# Abrogation of heparan sulfate synthesis in *Drosophila* disrupts the Wingless, Hedgehog and Decapentaplegic signaling pathways

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## Summary

**Studies in *Drosophila* and vertebrate systems have demonstrated that heparan sulfate proteoglycans (HSPGs) play crucial roles in modulating growth factor signaling. We have isolated mutations in *sister of tout velu* (*sotv*), a gene that encodes a co-polymerase that synthesizes HSPG glycosaminoglycan (GAG) chains. Our phenotypic and biochemical analyses reveal that HS levels are dramatically reduced in the absence of Sotv or its partner co-polymerase Tout velu (Ttv), suggesting that both copolymerases are essential for GAG synthesis. Furthermore, we find that mutations in *sotv* and *ttv* impair Hh, Wg and Decapentaplegic (Dpp) signaling. This contrasts with previous studies that suggested loss of *ttv* compromises only Hh signaling. Our results may contribute to understanding the biological basis of hereditary multiple exostoses (HME), a disease associated with bone overgrowth that results from mutations in *EXT1* and *EXT2*, the human orthologs of *ttv* and *sotv*.**

Key words: Growth factor signaling, Heparan sulfate proteoglycan, Hedgehog, Wingless, Decapentaplegic, Tout velu, Sister of tout velu, Hereditary multiple exostoses, EXT1, EXT2

## Introduction

There is mounting evidence that extracellular proteoglycans critically affect the signaling efficiency of secreted growth factors by modulating ligand stability, transport and ligand-receptor interaction. Proteoglycans are cell surface and extracellular matrix molecules consisting of a protein core modified at specific serine residues by the attachment of GAG chains – long unbranched stretches of repeating sugar subunits. GAG chains can vary in length and composition and are further diversified by N-deacetylation/N-sulfation, epimerization and O-sulfation of a subset of the residues. The ability of proteoglycans to affect signal transduction depends on chain composition and the extent and type of sugar modifications. As these characteristics can vary dramatically in a tissue-specific fashion, a given proteoglycan may have different effects in distinct cellular contexts (Lander and Selleck, 2000; Nybakken and Perrimon, 2002; Selleck, 2000).

Proteoglycans can be divided into several classes based on the sugar subunits incorporated into the GAG chains. Among these, HSPGs are composed of glucuronic acid-N-acetylglucosamine (GlcA-GlcNAc) repeating disaccharide units, whereas chondroitin sulfate (CS) proteoglycans contain glucuronic acid-N-acetylgalactosamine (GalNAc-GlcA) repeats. The initial steps of both CS and HS synthesis share a common pathway: nucleotide sugar precursors are transported to the golgi where a tetrasaccharide linker is added to the core protein. Following this, the two pathways diverge. If the proteoglycan is an HSPG, the next residue added is GlcNAc, after which the sugar chain is extended by HS polymerase. If instead GalNAc is added, chondroitin synthesis ensues.

Biochemical studies suggest that human HS polymerase is a complex of two related proteins, EXT1 and EXT2, encoded by the causative genes for the bone overgrowth syndrome, HME (Lind et al., 1998; McCormick et al., 1999). Individuals with HME develop bony nodules (exostoses) at the ends of bones, particularly in limbs, and are at increased risk for malignancy, suggesting that *EXT1* and *EXT2* are tumor suppressors (Ahn et al., 1995; Stickens et al., 1996; Wuyts et al., 1996). *Drosophila* contains homologs for both *EXT1* (*ttv*) and *EXT2* (*sotv*; *Ext2* – FlyBase) (Bellaiche et al., 1998; The et al., 1999). Direct evidence that Ttv (and by extension vertebrate EXT1) is required for HS biosynthesis in the organism comes from the finding that HS-derived GAG levels are greatly reduced in *ttv* mutant larvae, while CS levels remain essentially unchanged (Toyoda et al., 2000a; Toyoda et al., 2000b). These data suggest that HS biosynthesis in *Drosophila* and vertebrates takes place through conserved mechanisms.

Although the biochemical assays argue that Ttv plays an integral role in GAG chain synthesis, phenotypic analysis of the *ttv* mutants yielded surprising results. Loss of *ttv* function was reported to impair only Hedgehog (Hh) signaling without affecting the Wingless (Wg) or the Fibroblast Growth Factor (FGF) pathways (Bellaiche et al., 1998; The et al., 1999). This result was puzzling, as mutations in *sugarless* (*sgl*) and *sulfateless* (*sfl*), two other genes involved in HS biosynthesis, compromise signaling by all three ligands. Sgl is a UDP-glucose dehydrogenase that generates a nucleotide sugar donor

essential for both HS and CS synthesis. Thus, abolishing Sgl function might be expected to cause more dramatic defects than loss of HS synthesis alone. However, loss of Sfl should not cause more severe defects than *ttv*, as Sfl is an HS-specific N-deacetylase/N-sulfotransferase: without Sfl certain GAG modifications are lost, but without Ttv, the GAGs themselves are not synthesized (Toyoda et al., 2000a). To explain this apparent inconsistency, it was postulated that Sotv might act independently of Ttv to provide HS polymerase activity at levels sufficient for normal Wg and FGF function but too low to support Hh signaling (Bellaiche et al., 1998; The et al., 1999). This hypothesis predicts that the Hh pathway is more sensitive to loss of HSPGs than are the other two pathways. Alternatively, it has been proposed that *ttv* activity might be involved in generating a specific HSPG required only for Hh signaling (Bellaiche et al., 1998; The et al., 1999).

Our data do not support these models and argue against a Hh-specific role for *ttv*. We have isolated mutations in *sotv* and have found that the loss of either *sotv*, *ttv* or both genes compromises Hh, Wg and Dpp signaling pathways in the wing imaginal disc. In each instance, there were no obvious differences in the nature or severity of the effects in the single or the double mutants, suggesting that both co-polymerases are required for the biologically relevant activity. Our biochemical data also support this conclusion, because we find that HS levels drop dramatically in *ttv* or *sotv* homozygous mutant larvae. Finally, we also observed that Hh protein levels are reduced in Hh-expressing cells that lack HS polymerase activity. This result suggests that the reduced range of Hh signaling in the absence of HSPGs may be due to increased lability of the Hh ligand. Taken together, our data argue that *ttv* and *sotv* are equally required for HS synthesis and that HS is required in at least three major signal transduction pathways.

## Materials and methods

### Isolation of mutants

Five alleles of *sotv* were isolated in a previously described ethylmethanesulfonate mutagenesis screen (Lei and Warrior, 2000; Schindelholz et al., 2001). The *sotv* complementation group was identified by transgenic rescue and sequencing of mutant alleles.

### Generation of germline and somatic clones

The $sotv^{1.8.1}$ allele was used to generate FRT-G13 $sotv^{1.8.1}$ and FRT-G13 $ttv^{00681}$, $sotv^{1.8.1}$ lines by recombination. FRT-G13 $ttv^{02055}$ was kindly provided by Norbert Perrimon. Embryos lacking maternal and zygotic *ttv* or *sotv* activity were generated essentially as described (The et al., 1999). Mosaic mothers were mated to heterozygous males as follows: $ttv^{02055}$ crossed to $ttv^{02055}$/CyO-Ftz-*lacZ*; $sotv^{1.8.1}$ crossed to Df(2R)Jp8 Ftz-*lacZ*; and $ttv^{00681}$, $sotv^{1.8.1}$ crossed to $ttv^{00681}$, $sotv^{1.8.1}$/CyO Ftz-*lacZ*. Imaginal disc clones were generated by crossing male flies from the FRT lines described above to females homozygous for *hs*>FLP; FRT-G13 2XUbi-GFPnls. Two-to 3-day-old progeny were heat-shocked for 2 hours at 37°C and reared at room temperature until dissection 4-5 days later.

### Immunohistochemistry

Antibodies were used at the concentrations listed: mAb anti-Wg (Developmental Studies Hybridoma Bank, DSHB), 1:3 for extracellular (Baeg et al., 2001) and 1:1000 for cytoplasmic Wg (Brook et al., 1996); mAb anti-Ac (DSHB) (Skeath and Carroll, 1991), 1:100; rabbit anti-Sal provided by Rheinhard Schuh (Kuhnlein et al., 1994), 1:300; rabbit anti-pMad (PS1) from Carl-Henrik Heldin (Persson et al., 1998), 1:1000; rabbit anti-Hh from Philip Ingham (Taylor et al., 1993), 1:200; rat anti-Ci provided by Robert Holmgren (Motzny and Holmgren, 1995), 1:5; Cy3-conjugated secondary antibodies (Jackson ImmunoResearch, 1:200; and Alexa-568 secondary antibodies (Molecular Probes), 1:500. mAbs from the DSHB were developed under the auspices of the NICHD and maintained by the University of Iowa, Department of Biological Sciences, Iowa City, IA 52242.

