Three *Drosophila* EXT genes shape morphogen gradients through synthesis of heparan sulfate proteoglycans

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Summary

The signaling molecules Hedgehog (Hh), Decapentaplegic (Dpp) and Wingless (Wg) function as morphogens and organize wing patterning in *Drosophila*. In the screen for mutations that alter the morphogen activity, we identified novel mutants of two Drosophila genes, *sister of tout-velu* (*sotv*) and *brother of tout-velu* (*botv*), and new alleles of *tout-velu* (*ttv*). The encoded proteins of these genes belong to an EXT family of proteins that have or are closely related to glycosyltransferase activities required for biosynthesis of heparan sulfate proteoglycans (HSPGs). Mutation in any of these genes impaired biosynthesis of HSPGs in vivo, indicating that, despite their structural similarity, they are not redundant in the HSPG biosynthesis. Protein levels and signaling activities of Hh, Dpp and Wg were reduced in the cells mutant for any of these EXT genes to a various degree, Wg signaling being the least sensitive. Moreover, all three morphogens were accumulated in the front of EXT mutant cells, suggesting that these morphogens require HSPGs to move efficiently. In contrast to previous reports that *ttv* is involved exclusively in Hh signaling, we found that *ttv* mutations also affected Dpp and Wg. These data led us to conclude that each of three EXT genes studied contribute to Hh, Dpp and Wg morphogen signaling. We propose that HSPGs facilitate the spreading of morphogens and therefore, function to generate morphogen concentration gradients.

Key words: EXT genes, *tout-velu*, *sister of ttv*, *brother of ttv*, Morphogen, Gradient formation, Hedgehog, Decapentaplegic, Wingless, *Drosophila*

Introduction

Morphogens emanate from discrete sources and generate concentration gradients that elicit concentration-dependent responses in target cells (Wolpert, 1969). Secreted signaling molecules such as Hedgehog (Hh), Decapentaplegic (Dpp) and Wingless (Wg), have been identified as morphogens and their mode of action has been well-documented in *Drosophila* wing development (reviewed by Gurdon et al., 1998; Lawrence and Struhl, 1996; Neumann and Cohen, 1997a; Tabata, 2001).

Although the influence of Dpp and Wg extends to the edges of the wing disc (Capdevila and Guerrero, 1994; Neumann and Cohen, 1997b; Zecca et al., 1995) and Hh can signal across long distances as well (Chen and Struhl, 1996), these proteins share an unexpected characteristic; they are not readily soluble. The active form of Hh has cholesterol covalently bound at its C-terminus (Porter et al., 1996) and an N terminus that is palmitoylated (Pepinsky et al., 1998). Both modifications will probably anchor these proteins in the membranes of the cells in which they are produced. Both Wg and Dpp homolog TGF-β bind to matrix proteins (Reichsman et al., 1996; Taipale and Keski-Oja, 1997). In addition, Wg can also undergo lipid modification (Willert et al., 2003). Simple diffusion thus appears inadequate to distribute these proteins over even short distances.

Recently, several reports suggest that heparan sulfate proteoglycans (HSPGs) play a key role in morphogen transport and/or signaling (reviewed by Perrimon and Bernfield, 2000). HSPGs are abundant cell surface molecules and are part of the extracellular matrix. HSPGs consist of a protein core (such as syndecans and glypicans) to which heparan sulfate glycosaminoglycan (HS GAG) chains are attached. GAG chains are long unbranched polymers consisting of many sulfated disaccharides. (A GAG synthesis diagram is shown in Fig. 1G).

Genetic screens for mutations affecting morphogen signaling pathways in *Drosophila* have heretofore identified three genes that have sequence homologies with genes encoding vertebrate GAG biosynthetic enzymes. These are *sugarless* (*sgl*) (Binari et al., 1997; Hacker et al., 1997; Haerry et al., 1997), *sulfateless* (*sfl*) (Lin et al., 1999), and *tout-velu* (*ttv*) (Bellaiche et al., 1998; The et al., 1999), which encode proteins with homology to UDP-glucose dehydrogenase, N-deacetylase/N-sulfotransferase, and HS copolymerase with GlcAT-II and GlcNAcT-II activities, respectively. *sgl* mutations compromise signaling mediated by Wg (Binari et al., 1997; Hacker et al., 1997; Haerry et al., 1997) and Dpp (Haerry et al., 1997). The *sfl* mutation affects Wg and Hh signaling (Lin et al., 1999; The et al., 1999), and in somatic *sfl* mutant clones, Wg protein levels are diminished (Baeg et al., 2001). The *ttv* mutant was reported to selectively affect Hh signaling (Bellaiche et al., 1998; The et al., 1999). In addition to these three genes, *notum*, a gene that encodes a member of the α/β-hydrolase superfamily, has been reported to influence Wg protein distribution by destabilizing the HSPGs (Gerlitz and Basler, 2002; Giraldez et al., 2002). Finally, *dally*, which is proposed to encode a protein core of the HSPGs, has been shown to be required for Wg and Dpp activity (Jackson et al.,

