Distinct and collaborative roles of *Drosophila* EXT family proteins in morphogen signalling and gradient formation

Chun Han[1,2,*], Tatyana Y. Belenkaya[1,*], Marat Khodoun[1], Miyuki Tauchi[1,3], Xinda Lin[1] and Xinhua Lin[1,2,3,†]

[1]Division of Developmental Biology, Cincinnati Children's Hospital Medical Center, Cincinnati, OH 45229, USA
[2]Graduate Program in Molecular and Developmental Biology, University of Cincinnati College of Medicine, Cincinnati, OH 45229, USA
[3]Graduate Program in Neuroscience, University of Cincinnati College of Medicine, Cincinnati, OH 45229, USA
*Both authors contributed equally to this work
†Author for correspondence (e-mail: linyby@chmcc.org)

Accepted 15 December 2004

Development 131, 1563-1575
Published by The Company of Biologists 2004
doi:10.1242/dev.01051

Summary

Heparan sulfate proteoglycans (HSPG) have been implicated in regulating the signalling activities of secreted morphogen molecules including Wingless (Wg), Hedgehog (Hh) and Decapentaplegic (Dpp). HSPG consists of a protein core to which heparan sulfate (HS) glycosaminoglycan (GAG) chains are attached. The formation of HS GAG chains is catalyzed by glycosyltransferases encoded by members of the EXT family of putative tumor suppressors linked to hereditary multiple exostoses. Previous studies in *Drosophila* demonstrated that *tout-velu* (*ttv*), the *Drosophila EXT1*, is required for Hh movement. However, the functions of other EXT family members are unknown. We have identified and isolated the other two members of the *Drosophila* EXT family genes, which are named *sister of tout-velu* (*sotv*) and *brother of tout-velu* (*botv*), and encode *Drosophila* homologues of vertebrate EXT2 and EXT-like 3 (EXTL3), respectively. We show that both Hh and Dpp signalling activities, as well as their morphogen distributions, are defective in cells mutant for *ttv*, *sotv* or *botv* in the wing disc. Surprisingly, although Wg morphogen distribution is abnormal in *ttv*, *sotv* and *botv*, Wg signalling is only defective in *botv* mutants or *ttv-sotv* double mutants, and not in *ttv* nor *sotv* alone, suggesting that Ttv and Sotv are redundant in Wg signalling. We demonstrate further that Ttv and Sotv form a complex and are co-localized in vivo. Our results, along with previous studies on Ttv, provide evidence that all three *Drosophila* EXT proteins are required for the biosynthesis of HSPGs, and for the gradient formation of the Wg, Hh and Dpp morphogens. Our results also suggest that HSPGs have two distinct roles in Wg morphogen distribution and signalling.

Key words: Heparan sulfate proteoglycans, Exostosin (EXT), *tout-velu* (*ttv*), *botv of tout-velu* (*botv*), *sister of tout-velu* (*sotv*), Wingless (Wg), Hedgehog (Hh), Decapentaplegic (Dpp), *Drosophila*

Introduction

Secreted signalling molecules of the Wnt/Wingless (Wg), Hedgehog (Hh) and transforming growth factor β (TGF-β)/Decapentaplegic (Dpp) families function as organizers to control growth and pattern formation of tissues during animal development (Cadigan, 2002; Gurdon and Bourillot, 2001; Lawrence and Struhl, 1996; Teleman et al., 2001; Vincent and Dubois, 2002). Studies in *Drosophila* have demonstrated that Wg, Hh and Dpp act as morphogens to specify positional information during wing development. In the wing disc, Wg is required for dorsal (D)/ventral (V) patterning and wing margin specification, whereas Hh and Dpp are responsible for anterior (A)/posterior (P) patterning. In the DV axis, Wg is expressed in a narrow stripe of cells at the DV border and emanates from the DV border to form an extracellular gradient (Strigini and Cohen, 2000). Wg acts in a concentration-dependent manner to directly trigger a graded transcriptional response of its target genes (Neumann and Cohen, 1997; Zecca et al., 1996). In the AP axis, Hh and Dpp function as short- and long-range morphogens, respectively, to organize the anteroposterior patterning of the wing. Hh protein is exclusively expressed in the P compartment and moves into a stripe of A cells adjacent to the AP border to induce expression of its target genes including *dpp* (Basler and Struhl, 1994; Capdevila and Guerrero, 1994; Posakony et al., 1990; Tabata and Kornberg, 1994). Dpp moves bi-directionally into both the A and P compartments, and functions as a long-range morphogen to activate the expression of its target genes in a concentration-dependent manner (Entchev et al., 2000; Lecuit et al., 1996; Nellen et al., 1996; Tanimoto et al., 2000; Teleman and Cohen, 2000). Although the functions of Wg, Hh and Dpp as morphogens are well established, little is known about how their extracellular gradients are generated and how their morphogen concentrations are interpreted into their signalling outputs.