### Sugar chain biochemistry

Profiling of HS and CS-derived disaccharides was conducted according to published procedures (Toyoda et al., 2000b) with the following modifications: (1) the chromatographic equipment included a Hitachi L-7250 autosampler and Hitachi D-7000 computer interface, permitting disaccharide levels to be determined by comparing the area of each peak with reference standards; and (2) HPLC profiles in Fig. 8A and Fig. 9A were derived from 100 third instar larvae, whereas experiments in Fig. 8C and Fig. 9B used 300 third instar larvae. Standard unsaturated HS and CS disaccharides were purchased from Sigma and Seikagaku America (Falmouth, MA), respectively.

## Results

### Generation and identification of mutations in *sotv*

We isolated five alleles of *sotv* in the course of a genetic screen for essential loci contained in the deficiency *Df(2R)Jp6* (Lei and Warrior, 2000). The *sotv* complementation group was identified by transgenic rescue using a 7.6 kb genomic DNA fragment that includes the *sotv* open reading frame (ORF). Transformants in which the *sotv*-coding region was placed under the control of the Hsp70 promoter also rescued lethality.

The *sotv* transcription unit encodes a protein of 717 residues that contains a strongly hydrophobic domain (residues 26 to 47) indicative of a type II transmembrane protein and three aspartic acid residues (DDD) surrounded by a stretch of hydrophobic residues in the C-terminal third of the protein (Fig. 1). This DXD sequence is a signature motif for UDP-sugar-dependent glycosyltransferases and is important for their catalytic activity (Negishi et al., 2003; Pedersen et al., 2003). Comparison of Sotv with human EXT-related genes using clustal analysis revealed that Sotv is most homologous to EXT2 (47% amino acid identity), suggesting it is likely to be a glycosyltransferase resident in the endoplasmic reticulum or golgi (McCormick et al., 2000).

We sequenced all five *sotv* mutant lines and identified single nucleotide changes that introduce premature termination codons in each of them (see Fig. 1). The $sotv^{1.8.1}$ allele is expected to be a null or strong loss-of-function mutation, as it encodes a protein truncated at residue 184 that lacks the catalytic motif as well as several domains conserved throughout the Ext family (Zak et al., 2002).

### Embryos lacking *sotv* activity show defects suggestive of impaired Hh and/or Wg signaling

Homozygotes for *sotv* die as pupae, consistent with the presence of substantial amounts of maternally contributed *sotv* transcript in 0- to 2-hour-old embryos (data not shown). To determine if *sotv* is required in the early embryo, we generated females lacking *sotv* function in the germline. When these females were crossed to *Df(2R)Jp6/CyO* hemizygous males, embryos lacking both maternal and zygotic *sotv* activity failed to hatch and showed a strong segment polarity phenotype

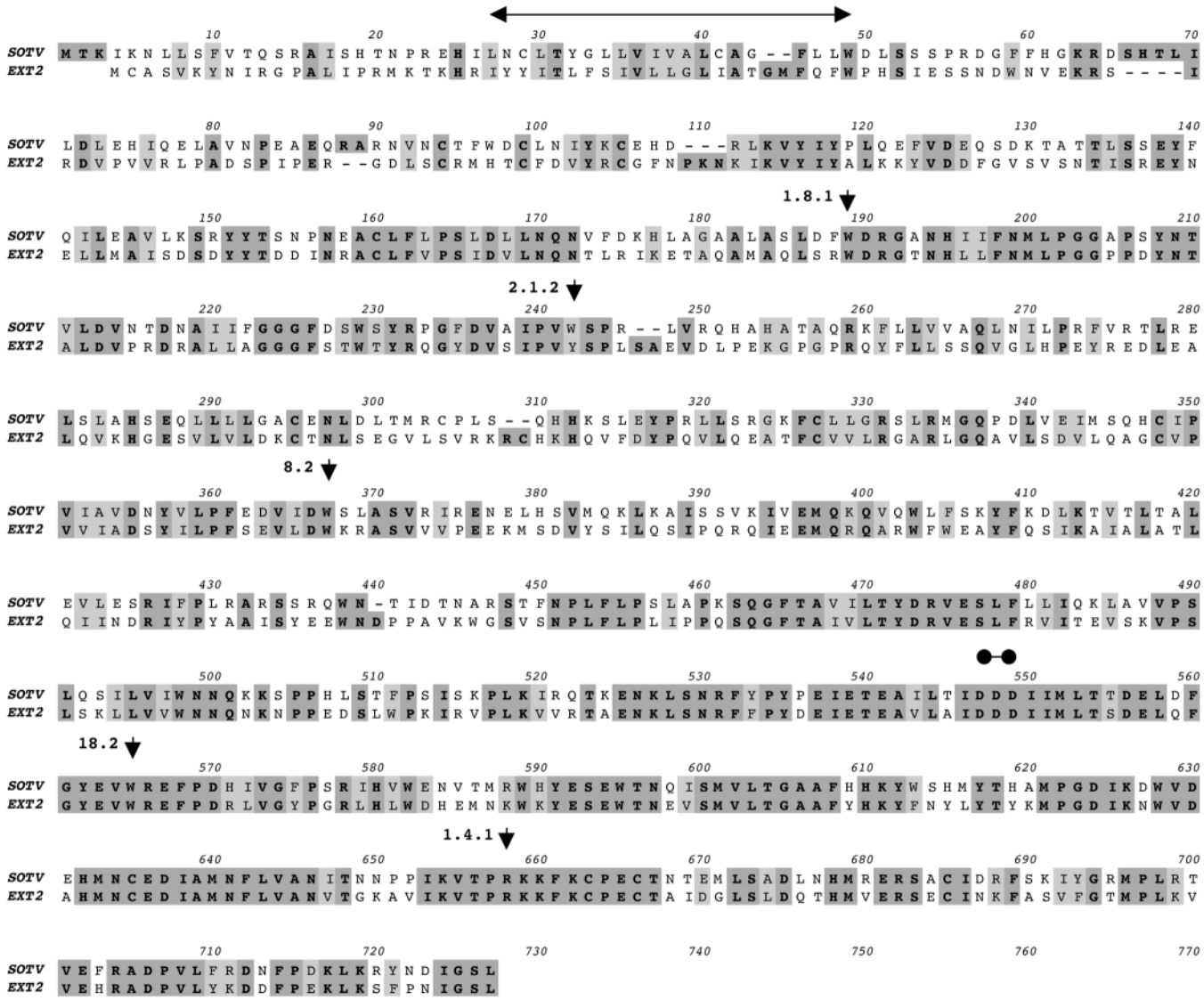



**Fig. 1.** Clustal alignment detects 47% sequence identity between Sotv and human EXT2. Identical residues are bold and boxed in dark gray, while similarities are boxed in light gray. A strongly hydrophobic region likely to represent a transmembrane domain is indicated by a double-headed arrow. Vertical arrows indicate positions of nonsense mutations in the five characterized *sotv* alleles. The dumbbell highlights a DXD motif conserved in UDP-sugar-dependent glycosyltransferases.

(Fig. 2). The ventral surface of the wild-type larval cuticle bears rows of denticles at the anterior of each segment interspersed with posterior naked regions. Embryos mutant for *sotv* lack naked cuticle and instead display a near contiguous lawn of denticles, a phenotype resembling, but less severe than, that of null mutations in *hh* or *wg* (Fig. 2B-D). Loss of both maternal and zygotic *ttv* activity results in similar segmentation defects (The et al., 1999). The phenotype of *sotv* germ line clone embryos appears indistinguishable from *ttv* null germline clones (Fig. 2E). Thus, the two genes do not have independent functions that can be identified at this level of analysis. To examine whether *sotv* and *ttv* have additive effects on patterning, we generated animals that lacked both gene activities. These double mutant embryos were phenotypically equivalent to either single mutant alone, arguing that *ttv* and *sotv* do not have partially redundant functions (Fig. 2F).

