Heparan sulfate proteoglycans regulate various physiological and developmental processes through interactions with a number of protein ligands. Heparan sulfate (HS)-ligand binding depends on the amount and patterns of sulfate groups on HS, which are controlled by various HS sulfotransferases in the Golgi apparatus as well as extracellular 6-O-endosulfatases called “Sulfs.” Sulfs are a family of secreted molecules that specifically remove 6-O-sulfate groups within the highly sulfated regions on HS. Vertebrate Sulfs promote Wnt signaling, whereas the only Drosophila homologue of Sulfs, Sulf1, negatively regulates Wingless (Wg) signaling. To understand the molecular mechanism for the negative regulation of Wg signaling by Sulf1, we studied the effects of Sulf1 on HS-Wg interaction and Wg stability. Sulf1 overexpression strongly inhibited the binding of Wg to Dally, a potential target heparan sulfate proteoglycan of Sulf1. This effect of Drosophila Sulf1 on the HS-Wg interaction is similar to that of vertebrate Sulfs. Using in vitro, in vivo, and ex vivo systems, we show that Sulf1 reduces extracellular Wg protein levels, at least partly by facilitating Wg degradation. In addition, expression of human Sulf1 in the Drosophila wing disc lowers the levels of extracellular Wg protein, as observed for Drosophila Sulf1. Our study demonstrates that vertebrate and Drosophila Sulfs have an intrinsically similar activity and that the function of Sulfs in the fate of Wnt/Wg ligands is context-dependent.

Heparan sulfate proteoglycans (HSPGs) are carbohydrate-modified proteins that play important roles in a variety of biological processes, such as growth factor signaling and cell adhesion. HSPGs are composed of a core protein to which heparan sulfate (HS) chains are covalently attached. HS chains are long, unbranched polysaccharides composed of repeating glucosamine and uronic acid subunits. Genetic studies have shown that mutations affecting HSPG core proteins or HS biosynthetic enzymes cause defects in growth factor signaling in vertebrates as well as in Drosophila (3). Thus, the biological function of HSPGs is believed to be dependent on both core protein structure and the heterogeneous fine structures of their sugar chains (4).

HS chain biosynthesis begins with the formation of a tetrasaccharide linker to specific serine residues of a core protein (5). The HS chain is polymerized by enzymes of the exostosin EXT family. The polymerizing chain is first modified by N-deacetylase/N-sulfotransferase. N-Deacetylase/N-sulfotransferase removes the N-acetyl group from N-acetylgalcosamine and adds a sulfate group using the sulfate donor 3′-phosphoadenosine 5′-phosphosulfate. At this point, glucuronic acid can be converted to iduronic acid by C-5 epimerization through the activity of HS C-5 epimerase (Hsepi). The nascent polysaccharide subsequently undergoes a series of O-sulfation events at different ring positions, including 2-O-sulfation on uronic acid and 6-O-sulfation on glucosamine. These reactions are catalyzed by HS 2-O-sulfotransferase (Hs2st) and 6-O-sulfotransferase (Hs6st), respectively.

After the HS biosynthesis/modification steps occur in the Golgi, HS can be further modified extracellularly by a family of enzymes: the extracellular HS 6-O-endosulfatasases (Sulfs) (6–8). Sulfs have been shown to remove a specific subset of 6-O-sulfate groups within the highly sulfated domains, in particular, trisulfated disaccharide units, 2-O-sulfo-iduronic acid-N-sulfoglucosamine-6-O-sulfate, within HS chains (9–12). Functional studies of Sulfs in vertebrate systems have shown that they promote Wnt signaling (6, 10). It has been proposed that the activity of Sulfs reduces the binding between HS and the Wnt ligand, in turn promoting the access of Wnt to Frizzled receptor for signaling (10, 13–15). Thus, HS structure and function can be controlled by a post-synthetic remodeling process.

Drosophila Sulf1, which is the only Drosophila Sulf, shows the same enzymatic activity with a similar target specificity (2-O-sulfo-iduronic acid-N-sulfoglucosamine-6-O-sulfate) to
its vertebrate homologues in vivo (12, 16). Nevertheless, its effect on Wnt/Wg signaling is opposite to its vertebrate counterparts; Drosophila Sulf1 is a negative regulator of Wg signaling (12, 17). The molecular nature for this opposite activity of Drosophila Sulf1 is unknown. There are two major possibilities to explain this differential effect of vertebrate and Drosophila Sulfs. First, it is possible that Sulf1 has an intrinsically distinct function on Wg from that of vertebrate Sulfs. For example, it has not been determined whether or not the removal of 6-O-sulfate groups by Drosophila Sulf1 releases Wg from HS on the cell surface as proposed for vertebrate Sulfs. Alternatively, the difference may result from environmental factors that affect the fate of Wnt/Wg protein once they are released from the cell surface HS by Sulfs. It is not known how the fate of Wg protein, after release from HS, is regulated in Drosophila. We have previously shown that overexpression of Sulf1 in the developing wing reduced extracellular Wg protein levels without affecting its gene transcription and secretion (12).

