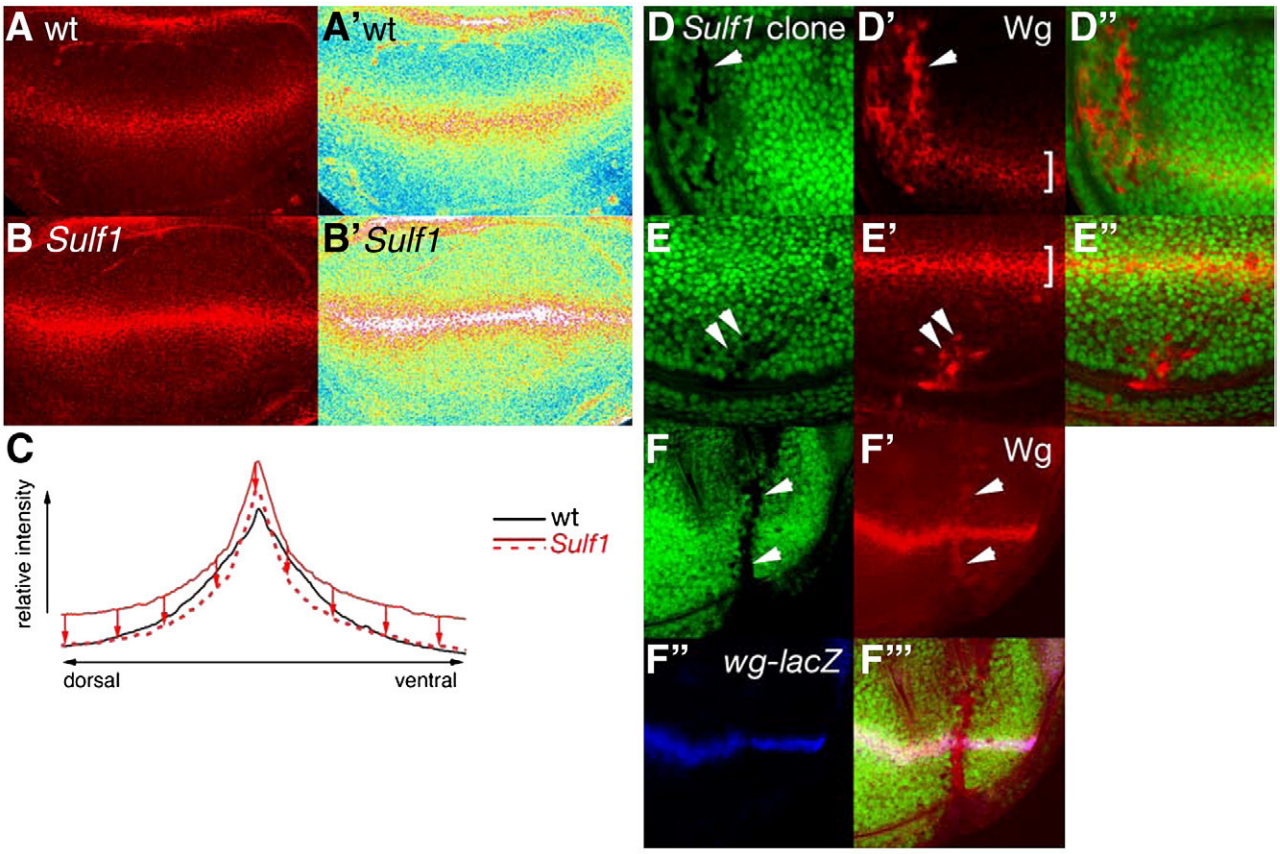

Fig. 4. The level of extracellular Wg protein is elevated in *Sulf1* mutant wing discs. (A–B′) Immuno-staining for extracellular Wg protein in wild-type (A and A′) and *Sulf1* (B and B′) discs. Signal intensity in A and B is shown in pseudocolor to compare intensity in A′ and B′, respectively. Pseudocolor scale ranges from white (highest signal intensity) to dark blue (lowest signal intensity). Note the higher intensity levels of Wg protein throughout the wing disc as a whole in the *Sulf1* homozygous mutant. (C) Signal intensity of extracellular Wg was obtained from raw confocal images by Image J software. Intensity plots were generated by averaging intensity values for wild-type (continuous black line, n = 29) and *Sulf1* (continuous red line, n = 18) wing discs. For a more direct comparison of gradient shape, a downward translation of the *Sulf1* plot (red arrows/dashed red line) was transposed directly on top of wild-type. *Sulf1* wing discs exhibited a steeper gradient of Wg signal intensity when compared to wild-type. (D–E″) Two examples are shown for the effect of small *Sulf1* mutant clones on the distribution of extracellular Wg. GFP signal and extracellular staining of Wg are shown in green (D and E) and red (D′ and E′), respectively. D″ and E″ show merged images. Abnormally high levels of Wg protein accumulate in small *Sulf1* clones (arrowheads). The brackets indicate the extracellular Wg signal located at the DV border. (F–F‴) A wing disc with small *Sulf1* clones stained with anti-β-gal antibody to mark *wg-lacZ* expression. GFP signal, extracellular staining of Wg, and *wg-lacZ* are shown in green (F), red (F′), and blue (F″), respectively. F‴ shows a merged image of F, F′, and F″. *wg-lacZ* is not induced in small clones in which extracellular Wg protein accumulates (arrowheads).