## Loss of *sotv* or *ttv* activity results in defects in the adult wing indicative of aberrant Wg signaling

Expression of Hh and Wg in the embryo is maintained through a mutually dependent positive feedback loop, so disruption of either pathway results in similar phenotypes (see Fig. 2) (DiNardo et al., 1988). Thus, it is difficult to determine whether loss of *sotv* interferes with Hh, Wg or both signaling pathways. Therefore, to resolve this issue, we turned to the wing imaginal disc, where transcription of the ligands is unlinked. Using the FRT/FLP technique (Xu and Rubin, 1993), we generated clones of cells that lack *sotv* function in a heterozygous mutant background. Clones mutant for *ttv* or both *ttv* and *sotv* were examined in parallel. Previous studies reported that mutations in *ttv* affect the Hh pathway without affecting Wg signaling (Bellaiche et al., 1998; The et al., 1999). Therefore, if *sotv* and *ttv* play equivalent roles, removing *sotv* activity should

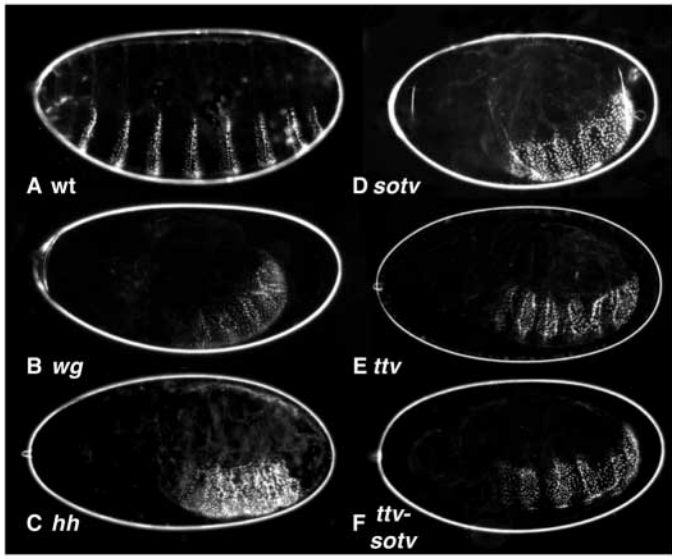



**Fig. 2.** Cuticle preparations of *ttv* and *sotv* germline clone embryos resemble *hh* and *wg* mutants. The ventral surface of wild-type embryos is marked by bands of denticles separated by naked cuticle (A). Zygotic removal of either *wg* (B) or *hh* (C) results in loss of naked cuticle and a lawn of ventral denticles. Embryos lacking maternal and zygotic *sotv* (D), *ttv* (E), or both *ttv* and *sotv* (F) also show a reduction in naked cuticle. The phenotypes in D-F are of similar severity, but less severe than *hh* or *wg* nulls.

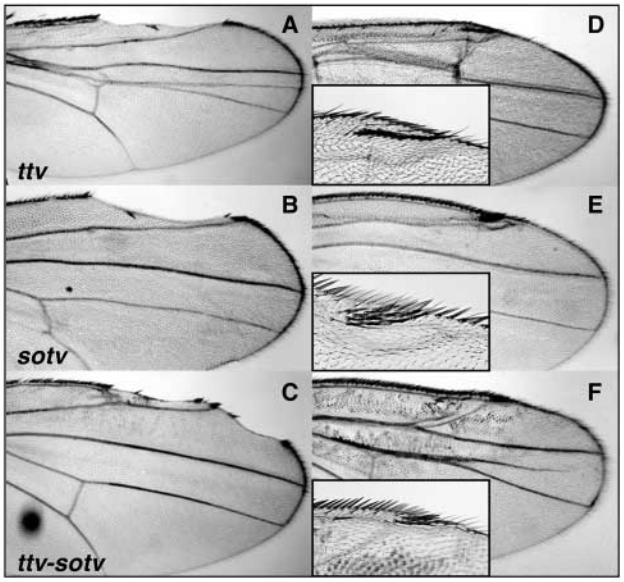



**Fig. 3.** Wings mosaic for homozygous patches of *ttv* (A,D), *sotv* (B,E), and *ttv, sotv* (C,F) mutant cells show notching (A-C) and ectopic bristles (D-F) in the vicinity of the wing margin. The insets in D-F are higher magnification views. Clones are unmarked in these examples, but no notching or ectopic bristles were observed in non-mosaic control siblings.

similarly affect only Hh-dependent patterning. In fact, flies mosaic for all three genotypes showed defects in the region encompassing the third and fourth wing veins, which is patterned in response to Hh (Fig. 3 and data not shown). This result suggests that *sotv* is also required for Hh function. Surprisingly, however, all three genotypes also showed defects in the wing margin, which is specified by Wg. Wings containing *sotv, ttv* and *ttv, sotv* clones showed two classes of defects. A majority of the affected wings contained large notches that deleted margin tissue (Fig. 3A-C), and at a lower frequency we recovered wings containing extra margin bristles, another hallmark of impaired Wg signaling (Fig. 3D-F) (Couso et al., 1994; Neumann and Cohen, 1996; Zhang and Carthew, 1998). Clones mutant for *sotv*, *ttv* or both showed no obvious differences in the severity of the wing defects, suggesting that Wg signaling is equally affected by loss of either co-polymerase. Analysis of marked adult clones for *sotv* showed that the ectopic bristles associated with a mutant clone can include both mutant and wild-type cells, suggesting that this effect is partially cell non-autonomous (data not shown; see below).

### The Wg gradient and Wg target gene expression are perturbed in the absence of *sotv* or *ttv*

To determine the developmental basis of the wing margin phenotypes, we examined the expression of Wg in the wing imaginal discs. In the late third instar larva, Wg is transcribed in a stripe 3-6 cells wide along the dorsoventral (DV) boundary of the wing disc (Fig. 4), where it is thought to form an instructive gradient that controls growth and patterning (Couso et al., 1995; Diaz-Benjumea and Cohen, 1995; Neumann and Cohen, 1997). The distribution of Wg protein in the imaginal disc can be assayed both in the cytoplasm and in the extracellular space. Conventional fixation protocols visualize cytoplasmic Wg at high but relatively uniform levels in a narrow stripe of 3-5 cells along the DV margin and in an irregular pattern of dots in nearby cells (Fig. 4G-I). By contrast, incubation of discs with antisera prior to fixation reveals a broader, shallower and less punctate gradient of extracellular Wg (Greco et al., 2001; Strigini and Cohen, 2000; van den Heuvel et al., 1989). We found that in the absence of *sotv* or *ttv*, extracellular Wg could not be detected in mutant cells flanking the DV boundary, and ligand levels in the cells where *wg* is normally transcribed were greatly reduced (see Fig. 4A-F). The lower extracellular Wg levels would be expected to reduce target gene activation and could result from decreased stability of the ligand, reduction in its ability to bind mutant cells or defects in transport. In the majority of mutant clones that crossed the DV compartment boundary, cytoplasmic Wg could still be detected in its normal domain, but at reduced levels (Fig. 4G-I and data not shown). In addition, clones along the future wing margin occasionally resulted in ectopic Wg expression extending up to six cells from the endogenous stripe (data not shown). The reduction in extracellular Wg and the occurrence of ectopic Wg expression in clones near the DV margin are both consistent with compromised Wg signal transduction. Ectopic Wg expression is probably due to loss of negative regulation by *wg* of its own transcription in cells on either side of its normal domain (Nakagoshi et al., 2002; Rulifson et al., 1996). As in the embryo, there was no detectable difference in the severity of the effects on cytoplasmic or extracellular Wg staining in *sotv* mutant, *ttv* mutant and double mutant clones, arguing that the two genes have equivalent and non-redundant activities.