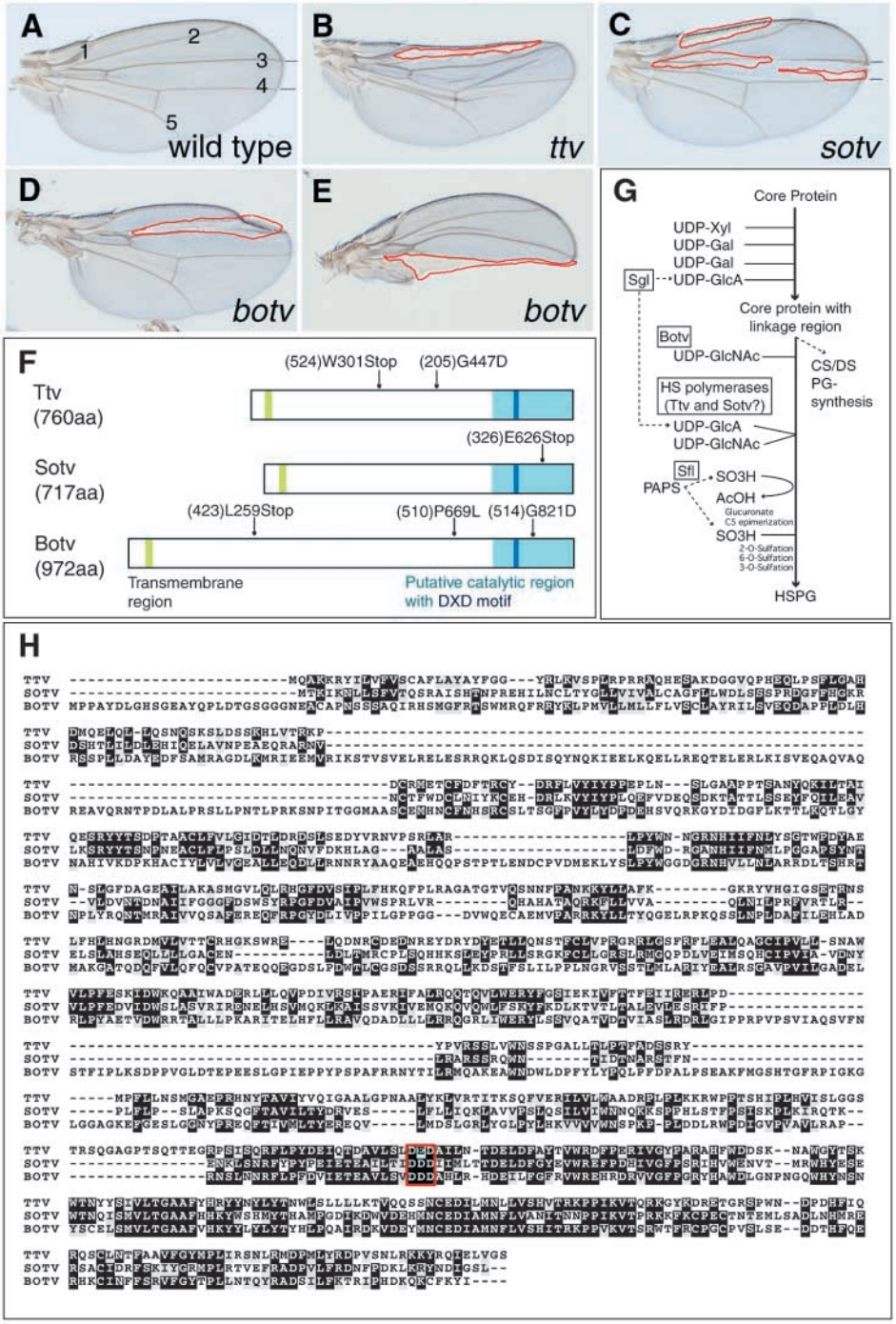

Fig. 1. *sotv* and *botv* are novel *Drosophila* EXT genes. All clones are marked by *shavenoid*$^{-}$ in *Drosophila* adult wing blade. (A) Wild-type wing. Longitudinal veins 1 to 5 (L1 to L5) are shown. (B) *ttv* mutant clone made between L1 and L2. The tissues within the clone are deleted and wing blade becomes narrower in A/P orientation. (C) *sotv* mutant clones. Note that veins L3 and L4 are abnormally close to each other. The region between L3 and L4 is patterned by Hh, whereas the rest of the wing blade is patterned by Dpp. *sotv* phenotype is relatively weaker than other EXT mutants *ttv* and *botv*. (D,E) *botv* mutant clones located between L2 and L3 or behind L4. The tissues within the clone are deleted. (F) Structures of *Drosophila* EXTs, Ttv, Sotv and Botv deduced from genome sequence. These proteins have three domains as indicated: light green, transmembrane region; light blue, putative catalytic region; blue, DXD motif. Amino acid sequence alterations in Ttv, Sotv and Botv caused by EMS mutation are indicated by arrows. (G) Glycosaminoglycan synthesis in *Drosophila* (from Toyoda et al., with modification) (Toyoda et al., 2000). (H) Sequence alignment of Ttv, Sotv and Botv proteins. Black boxes indicate amino acids that are identical in these proteins. The overall similarity between them is 26-32%. The homology is especially high in their C-terminal regions. Conserved DXD motif is indicated in red. Gal, galactose; GlcA, glucuronic acid; GlcNAc, N-acetylglucosamine; Xyl, xylose.

1997; Lin and Perrimon, 1999; Nakato et al., 1995; Tsuda et al., 1999; Fujise et al., 2003). Dally and related Dally-like (Dlp) have been shown to bind and stabilize Wg at the cell surface (Baeg et al., 2001; Strigini and Cohen, 2000) and may provide a pool of Wg protein that can become available for receptor binding upon release from the HSPG.

Among the three GAG-biosynthetic genes, the *ttv* mutant is unique in its selective effects on Hh signaling. Wg-directed events were unaffected in *ttv* embryos and larvae (Bellaiche et al., 1998; The et al., 1999). A possible explanation accounting for this apparent selectivity is that HS GAGs synthesized by other EXT genes may be sufficient to allow Wg pathways to function (‘quantitative model’). Alternatively, Hh-specific HSPGs may exist and *ttv* might be required for their synthesis, whereas other EXT genes might synthesize HSPGs specific for Wg signaling (‘qualitative model’).

Here we describe novel mutants of *sister of tout-velu* (*sotv*) and *brother of tout-velu* (*botv*), as well as new alleles of *ttv*. *sotv* and *botv*, like *ttv*, encode EXT family proteins. In the clones of mutant cells, HSPGs biosynthesis was severely affected, and the morphogen signaling activities of Hh, Dpp and Wg were impaired to various degrees. Morphogen molecules were rarely seen in mutant clones, and had been accumulated at the wild-type cells that reside next to a mutant

clone. These results suggest that HSPGs are required for proper transport of morphogens.

Materials and Methods

Fly strains

y w; *smo stc* FRT^{42D} y^{+} *sha*/CyO males were mutated with ethylmethanesulfonate (EMS), crossed first to *y w*; Pin/CyO and then individually to *y w* FLP122; FRT^{42D} y^{+}. Males with phenotypes in wing development were retained and maintained as balanced stocks. The mutants and transgenic animals used were as follows: *ttv*^{524}, *ttv*^{205}, *sotv*^{326}, *botv*^{423}, *botv*^{510} and *botv*^{514} (isolated in this study); *ttv*$^{l(2)00681}$ (Bellaiche et al., 1998); UAS-Dpp-GFP, a biologically active form of Dpp tagged with GFP (Entchev et al., 2000); *dpp*-GAL4 (Teleman and Cohen, 2000); and UAS-sotv (made by injection of pUAST construct containing *Not*I-*Asp*718 fragment of the sotv cDNA, this study).

Mapping

One of three complementation groups that had similar phenotypes, failed to complement the lethality of *ttv*, which has been reported to be a *Drosophila* EXT family gene (Bellaiche et al., 1998) and two other groups failed to complement Df(2R)Jp8 and Df(2R)P34 which delete 52F5-9; 53A1 and 55E6-F3; 56C1, respectively. In and around these regions there are two other *Drosophila* EXT genes, *DEXT2* (Han et al., 2002; The et al., 1999) and *DEXT3* (Han et al., 2002; Han et al., 2001; Kim et al., 2002). We sequenced all predicted coding regions of their genomic DNA and identified SNPs in *DEXT2* and *DEXT3*.

Clonal analysis

Clones of mutant cells were induced by Flippase-mediated mitotic recombination (Xu and Rubin, 1993). First instar larvae were heat-shocked at 37°C for 1 hour and dissected 2-4 days after the heat-shock. Mutant clones were identified by the loss of GFP expression. Genotypes of the flies used for making mitotic clones were as follows: *y w* FLP122/+; FRT^{42D} y^{+} *sha EXT gene(s)*/FRT^{42D} y^{+} Ub-GFP or *wg-lacZ* FRT^{42D} Ub-GFP or *dpp-lacZ* FRT^{42D} Ub-GFP. For monitoring the Dpp-GFP distribution in the EXT mutant clone, we dissected wing discs of *y* FLP122/+; FRT^{42D} *ttv*^{524} *botv*^{510}/FRT^{42D} hs-CD2; *UAS-dpp-GFP*/*dpp-GAL4* larvae.