effect on *Hs6st* mutant GAG chains or on the adult phenotype. Indeed, this proved to be the case: *Hs6st Sulf1* double null animals showed wing margin phenotypes indistinguishable from those of *Hs6st* null animals (Table 1). In addition, the double mutants and *Hs6st* single mutants did not display the ectopic mechanosensory bristles that were observed in *Sulf1* mutants (data not shown). These observations provide genetic evidence for the post-synthetic activity of Sulf1. They also demonstrate that the *Sulf1* mutant phenotypes are dependent on 6-*O* sulfation, confirming the substrate specificity of this enzyme.

Genetic interactions between Sulf1 and glypican genes

We next asked which proteoglycan core protein(s) are regulated by *Sulf1*. Since both *dlp* and *Sulf1* have a negative effect on Wg signaling at the DV border, we examined the genetic interactions between these two genes. As expected, each of these mutations increased the number of wing margin bristles. *Sulf1 dlp* double mutants had a moderately increased number of bristles (data not shown), but we did not observe an obvious synergistic effect in this experiment. We also tested the genetic relationship between *Sulf1* and *dally* using the same assay system. We found that *Sulf1 dally* double mutants had a reduced number of both chemosensory and mechanosensory bristles, which mimics the phenotypes of *dally* single mutants (Table 2). In addition, *dally* heterozygosity partially suppressed the ectopic mechanosensory bristle phenotype of *Sulf1* mutants. These results suggest that *Sulf1* acts in the same pathway as *dally* and that *dally* is epistatic to *Sulf1*. Previous studies showed that *dally* is epistatic to *dlp* in bristle formation at the wing margin (Han et al., 2005). Therefore, our genetic interaction experiments support, but do not provide definitive evidence for, the idea that Sulf1 modifies HS chains of Dally and/or Dlp.

Effect of Sulf1 on glypican overexpression phenotypes

To further analyze the substrate specificity of Sulf1, we examined the effect of *Sulf1* overexpression on the overexpression phenotypes of *dlp* and *dally*. Overexpression of *dally* in the posterior compartment using *en-Gal4* or *hh-Gal4* increases the levels of Wg protein due to stabilization (Fig. 6A; Han et al., 2005). *dlp* expression also results in a similar accumulation of Wg (Figs. 6B; Baeg et al., 2001; Han et al., 2005). To determine if Sulf1 affects these phenotypes, we co-expressed *Sulf1* with *dally* or *dlp*, and extracellular Wg levels were observed in each wing disc. As depicted in Figs. 6C and D, co-expression of *Sulf1* with *dally* or *dlp* completely blocked the accumulation of Wg induced by glypican expression, leading to a dramatic loss of Wg protein. The signal intensity of extracellular Wg in the P compartment was lower than the A compartment (Figs. 6C and D), indicating that Sulf1 not only affected the overexpressed molecules but also endogenous HSPGs. Thus, Sulf1 suppressed the phenotype produced by overexpression of both glypicans, suggesting that Sulf1 can modulate HS structures of