To determine if the altered distribution of Wg in mutant cells

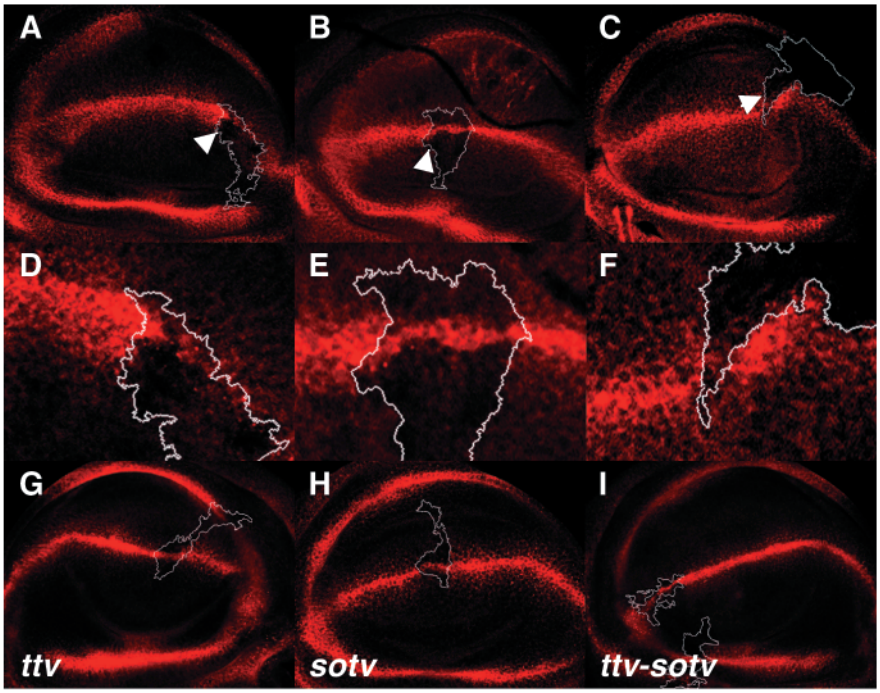



**Fig. 4.** Formation of the Wg gradient is severely affected in cells that are homozygous mutant for *ttv*, *sotv* and *ttv*, *sotv*. In this and subsequent figures, antibody staining is in red. The boundaries of informative clones are outlined in white. Staining for extracellular Wg reveals a shallow gradient in the wing pouch that is strongly reduced in clones of *ttv* (A,D), *sotv* (B,E) and *ttv*, *sotv* (C,F) that cross the Wg expression domain. Arrows draw attention to regions near the prospective wing margin, where a sharp decrease in extracellular wingless levels is visible across the clone boundary. Mutant clones of *ttv* (G), *sotv* (H) and *ttv*, *sotv* (I) that cross the Wg stripe cause a less dramatic reduction in cytoplasmic Wg levels.

compromises the ability of the ligand to signal, we examined the expression of *achaete* (*ac*), a high-threshold target for Wg. In wild type, Ac can be detected in the anterior compartment close to DV boundary on either side of the Wg domain (Fig. 5). We found that in cells lacking *sotv* or *ttv*, Ac staining was strongly reduced, except at the clone margins. Interestingly, when *ttv* or *sotv* mutant clones were present adjacent to the endogenous Wg domain, small groups of ectopic Ac-expressing cells could be detected (Fig. 5), probably in response to the ectopic Wg expression domains described above.

### Hh levels and Hh signaling are reduced in cells lacking *sotv* or *ttv*

In the third instar wing imaginal disc, Hh is expressed only in the posterior compartment. From there it signals to induce transcription of *dpp* and *patched* (*ptc*), and stabilizes the transcription factor Cubitus interruptus (Ci) in cells along the anteroposterior (AP) boundary (Aza-Blanc et al., 1997; Strigini and Cohen, 1997) (Fig. 6). As loss of *ttv* has been shown to impair Hh distribution and signaling activity (Bellaiche et al., 1998; The et al., 1999), we investigated whether loss of *sotv* would have similar effects. First we examined whether *sotv* mutant clones affected the range over which Hh stabilized Ci. In wild type, Ci can be detected at low levels throughout the anterior compartment, with more intense staining in a band of cells along the AP boundary, where it is stabilized by Hh. The width of this band is substantially reduced in *ttv* mutant clones located in the anterior compartment (Bellaiche et al., 1998). The zone of Ci stabilization was similarly compressed in *sotv* and *ttv, sotv* double mutant clones (Fig. 6B,C). The reduction in the number of cells that show Ci stabilization demonstrates that loss of either *ttv* or *sotv* reduces the effective range of Hh signaling in the anterior compartment.

We next examined whether Hh distribution was altered in these mutants. In wild type, Hh can be detected at high levels throughout the posterior compartment and at low levels in anterior cells adjacent to the compartment boundary. In *ttv* clones located in the anterior compartment, Hh staining cannot be detected (Bellaiche et al., 1998). We observed a similar loss of ligand in clones in the anterior region that are mutant for *sotv* or both *sotv* and *ttv* (Fig. 6) (data not shown). Unexpectedly, Hh levels were also strongly decreased in mutant clones located in the posterior compartment (Fig. 6E-G). However, Hh expression (assayed using an Hh-*lacZ* reporter construct) was unaffected in *sotv* mutant clones (Fig.

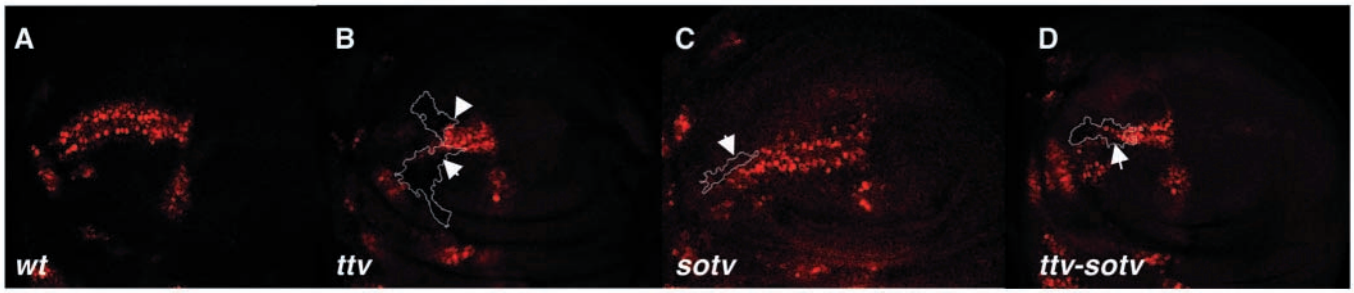



**Fig. 5.** Expression of the Wg target gene *ac* is altered in clones lacking HS polymerase activity. In wild type (A), Ac is expressed in the anterior compartment in cells on either side of the Wg expression domain. Ac expression is reduced or lost in *ttv* (B), *sotv* (C) or both *ttv*, *sotv* (D) mutant clones (see arrows). Occasionally, ectopic Ac is observed in the vicinity of the mutant clone (B, arrowhead), which correlates with the location of ectopic margin bristles in wings from mosaic adults (see Fig. 3D-F).

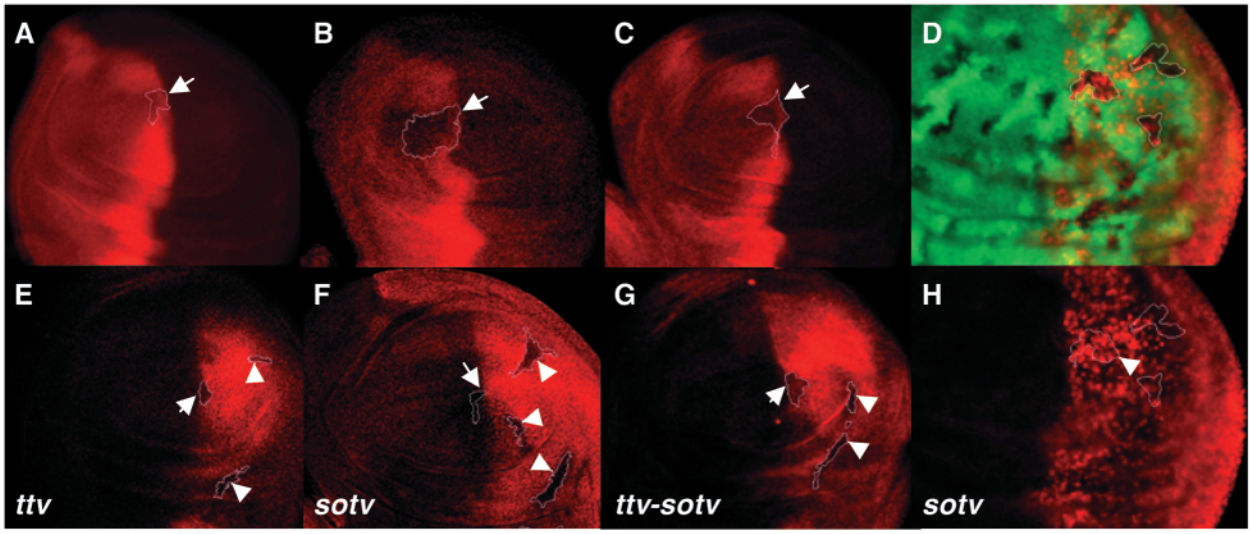



**Fig. 6.** Stabilization of the Hh target Ci is restricted, and Hh levels are reduced in mutant clones. Hh dependent inhibition of Ci proteolysis appears as a band of intense staining 8-10 cells wide anterior to Hh-expressing cells in the posterior compartment. The remaining cells in the anterior compartment show low levels of Ci staining. In *ttv* (A), *sotv* (B) or double mutant clones (C), the domain of Ci stabilization is reduced to 1-2 cells in width. Hh is uniformly distributed in the posterior compartment except in clones lacking *ttv* (E), *sotv* (F) or *ttv* and *sotv* (G). Reduced ligand levels are apparent in clones located along the AP boundary (arrows) as well as deep within the posterior compartment (arrowheads). (D,H) By contrast, Hh transcription, visualized using a Hh-lacZ reporter (H), is unaffected in posterior clones mutant for *sotv* (arrowhead). Mutant clones were visualized by the absence of GFP expression (D).