In the past few years, genetic studies in *Drosophila* have demonstrated the crucial roles of heparan sulfate proteoglycans (HSPG) in signalling events controlled by secreted Wg, Hh and Dpp morphogens (Lander and Selleck, 2000; Lin and Perrimon, 2000; Nybakken and Perrimon, 2002; Perrimon and Bernfield, 2000). HSPGs consist of a protein core to which heparan sulfate (HS) glycosaminoglycan (GAG) chains are

attached (Bernfield et al., 1999; Esko and Selleck, 2002; Perrimon and Bernfield, 2000). The biosynthesis of HS GAG chains is initiated by the formation of a GAG-protein linkage region consisting of a tetrasaccharide (-GlcAβ1-3Galβ1-3Galβ1-4Xylβ-*O*-) attached to specific serine residues in a proteoglycan core protein (Bernfield et al., 1999; Esko and Selleck, 2002). Following the transfer of α-GlcNAc as the first N-acetylhexosamine unit to this linkage region, HS co-polymerases add alternating β1-4-linked GlcA and α1-4-linked GlcNAc residues, generating HS GAG chains of 100 or more sugar units in length (Esko and Selleck, 2002). Biochemical studies have demonstrated that both the attachment of the first α-GlcNAc to the GAG-protein linkage region and the subsequent polymer formation are catalyzed by members of the hereditary multiple exostoses (EXT) gene family of tumor supressors (Esko and Selleck, 2002; Zak et al., 2002). In vertebrates, the EXT gene family consists of *EXT1*, *EXT2*, and three EXT-like genes designated *EXTL1*, *EXTL2* and *EXTL3* (Zak et al., 2002). Human mutations in *EXT1* and *EXT2* are associated with hereditary multiple exostoses (HME), a benign bone tumor characterized by multiple cartilage-capped outgrowths of various bones (Ahn et al., 1995; Stickens et al., 1996). However, three EXT-like genes have not been demonstrated to be linked to genetic disorder(s). A number of biochemical studies have shown that EXT1 and EXT2 function as HS co-polymerases involved in HS polymerization (Lind et al., 1998; McCormick et al., 2000; McCormick et al., 1998; Senay et al., 2000; Wei et al., 2000). Recent biochemical studies also demonstrated that both *EXTL2* and *EXTL3* proteins possess enzymatic activities that can transfer α-GlcNAc to the GAG-protein linkage region and to intermediates of chain polymerization, suggesting roles for these proteins in initiation and polymerization reactions (Kim et al., 2001; Kitagawa et al., 1999; Zak et al., 2002). Despite intensive biochemical studies of the EXT family proteins in the HS GAG biosynthesis, their relationship in HS GAG biosynthesis and, in particular, their respective in vivo roles in development are largely unknown.