Histochemistry

Imaginal disc staining was performed as described previously (Tanimoto et al., 2000), except for 3G10 staining: treatment of imaginal discs to expose the epitope recognized by the 3G10 monoclonal antibody (Seikagaku) was done by incubating fixed imaginal discs with 20 mU heparitinase I (Seikagaku) per 1 ml 100 mM NaOAc, 3.3 mM $CaCl_2$ for 1 hour at 37°C. After washing three times with PBT, the samples were stained with 3G10 antibody, 1:100 in 2% BSA/PBT.

The primary antibodies used were: rabbit anti-β-gal antibody, 1:2000 (Cappel); mouse anti-CD2 antibody, 1:1000 (Cederlane); rat anti-Ptc antibody, 1:150 (gift from R. Johnson); rat anti-Sal antibody, 1:250 (gift from R. Barrio); rabbit anti-p-Mad antibody PS1, 1:20,000 (Persson et al., 1998); rabbit anti-Dll antibody, 1:300 (Panganiban et al., 1995); mouse anti-Wg antibody 4D4, 1:5000 (Developmental Studies Hybridoma Bank); rabbit anti-Hh antibody NHhI, 1:1000 (raised against the N-terminal amino acids 89-306 of Hh); rabbit anti-Dpp antibody, 1:5000 (gift from M. Hoffmann).

Secondary antibodies used were anti-mouse Cy3, anti-rabbit Cy3, anti-Rat Cy3, anti-rabbit Cy5 and anti-rat Cy5 (Jackson).

Immunofluorescent images were observed on a Zeiss confocal laser microscope 510.

Results

Isolation of *Drosophila* EXT mutants

We performed a screen for mutations that affect wing patterning. Clones of cells homozygous for mutagenized chromosomes were generated, and adult wings were examined for patterning defects. In the wing blade, the area between longitudinal veins 3 and 4 is patterned by Hh signaling (Mullor et al., 1997; Strigini and Cohen, 1997) and the rest of the area is patterned by Dpp signaling (Bier, 2000; Sturtevant et al., 1997). We identified three independent loci on the right arm of chromosome 2 that delete tissue within the clone (Fig. 1B-E). This tissue-deletion phenotype was seen in all areas of the wing blade. These mutants were classified into three complementation groups. Two mutants of the first complementation group, *ttv*^{524} and *ttv*^{205}, failed to complement *ttv* (Bellaiche et al., 1998) and are presumed to represent *ttv* mutant alleles. We sequenced all predicted coding regions of the *ttv* gene and identified SNPs in exons of *ttv* (Fig. 1F). *ttv*^{524} mutant has a nonsense mutation at Trp 301 that truncates 460 C-terminal residues, and *ttv*^{205} has a missense mutation at Gly

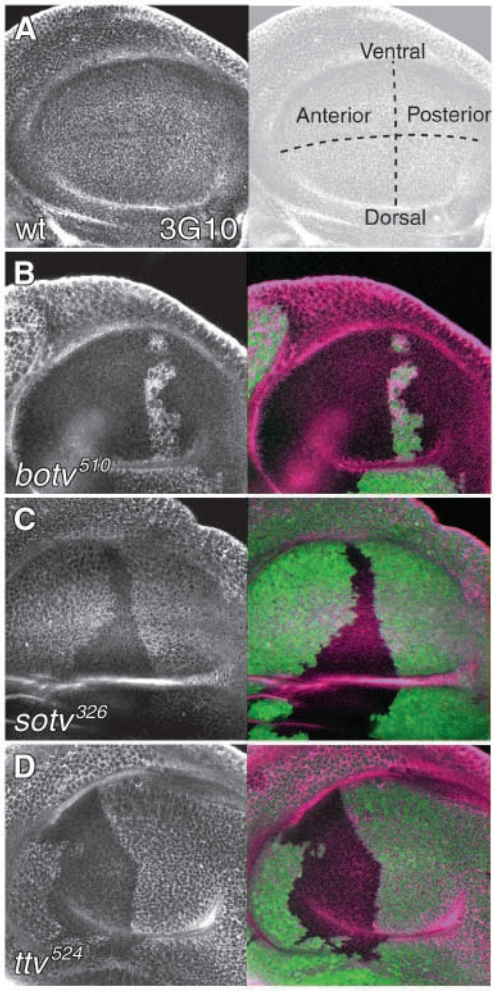

Fig. 2. All three EXT genes are involved in HSPG biosynthesis in vivo. HSPG levels at the lateral surface of cells in the wild-type and mutant wing imaginal discs. (A) In the wild-type disc, uniform staining is detected (gray on the left panel). (B-D) Clones of cells mutant for the EXT genes are marked by the absence of GFP (green on the right panel). HSPG levels are severely reduced in cells mutant for any of the EXT genes. Here, and on all Figures in this report, imaginal discs are oriented anterior to the left and dorsal to the bottom.