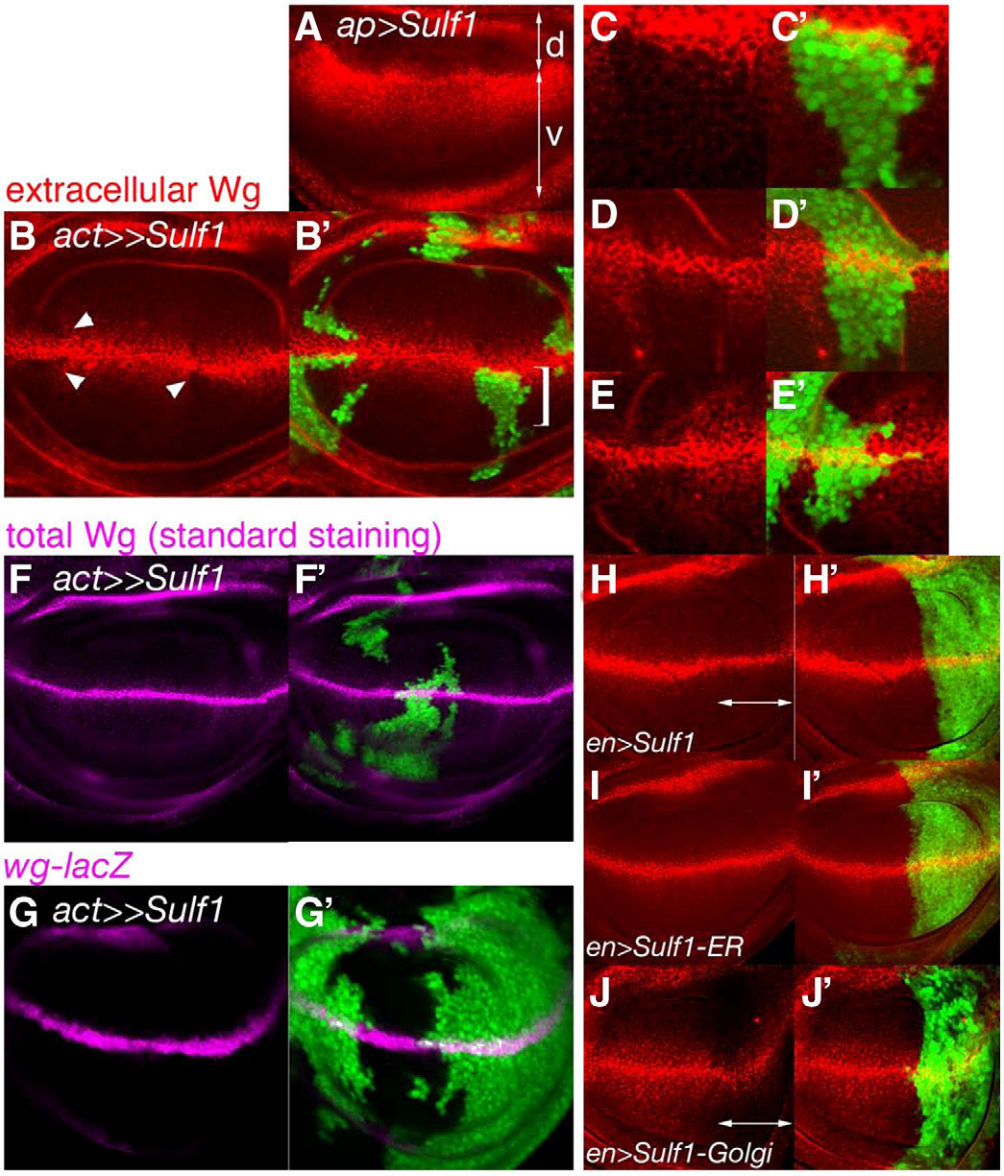

Fig. 5. Effects of *Sulf1* overexpression on the distribution of extracellular Wg protein. (A) Extracellular Wg staining (red) of an *ap>Sulf1* wing disc. Arrows indicate the dorsal (d) and ventral (v) compartments. (B–E′) Immuno-staining of extracellular Wg (red) in a wing disc bearing *Sulf1*-overexpressing FLP-OUT clones. (B and B′) Extracellular levels of Wg protein are reduced in the FLP-OUT clones (arrowheads in B) marked with GFP (B′). (C and C′) A high magnification view of the region marked by the bracket in B′, showing the cell autonomous nature of *Sulf1*'s effect on Wg levels. (D–E′) Two examples are shown for FLP-OUT clones located on the Wg-expressing domain. (F and F′) A wing disc with *Sulf1*-overexpressing clones stained with anti-Wg antibody using the standard immuno-staining protocol. (G and G') A wing disc with *Sulf1* overexpressing clones stained with anti-β-gal antibody to mark *wg-lacZ* expression. (H–J′) Overexpression of modified forms of *Sulf1*. Extracellular levels of Wg protein (red) were examined in wing discs from *en>Sulf1* (H and H′), *en>Sulf1-ER* (I and I′), and *en>Sulf1-Golgi* (J and J′). The posterior compartment is marked by GFP (green in H′, I′, and J′).

Table 1
Hs6st is epistatic to *Sulf1*. The number of chemosensory bristles (CSB) at the anterior dorsal wing margin of adult female flies for each genotype is shown. $Hs6st^{d770}$ was used as an *Hs6st* null mutation. Values represent mean ± standard error for bristle numbers counted for 20 or more wings for each genotype.