Mammalian Sulfs play central roles in tumor formation through modulation of Wnt signaling and are novel therapeutic targets for various cancers (18, 19). For this purpose, it is critical to understand the molecular basis for differential effects of Sulfs on Wnt/Wg signaling in different contexts. In this study, to elucidate the mechanism for the negative regulation of Wg signaling by Drosophila Sulf1, we studied the effect of Sulf1 overexpression on Wg-HS binding and the fate of Wg released from the cell surface. We found that Dally, a Drosophila member of the glypican family of HSPGs, isolated from Sulf1-overexpressing cells showed a weaker affinity to Wg than that from wild-type cells, suggesting that Sulf1 removes the Wg binding site from Dally HS chains. We also devised various assay systems (in vitro, in vivo, and ex vivo) to monitor the fate of Wg protein bound to the cell surface in the presence or absence of overexpressed Sulf1. These assays consistently supported the model that Sulf1 reduces extracellular Wg protein levels and that Wg degradation contributes to this reduction. We propose that Sulf1 negatively regulates Wg signaling by removing the Wg binding site on the cell surface HS, thus affecting Wg turnover rate. Furthermore, we show that human Sulf1 expressed in the Drosophila wing disc decreases the level of extracellular Wg protein, confirming that Sulfs exert different effects on Wnt/Wg signaling in a context-dependent manner.

**Experimental Procedures**

Fly Strains—Detailed information for the fly strains used is described in Flybase flybase.bio.indiana.edu) except where noted. The wild-type strain used was Oregon R. Other strains used were: hedgehog (hh)-GAL4; UAS-GFP, UAS-Sulf1-HA (12); and tub-GAL80(20).

UAS-human Sulf1 (hSulf1) was constructed by cloning hSulf1 cDNA (9) into the vector pUASg.attB (a gift from K. Basler), and transgenic strains bearing this construct were made by BestGene Inc. using dC31-mediated integration of the plasmid DNA into Basler ZH line 68E (21) as has been done for UAS-Sulf1 (12). hSulf1 cDNA was obtained from Addgene. FLP-OUT clones overexpressing Sulf1 or hSulf1 were generated as described previously (22, 23) using a Act5C>CD2>Gal4 transgene cassette.

Immunostaining—Larval wing discs were fixed with 3.7% formaldehyde in PBS for 20 min at room temperature. Antibody staining was performed according to standard procedures (24). The following antibodies were used: rabbit anti-GAL4 (1:100, Santa Cruz Biotechnology), rat anti-HA (1:200, Roche Applied Science), and mouse anti-Wg (1:100, 4D4, Developmental Studies Hybridoma Bank (DSHB)). Extracellular labeling of Wg protein was performed according to Strigini and Cohen (25) using the anti-Wg antibody (4D4) at 1:3 dilution (25). Secondary antibodies were from the Alexa Fluor series (1:500; Molecular Probes).

Wg Binding Assay—In this and the following in vitro assays, we used the S2R+ cell line obtained from the Drosophila Genomics Resource Center (DGRC). S2 cells do not respond to soluble Wg because they do not express Dfizzled-2, the predominant Wg receptor (26). In contrast, S2R+ cells express Dfizzled-1 and Dfizzled-2 and can mimic behaviors of Wg-receiving cells (27).

We established a Sulf1-overexpressing S2R+ cell line (S2R+ -pAW-Sulf1-HA) by stably transfecting S2R+ cells with an actin promoter-driven Sulf1-HA cDNA (pAW-Sulf1-HA). S2R+ control cells and S2R+ -pAW-Sulf1-HA cells were transiently transfected with empty vector, wg, and/or Myc-sec-dally cDNA. To compare the amount of Wg bound to Dally, Myc-Sec-Dally was immunoprecipitated with anti-Myc antibody from culture media of both cell lines. Immunoprecipitation was carried out by incubating the media with anti-c-Myc-conjugated agarose (Sigma) overnight with agitation at 4 °C. Precipitates were washed five times with TBS (50 mM Tris-HCl pH 7.4, 150 mM NaCl). Immunoprecipitated protein was eluted with 1× SDS loading buffer, and relative Wg levels in the precipitates were analyzed by immunoblot analysis using anti-Wg antibody.