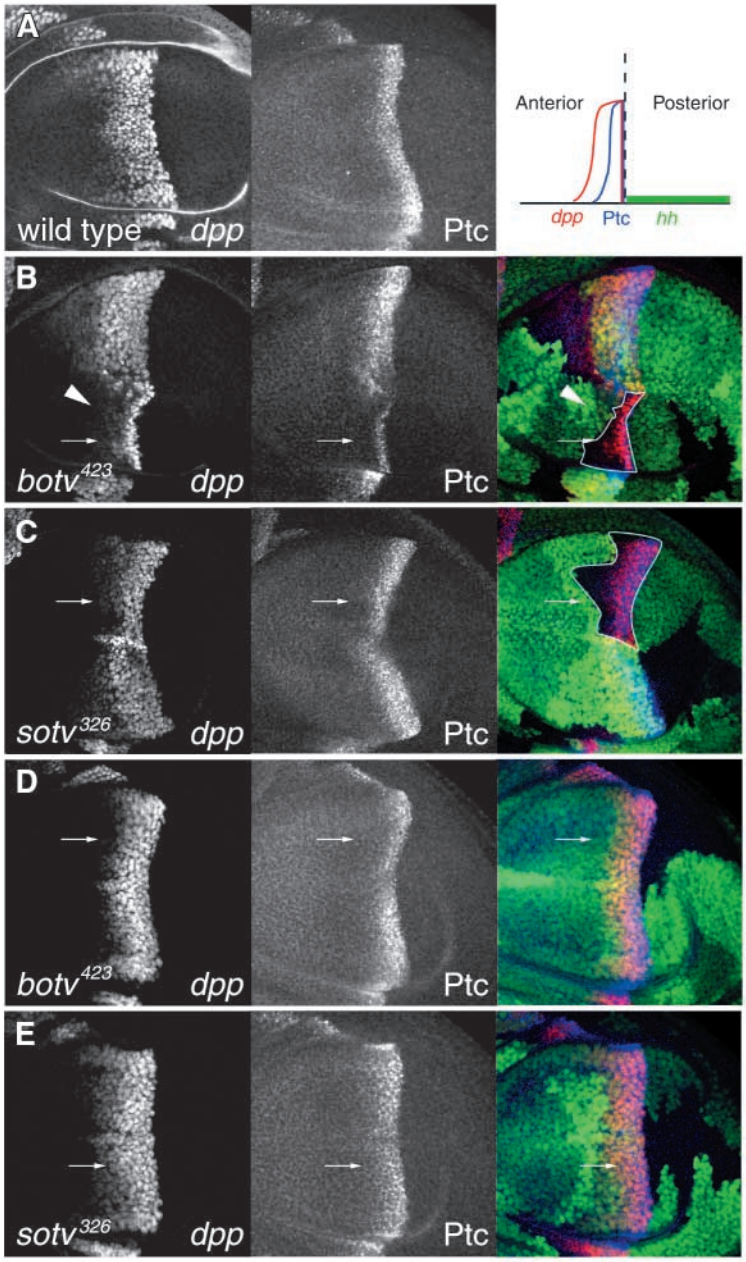

Fig. 3. EXT genes are required for Hh signaling. *dpp-lacZ* expression (gray on the left panel) and Ptc protein level (gray on the middle panel) in the wild-type and mutant wing discs. Some of mutant clones are marked by light-blue lines for clarification. (A) In the wild-type wing discs, *dpp* expression is induced by Hh that emanates from the posterior compartment and is detected as far as 15 cells away from the anterior-posterior boundary, and Ptc protein can be detected as far as five cells away. A schematic diagram is shown on the right panel. (B) In the $botv^{423}$ clone (marked by the absence of GFP on the right panel), *dpp* (red in the right panel) and Ptc (blue in the right panel) levels are reduced (arrows), and cells located at the posterior part of the clone still respond to Hh signal. The reduction in *dpp* expression extends to the wild-type cells anterior to the mutant clone (arrowhead). (C) In the $sotv^{326}$ mutant clone, Ptc protein and *dpp* expression levels are reduced slightly (arrow), indicating that Hh signaling is not severely impaired. (D,E) Loss of *botv* (D) or *sotv* (E) activity in the posterior compartment does not affect *dpp* level in anterior cells (arrow). However, Ptc protein level was sometimes slightly decreased in the anterior cells along the A-P boundary (D,E).

447 Asp (Fig. 1F). *ttv* is the *Drosophila* EXT family gene with highest homology to human EXT1 (amino acid sequence identity 50.4%) which has GlcNAcT-II and GlcAT-II activities that are required for the elongation of HS chains (Fig. 1G) (reviewed by Sugahara, 2000). *Drosophila ttv* mutant embryos exhibit strongly impaired HS biosynthesis (The et al., 1999; Toyoda et al., 2000), but it has not been demonstrated directly whether Ttv protein has GlcNAcT-II and GlcAT-II activities.

Crosses to deficiency strains mapped the other two loci to the interval 52F5-9; 53A1 and 55E6-F3; 56C1, respectively. Near these regions, two EXT-related genes have been identified by homology search: *DEXT2*, *sotv* (CG8433, 52E11) (Han et al., 2002; The et al., 1999) and *DEXT3*, *botv* (CG15110, 56B5) (Han et al., 2002; Han et al., 2001; Kim et al., 2002). However, evidence for the function of these genes in an intact organism has been lacking because no mutant has been identified. We sequenced all predicted coding regions of these genes and identified deletions and SNPs in exons of both *DEXT2* and *DEXT3* (Fig. 1F).

The predicted ORF of *sotv* encodes a protein with 717 amino acids. Our *Drosophila* $sotv^{326}$ mutant has a nonsense mutation at glutamic acid 626 that cuts off 92 C-terminal residues (Fig. 1F). This candidate gene must represent *sotv*, because a transgene harboring a full-length *sotv* cDNA driven by the ubiquitous promoter of the *tubulin alpha1* gene was found to completely rescue the lethality of sotv326/Df(2R)Jp8 and other phenotypes associated with clones of *sotv* mutation (data not shown). The Sotv sequence is similar to human EXT2 (44.8% amino acid similarity), which also has GlcNAcT-II and GlcAT-II activities (Senay et al., 2000).

The predicted *botv* encodes a protein with 972 residues and shares 45.8% sequence identity with human EXTL3. Three mutants of our third complementation group, $botv^{423}$, $botv^{510}$ and $botv^{514}$ have nonsense mutation at Leu 259, missense mutation at Pro 669 Leu, and Gly 821 Asp, respectively (Fig. 1F). Human EXTL3 has GlcNAcT-I and -II activities and is required for initiation of HS biosynthesis. Previous studies have shown that a truncated soluble form of *Drosophila* Botv protein has GlcNAcT-I and GlcNAcT-II activities when expressed in COS-1 cells (Kim et al., 2002).

Like most EXT family members, including Ttv, Sotv and Botv have three apparent domains: an N-terminal transmembrane domain; a DXD motif which is characteristic of glycosyltransferases and is required for binding divalent cations; and a large globular domain in the C-terminal region that will probably have enzymatic activity (Fig. 1F). Amino acid sequences of these *Drosophila* EXTs are related, with 26-32% amino acid overall similarity, and especially high homology in their C-terminal regions (Fig. 1H).

Animals transheterozygous for each EXT mutant (ttv^{524}/ttv^{205}, $botv^{423}/botv^{510}$, $sotv^{326}$/Df(2R)Jp8) die as pupae with small eyes and legs (data not shown). In all cases, third