Genotype	CSB
Wild-type	16.7 ± 0.2
Sulf1	21.3 ± 0.3
Hs6st	16.3 ± 0.2
Sulf1 +/+ Hs6st	17.7 ± 0.2
Sulf1 Hs6st/+ Hs6st	16.4 ± 0.7
Sulf1 Hs6st/Sulf1 +	20.9 ± 0.6
Sulf1 Hs6st	16.9 ± 0.2

Table 2
Genetic interactions between *Sulf1* and *dally*. The numbers of chemosensory bristles (CSB), mechanosensory bristles (MSB), and ectopic mechanosensory bristles (ectopic MSB) at the anterior dorsal wing margin are shown for the indicated genotypes. $dally^{gem}$ was used as a *dally* loss-of-function mutant allele. Values represent mean ± standard error for bristle numbers counted for 20 or more wings for each genotype.

Genotype	CSB	MSB	Ectopic MSB
Wild-type	16.7 ± 0.2	80.9 ± 0.6	0
Sulf1	21.3 ± 0.3	93.2 ± 0.7	1.2 ± 0.2
dally	8.6 ± 0.1	50.6 ± 0.5	0
dally Sulf1/dally +	9.0 ± 0.1	55.3 ± 0.3	0
dally Sulf1/+ Sulf1	20.6 ± 0.5	93.5 ± 1.6	0.5 ± 0.2
dally Sulf1	8.9 ± 0.2	56.9 ± 0.5	0

both Dlp and Dally in this artificial in vivo system. As discussed later, since expression patterns of *Sulf1* are closely related to those of *dally*, but not *dlp*, Dally may be the major direct substrate of Sulf1 in the developing wing.

HS disaccharide profiles of Sulf1 mutants

To characterize the enzymatic activity of Sulf1, we studied the disaccharide structures of HS isolated from *Sulf1* mutants. HS was purified from adult flies and completely digested into disaccharide units by heparin lyases. Differently modified disaccharides were separated by reverse-phase HPLC. The disaccharide profile of *Sulf1* mutant HS revealed abnormally high levels of the tri-S disaccharide unit (ΔUA2S-GlcNS6S) (Fig. 7). Additionally the levels of the NS2S unit (ΔUA2S-GlcNS) were significantly reduced in the *Sulf1* mutants. These observations confirmed that HS is normally synthesized in a hypersulfated form and excess 6-*O* sulfate groups are later trimmed by Sulf1. Notably, there was no significant increase in the amount of 6S (ΔUA-GlcNAc6S) or NS6S (ΔUA-GlcNS6S) disaccharide units, indicating a high substrate specificity for *Sulf1*. This enzyme selectively removes 6-*O* sulfate groups from tri-S ΔUA2S-GlcNS6S disaccharide units of HS. This substrate specificity of Sulf1 is similar to that of vertebrate homologues reported previously (Ai et al., 2006; Ai et al., 2003).

Expression of the Sulf1 gene is controlled by Wg signaling

Previous studies have shown that some HS modifying enzymes are controlled at the transcriptional level, thus providing a mechanism for temporal and spatial regulation of HS fine structure (reviewed in Gorsi and Stringer, 2007). *In situ* hybridization revealed that *Sulf1* expression exhibits specific patterns in different larval tissues. *Sulf1* mRNA is expressed at high levels in the morphogenetic furrow of the eye disc (Fig. 8A) and in selective sets of cells in the central brain region of the larval CNS (Fig. 8B). *Sulf1* is also expressed in tracheal precursor cells (data not shown). This is interesting given that these

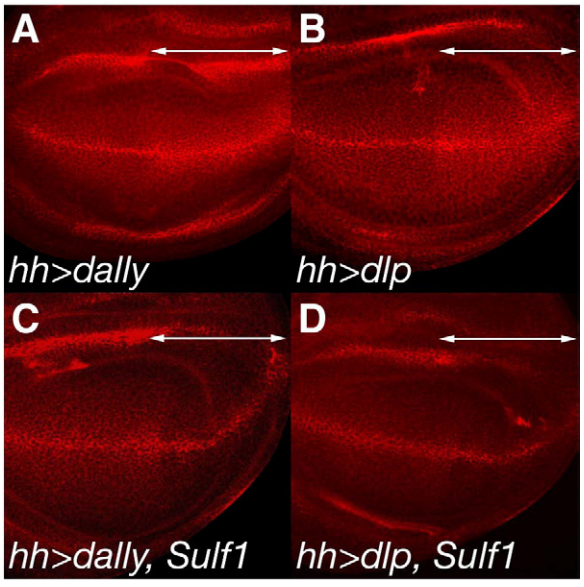

Fig. 6. *Sulf1* affects *dlp*- and *dally*-dependent Wg accumulation. (A and B) Extracellular Wg staining of *UAS-dally/+; hh-Gal4/+* (A) and *UAS-dlp/+; hh-Gal4/+* (B) wing discs. Arrows show the posterior compartment where UAS transgenes were driven by *hh-Gal4*. (C and D) Effect of *Sulf1* co-expression on the *dally* and *dlp* overexpression phenotypes. *UAS-dally/+; hh-Gal4/UAS-Sulf1* (C) and *UAS-dlp/+; hh-Gal4/UAS-Sulf1* (D) wing discs were stained for extracellular Wg. Co-expression of *Sulf1* with *dally* or *dlp* blocked the overexpression phenotypes of both glypicans.