We also established a second stably transfected cell line, S2R+ -pMt-Sulf1-HA, in which Sulf1 may be induced (27). The inducible cell line was created by stably transfecting cells with a Sulf1-HA transgene driven by an inducible metallothionein promoter (pMt-Sulf1-HA). S2R+ -pMt-Sulf1-HA cells were transiently transfected with empty vector, wg, and/or sec-dally-myc cDNA. At 24 h after transfection, cells were treated with CuSO4 (27) at a final concentration of 350 μM to induce Sulf1 expression. Control cells were given an equal volume of culture medium. At 72 h after the CuSO4 treatment, the binding of Wg to Dally was monitored by the same immunoprecipitation method and immunoblot analysis as above.

In Vitro Wg Protein Assay—Exogenous Wg, in the form of Wg-containing conditioned medium, was prepared by an S2 cell line stably transfected with Wg cDNA (S2-tub-Wg, DGRC). The Wg conditioned medium was incubated with control S2R+ and S2R+ -pMt-Sulf1-HA cells at 4 °C for 1 h to allow binding, whereas preventing internalization of Wg protein (28). Expression of Sulf1 was induced at t = 0, after exogenous Wg exposure allowing equal Wg protein binding. After Wg binding, excess Wg was washed away, and Sulf1-HA expression was induced by the addition of CuSO4. An equal volume of medium was added to control cells. Cell aliquots were taken over the course of 6 h and subjected to immunoblot analysis to assess Wg protein levels over time. α-Tubulin was used as a loading...
control for each sample and was further used to normalize relative Wg protein levels. Normalized Wg protein levels from four independent experiments were averaged as a measure of Wg protein stability with and without Sulf1-HA induction.

To inhibit lysosomal activity, S2 cells were treated with bafilomycin A1 (29–31). S2 cells were pretreated with 25 nm bafilomycin A1 for 30 min before the incubation with Wg conditioned medium. After excess Wg was washed away, the cells were incubated for 0–2 h in the presence of 25 nm bafilomycin A1. RNAi treatment of S2 cells was performed as described (32). The recovery of proteins from the conditioned medium by tri-chloroacetic acid (TCA) precipitation was carried out as described previously (33).

In Vivo Wg Gradient Time Course Assay—The Gal4-Gal80 system was used to induce Sulf1 expression specifically in the posterior compartment of the developing wing. Animals with the genotype of tub-GAL80$^{+}/+$; hh-GAL4 UIAS-GFP/UIAS-Sulf1-HA were raised at the GAL80$^{+}$ permissive temperature (18°C). At mid-third instar, the temperature was shifted to the GAL80$^{+}$ restrictive temperature (30°C) and incubated for an additional 2–8.5 h. Wing discs were dissected at each respective time point and stained for GFP or with anti-GAL4, anti-HA, or anti-Wg antibodies. To examine the Wg gradient in wing discs, we used a protocol that specifically detects the extracellular fraction of Wg protein (25). Images were analyzed by confocal microscopy (Zeiss LSM710).

The phenotypic spectrum of Wg staining was classified into three categories: wild-type, mild, and strong extracellular Wg phenotypes. The three phenotypic classes were based on the difference in levels of extracellular Wg protein between the anterior (control) and posterior (Sulf1-overexpressing) compartments. The categories were defined as follows: wild type, there is no difference; mild, the difference is detectable but not substantial; strong, there is significant difference.

Ex Vivo Wg Stability Assay—Animals with the genotype of tub-GAL80$^{+}/+$; hh-GAL4 UIAS-GFP/UIAS-Sulf1-HA were raised at 18°C. At mid-third instar, the temperature was shifted to 30°C to induce Sulf1-HA expression. At 2 h after the temperature shift, wing discs were dissected and stained with anti-Wg antibody for 1 h in ice-cold Schneider’s Drosophila tissue culture medium to allow the antibody to bind extracellular Wg. After excess antibody was washed off, wing discs were further incubated at 30°C in the culture medium containing antibiotics (penicillin-streptomycin, Invitrogen) for 0–3 h. At each respective time point, the discs were fixed, and Wg levels were indirectly measured by the detection of anti-Wg antibody with secondary antibody. Stained tissues were analyzed by confocal microscopy. Wing discs were phenotypically classified based on the severity of the reduction of extracellular Wg in the posterior compartment when compared with its anterior control compartment, as described under “In Vivo Wg Gradient Time Course Assay.”