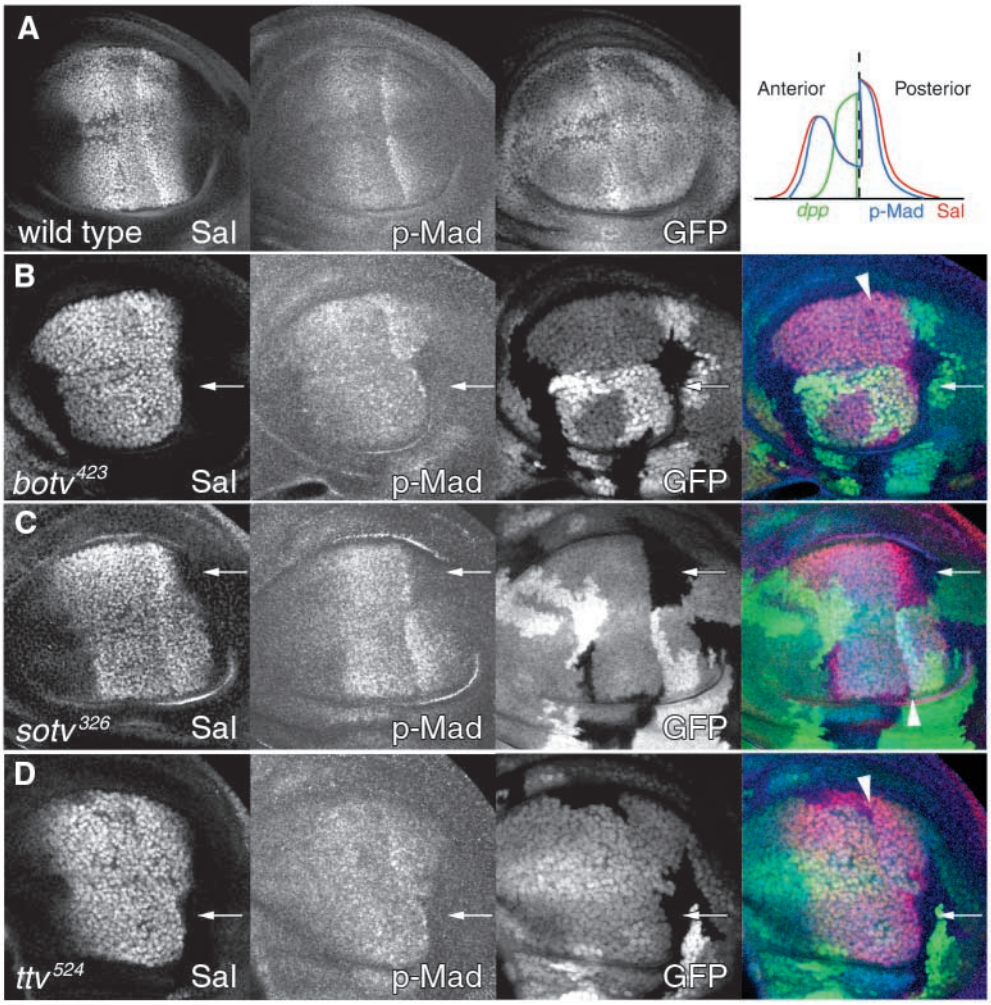

Fig. 4. EXT genes are required for Dpp signaling. Levels of Sal and p-Mad in the wild-type and mutant discs. (A) The Sal and p-Mad levels are normally high in the central region of the wild-type wing disc. In A cells close to the A-P boundary, the Sal and p-Mad levels are much lower than those in the adjacent P cells. A schematic diagram is shown on the right panel. (B-D) In *botv*^{423} (B), *sotv*^{326} (C) and *ttv*^{524} mutant cells (marked by the absence of GFP), Sal and p-Mad levels are decreased (red and blue on the right panel, respectively). The A-P boundary is indicated by white arrowheads in B-D, right panel.

instar larvae have smaller discs than the wild type, and in particular, their wing discs were narrower in anterior-posterior direction (data not shown). In situ hybridization revealed that third instar larvae express *sotv* and *botv* ubiquitously in wing discs as well as *ttv* (data not shown).

HSPG biosynthesis is defective in EXT mutants

To test whether *sotv* and *botv* function in HSPG biosynthesis in flies, we stained wing imaginal discs with antibody 3G10 that recognizes an epitope produced by heparitinase I digestion of HSPGs (David et al., 1992). In the wild-type wing discs, uniform staining was detected (Fig. 2A). However in discs with mutant clones, 3G10 staining was severely reduced in cells mutant for any of the EXT genes (Fig. 2B-D). These results indicate that HSPG biosynthesis in *Drosophila* requires each of these genes.

This and the following phenotypes described in this report are unlikely to be caused by possible secondary lesions on the mutated chromosome because the same phenotypes are associated with all *ttv* and *botv* alleles independently isolated in this screen.

Hh, Dpp and Wg signaling is defective in EXT mutants

Hh signaling

In the wing imaginal disc, Hh is only expressed in the posterior compartment cells but diffuses into the anterior compartment (Basler and Struhl, 1994; Chen and Struhl, 1996; Lee et al., 1992; Tabata and Kornberg, 1994) where it upregulates expression of *dpp* and *patched* (*ptc*). *dpp* responds to low Hh activity and is observed as far as 15 cells away from the A/P boundary; *ptc* responds to higher Hh-signaling activity and can be detected only up to five cells away (Fig. 3A). We used *dpp* and Ptc expression as reporters of the Hh signaling in EXT mutants.

In *botv* mutant clones located in the anterior compartment near the A/P boundary, Hh signaling was partially impaired, as indicated by reduced levels of Ptc protein and *dpp* expression in most parts of the clone (Fig. 3B, arrow). However, Ptc remained at the posterior edge of the clone next to the A/P boundary, and *dpp* expression in these cells also remained and was sometimes higher than in the corresponding wild-type cells (Fig. 3B). The reduction in *dpp* expression extended to the wild-type cells anterior to the clone (Fig. 3B, arrowhead). Similar observations were described in *ttv* mutant clones (Bellaiche et al., 1998) (data not shown). In *sotv* clones, Ptc and *dpp* levels were also reduced, but the decrease was not as significant as in *botv* or *ttv* clones (arrow in Fig. 3C). We interpret this as reflecting the remaining activity of Sotv protein encoded by *sotv*^{326} which has a mutation at the very C-terminus of the protein. These observations are consistent with the reduced distance between veins L3 and L4 that is associated with small mutant clones in this region of the wing blade (Fig. 1C).

Almost no change in level of *dpp* expression was detected when mutant clones grew along the boundary in the posterior compartment (arrow in Fig. 3D,E). This result suggests that neither *sotv* nor *botv* is required for Hh secretion, but rather that their functions are necessary for efficient movement of Hh. However on the same occasion, Ptc protein level was sometimes slightly decreased in the anterior cells along the A/P

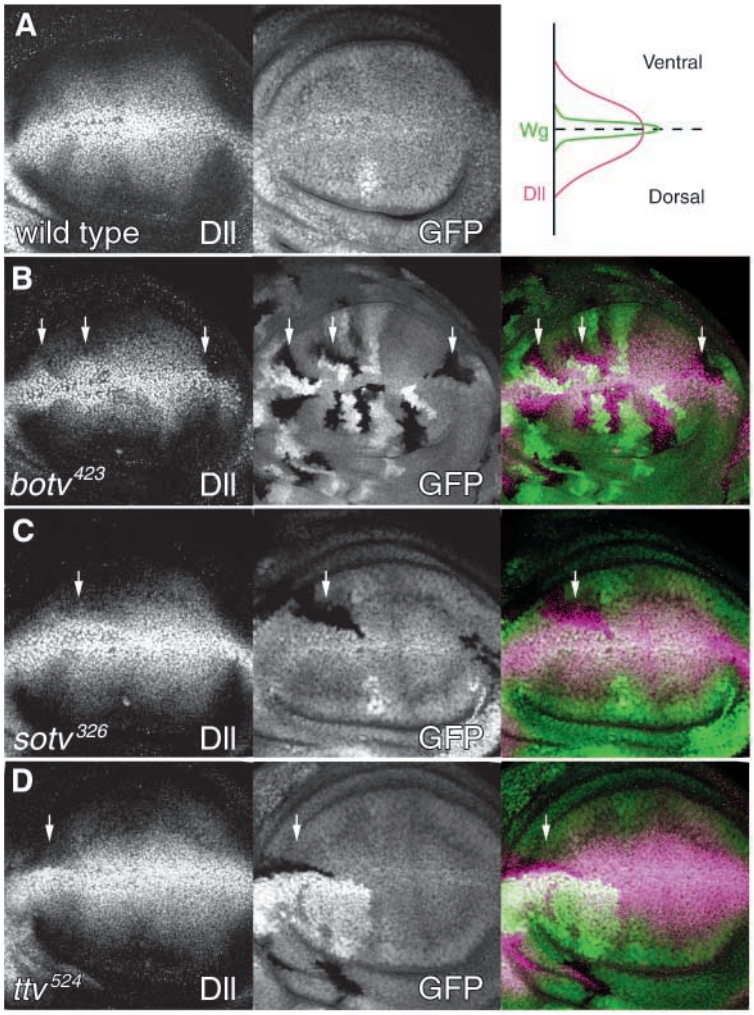

Fig. 5. EXT genes are required for Wg signaling. Dll protein levels in the wild-type and mutant wing discs. (A) Wg is expressed at the wing margin and regulates the Dll expression (gray in the left panel) in a concentration-dependent manner. Schematic diagram is shown on the right panel. (B-D) In *botv*^{423} (B), *sotv*^{326} (C) and *ttv*^{524} (D) mutant cells, Dll expression is slightly decreased (arrows). The reduction in Dll protein level is extended to the wild-type cells located on the far side of the mutant clones (C,D).