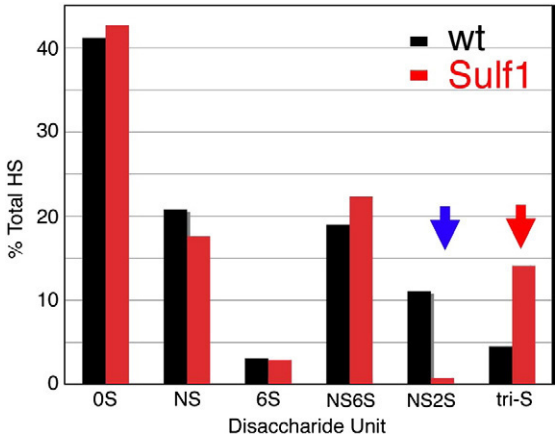

Fig. 7. HS disaccharide profiling of *Sulf1* mutants. Graphical depiction of disaccharide composition of HS from wild-type (black) and *Sulf1* mutants (red), represented as percent of total HS. The *Sulf1* mutant has a decrease in NS2S groups (blue arrow) and a concomitant increase in NS2S6S groups (red arrow). Abbreviations for disaccharides are: 0S, ΔUA-GlcNAc; NS, ΔUA-GlcNS; 6S, ΔUAGlcNAc6S; NS6S, ΔUA-GlcNS6S; NS2S, ΔUA2S-GlcNS; tri-S, ΔUA2S-GlcNS6S.

cells also express high levels of *Hs6st*, which transfers 6-*O* sulfate groups onto HS (Kamimura et al., 2001).

One intriguing pattern of *Sulf1* transcripts is the high level of expression near the AP and DV boundaries of the wing disc (Figs. 8C and D). Since Wg signaling regulates expression of a number of genes, including components of the Wg pathway at the DV boundary (Cadigan et al., 1998; Gerlitz and Basler, 2002; Giraldez et al., 2002), the *Sulf1* expression pattern prompted us to determine if *Sulf1* expression is regulated by Wg signaling. To address this question, we modulated Wg signaling using the Gal4/UAS system and monitored the effect on *Sulf1* expression by *in situ* hybridization with fluorescent probes. Expression of *UAS-GPI-Dfz2*, a dominant negative form of the Wg receptor *Dfrizzled2* (*Dfz2*), partially compromises Wg signaling (Cadigan et al., 1998; Rulifson et al., 2000). We observed that *GPI-Dfz2* expression by *hh-Gal4* eliminated the DV border expression of *Sulf1* mRNA (Figs. 8E–E″). Expression of a constitutively active form of Armadillo (Arm), *UAS-Arm*^{S10}, can induce ectopic activation of Wg signaling (Pai et al., 1997). *Arm*^{S10} is highly lethal when expressed in large domains, but we found that its expression at the AP compartment boundary driven by *dpp-Gal4* is partially penetrant, allowing us to obtain wing discs from surviving animals. Fluorescent *in situ* staining of *Sulf1* transcripts in *dpp>Arm*^{S10} displayed a massive accumulation of *Sulf1* mRNA in the *dpp*-expressing cells, where *Sulf1* expression is normally low (Figs. 8F–F″). Together, these results indicate that Wg signaling at the DV boundary induces expression of *Sulf1*, which acts as a negative regulator of Wg signaling.

Discussion

Although roles for HSPGs in morphogen signaling and distribution have been well established, the molecular basis of these activities remains to be elucidated. A number of genetic and in vitro analyses have demonstrated the critical importance of HS moieties for HSPG function during developmental patterning (Bornemann et al., 2004; Han et al., 2004; Nakato and Kimata, 2002; Takei et al., 2004). Recent reports using mutant forms of Dally and Dlp that lack all HS attachment sites have revealed the essential contribution of the core protein to regulating growth factor binding and signaling activity (Kirkpatrick et al., 2006; Yan et al., 2009). Thus, the regulatory function of HSPGs is likely to be affected by a combination of both HS and core protein structures.