RESULTS

Sulf1 Affects Levels of Wg Bound to Heparan Sulfate—Wg protein strongly adheres to the cell surface and the extracellular matrix through association with HSPGs (34). One of the predictions regarding Sulf1 function from previous studies is that Sulf1 removes Wg binding sites from HS on the cell surface (12). To test this idea, we used two stably transfected S2R$^{+}$ cell lines overexpressing Sulf1. In the first line, S2R$^{+}$-pAW-Sulf1-HA, Sulf1 is overexpressed under a constitutive actin promoter. S2R$^{+}$ control and S2R$^{+}$-pAW-Sulf1-HA cells were transiently transfected with $wg$ cDNA. To analyze interactions between Wg protein and a specific cell surface HSPG molecule, we chose to use Dally, a Drosophila glypican. Dally has been shown to modulate Wg signaling (35–37) and is a potential target substrate for Sulf1 (12, 17). To detect Wg protein bound to Dally, we co-expressed a secreted form of Dally (Myc-sec-dally), which encodes a truncated form of Dally lacking the signal sequence for the glycosylphosphatidylinositol linkage at its C terminus (38). This construct allowed us to directly immunoprecipitate Dally from the cultured medium with anti-Myc antibody. The amount of Wg in the precipitates was analyzed by immunoblot analysis using anti-Wg antibody. In both S2R$^{+}$ control and S2R$^{+}$-pAW-Sulf1-HA cells, a similar amount of Myc-sec-Dally and Wg protein input into the immunoprecipitation assay was detected (Fig. 1A). Sec-Dally was detected as smear bands with comparable size in both cell lines, indicating that Sec-Dally expressed in the two lines has a similar degree of HS modification. Nevertheless, a significantly smaller amount of Wg protein was recovered in the Sec-Dally immunoprecipitate from the Sulf1-expressing S2R$^{+}$ cells. This result shows that less Wg was able to bind Dally HS that has been previously modified by Sulf1.
Sulf1 in Wg Degradation

Similar results were obtained using a second stably transfected cell line (S2R+/-pMt-Sulf1-HA), in which Sulf1 may be induced. At 72 h after induction of Sulf1 expression by CuSO4 treatment, Wg binding to Dally was assayed as above. Consistent with the result with S2R+-pAW-Sulf1-HA, immunoblot analysis revealed that less Wg protein bound to Sulf1-modified Dally, whereas there was no detectable difference in the degree of HS attachment to Sec-Dally (Fig. 1B). Together these results show that the enzymatic modification of 6-O-sulfate groups on HS reduces the amount of Wg protein binding.

**Sulf1 Reduces Wg Protein Levels in Cultured Cells**—It has been well established that endocytosis of Wg followed by degradation plays a major role in controlling extracellular Wg levels as well as Wg signaling in the wing disc (25, 39–44). In a previous study, we have shown that in vivo expression of Sulf1 reduces the levels of extracellular Wg protein (12). To ask whether this observation is due to reduced Wg stability, we designed an in vitro pulse-chase Wg protein assay. We have shown that the presence of Sulf1 can reduce Wg binding to HS (Fig. 1). Thus, to examine the effect of Sulf1 on the fate of cell surface-bound Wg, it was important to begin the time course assay with the same amount of Wg bound to the control and test cells. We therefore used the S2R+-pMt-Sulf1-HA cell line, which allowed us to conditionally induce Sulf1 expression at time 0, after exogenous Wg exposure allowing equal Wg protein binding. After exogenous Wg was bound to the cell surface and excess Wg was washed off, Sulf1 expression was induced by CuSO4. Cells without CuSO4 treatment were used as a control. Cell aliquots were taken over the course of 6 h after induction, and Wg protein levels were monitored over time. In this experiment, no exogenous HSPG was expressed.

As expected, the amount of Wg protein bound to the cell surface was indistinguishable between control and Sulf1-induced cells at time 0 (Fig. 2A). In control cells, Wg protein levels started to gradually decline after 45 min (Fig. 2A and B). In cells induced for Sulf1-1HA, Wg protein started to drop earlier (around 30 min) and reached much lower levels after 2 h of incubation. Over the time course of 6 h, the level of Wg protein decreased much more rapidly in cells expressing Sulf1 when compared with control cells. Quantification of four independent experiments is shown in Fig. 2B.