boundary (Fig. 3D,E). It is probably because *ptc* expression requires higher Hh signal than *dpp* expression, and is more sensitive to reduction in Hh protein level in posterior compartment, as described below (Fig. 6A). The same results were observed in *ttv*^{524} mutant clones (data not shown).

Dpp signaling

Effects on Dpp signaling were monitored by cataloging the distribution of Dpp target gene Spalt (Sal) protein, and the phosphorylated level of the cytoplasmic signal transducer, Mothers against dpp (p-Mad) (Tanimoto et al., 2000). The Sal and p-Mad levels are normally high in the central region of the wing disc (Fig. 4A); they are regulated by Dpp which is expressed by anterior compartment cells at the A/P boundary.

As shown in Fig. 4, Sal and p-Mad levels were decreased when any of the three EXT genes were mutated (Fig. 4B-D). Sal and p-Mad levels remained at the edge of the clone nearest to the Dpp source, and moreover their levels were sometimes higher, as in the case of Hh target genes (Fig. 4B-D). It should be noted that the Dpp reporter levels were reduced even in *ttv* mutant clones (Fig. 4D). As mentioned above, *ttv* has previously been reported to be exclusively involved in Hh signaling, thus it is the first evidence that *ttv* is also required for other morphogen signaling. These effects were observed both in anterior and posterior compartments, indicating that the observed effect on Dpp signaling was direct and did not reflect the reduction in Hh signaling, which is only active in the anterior compartment.

Wg signaling

A functional connection between Wg and HSPGs has been suggested by the observed strong association of Wg proteins and sulfated proteoglycans in tissue culture cells (reviewed by Lin and Perrimon, 2000; Selleck, 2001). In addition, Wg signaling is affected by defective HSPG biosynthesis in *sgl* and *sfl* mutants, and in *dally* and *dlp* mutants that alter HSPG core proteins. Wg is normally expressed at the wing margin and controls patterning along the dorsal/ventral axis by regulating target genes such as *distalless* (*dll*) and *vestigial* (*vg*) in a concentration-dependent manner (Neumann and Cohen, 1997b; Zecca et al., 1996). We investigated Wg signaling in EXT mutant cells by monitoring the distribution of these proteins.

Levels of Dll protein were slightly reduced in the clones homozygous for *botv*^{423} or *ttv*^{524} (Fig. 5B,D). In the cells mutant for *sotv*^{326}, the effect was too subtle to assess definitively, probably because the *sotv*^{326} allele is weak, as described above. The reduction in Dll protein level extended to the wild-type cells near the clone (Fig. 5C,D, arrows). Furthermore, the Dll protein levels were sometimes higher at the edge of the clone nearest to the Wg source, as in the case of Hh and Dpp target genes (Fig. 5B-D). The similar phenotype was also observed for another target, Vg in *botv*^{423} and *ttv*^{524} mutant clones, but too subtle to assess in *sotv* mutant clones (data not shown). The previous study did not detect any effects of Ttv on the Wg signaling (The et al., 1999). Because the difference could be ascribed to the alleles used, we re-examined this finding using the same allele *ttv*$^{l(2)00681}$ that was used in the previous study (The et al., 1999) or our weak allele *ttv*^{205}. We found that in these mutant clones Wg signaling was also slightly decreased. Thus, these results suggest that *Drosophila* EXT genes contribute to Wg signaling in the wing imaginal disc.

Morphogen distribution depends on EXT activity

In addition to monitoring signaling in EXT mutant cells, we used antibodies that recognize Hh, Dpp and Wg, and a GFP-tagged version of Dpp to analyze whether the levels or distribution of these morphogens had been affected. We found that levels of each of these proteins were significantly reduced in the mutant, both in the morphogen-expressing region and in the receiving region (Fig. 6A-F). For Hh, Dpp and Wg, similar results were observed in cells mutant singly for any of the EXT