HS biosynthesis is a complex, multi-step process catalyzed by Golgi enzymes in a highly organized fashion (reviewed in Gallagher,

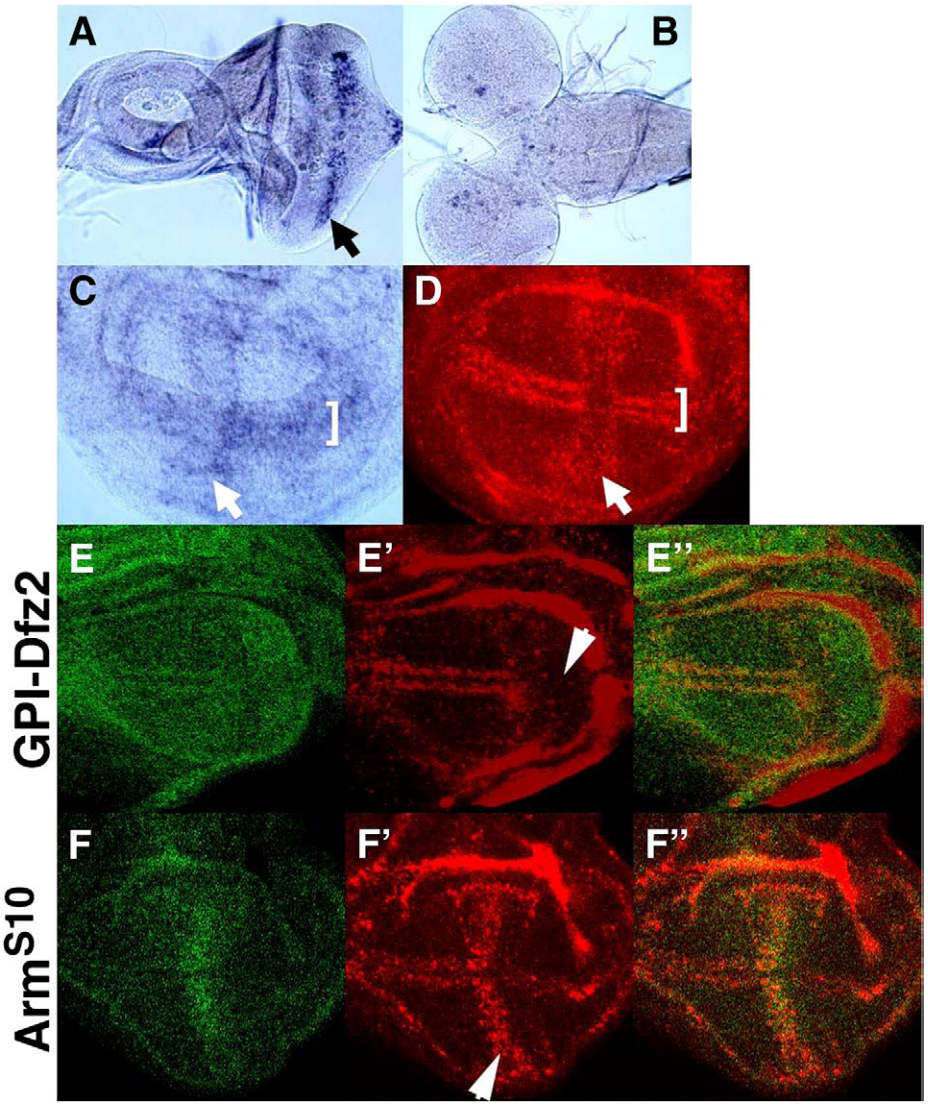

Fig. 8. Regulation of *Sulf1* expression by Wg signaling. (A–D) *Sulf1* mRNA expression in the developing imaginal tissues was analyzed by *in situ* hybridization. High levels of *Sulf1* expression were detected in the morphogenetic furrow in the eye disc (arrow in A) and in specific sets of cells in the central brain (B). In the wing discs, hybridized probe was detected with a colorimetric reaction (C) or fluorescent dye (D). *Sulf1* is expressed at high levels near the AP compartment boundary (arrows) and the DV border (brackets) of the wing disc. (E–F″) *Sulf1* mRNA expression was examined in *hh-Gal4 UAS-GFP/UAS-GPI-Dfz2* (E–E″) and *dpp-GAL4 UAS-GFP/UAS-Arm^{S10}* (F–F″) wing discs. GFP signal from *UAS-GFP* is shown in green (E, E″, F and F″). (E–E″) A dominant negative form of the Wg receptor, *GPI-Dfz2*, was expressed in the posterior compartment by *hh-Gal4*. *Sulf1* expression was reduced by *GPI-Dfz2* (arrowhead and red in E′). (F–F″) Expression of a constitutively active form of Arm, *Arm^{S10}*, by *dpp-Gal4*, up-regulated *Sulf1* expression (arrowhead and red in F).