The *Drosophila* genome contains three EXT family members. Previous studies have shown that Tout-velu (Ttv), the *Drosophila* homologue of mammalian EXT1, is required for Hh signalling (Bellaiche et al., 1998; The et al., 1999). Hh movement in the wing disc is defective in cells mutant for *ttv.* Further study demonstrated that only cholesterol-modified Hh (Hh-Np), and not cholesterol-unmodified (Hh-N), is dependent on Ttv function for its movement (The et al., 1999). Consistent with this observation, a recent study showed that the movement of large punctate structures containing Hh-Np across cells is contingent upon the activity of Ttv (Gallet et al., 2003). The involvement of Ttv in HS GAG biosynthesis was also demonstrated. HS GAG is strikingly reduced, but not completely eliminated, in the *ttv* null embryo (The et al., 1999). Biochemical analysis further showed that HS GAG is markedly reduced in *ttv* mutant larvae (Toyoda et al., 2000). Together, these studies have demonstrated that Ttv is required for Hh movement and involved in the HS GAG biosynthesis. Interestingly, it was shown that Ttv is required specifically for Hh, but not for Wg and Fgf signalling (The et al., 1999), raising the question of whether the other two *Drosophila* EXT members play partially redundant roles with Ttv in signalling pathways other than Hh.

To understand the molecular mechanisms by which Wg, Hh and Dpp morphogen gradients are regulated during wing development, we have conducted a genetic screen for mutations associated with specific wing patterning defects (Belenkaya et al., 2002). In this paper, we report the identification and characterization of *sister of tout-velu* (*sotv*; *Ext2* – FlyBase) and *brother of tout-velu* (*botv*), encoding *Drosophila* homologues of mammalian EXT2 and EXTL3, respectively. We show that Hh signalling and its distribution are defective in either *sotv* or *botv* mutant cells. We further demonstrate that all three *Drosophila* EXT proteins (*ttv*, *botv* and *sotv*) are essential for Dpp signalling and its morphogen distribution. Surprisingly, although all three *Drosophila* EXT proteins are required for the proper extracellular Wg distribution, Wg signalling is only defective in *botv* mutant or *ttv-sotv* double mutant cells, but not in *ttv* nor *sotv* mutant cells. We provide further biochemical evidence that Ttv and Sotv form a complex and are co-localized in vivo. Our results provide new insights into the functions of the EXT family proteins in morphogen signalling during development.

Materials and methods

Genetic screen, mapping and identification of *botv* and *sotv* mutations

tout-velu (*ttv*), *brother of tout-velu* (*botv*) and *sister of tout-velu* (*sotv*) were isolated from a F1 genetic screen (Belenkaya et al., 2002). Approximately 200,000 F1 flies were screened, leading to the isolation of 9 *ttv*, 23 *botv* and 4 *sotv* alleles. ttv^{63} is a putative null, containing a C to T transition, which leads to a nonsense mutation at 224R and therefore a deletion of most of the protein. *botv* was mapped to the cytological interval 56A-56C by using deficiencies Df(2R)P34 and Df(2R)PC4. Mutations of *botv* alleles in CG15110 were subsequently identified by sequencing DNA from homozygous *botv* larvae. $botv^{103}$ is a putative null and has a C to T transition, resulting in a nonsense mutation at 169Q. *sotv* failed to complement deficiencies Df(2R)Jp8 and Df(2R)Jp4, and was mapped to 52F. Mutations in CG8433 were identified from *sotv* alleles by sequencing. $sotv^{44}$ has a G to A transition at the start codon of CG8433. The second Met (ATG) of CG8433 in $sotv^{44}$ is at AA195, and is unlikely to be an alternative start codon for a functional protein as it is located after the signal peptide and the transmembrane domain. Therefore $sotv^{44}$ is considered as a null. Consistent with this, cuticle defects associated with $sotv^{44}$ null embryos are as severe as those of $sotv^{44}$/Df(2R)Jp8 or $sotv^{44}$/Df(2R)Jp4.

Generation of marked clones for phenotypic analysis

Females with germline clones were generated using the autosomal 'FLP-DFS' technique (Chou and Perrimon, 1996) as described (Belenkaya et al., 2002; Hacker et al., 1997). Imaginal disc clones of mutant cells were generated as described (Belenkaya et al., 2002; Hacker et al., 1997). To induce the expression of DsRed marker, third-instar larvae were subsequently subjected to a second heat shock for 90 minutes at 37°C and allowed to recover for 5 hours at room temperature before fixation and immunostaining. Below, we list the genotypes used in our analyses.