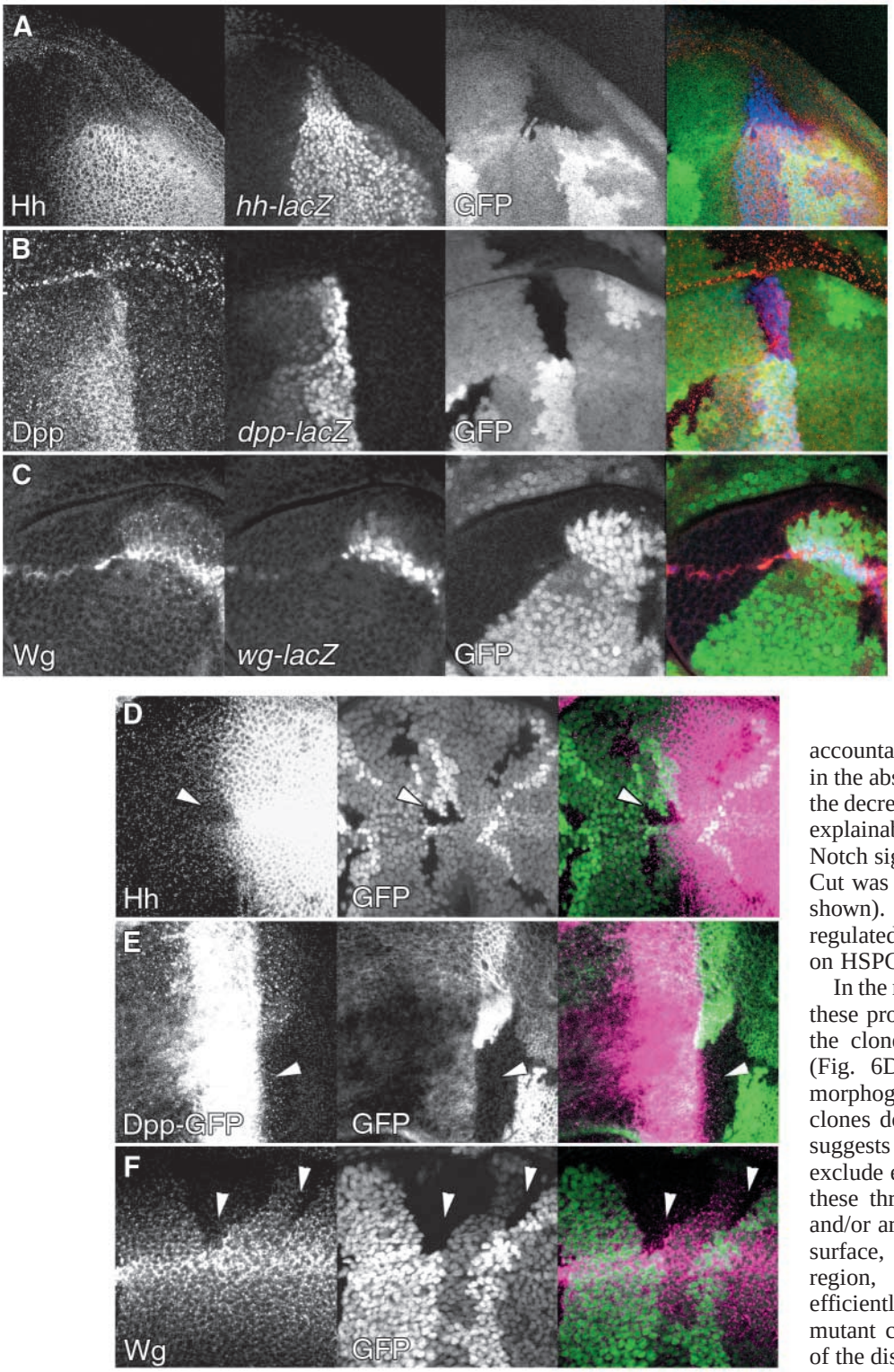

Fig. 6. Morphogen distribution in the EXT (*ttv*^{524}*botv*^{510}) mutant clones. Hh, Dpp and Wg proteins are significantly reduced in the mutant clones, both in the morphogen-expressing region (A-C) and in the receiving region (D-F). (A) In *ttv*^{524}*botv*^{510} mutant clones (marked by the absence of GFP), Hh protein is severely reduced, whereas *hh-lacZ* expression was not downregulated. (B) Both Dpp protein and *dpp* expression levels are decreased in the *ttv*^{524}*botv*^{510} clones. (C) Both Wg protein and *wg* expression levels are decreased in the *ttv*^{524}*botv*^{510} clones. In the morphogen-receiving region, each of Hh, Dpp-GFP and Wg protein was significantly decreased in the mutant clones (arrowheads; D-F).

genes, although we only show the results with the *ttv*^{524} *botv*^{510} double mutant clones in Fig. 6A-D,F. Single mutation was not tested for the distribution of Dpp-GFP (Fig. 6E).

In the morphogen-expressing region, *hh* expression was not downregulated, however levels of Hh protein were significantly decreased (Fig. 6A). This may indicate that Hh protein is destabilized and/or not retained efficiently on the cell surface in the absence of HSPGs. In contrast to *hh*, expression of the *wg* and *dpp* and levels of Wg and Dpp were decreased in the EXT clones (Fig. 6B,C). The decrease in *dpp* expression is easily accountable because Hh signaling is impaired in the absence of HSPGs (Fig. 3). In contrast, the decrease in *wg* expression is not as readily explainable: *cut* and *wg* are both targets of Notch signaling, however the protein level of Cut was not altered in EXT clones (data not shown). This suggests that *wg* is also regulated by unknown mechanism dependent on HSPGs.

In the morphogen-receiving region, each of these proteins was significantly decreased in the clones of cells mutant for EXT genes (Fig. 6D-F), although a little leakage of morphogen molecules was seen even in the clones doubly mutant for *ttv* and *botv*. This suggests two possible mechanisms that do not exclude each other: in the absence of HSPGs these three morphogens are 1) destabilized and/or are not retained efficiently on the cell surface, like Hh in morphogen-expressing region, or 2) prevented from diffusing efficiently into the region consisting of EXT mutant cells. Intriguingly, close observation of the distribution of Hh strongly suggested a function for HSPGs in morphogen movement. In the wild-type discs, Hh protein synthesized in the posterior compartment appears to flow into the anterior compartment, with a moderate concentration gradient starting from the middle of the posterior compartment (Fig. 7A-C). However, Hh abnormally accumulated in the posterior compartment when the EXT mutant clone was in the anterior compartment along the A/P boundary (Fig. 7D-I). This effect was seen both in the ventral region (Fig. 7D-F) and in