6H). These results demonstrate that Hh stability is decreased in the absence of HSPGs, and raise the possibility that a decrease in stability may contribute to the shorter effective range of the ligands in *ttv* and *sotv* mutant cells.

### Dpp signaling is sensitive to the loss of HS polymerase activity

We next examined whether loss of *sotv* or *ttv* affects Dpp signaling, as both Dpp and its human homolog BMP-2 bind heparin with high affinity in vitro (Groppe et al., 1998; Ruppert et al., 1996). Furthermore, changes in the level of Division abnormally delayed (Dally), a GPI-anchored HSPG, affect the Dpp gradient in the wing disc and Dpp target gene expression in many tissues (Fujise et al., 2001; Fujise et al., 2003; Jackson et al., 1997). To monitor Dpp signaling, we followed phosphorylated Mothers against Dpp (pMad), which is detected in a graded pattern that reflects Dpp activity (Fig. 7). In wild type, the levels of pMad are highest close to the source of Dpp at the AP boundary, except in Dpp transcribing cells, where signaling is attenuated due to Hh-dependent downregulation of the Dpp receptor *thick veins* (Tanimoto et al., 2000). We found that pMad staining was strongly reduced in mutant cells that lacked either *sotv*, *ttv* or both genes (Fig. 7). As Hh regulates Dpp expression in cells adjacent to the AP boundary, the loss of pMad staining in posterior clones could be an indirect consequence of lowered Hh activity. However, pMad levels were also reduced in clones anterior to the Dpp stripe, where Hh is normally absent (Fig. 7C). Consequently, the reduction in pMad is a direct effect of *sotv* and *ttv* on the Dpp gradient. Furthermore, the lower pMad levels reflect diminished signal transduction, as shown by reduced expression of *spalt* (*sal*) a direct transcriptional target of the Dpp pathway in the wing pouch (de Celis et al., 1996). Sal levels were strongly diminished in mutant clones, consistent with the reduction in pMad levels described above (Fig. 7D-F).

### Both *ttv* and *sotv* are required for HS biosynthesis *in vivo*

Although *ttv* and *sotv* mutations clearly affect signaling by Wg, Hh and Dpp, phenotypic studies do not directly address their biochemical activities in the organism. Data from cell culture experiments suggest that both EXT1 and EXT2 are required to constitute a functional mammalian HS-GAG polymerase. However, EXT1 and EXT2 individually can transfer a single GlcNAc or GlcA residue to a suitable substrate in vitro (Lind et al., 1998), and purified EXT1 can catalyze multiple rounds of GlcNAc and GlcA addition onto an artificial substrate (Busse and Kusche-Gullberg, 2003). Thus, the possibility remained that either *sotv* (or *ttv*) could still synthesize HS by itself, albeit at levels below the threshold required for viability. We therefore carried out a structural profiling of HS and CS in both *ttv* and *sotv* single mutants.

To determine the effect of loss of *sotv* on HS synthesis, we examined all five *sotv* alleles as homozygotes (data not shown) and hemizygotes over *Df(2R)Jp8*. As a control, we tested a transheterozygous combination of the null *ttv* alleles ($ttv^{K06619}/ttv^{02055}$). GAGs prepared from third instar larvae were digested into disaccharide units using either heparin or chondroitin lyases and separated by HPLC with a reverse phase-ion pair column. The disaccharides were then labeled by covalent fluorescence derivatisation and measured with an in-line fluorescence detector. In wild type, six HS-derived disaccharide species are detected: the unsulfated uronic acid-GlcNAc, two monosulfated species, UA-GlcNS and UA-Glc6S, two disulfated disaccharides, UA-GlcNS6S, UA2S-GlcNS and a single tri-sulfated unit, UA2S-GlcNS6S (Fig. 8A). Fluorescence intensities permit reliable quantification of

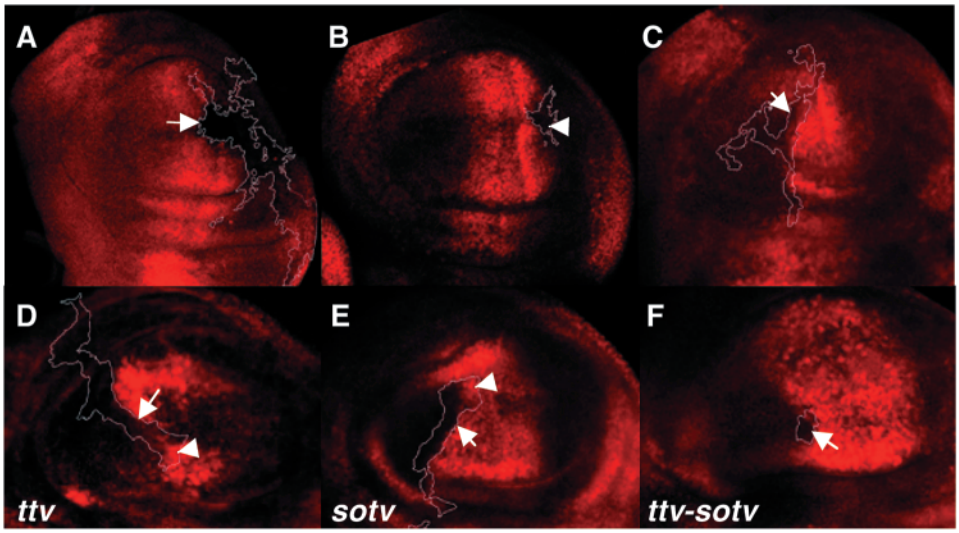



**Fig. 7.** Dpp signal transduction and Sal expression are inhibited in cells lacking HS polymerase activity. Staining with PS1 antisera allows visualization of pMad generated by Dpp signaling activity. Cells mutant for *ttv* (A), *sotv* (B) or *ttv* and *sotv* (C), show reduced pMad levels (arrows in A and C or arrowhead in B) regardless of whether the clones are situated in the anterior or posterior compartments, suggesting that Dpp signaling is compromised independently of Hh. Low levels of pMad can be detected within clones in the vicinity of the clone boundaries, suggesting that Dpp signaling can still occur in mutant cells, although with reduced effectiveness or range. Sal responds to a high threshold of Dpp signaling and is expressed in the wing pouch centered on the AP boundary. However, in *ttv* (D), *sotv* (E) and *ttv*, *sotv* (F) mutant cells, Sal expression is reduced independently of whether clones lie in the anterior or posterior compartment (arrows). Loss of Sal in anterior clones is a direct result of loss of Dpp signaling rather than an indirect consequence of compromised Hh signaling, since it occurs in a domain beyond the effective range of Hh. Sal staining persists in *ttv* and *sotv* clones that overlap the AP boundary (arrowheads), suggesting that Dpp can signal in an autocrine or paracrine fashion, even in mutant cells.

each of these disaccharides, with a sensitivity of ~1 ng/mg of dried tissue (Toyoda et al., 2000b; Toyoda et al., 1997).

We found that the HS-derived disaccharide levels were dramatically reduced in all *sotv* alleles. For example, homozygotes for the $sotv^{1.8.1}$ null allele had total disaccharide levels several hundred fold lower than in wild type (Table 1, Fig. 8). Larvae null for *ttv* also showed severely reduced disaccharide levels (~75 fold lower, Table 1, Fig. 8) (Toyoda et al., 2000b), with the residual HS probably resulting from perdurance of maternal product. Taken together, these findings establish that *sotv* is required for HS production in vivo and argue that a Ttv/Sotv complex constitutes the functional HS polymerase.