2001). Recent studies have demonstrated that extracellular Sulfs further modify the HS fine structures in a post-synthetic manner (reviewed in Gorsi and Stringer, 2007). Thus, Sulfs may contribute to generating structural diversity and modify the number of ligand binding sites on HS at the cell surface.

Models for the regulation of the Wg gradient by Sulf1

To better understand the importance of regulating HS sulfation during development, we investigated the role of *Drosophila Sulf1* in patterning and morphogenesis. *Sulf1* mutant wings show specific phenotypes characteristic of abnormally high levels of Wg signaling near the Wg-expressing cells. We demonstrated that extracellular levels of Wg protein were elevated throughout the *Sulf1* mutant wing discs, and decreased in cells overexpressing *Sulf1*. In addition, a *Sulf1* mutation caused Wg to accumulate near its source, altering the shape of the gradient. Thus, *Sulf1* is a novel regulator of Wg gradient formation. Disaccharide analysis of *Sulf1* mutant HS showed abnormally high levels of tri-S disaccharide units, indicating that *Sulf1* regulates Wg signaling by modulating HS fine structure. Given that Sulf1 decreases local levels of Wg protein in the extracellular space (Figs. 4 and 5), it is likely that the domain structure of HS to which the Wg ligand preferentially binds includes tri-S disaccharide unit(s) as a major component. Thus, Sulf1 may redefine the shape of the Wg gradient by removing some of the Wg-binding sites from HS on the cell surface.

It has been shown in *Drosophila* embryos that a significant fraction of Wg protein is retained on the expressing cells in a HSPG-dependent manner (Pfeiffer et al., 2002). High levels of *dally* expression near the DV boundary of the wing disc (Fujise et al., 2001) suggest that Wg may be also trapped by HS in the developing wing. In such a situation, Sulf1 activity could reduce the trapping of Wg by cell surface HSPGs near the expressing cells. Wg protein, thus released from HS, could have two possible fates. First, Wg ligand could be quickly internalized by nearby cells for degradation. Second, released Wg ligand could escape degradation and migrate away from the trapped site. Therefore, theoretically, Sulf1 can affect the Wg gradient through two differential activities: (1) destabilization of Wg and (2) enhancement of Wg re-distribution by facilitating Wg release from the HSPGs. We showed that *Sulf1* reduces extracellular levels of Wg protein without affecting *wg* expression (Fig. 5). In addition, Wg signal intensity plots for wild-

type and *Sulf1* mutant discs suggested that *Sulf1* affects Wg distribution near the DV boundary (Fig. 4C). Thus, our observations are consistent with the idea that Sulf1 indeed modulates the Wg gradient by influencing both Wg stability and distribution.

How can Sulf1 contribute to lateral distribution of Wg? Gallet et al. (2008) has proposed that Dlp mediates apicobasal trafficking of Wg, which is required for its long-range gradient formation (Gallet et al., 2008). A more recent study has shown that Dlp can act in a biphasic manner to potentiate Wg long-range signaling (Yan et al., 2009). In this model, Dlp either competes with the receptor or provides ligand to the receptor, dependent on its ratio to Wg and the receptor. In both models, however, since *dlp* expression is repressed at the DV compartment border (Kirkpatrick et al., 2004), an additional mechanism by which Wg reaches the *dlp*-expressing cells appears to be required. Wg secreted from cells at the DV boundary is likely to be first trapped by Dally, a glypican expressed at high levels in this region (Fujise et al., 2001). One possible function of Sulf1 is to facilitate the short-range movement of Wg from the expressing cells to Dlp (Fig. 9). In this model, Sulf1, which is also abundant near the source of Wg, removes 6-*O* sulfate groups from Dally HS chains. This enzymatic cleavage would lower the efficiency of Wg trapping by Dally, allowing it to migrate away from the DV boundary. Released Wg would now have a better chance to reach Dlp, which recaptures and facilitates further diffusion of Wg (Gallet et al., 2008; Yan et al., 2009). Thus, our study demonstrates that modification of HS fine structure provides a novel mechanism to shape morphogen gradients.

Given that vertebrate Sulfs are known to positively regulate Wnt signaling (Ai et al., 2003), it is surprising that *Drosophila* Sulf1 has an opposite effect on the Wg pathway. Our results suggest that *Drosophila* Sulf1 has a similar biochemical activity and we expect that a direct consequence of the function of Sulf enzymes on Wnt/Wg protein is also similar between vertebrate and invertebrate models: Sulfs release Wnt/Wg ligands from HSPGs. We propose that the fate of the released Wnt/Wg could be different dependent on extracellular environment. In vertebrate systems where Sulfs enhance Wnt signaling, released Wnt appears to have better chance to bind and activate receptors (Ai et al., 2003). On the other hand, a major fraction of Wg protein detached from HSPGs may be degraded in the *Drosophila* wing disc.