To determine whether the observed gradual decrease in the level of Wg protein reflects the destabilization of Wg or its release into the culture medium, we employed two strategies to disrupt Wg intracellular degradation. First, we examined the effect of treatment of S2 cells with bafilomycin A1, an inhibitor of endosomal acidification (29–31). In the absence of bafilomycin A1, the levels of Wg protein were decreased during a 2-h incubation as stated above, and this reduction was substantially enhanced by Sulf1 overexpression (Fig. 3A, upper panel). We observed that this decrease of Wg was significantly blocked by the treatment of the cells with bafilomycin A1, supporting the idea that lysosomal degradation contributes to the change in the level of Wg (Fig. 3A, lower panel). Second, we tested whether Rab7, a small GTPase required for late endosome function, is required for the reduction of Wg levels during incubation. It has been shown that Rab7 activity is critical for endocytosis and degradation of Wg (43). We found that rab7 RNAi treatment impaired the reduction of Wg protein during incubation in both Sulf1-induced and non-induced cells (Fig. 3). The result suggests that the observed decrease of Wg requires...
Rab7-mediated Wg membrane trafficking. The two treatments showed similar effects; the Wg protein levels in control and Sulf1-expressing cells after these treatments became almost indistinguishable (Fig. 3). These observations suggest that cellular degradation contributes to Sulf1-dependent decrease in the level of Wg.

To examine the impact of the release of Wg protein into the culture medium on the decreased levels of Wg in the cell fractions, we investigated Wg in the medium. Proteins were precipitated from the medium samples by TCA prepared from S2R cells after 0 and 2 h of incubation. We detected low levels of Wg by immunoblotting at time 0 in control, representing a background (Fig. 4, lane 4). No increase in signal intensity was observed after 2 h in control (lane 5), suggesting that negligible levels of Wg detached from the cell surface. Importantly, we detected indistinguishable levels of Wg in the media for control and Sulf1-expressing cells (lanes 6 and 7). Comparison with TCA-precipitated soluble Wg (lanes 1–3) showed that the Wg level detected in the media is equivalent to ~2.5% of total Wg protein attached to the cell surface at time 0. Consistent results were obtained from three independent experiments. Thus, the amount of Wg released into the media appears negligible and is not affected by Sulf1 overexpression.

The analyses of the effects of chemical and genetic inhibition of Wg membrane trafficking and lysosomal degradation as well as Wg protein in the medium fractions consistently supported that the cellular degradation of Wg has a major contribution to the gradual decrease in the level of Wg protein observed in Fig. 2. Altogether, our in vitro Wg protein assay showed that Sulf1 affects extracellular Wg levels in vitro by, at least in part, affecting the stability of Wg.

Induction of Sulf1 Reduces Wg Levels in Vivo—To confirm the in vitro Wg protein assay results, we attempted to study the effect of Sulf1 on Wg stability and degradation in more natural physiological conditions. Our ultimate goal was to establish a novel ex vivo pulse-chase Wg stability assay, which combines organ culture techniques and genetic tools, as we will describe later (see Fig. 6). Toward this goal, we needed to optimize a number of genetic and culture conditions. Therefore, we first developed an in vivo time course assay to monitor Wg levels in the extracellular space.

Using the GAL4/UAS system in conjunction with temperature-sensitive GAL80 (GAL80ts), transgenes can be both temporally and spatially controlled (20, 45, 46). The GAL80ts system blocks GAL4/UAS-induced transgene expression when tissue is exposed to the GAL80ts permissive temperature (18 °C), repressing target gene expression (Sulf1-HA). After shifting to the restrictive temperature (30 °C), GAL80ts loses its affinity to the GAL4 activation domain, and target gene expression is turned on. A, graphic depicting the GAL4/GAL80ts system. A temperature-sensitive transgenic allele of GAL80 (black oval) driven ubiquitously by the tubulin promoter (tub-GAL80ts) actively binds to GAL4 (gray oval) in a dimer–dimer interaction at the permissive temperature (18 °C), repressing target gene expression (Sulf1-HA). After shifting to the restrictive temperature (30 °C), GAL80ts loses its affinity to the GAL4 activation domain, and target gene expression is turned on.

B, immunostaining of mid-third instar larval wing discs showing the gradual induction (2–8.5 h) of Sulf1 after shifting whole larvae to the GAL80ts restrictive temperature. Expression of GAL4 protein and GFP at each time point is also shown. GAL4 protein was detected at a constant level throughout the time course. C, graphic depiction of Wg gradient phenotype penetrance. Bar graphs show the percentage of wing discs exhibiting wild-type (white), mild (gray), and strong (black) phenotypes at each respective time point after transferring whole larvae to the restrictive temperature. Examples of each phenotypic category are shown on the left. Signal intensity of three discs is shown in pseudocolor. Pseudocolor scale ranges from white (highest signal intensity) to dark blue (lowest signal intensity). The number of discs classified into each phenotypic category at respective time points is shown in Table 1. A, anterior; P, posterior.
partiment-specific induction of Sulf1 in a temporally controlled manner allows a time course observation of the change in Wg gradient. Furthermore, the anterior compartment serves as an excellent control in this system. At mid-third instar, the culture was transferred to the restrictive temperature and incubated for 2–8.5 h. We first tested the induction of Sulf1-HA at each respective time point. Immunostaining revealed that GAL4 is constitutively expressed independently of the GAL80ts system at the respective time point. We did not detect leaky expression of Sulf1-HA or GFP at the permissive temperature (data not shown) or the first 2 h after induction (Fig. 5B), further confirming that the assay system works.