Although all the *sotv* mutations severely affect HS synthesis, comparison of the total HS-derived disaccharide levels in different alleles revealed a progressive reduction that correlates with the predicted size of the polypeptide (Table 1). For example, $sotv^{1.8.1}$, which is truncated at residue 184, displayed total HS levels ~450 fold less than wild type, while $sotv^{1.4.1}$, which terminates at position 648, showed only ~30-fold lower levels, with all six disaccharides represented. These findings suggest that the longer mutant Sotv proteins retain residual function (see Table 1).

## Reducing *sotv* function alters the sulfation of HS

HSPG GAG chains undergo extensive modification during synthesis, including N-deacetylation, epimerization of some GlcA residues to iduronic acid, and N- and O- sulfation. Sulfation patterns vary in a tissue-specific manner and can differentially affect interactions with proteins such as bFGF, antithrombin III and HGF (Nakato and Kimata, 2002). Therefore, we analyzed the residual HS chains from *ttv* and *sotv* mutants to determine if the mutations impact GAG modification as well as synthesis. We found that the individual *sotv* alleles produced HS chains with strikingly different compositions (Fig. 8C). For example, as the length of the mutant protein increased, levels of the trisulfated disaccharide UA2S-GlcNS6S rose, with the mildest allele retaining more than 6% of wild-type levels. In general, milder alleles produced more sulfated disaccharides. These findings show that, in addition to affecting HS levels, mutations in *sotv* can affect the extent and perhaps the pattern of HS sulfation.

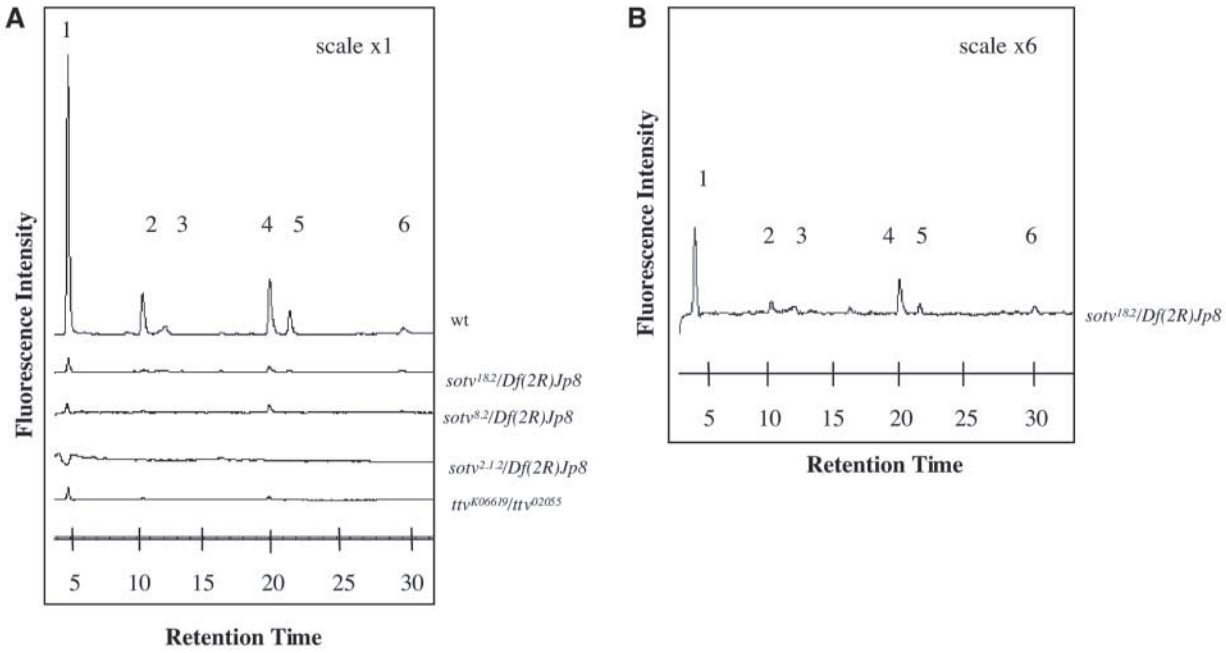
## Changes in *sotv* activity affect the levels and structure of CS polymers

Both HS and CS GAG-chain polymerization are initiated on an identical tetrasaccharide substrate GlcA-Gal-Gal-Xyl attached

**Table 1. HS Disaccharides from *sotv* and *ttv* mutants**

| ng/mg dry weight | UA-GlcNAc | UA-GlcNS | UA-GlcNAc6S | UA-GlcNS6S | UA2S-GlcNS | UA2S-GlcNS6S | Total |
|---|---|---|---|---|---|---|---|
| Wild type | 4.2561 | 2.0601 | 0.2964 | 2.5206 | 1.7781 | 1.0187 | 11.9300 |
| $sotv^{1.8.1}/Df(2R)Jp8$ | 0.0251 | ND | ND | ND | ND | ND | 0.0251 |
| $sotv^{2.1.2}/Df(2R)Jp8$ | 0.0754 | ND | ND | ND | ND | ND | 0.0754 |
| $sotv^{8.2}/Df(2R)Jp8$ | 0.0610 | 0.0403 | ND | 0.1232 | 0.0314 | 0.0437 | 0.2996 |
| $sotv^{18.2}/Df(2R)Jp8$ | 0.0949 | 0.0366 | 0.0131 | 0.1097 | 0.0380 | 0.0499 | 0.3423 |
| $sotv^{1.4.1}/Df(2R)Jp8$ | 0.0801 | 0.0306 | 0.0259 | 0.1197 | 0.0445 | 0.0652 | 0.3660 |
| $ttv^{K06619}/ttv^{02055}$ | 0.0710 | 0.0198 | ND | 0.0386 | 0.0209 | ND | 0.1503 |
| $ttv^{02055}/ttv^{K06619}$ | 0.0547 | 0.0172 | ND | 0.0377 | 0.0180 | 0.0251 | 0.1527 |

ND, not detectable.



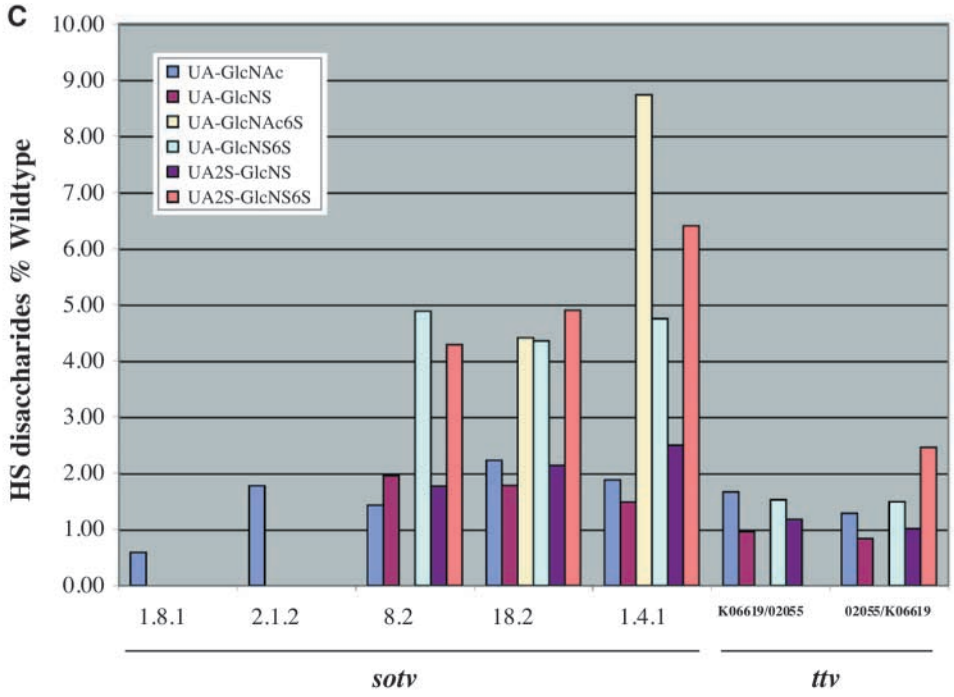




**Fig. 8.** HS-derived disaccharides from *sotv* and *ttv* mutants. HPLC profiles of GAGs from wild-type, *sotv* and *ttv* larvae are shown (A). Peaks 1-6 represent the following disaccharides: ΔUA-GlcNAc, ΔUA-GlcNS, ΔUA-GlcNAc6S, ΔUA-GlcNS6S, ΔUA-2S-GlcNS and ΔUA2S-GlcNs6S, respectively. (B) A representative HPLC trace from *sotv*$^{18.2}$*/Df(2R)Jp8* larvae at a six times more sensitive scale than in A. All six disaccharide species found in wild type can be detected in these animals, albeit at greatly reduced levels. (C) Disaccharides from *sotv/Df(2R)Jp8* larvae are shown as a percentage of wild-type controls. The *sotv* alleles are ordered according to the levels of HS produced, which correlates with the predicted lengths of the mutant proteins (see Fig. 1). The results for *ttv* mutants are from two different experiments. In one case *ttv*$^{K06619}$ was derived maternally and in the other *ttv*$^{02055}$ was contributed maternally. Different colored bars indicate individual HS disaccharides.