Although Sulfs are believed to function post-synthetically in the extracellular space, the effects of Sulf1 function were observed cell autonomously. In addition, our experiments using Sulf1-Golgi showed that this modified form retains the ability to decrease extracellular levels of Wg protein, indicating that Sulf1 does not have to be secreted into the extracellular space to function. Thus, Sulf1 may act in the Golgi and/or on the cell surface. If Sulf1 acts extracellularly, Sulf1 is likely to adhere to the surface of the secreting cells as has been shown in vertebrate models: previous studies reported that Sulf enzymes associate with the cell fraction and not the medium fraction of transfected cultured cells (Ai et al., 2003; Dhoot et al., 2001; Lai et al., 2003; Morimoto-Tomita et al., 2002; Ohto et al., 2002). The binding of QSulf1 to the cell fraction in CHO cells has shown to be dependent on a large hydrophilic domain (Dhoot et al., 2001). Since a similar conserved hydrophilic domain is found in *Drosophila* Sulf1, we hypothesize that Sulf1 may bind to a constituent of the ECM in close proximity to the expressing cells.

We found that small *Sulf1* clones show more severe phenotypes than large clones and *Sulf1* homozygous mutant discs (Figs. 4D–E″). This observation suggests that morphogen gradients are more severely disrupted in a developmental field with discontinuity of cell surface HS structures (e.g. discs with *Sulf1* small clones) compared to one where HS sulfation is uniformly altered (e.g. *Sulf1* homozygous mutant discs). However, the molecular mechanism behind this difference remains to be elucidated.

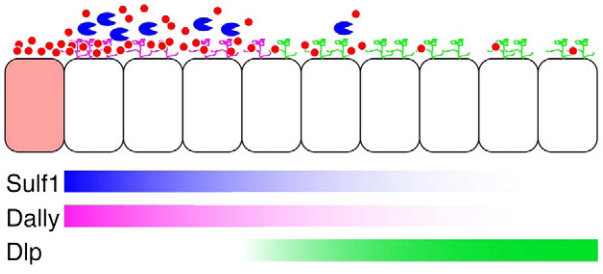

Fig. 9. A model of the role of Sulf1 in Wg gradient formation. Sulf1 (blue) functions by enzymatically modifying the number of 6-*O* sulfate groups on glucosamine residues of HS tri-sulfated disaccharides. This post-synthetic reduction of 6-*O* sulfate groups would release Wg (red circle) from HSPGs. Released Wg can undergo either degradation or re-distribution to the periphery of the wing pouch. Since Dally (magenta) is expressed at high levels near the Wg-expressing domain, Wg protein is likely to be first trapped by Dally. Sulf1, which is expressed at high levels in the same region as Dally, may function to release Wg from Dally. Wg released from Dally by Sulf1 can be degraded or re-captured by Dlp (green), of which expression domain is high in the peripheral cells of the wing disc. Wg secreting cells (pink) at the DV border are located on the left side of the figure opposite of Wg non-secreting cells (white) which expand into the periphery of the wing pouch. Expression domains of Sulf1, Dally, and Dlp are represented by blue, magenta, and green graded bars respectively.

Sulf1 forms a feedback loop of Wg signaling

In situ hybridization showed that *Sulf1* mRNA is expressed at high levels near both the AP and DV borders of the wing disc (Fig. 8). Interestingly, this feature is similar to the expression pattern of *dally* in the wing disc. The DV boundary expression of *dally* is induced by Wg signaling (Fujise et al., 2001). We showed that expression of *Sulf1*, like that of *dally*, is induced by Wg signaling. Thus, *Sulf1*, a negative regulator of the Wg pathway, participates in a negative feedback loop within this morphogen system. We previously showed that Dally is a component of the negative feedback loop for the Dpp signaling pathway, potentially stabilizing the shape of the Dpp gradient (Fujise et al., 2003). In addition, Wg also induces *Notum*, which is a secreted antagonist of Wg and functions through the posttranslational cleavage of glypicans at the wing margin (Gerlitz and Basler, 2002; Giraldez et al., 2002; Kreuger et al., 2004). Collectively, these results implicate HSPGs and HS biosynthetic machinery components as general constituents of morphogen feedback systems, supporting the stability and the robustness of morphogen signaling gradients.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2010.07.006.

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