We next examined extracellular Wg levels at each time point at the restrictive temperature (2–8.5 h). Wg gradient in the wing disc was detected using a protocol that specifically stains the extracellular fraction of Wg protein (25). We observed normal extracellular Wg gradient in both anterior and posterior compartments at time 0 and up to 2 h after the temperature shift to 30 °C (Fig. 5C). At time points 2–8.5 h, we observed a gradual phenotypic shift from a wild-type extracellular Wg gradient to a strongly reduced gradient in the posterior compartment. To quantify time-dependent changes in the level of extracellular Wg, the phenotypic spectrum was classified into three categories: wild-type, mild, and strong extracellular Wg phenotypes (see “Experimental Procedures” and Fig. 5C, left panel). Discs showing the strong phenotype were found after 4.5 h after temperature shift and predominantly observed after 7 h (Fig. 5C; Table 1). This strong Sulf1-induced extracellular Wg phenotypic data are consistent with previous overexpression results, which showed reduced Wg protein levels in cells over-expressing Sulf1 in the developing wing (12). Thus, induced expression of Sulf1 led to a gradual reduction of steady-state levels of extracellular Wg.

Sulf1 Reduces Wg Stability in Cultured Tissue—By modifying the established Sulf1 induction system, we next devised the tissue-based ex vivo Wg stability assay. The method is based on our previous study in which the stability of an extracellular ligand molecule was assayed by pulse-chase experiments using antibodies (33). The fate of the pulse-labeled ligand can be monitored as a ligand-antibody complex. To monitor the degradation of extracellular Wg, we pulse-labeled Wg protein with anti-Wg antibody on dissected mid-third instar larval wing discs. Animals bearing hh-GAL4, tub-GAL80ts, UAS-Sulf1-HA, and UAS-GFP transgenes were raised at 18 °C until mid-third instar when the temperature was shifted to 30 °C to induce

Sulf1-HA in the posterior compartment. As shown in the experiment in the previous section, there is no detectable Sulf1-HA expression or extracellular Wg phenotype after 2 h of in vivo incubation at 30 °C (Fig. 5B). We therefore incubated whole larvae for 2 h at 30 °C, and then wing discs were dissected and extracellular Wg was antibody pulse-labeled (Fig. 6A). This 2-h in vivo incubation was useful to minimize ex vivo incubation time to keep discs as healthy as possible. After washing away excess antibody, the discs were further incubated ex vivo for 0–3 h. Extracellular Wg was indirectly visualized by detecting anti-Wg antibody with a secondary antibody at each respective time point.

### TABLE 1

Phenotypes observed in the in vivo Wg gradient time course assay

| Time (hour) | Wild type | Mild | Strong | Total |
|-------------|-----------|------|--------|-------|
| 2           | 11        | 0    | 0      | 11    |
| 2.75        | 9         | 3    | 0      | 12    |
| 3.5         | 5         | 5    | 0      | 10    |
| 4.5         | 1         | 4    | 0      | 5     |
| 5.5         | 1         | 5    | 0      | 6     |
| 7           | 1         | 3    | 0      | 4     |
| 8.5         | 0         | 2    | 0      | 2     |

The number of wing discs classified into wild-type, mild, and strong extracellular Wg categories: wild-type, mild, and strong extracellular Wg phenotype after 2–3 h after the temperature shift and induced over time. Additionally, we did not detect leaky expression of Sulf1-HA or GFP at the permissive temperature (data not shown) or the first 2 h after induction (Fig. 5B), further confirming that the assay system works.