(1) ttv^{63}, $sotv^{44}$, and $botv^{103}$ clones marked by the absence of GFP (Figs 3, 4; Fig. 5B-D′, Fig. 6B-H″):

y w hsp70-flp/+ or *Y; FRT*^{G13} *ubiquitin-GFP M(2)58F / FRT*^{G13} ttv^{63};

y w hsp70-flp/+ or *Y; FRT*^{G13} *ubiquitin-GFP M(2)58F / FRT*^{G13} $sotv^{44}$; and

y w hsp70-flp/+ or *Y; FRT*^{G13} *ubiquitin-GFP M(2)58F / FRT*^{G13} $botv^{103}$.

(2) ttv^{63}-$sotv^{44}$ clones marked by the absence of GFP (Fig. 6I-J′):

y w hsp70-flp/+ or *Y; FRT^{G13} ubiquitin-GFP M(2)58F / FRT^{G13} ttv^{63} sotv^{44}*.

(3) For GFP-Dpp in a wild-type background (Fig. 5E):

w; UAS-GFP-Dpp/+; *dpp^{Gal4}*/+.

(4) For GFP-Dpp in wing discs bearing clones of *ttv^{63}*, *sotv^{44}* and *botv^{103}*, marked by the absence of DsRed (Fig. 5F-H′):

y w hsp70-flp; FRT^{G13} hsp70-DsRed / FRT^{G13} ttv^{63} UAS-GFP-Dpp; dpp^{Gal4}/+;

y w hsp70-flp; FRT^{G13} hsp70-DsRed / FRT^{G13} sotv^{44} UAS-GFP-Dpp; dpp^{Gal4}/+; and

y w hsp70-flp; FRT^{G13} hsp70-DsRed / FRT^{G13} botv^{103} UAS-GFP-Dpp; dpp^{Gal4}/+.

Antibody and X-gal staining

Fixation of embryos and imaginal discs as well as antibody staining procedure were performed as described (Belenkaya et al., 2002; Hacker et al., 1997). For antibody staining in *Drosophila Schneider's* S2 cells, cells were fixed in PBS with 2% formaldehyde for 15 minutes and then subjected to the same antibody staining procedure as for disc staining. X-gal staining was as described (Sullivan et al., 2000). HS GAG staining using 3G10 antibody was performed as described (The et al., 1999), except that the heparinase III treatment was shortened to 6 hours. Extracellular Wg staining was performed as described (Baeg et al., 2001; Strigini and Cohen, 2000). Primary antibodies were used at the following dilutions: rabbit anti-pMad (PS1) at 1:5000 (Persson et al., 1998; Tanimoto et al., 2000); rabbit anti-Wg at 1:500 (Reichsman et al., 1996); rat anti-Ci at 1:10 (Motzny and Holmgren, 1995); rabbit anti-Hh at 1:2000 (Taylor et al., 1993); guinea pig anti-Sens at 1:2000 (Nolo et al., 2000); mouse anti-Dll at 1:500 (Duncan et al., 1998); mouse anti-Engrailed 4D9 at 1:500 (Iowa Developmental Studies Hybridoma Bank; IDSHB); mouse anti-Wg 4D4 at 1:3 (IDSHB); rabbit anti-GFP Alexa Fluor 488 at 1:1000 (Molecular Probe); rabbit anti-DsRed at 1:4000 (Clontech); mouse anti-ΔHS 3G10 at 1:100 (Seikagaku Corporation); rat anti-HA 3F10 at 1:1000; mouse anti-Myc 9E10 at 1:500 (Roche Molecular Biochemicals); mouse anti-V5 at 1:200 (Invitrogen); rabbit anti-canine Calnexin at 1:100 (Stressgen); and mouse anti-*Drosophila* Golgi at 1:100 (Calbiochem).

Molecular biology

The *botv* full-length cDNA was isolated by screening a 0-to-4 hour *Drosophila* embryonic cDNA library (Brown and Kafatos, 1988). The coding region of *botv* cDNA with three haemagglutinin (HA) tags in-frame at its C terminus was then cloned into the pUAST vector to generate pUAST-botv-HA. The *sotv* cDNA was obtained from EST cDNA clone GH02288 (Invitrogen). The V5-tagged Sotv construct was generated by cloning the coding region into the *Kpn*I-*Eco*RV site of pAc5.1 V5-His C vector (Invitrogen), in-frame with the V5 tag. To generate the pAc5.1-ttv-myc construct, the *ttv-myc* fragment was amplified by PCR from the genomic DNA of the *UAS-ttv-myc* line (The et al., 1999) and subcloned into the pAc5.1V5-His A vector. HS-DsRed was generated by cloning the full-length (*Bam*HI-*Spec*I) DsRed T1 coding region from pDsRed expression vector (Clontech) into *Bgl*II-*Xba*I sites of the pCasperR-hs vector.