to the core protein. Consequently, disruption of the synthesis of one class of GAG chains could affect the production of the other. Consistent with this idea, chondroitin synthesis increases in somatic cells bearing mutations in *EXT1* (Wei et al., 2000). We therefore examined CS levels and disaccharide structure in *ttv* and *sotv* mutants (Fig. 9). As previously reported, *ttv*-null mutants display only modestly reduced levels of CS, demonstrating the selectivity of this *EXT1*-related gene for HS biosynthesis. However, the *sotv* allelic series revealed an unexpected relationship between HS and CS biosynthesis. Like the *ttv* nulls, the *sotv*$^{1.8.1}$ null reduced chondroitin-derived disaccharides by a modest amount. However, in the remaining *sotv* alleles, the proportion of sulfated disaccharides increased progressively through the allelic series, *sotv*$^{2.1.2}$ to *sotv*$^{1.4.1}$ (Fig. 9B). Thus, both the composition and levels of CS were altered when *sotv* function was partially compromised. Moreover, *sotv/+* heterozygotes displayed similar changes in chondroitin levels and structure, albeit to a lesser degree (data not shown). These findings emphasize that partial-loss of *EXT2* function may alter the levels and sulfation of CS as well as HS.

## Discussion

### HS synthesis is essential for Hh signaling

The involvement of HSPGs in Hh signaling was first demonstrated when Ci stabilization and Ptc expression were

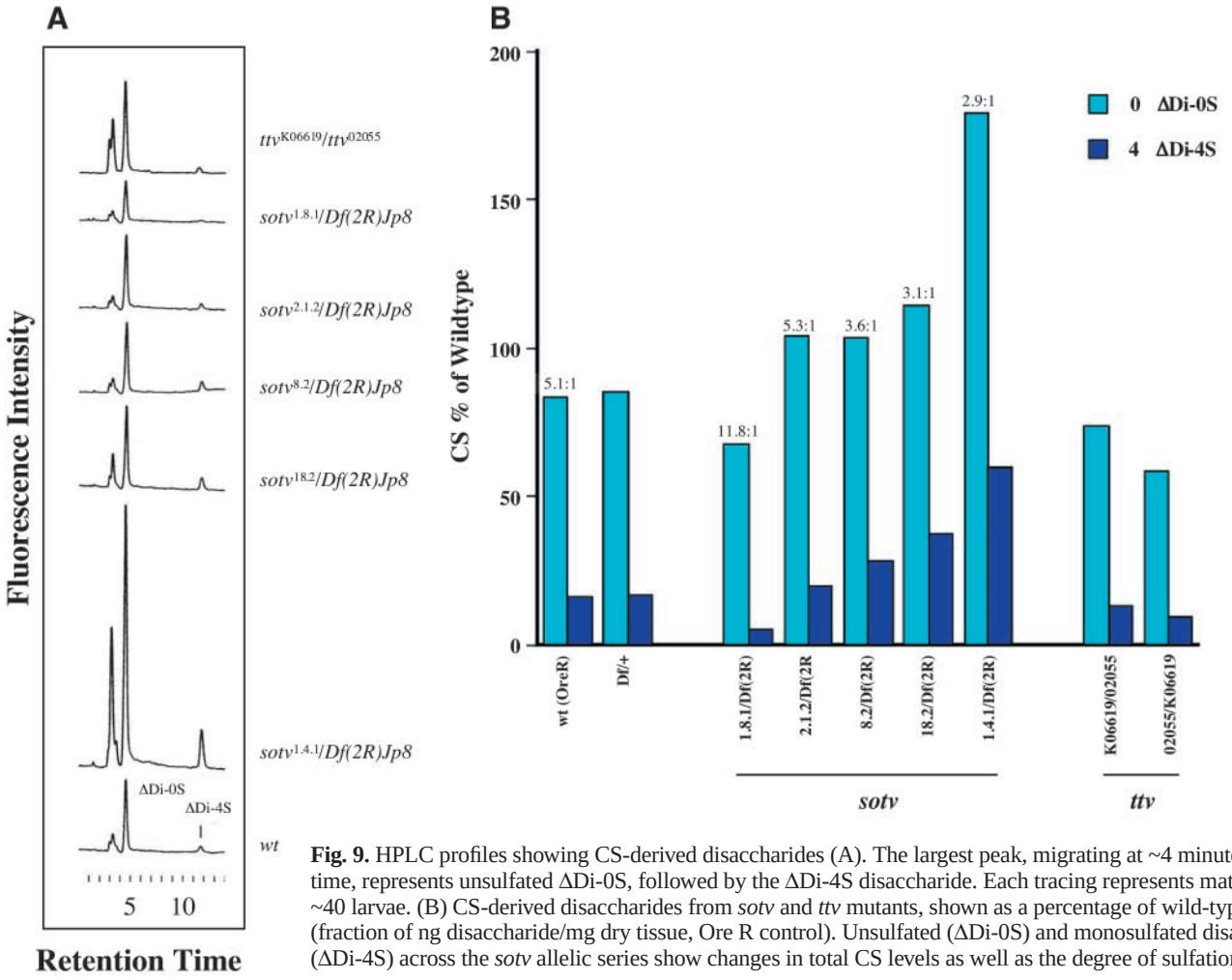



**Fig. 9.** HPLC profiles showing CS-derived disaccharides (A). The largest peak, migrating at ~4 minutes retention time, represents unsulfated ΔDi-0S, followed by the ΔDi-4S disaccharide. Each tracing represents material from ~40 larvae. (B) CS-derived disaccharides from *sotv* and *ttv* mutants, shown as a percentage of wild-type controls (fraction of ng disaccharide/mg dry tissue, Ore R control). Unsulfated (ΔDi-0S) and monosulfated disaccharides (ΔDi-4S) across the *sotv* allelic series show changes in total CS levels as well as the degree of sulfation.

shown to be reduced in *ttv* mutant clones in the wing disc (Bellaiche et al., 1998). The authors found that, in clones at the AP boundary, Ci and Ptc levels were maintained in only a single row of mutant cells along the posterior edge of the clone. Thus, it was proposed that cells lacking HSPGs are competent to receive, but are impaired in propagating, the Hh signal. Interestingly, *ttv* mutations only affect Hh-Np, the mature cholesterol-modified form of the ligand (Burke et al., 1999; Porter et al., 1996). In *ttv* mutant embryos, Hh-Np distribution is curtailed, while Hh-N, which lacks cholesterol, is unaffected. Based on these findings, it has been suggested that HSPGs may enhance the targeting of Hh-Np to 'lipid rafts' where the ligand can be then be transported from cell to cell (Kooyman et al., 1995; Nybakken and Perrimon, 2002; The et al., 1999).