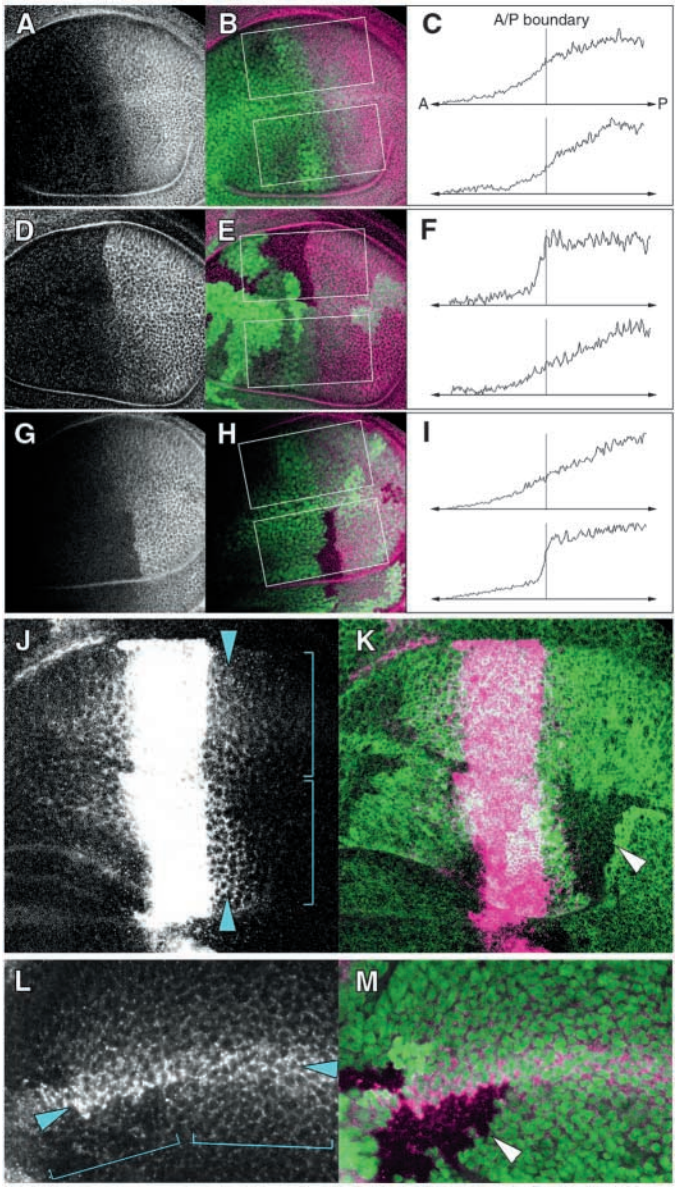

Fig. 7. Morphogens are accumulated in front of the mutant clones. (A-I) Hh diffusion patterns in the wild-type disc (A-C) and disc with $ttv^{524}botv^{510}$ clone (D-I). The staining intensity of Hh in the selected area (white boxes in B,E,H) was integrated along the A-P axis, plotted using NIH Image software and presented schematically (C,F,I). (A-C) In the wild-type disc, Hh flows into the anterior compartment with a moderate gradient starting from the middle of the posterior compartment. The distribution pattern of Hh in the ventral region (upper graph in C) is similar to that in the dorsal region (lower graph in C). (D-F) When the mutant clone was located in the anterior-ventral region along the A-P boundary, Hh was accumulated in the posterior-ventral cells. Compare the levels of Hh at the A-P boundary in the ventral region with that in the dorsal region (graphs in F). (G-I) Likewise, when the EXT clone was in the anterior-dorsal compartment, Hh accumulated in the posterior-dorsal cells (graphs in I). (J,K) Dpp-GFP accumulated in front of the EXT mutant clone. In the ventral compartment, Dpp-GFP diffuses with moderate gradient (upper bracket in J). Dpp-GFP accumulated on the cell surface of the wild-type cells adjacent to the mutant clone (lower bracket in J). Compare the levels of Dpp-GFP in the region with mutant clone (lower arrowhead in J) with that in the region without clone (upper arrowhead in J). (L,M) Wg accumulated in front of the EXT mutant clone. Compare the levels of Wg in the region with mutant clone (left bracket and arrowhead in L) with that in the region without clone (right bracket and arrowhead in L). For Hh and Wg, the same results were obtained in cells mutant singly for any of the EXT genes. Single mutation was not tested for the distribution of Dpp-GFP.

the dorsal region (Fig. 7G-I). This suggests that Hh failed to move into the mutant cells and as a consequence accumulated in posterior cells instead. Dpp-GFP and Wg accumulation in front of the mutant clones was also apparent, however less pronounced compared with the case of Hh (Fig. 7J-M). Therefore we conclude that the HSPG-dependent diffusion is the common mechanism for the movement of these three morphogens.

Discussion

In this report we identified novel *Drosophila* mutants of *sotv* and *botv*, and new alleles of *ttv*. Based on sequence homology, we suggest that *sotv* is a homologue of human EXT2 and that *botv* is a homologue of human EXTL3. We showed that each of these three mutants affected biosynthesis of HSPGs and also impaired signaling of the Hh, Dpp and Wg morphogens. Furthermore, we found that HSPGs are required for normal movement of morphogens across the disc epithelium.

HSPGs are required for Hh, Wg and Dpp signaling

Extensive studies of *sgl*, *sfl* and *ttv* showed that morphogen signaling and morphogenesis were affected in these HSPG mutants (reviewed by Lin and Perrimon, 2000; Selleck, 2001). *sgl* and *sfl* mutants had the broadest effects on signaling, compromising Wg- and Hh-regulated processes. In contrast, *ttv* seemed to have affected only Hh signaling, leaving Wg-mediated patterning unaffected in both embryonic and imaginal development (Bellaiche et al., 1998; The et al., 1999). These phenotypes suggested that HSPGs can distinguish between these different morphogens.

Findings on the three EXT mutants identified in this work, however, argue for a more general role for EXT genes in cell-cell signaling. We found that three EXT family genes, including *ttv*, affected Hh and Dpp signaling in a similar way. The effect on Wg signaling was not so pronounced compared with that on Hh and Dpp, but the subtle effect on target gene expression and clear reduction in the protein level in the mutant clones also suggest a role for the EXT genes in Wg signaling. The explanation for this contradiction might be that, as seen in Fig. 5, Wg signaling appears to depend on the EXT activity to a lesser degree than Hh and Dpp signaling, so previous study might have missed the role of EXT genes in Wg signaling. We therefore suggest that differences in the behavior of Hh, Wg and Dpp may be more quantitative than qualitative.

ttv and *sotv* do not encode redundant functions

Although Ttv and Sotv are expected to have similar enzymatic activities, mutants defective in either of the *ttv* or *sotv* genes had dramatically impaired HSPG biosynthesis and morphogen

signaling. Therefore, despite their similarities, they cannot compensate for each other. They apparently share this property with their human homologues.

EXT1 and EXT2, the human homologues of *ttv* and *sotv*, do not compensate for each other in vitro or in vivo (McCormick et al., 2000; Senay et al., 2000). In cultured cells, GFP-tagged EXT1 and EXT2 localize predominantly to the endoplasmic reticulum when expressed independently. However, GFP-tagged EXT1 and EXT2 formed hetero-oligomeric complexes that accumulated in the Golgi when expressed together in the same cell. Moreover, the Golgi-localized EXT1-EXT2 complex has substantially higher glycosyltransferase activity than EXT1 or EXT2 alone, which suggests that this complex represents the biologically relevant form. These findings provide a rationale to explain how inherited mutations in either of the two EXT genes can cause loss of activity, resulting in hereditary multiple extosis. In *Drosophila*, the observed lack of complementation is also consistent with an active enzymatic complex that consists of both Ttv and Sotv.

HSPGs are required for morphogen movement

Gradients are formed by spread of morphogen from a localized source, but whether this occurs by simple diffusion or by more elaborate mechanisms is not known. Arguments against morphogen movement by diffusion have been raised by many, including Kerszberg and Wolpert (Kerszberg and Wolpert, 1998) who proposed that morphogens use a 'bucket brigade' mechanism in which receptor-bound morphogen on one cell is transferred to receptors on an adjacent cell. Alternatively, Entchev et al. (Entchev et al., 2000) proposed transport through 'planar transcytosis', a process by which morphogens move by repeated cycles of endocytosis and exocytosis in the plane of an epithelium. The notion that Dpp and other morphogens, such as Wg and Hh, all pass through tissues by transcytosis or similar processes has been championed by many (e.g. Greco et al., 2001; Moline et al., 1999; Narayanan and Ramaswami, 2001; Pfeiffer and Vincent, 1999), albeit not all (McDowell et al., 2001; Strigini and Cohen, 2000) investigators. The third mechanism that has been considered includes serial passage through neighboring cells through GPI-anchored proteoglycans (glypicans) (The et al., 1999). GPI-linked proteins are inserted in only the outer leaflet of the plasma membrane, and have been shown to transfer from the plasma membrane of one cell to another when these cells are in contact, by flip-flap between adjacent outer leaflets.

We still do not know by which mechanism morphogen diffusion occurs in an intact organism. Our observation that morphogen signaling and protein levels are reduced in the mutant clone, and morphogens accumulated in front of the mutant clones, led us to suppose that HSPGs create an environment that supports the efficient movement of much of the morphogen molecules as scaffolds. However, we cannot exclude the possibility that other systems such as 'bucket brigade' or 'planar transcytosis' would require the recognition by HSPGs on cell surfaces for cell-to-cell transfer. In addition, we also observed a little leakage of morphogen molecules and signaling in the EXT mutant clones. A possible explanation accounting for these observations might be that in our EXT mutant clones a little amount of HSPGs is still produced, and they barely contribute the morphogen diffusion. Alternatively, other transport mechanisms that do not require HSPGs may play supplementary roles. In either way, all movement of the morphogens must occur within the contiguous plane of this surface environment, because tissues such as wing imaginal discs are folded in numerous places, and no evidence for skipping of morphogens over folds of epithelia was found. More work is necessary to find out how the morphogen gradient is established and maintained in developing organisms.

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