We next examined extracellular Wg levels at each time point at the restrictive temperature (2–8.5 h). Wg gradient in the wing disc was detected using a protocol that specifically stains the extracellular fraction of Wg protein (25). We observed normal extracellular Wg gradient in both anterior and posterior compartments at time 0 and up to 2 h after the temperature shift to 30 °C (Fig. 5C). At time points 2–8.5 h, we observed a gradual phenotypic shift from a wild-type extracellular Wg gradient to a strongly reduced gradient in the posterior compartment. To quantify time-dependent changes in the level of extracellular Wg, the phenotypic spectrum was classified into three categories: wild-type, mild, and strong extracellular Wg phenotypes (see “Experimental Procedures” and Fig. 5C, left panel). Discs showing the strong phenotype were found after 4.5 h after temperature shift and predominantly observed after 7 h (Fig. 5C; Table 1). This strong Sulf1-induced extracellular Wg phenotypic data are consistent with previous overexpression results, which showed reduced Wg protein levels in cells over-expressing Sulf1 in the developing wing (12). Thus, induced expression of Sulf1 led to a gradual reduction of steady-state levels of extracellular Wg.

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Sulf1-HA in the posterior compartment. As shown in the experiment in the previous section, there is no detectable Sulf1-HA expression or extracellular Wg phenotype after 2 h of in vivo incubation at 30 °C (Fig. 5B). We therefore incubated whole larvae for 2 h at 30 °C, and then wing discs were dissected and extracellular Wg was antibody pulse-labeled (Fig. 6A). This 2-h in vivo incubation was useful to minimize ex vivo incubation time to keep discs as healthy as possible. After washing away excess antibody, the discs were further incubated ex vivo for 0–3 h. Extracellular Wg was indirectly visualized by detecting anti-Wg antibody with a secondary antibody at each respective time point.

![FIGURE 6. Pulse-chase analysis of extracellular Wg using ex vivo culture system.](image-url)

**A**. A graphic depicting Wg ex vivo pulse-chase assay system. **B**. Time (h) for in vivo incubation and ex vivo incubation. **C**. The extracellular Wg gradient exhibited a predominantly wild-type phenotype in the posterior compartment at 0 and 1 h, whereas the majority of sample discs exhibited a mild phenotype and a strong phenotype at 2 and 3 h, respectively. C, graphical depiction of the extracellular Wg phenotype observed at 0–3-h time points of the ex vivo Wg pulse-chase assay. Bar graphs show the percentage of wing discs exhibiting wild-type, mild, and strong phenotypes at each respective time point. The number of discs classified into each phenotypic category at respective time points is shown in Table 2.
At time 0 after the pulse labeling (2 h after the temperature shift), we detected no GFP or Sulf1-HA and no difference in the levels of extracellular Wg between the anterior and posterior compartments (Fig. 6B). The levels of extracellular Wg (detected as Wg-antibody complex) are only marginally reduced in the anterior compartment in the time frame of 3 h following time 0. In contrast, its signal intensity in the posterior compartment dropped at a much faster rate over 3 h (Fig. 6B).

For the quantification of the results, wing discs were phenotypically classified into three classes based on the severity of the reduction of extracellular Wg in the posterior compartment when compared with its anterior control compartment as described under “In Vivo Wg Gradient Time Course Assay” (Fig. 6C; Table 2). At time 0, we observed no difference in Wg levels between the anterior and posterior compartments in virtually all discs (97%). The averaged phenotype gradually shifted to more severe ones over the time course of 3 h. The number of discs showing the “mild phenotype” increased in 2 h. By 3 h, most discs (88%) were categorized into mild or strong phenotypes, in which we observed a reduction of extracellular Wg specifically in the posterior compartment. Thus, in cells over-expressing Sulf1, extracellular levels of Wg are decreased at a much more accelerated rate when compared with wild-type cells. Together with the results from the in vitro Wg protein assay, these results suggest that Sulf1 reduces extracellular Wg protein levels, at least partly by facilitating Wg degradation.

Human Sulf1 Decreases the Levels of Extracellular Wg Protein in the Drosophila Wing Disc—The results described above suggest that vertebrate and Drosophila Sulfs have an intrinsically similar enzymatic activity, but exert different effects on Wnt/Wg signaling in a context-dependent manner. If this is the case, one would expect that expression of vertebrate Sulfs, which is known to enhance Wnt signaling, may have a negative effect on Wg stability when expressed in a Drosophila tissue. To test this idea, we generated fly strains bearing an hSulf1 transgene and expressed hSulf1 in the wing disc using the Ga4/UAS system. The effects of Drosophila and human Sulf1 on extracellular Wg protein were examined.

First, expression of Drosophila and human Sulf1 was induced in the posterior compartment by hh-Gal4. As shown previously (12), Drosophila Sulf1 reduced the levels of Wg protein in the posterior compartment (Fig. 7, A and A’). A similar reduction of Wg protein was observed by overexpression of hSulf1 (Fig. 7, B and B’), suggesting that the activity of hSulf1 on Wnt/Wg signaling is fundamentally similar to that of the Drosophila homologue.