We have shown that, like *ttv* clones, *sotv* and *ttv, sotv* double mutant clones limit the domain of Ci stabilization, indicating that the range of Hh signaling is impaired (Bellaiche et al., 1998). This reduction is consistent with a requirement for HSPGs in Hh transport. However, our observations that Hh levels are reduced in posterior compartment clones (Fig. 6) suggest an alternative (or additional) mechanism by which HSPGs could affect Hh signaling: by altering ligand stability. In wild type, *hh* is transcribed and expressed uniformly throughout the posterior compartment. Therefore, the weaker staining in posterior clones is unlikely to be due to failure of concentration-dependent transport mechanisms. Moreover, lower Hh levels are not caused by reduced expression, as *hh* transcription (monitored through expression of a *hh-lacZ* transgene) is unaffected in *sotv* mutant clones (see Fig. 6). By extension, Hh ligand instability in cells lacking HSPGs could also contribute to the reduced effectiveness of signaling in clones along the AP boundary. HSPGs could bind to and stabilize the ligand directly, or alternatively may act indirectly to reduce the activity of extracellular proteases. Consistent with the latter model, heparin is known to promote inhibition of thrombin by acting through the protease inhibitor serpin AT III (Bernfield et al., 1999). Reduced Hh ligand stability would lower the distance over which the growth factor can signal in a manner difficult to distinguish from compromised ligand transport. Although these models presume that loss of HS impacts growth factor signaling by disrupting protein interaction with GAG chains, it is also possible that without GAG synthesis HSPG core proteins are mislocalized or less stable, contributing to the observed phenotypes.

It has been suggested that disruption of intracellular transport should lead to a diagnostic accumulation of ligand on the side of a clone closest to the morphogen source (Teleman et al., 2001). Although we do not observe ligand accumulation at the boundaries of *ttv* or *sotv* clones, consistent with a failure to affect transport, we interpret these results cautiously. As Hh

stability is compromised in mutant cells, it is possible that the rate of ligand degradation simply exceeds the rate of accumulation. Additionally, it has been argued that ligand accumulation may not be a reliable indicator of a blockage in transport, and instead could reflect increased expression of ligand-binding factors (such as receptors or HSPGs) (Lander et al., 2002).

A recent cell culture-based screen to identify novel components in the Hh pathway demonstrated that RNAi-based degradation of Ttv, Sotv and Botv did not reduce the transcriptional response to exogenously added Hh ligand (Lum et al., 2003). These results are consistent with our findings, as addition of exogenous ligand bypasses any requirement for ligand stabilization or transport. These data underscore the idea that GAG chain synthesis in receiving cells is not essential for transduction of the Hh signal. Loss of Hh in posterior compartment clones lacking *ttv* or *sotv* (see Fig. 6) also argues against a current model that implicates HSPGs and the transmembrane protein Dispatched (Disp) in promoting ligand transport (Burke et al., 1999; Ingham and McMahon, 2001). Disp is required to release Hh from the membrane of expressing cells. Thus, *disp* mutant cells show high levels of Hh accumulation. A Ttv-modified HSPG has been proposed to aid in releasing Hh-Np from Disp and prevent reinsertion of the ligand into the membrane, thereby enhancing its diffusion. However, if a Ttv-modified HSPG were essential for Hh-Np release from Disp, then *ttv* and *sotv* mutant clones in the posterior compartment should accumulate high levels of Hh, similar to *disp* clones. Finally, our results conflict with the proposal that Ttv could synthesize HSPGs that promote Hh signaling specifically (Bellaiche et al., 1998; The et al., 1999), as the Dpp and Wg pathways are also affected by loss of Ttv or Sotv. We have only examined Wg and Dpp signaling in wing discs, and it remains possible that these ligands have differential requirements for HS during embryogenesis.

### Wg and Dpp signaling are sensitive to loss of HS synthesis

A role for HSPGs in Wg signaling in *Drosophila* is well established. Addition of HS or CS GAGs to culture medium causes S2 cells to release secreted Wg protein, while heparinase treatment reduces their ability to respond to exogenous Wg (Reichsman et al., 1996). Furthermore, both Wg levels and signaling efficiency are sensitive to HSPG concentration, as Wg activity is reduced when either HS synthesis or the core proteins for the glypicans Dally and Dlp are compromised (Baeg et al., 2001; Binari et al., 1997; Hacker et al., 1997; Haerry et al., 1997; Lin and Perrimon, 1999; Tsuda et al., 1999). Severe disruption of extracellular Wg distribution and the altered expression of the downstream target Ac, in *ttv* and *sotv* mutant clones demonstrates that HS chains are essential for establishing and/or maintaining the Wg gradient.

The effects of HSPGs on signaling are further modulated by secondary modifications to the GAG chains. For example in *Drosophila*, loss of either *sfl* or *slalom* (*sll*) compromises both Wg and Hh signaling (Lin and Perrimon, 1999; Luders et al., 2003). Sfl is an N-deacetyl/N-sulfotransferase, and Sll is required to transport a high-energy sulfate donor molecule to the golgi, where HSPGs such as *dally* and *dlp* are sulfate modified. Loss of GAG sulfation results in signaling defects, despite the fact that unsulfated HS-GAGs are still present in *sfl* mutants (Toyoda et al., 2000a). Consequently, not only are HS-GAG chains required for signaling, but the extent of sulfation is also crucial. In vertebrates, Qsulf1 promotes Wg signaling by catalyzing removal of 6-O sulfate groups from HS chains of HSPGs, including the Dally homolog glypican 1 (Ai et al., 2003; Dhoot et al., 2001). It has been proposed that in the absence of Qsulf1, Wg stays tightly bound to HSPGs, which prevents the ligand from interacting with receptors. However, in Qsulf1-expressing cells, selective 6-O desulfation reduces the binding affinity sufficiently to permit ligand-receptor interaction. In this context, the altered HS disaccharide distribution and abnormal sulfation patterns encountered in hypomorphic *sotv* alleles is intriguing (see Fig. 8, Table 1). If partial loss of function EXT2 mutations in individuals with HME causes similar disruption of GAG modifications, they could also have complex and allele-specific effects on signaling.

Our data also provide direct evidence that HS chains are required for Dpp signaling in vivo. The core protein of the Glypican Dally has been implicated in Dpp signaling based on genetic interactions and recent studies demonstrating its role in regulating the Dpp morphogen gradient in the wing (Jackson et al., 1997; Fujise et al., 2003). However, the contribution of HS chains to Dpp signaling has remained unclear. We have shown that Dpp signaling in the wing disc is reduced in *ttv* or *sotv* mutant clones independent of effects on Hh signaling, establishing that HS GAG chains are required for optimal activity of the Dpp pathway. Although Dpp activity is clearly compromised in mutant tissue, signaling is still detectable in mutant clones located where ligand levels are the highest, such as near the AP compartment boundary (see Fig. 7). These results imply that, like Wg, Dpp can signal in the absence of HSPGs, albeit at lower efficiency.

### A *Drosophila* HS co-polymerase complex

Initial reports that *ttv* was required for Hh, but not Wg or FGF, signaling, prompted speculation that Ttv might generate a Hh-specific HSPG, and that, unlike its mammalian orthologs, *Drosophila* Sotv might retain significant functional activity in the absence of its partner (Bellaiche et al., 1998; The et al., 1999; Toyoda et al., 2000a; Toyoda et al., 2000b). However, our demonstration that Hh, Wg and Dpp signaling are affected in single mutants for *ttv* and for *sotv*, together with the biochemical analysis presented here, suggest that the mammalian model of EXT1 and EXT2 as obligate co-polymerases applies equally well to *Drosophila*. The severe and comparable reductions in HS disaccharides observed in *ttv* and *sotv* null mutant larvae lend additional support to the co-polymerase model. Moreover, the fact that the phenotype of both single and *ttv, sotv* double mutants is indistinguishable strongly suggests that any residual partner activity is not biologically significant.

In contrast to the null alleles, hypomorphic alleles of *sotv* that result in C-terminal truncations retain some biosynthetic activity that varies directly with the predicted length of the protein (see Fig. 8, Table 1). Thus, $sotv^{1.4.1}$, which encodes the longest mutant product, generates HS at 3.1% the wild-type level compared with 0.21% in the null allele $sotv^{1.8.1}$ (see Table 1). Retention of similar levels of function by truncated human EXT2 proteins could explain why all 27 of the disease-causing dominant mutations in *EXT2* are clustered within the N-

terminal two-thirds of the protein, rather than spread throughout its length (Zak et al., 2002). Perhaps C-terminal truncations retain sufficient function to supplement the HSPG levels from the wild-type copy and achieve the critical threshold required for normal bone growth. Alternatively, reduced levels or altered ratios of proteoglycans bearing specific sulfate modifications may contribute to the clinical manifestation of the disease. We note, for example, that the residual HS produced from null and $sotv^{8.2}$ alleles contain little or no UA-GlcNAc6S. However, that species constitutes a substantial fraction of the HS produced by the less truncated alleles $sotv^{18.2}$ and $sotv^{1.4.1}$ (see Fig. 8 and Table 1).

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