Immunoprecipitation and western blotting

Drosophila Schneider's S2 cells (1×10^7) were transfected with 10 μg of corresponding expression vectors by the calcium phosphate precipitation method. For the induction of Botv-HA cloned in pUAST vector, 10 μg pUAST-botv-HA and 10 μg of pArmadillo-Gal4 (Klueg et al., 2002) was co-transfected. Cells were harvested 60 hours later, and lyzed in 1.5 ml of 20 mM Tris-HCl (pH 7.4), 2% Triton X-100, 150 mM NaCl and 7.5 ml proteinase inhibitor tablet (Roche Molecular Biochemicals) on ice for 20 minutes. After clearance, one half of each lysate was used for immunoprecipitation with 1 μg antibodies for 3 hours at 4°C and then incubated for an additional 1.5 hours in the presence of 12.5 μl bed volume of protein G sepharose (Amersham Pharmacia). Immunoprecipitates were washed three times with 10 mM Tris-HCl (pH 7.4), 0.2% Triton X-100, 150 mM NaCl, 2 mM EDTA and 1 μl proteinase inhibitor (Sigma), and twice with 10 mM Tris-HCl (pH 7.4). Western blotting was carried out as described (Belenkaya et al., 2002).

Results

brother of tout-velu (*botv*) and *sister of tout-velu* (*sotv*) are two new segment-polarity genes

To identify novel genes involved in the signalling events mediated by the Wg, Hh and Dpp morphogens during wing patterning, we conducted a genetic screen using a wing specific Flpase/FRT system (Belenkaya et al., 2002). Three independent loci on the second chromosome were recovered and exhibit similar wing defects, including fusion of longitudinal veins L3 and L4, vein deletions (Fig. 1A-D) and wing nicks (data not shown). One of loci was identified as *tout-velu* (*ttv)* (Fig. 1B) (see Materials and methods). On the basis of similar wing phenotypes associated with these loci, we named other two loci as *brother of tout-velu* (*botv*) and *sister of tout-velu* (*sotv*).

Previous studies have demonstrated that Ttv is required for Hh signalling during embryogenesis and development of the wing (Bellaiche et al., 1998; The et al., 1999). To further characterize *botv* and *sotv*, we examined the embryonic cuticle patterns associated with these mutants using null alleles (see Materials and methods). Animals zygotically mutant for either *botv* or *sotv* survive until the third instar larval stage and appear to have normal cuticle patterning (data not shown). However, homozygous mutant embryos derived from females lacking germline *botv* activity (referred to as *botv* null embryos) die with a strong segment polarity phenotype (Fig. 1G). Homozygous mutant embryos derived from females lacking germline *sotv* activity (referred to as *sotv* null embryos) also die with segment polarity phenotypes, albeit relatively weak and variable (Fig. 1H,I). In the ventral embryonic ectoderm, Wg and Hh signalling are required for normal expression of *engrailed* (*en*) and *wg* (Hatini and DiNardo, 2001). In *botv* and *sotv* null embryos, stripes of En and Wg expression were lost from the ectoderm by stage 11 (Fig. 1K,L,N,O). Taken together, these results suggest that both *botv* and *sotv* are segment-polarity genes, and are likely to be involved in Hh and/or Wg signalling.