We have also previously reported that extracellular levels of Wg protein were reduced in Sulf1-overexpressing clones at random positions of the wing disc (Fig. 7, C and C’ (12)) generated by the FLP-OUT system (22). Therefore, we next examined the effect of hSulf1-expressing FLP-OUT clones on Wg distribution. We observed a reduction of extracellular Wg in such clones (Fig. 7, D and D’) similar to the one observed in clones expressing the Drosophila homologue, confirming the result of hh-Gal4-driven hSulf1. These results suggest that hSulf1 expressed in the Drosophila wing disc can affect the stability of Wg protein, as observed in Drosophila Sulf1. Together, our findings consistently support the idea that the function of Sulfs is context-dependent, rather than homologue-specific.

DISCUSSION

Based on the enzymatic activity of Sulf1, which removes HS 6-O-sulfation, we hypothesized that a direct consequence of this reaction is the decrease of affinity of Wg ligand to cell surface HS. Our Wg binding assay demonstrated that this was indeed the case. Daily HS derived from Sulf1-overexpressing cells showed a lower affinity to Wg, suggesting that modulation of 6-O-sulfation by Sulf1 affects the number of the Wg binding sites on HS. The fact that Sulfs regulate signaling mediated by various HS-dependent signaling molecules, including FGFs (47), Wnt/Wg (6, 10), Noggin (11), and Hedgehog (48), suggests that the 6-O-sulfate group is a critical component of binding sites on HS for a number of protein ligands.

In addition to a cell line overexpressing Sulf1 ubiquitously, we also established a line in which Sulf1 expression can be induced through a metallothionein promoter. This inducible system was critical for use in a pulse-chase experiment to examine the fate of Wg bound to the cell surface. In our in vitro degradation assay using an inducible Sulf1 construct, we demonstrated that Wg was cleared from the system more rapidly when Sulf1 was overexpressed. Experiments with chemical and genetic inhibition of Wg membrane trafficking and lysosomal degradation as well as monitoring Wg protein in the medium suggested that the cellular degradation of Wg has a major contribution to the decrease in the level of Wg.

The results obtained from the in vitro protein assay were further confirmed in a more physiologically relevant circum-

TABLE 2

| Time (hour) | 0 | 1   | 2   | 3   |
|------------|---|-----|-----|-----|
| Wild type  | 28| 17  | 12  | 4   |
| Mild       | 1 | 10  | 17  | 12  |
| Strong     | 0 | 0   | 1   | 17  |
| Total      | 29| 27  | 30  | 33  |

FIGURE 7. Human Sulf1 reduces extracellular Wg in the Drosophila wing disc. A, A’ , B, and B’, extracellular Wg staining (magenta) of wing discs over-expressing Drosophila (A and A’) and human (B and B’) Sulf1. Expression of UAS-Sulf1 and UAS-hSulf1 was induced in the posterior compartment by an hh-Gal4 driver. The posterior compartment is marked by GFP expression (A’ and B’, arrows in A and B). C, C’, D, and D’, immunostaining of extracellular Wg (magenta) in wing discs bearing FLP-OUT clones over-expressing Drosophila (C and C’) and human (D and D’) Sulf1. Extracellular levels of Wg protein are reduced in the FLP-OUT clones (arrows) marked with GFP (B’ and C’).
Sulf1 in Wg Degradation

In vertebrates, Wnt ligands (red) show high affinity to a binding site on HS, which presumably includes a 6-O-sulfate group. 6-O-desulfation by Sulf1 (blue) converts HS to a low affinity binding state, which can present Wnt to receptor (R) (magenta) (10). In Drosophila, Sulf1 activity releases Wg from cell surface HS similarly to vertebrate systems. However, a majority of Wg dissociated from HS is more quickly internalized and releases Wg from cell surface HS similarly to vertebrate systems. Consequently, the molecular ratio of the ligand/receptor/HSPGs in these systems may have a major impact. In addition, the differential composition of secreted molecules that affect Wnt/Wg signaling in the extracellular milieu, such as Wnt inhibitor, would contribute to the fate of ligands. Furthermore, Sulf1 may affect Wg turnover through the regulation of the affinity of HSPG to other components that mediate endocytosis and lysosomal transport of Wg. Two major candidates for such components are Dfz2 and Arrow (the Drosophila LRP6 homologue) (40, 41). Further studies to test these possibilities will provide novel insights into in vivo function of Sulfs, which are attractive therapeutic targets of various cancers.

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