botv and *sotv* encode the *Drosophila* homologues of mammalian EXTL3 and EXT2, respectively, and are required for HS GAG biosynthesis

botv and *sotv* were subsequently mapped to the cytological positions 56A-56C and 52F, respectively (see Materials and methods). Searches of annotated *Drosophila* genome databases identified a *Drosophila* EXT-like gene (CG15110) in 56A-56C and a *Drosophila* EXT2 (*DEXT2*; CG8433) in 52F. The *Drosophila* genome contains three EXT genes including *ttv*, CG15110 and CG8433. Based on the similarities of *botv* and *sotv* with *ttv* in both wing and embryonic cuticle defects, we suspected that the CG15110 and CG8433 transcripts may encode Botv and Sotv, respectively. Two lines of evidence strongly suggest that this is indeed the case. First, we used the RNA interference (RNAi) method (Kennerdell and Carthew, 1998) to perturb

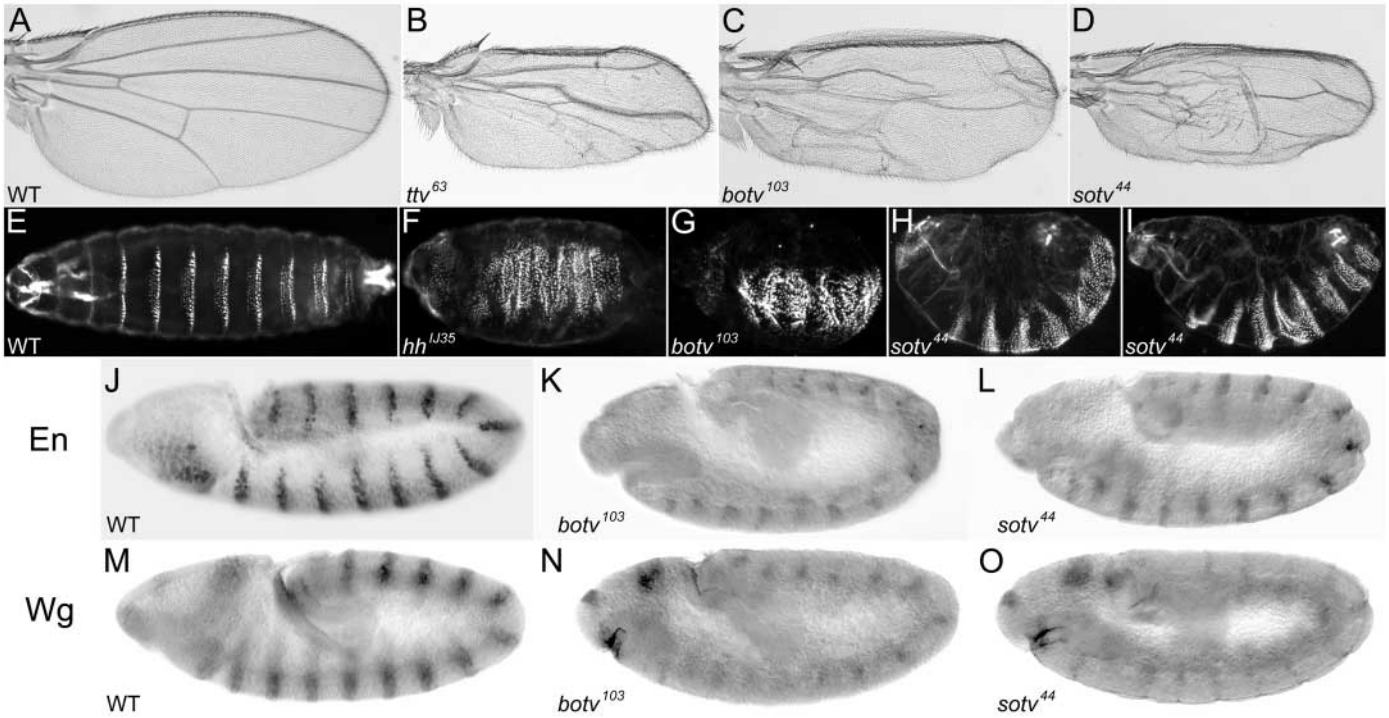


Fig. 1. Identification of *botv* and *sotv* as two novel segment-polarity genes. (A-D) Wings are oriented proximal to the left, anterior up. (A) A wild-type wing. (B-D) Wings bearing somatic clones of ttv^{63} (B), $botv^{103}$ (C) and $sotv^{44}$ (D). Adult wings with clones of these mutations exhibit a variety of phenotypes, including vein loss, vein fusion, blister, narrowed wing and wing notching (data not shown). (E-I) Cuticle preparations of a wild-type embryo (E), a hh^{IJ35} homozygous embryo (F), and embryos derived from mutant germ-line clones of $botv^{103}$ (G) and $sotv^{44}$ (H,I). All embryos are oriented anterior to the left. Embryos lacking both maternal and zygotic activities of *botv* or *sotv* exhibit typical segment-polarity phenotypes; however, the defects of *sotv* mutants can be relatively weak (I). (J-L) En staining of a stage 10 wild-type embryo (J), and embryos derived from mutant germ-line clones of $botv^{103}$ (K) and $sotv^{44}$ (L). (M-O) Wg staining of a stage 10 wild-type embryo (M), and embryos derived from mutant germ-line clones of $botv^{103}$ (N) and $sotv^{44}$ (O).

CG15110 and CG8433 transcripts. Embryos injected with either CG15110 or CG8433 double-stranded RNA showed segment-polarity defects (data not shown). Second, all the sequenced alleles of *botv* and *sotv* have mutations in the CG15110 and CG8433 genes, respectively (selected alleles are shown in Fig. 2A).

We generated a phylogenetic tree of the EXT family members among humans, mouse and *Drosophila* based on their amino acid sequences (Fig. 2B). Ttv is most similar to EXT1, whereas Sotv and Botv are more closely related with EXT2 and EXTL3, respectively. Ttv is 31.4% and 33.5% identical to Botv and Sotv proteins, respectively. In particular, all three *Drosophila* EXT members shared high amino acid identity in their C-terminal regions (Fig. 2C).

Biochemical studies have demonstrated that EXT family proteins are required for HS GAG biosynthesis (Esko and Selleck, 2002; Zak et al., 2002). Biosynthesis of HS GAG chains is strikingly reduced in *ttv* mutant embryos (The et al., 1999) and larvae (Toyoda et al., 2000). We analyzed levels of HS GAG chains in clones mutant for *ttv*, *botv* and *sotv* in the wing disc. In wild-type cells, HS GAG staining was found in punctate particles as well as on the membrane (Fig. 3). Consistent with previous studies in embryos, HS GAG staining in *ttv* mutant clones was strikingly reduced (Fig. 3A,A′), suggesting that Ttv is required for HS GAG biosynthesis in the wing disc as well. We also observed similar reductions in HS GAG staining in cells mutant for *sotv* (Fig. 3B,B′) or *botv* (Fig. 3C,C′). On the basis of these observations, we conclude that all three *Drosophila* EXT proteins are indispensable for the biosynthesis of HS GAG chains.

Botv and Sotv are required for Hh signalling in the wing disc

Previous studies have demonstrated the essential role of Ttv in Hh signalling in the wing disc (Bellaiche et al., 1998). Hh movement is blocked in A compartment cells mutant for *ttv* (Bellaiche et al., 1998). Two lines of evidence support the idea that Botv and Sotv are also required for Hh signalling. First, the level of Cubitus interruptus (Ci) is stabilized by Hh signalling in about 8-10 cells in the AP border (Aza-Blanc et al., 1997; Methot and Basler, 1999; Motzny and Holmgren, 1995). However, Ci stabilization is strikingly reduced in anterior cells mutant for *sotv* (Fig. 4B,B′) or *botv* (Fig. 4C,C′). Second, whereas Hh proteins are present as punctate particles in anterior cells at the AP border, these punctate particles are absent within *sotv* (Fig. 4E,E′) and *botv* clones (Fig. 4F,F′), except in the first row of cells facing the P compartment. These results suggest that similar to Ttv, Sotv and Botv activities are required for Hh movement in its receiving cells. In the absence of either Sotv or Botv, Hh can only move into the first row of cells immediately adjacent to the Hh-expressing cells, and fails to move further.

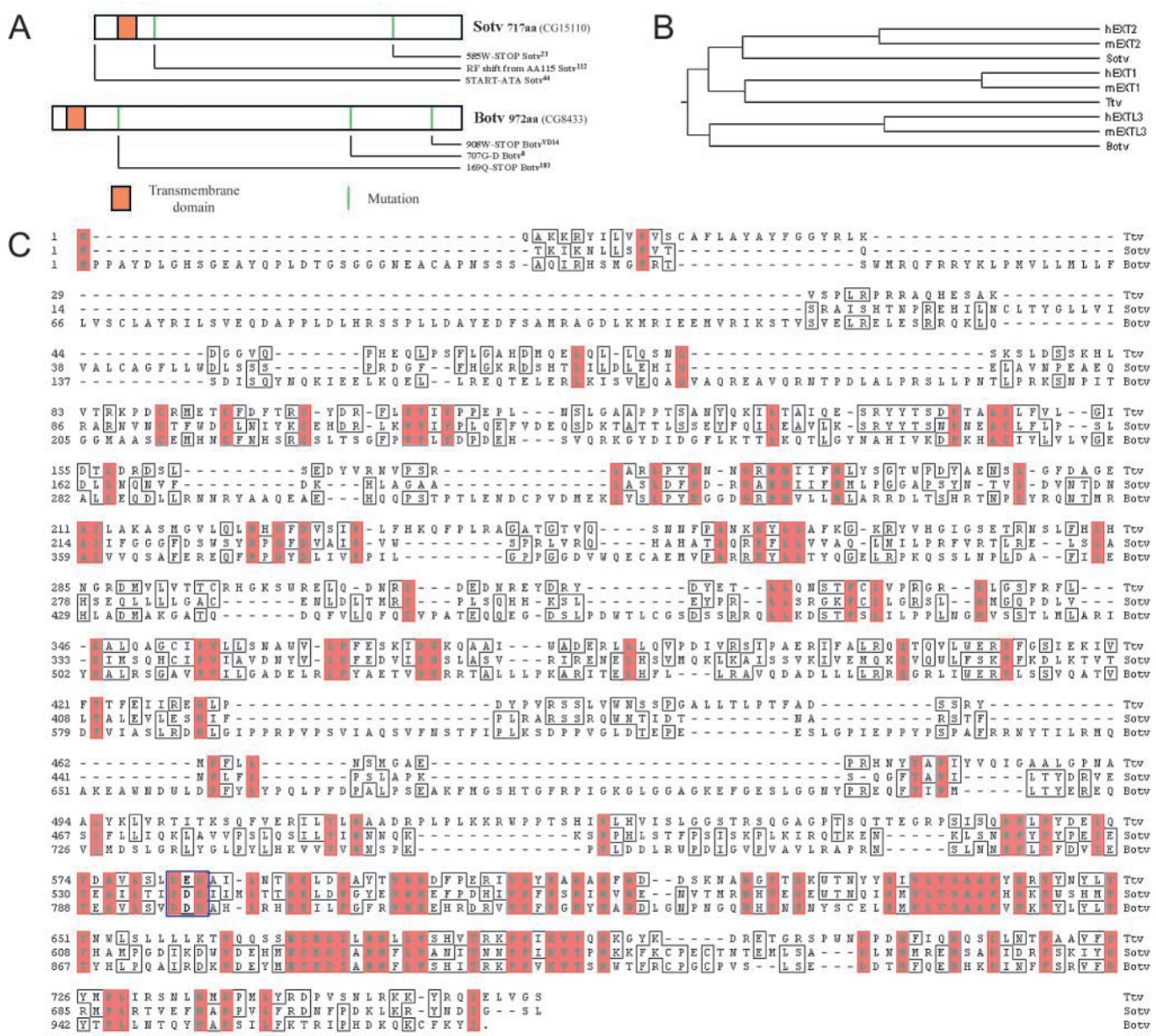


Fig. 2. Sotv and Botv are members of the *Drosophila* EXT family of tumor suppressors. (A) Structures and mutations of Sotv and Botv. Sotv and Botv are putative type II transmembrane proteins. Three mutations of *botv* and *sotv* are shown in green bars (except *sotv*^{44}, which harbors a G to A transition at the start codon). (B) Phylogenetic tree of *Drosophila*, mouse (m), and human (h) EXT family proteins. Ttv, Sotv and Botv are the *Drosophila* EXT1, EXT2 and EXTL3 proteins, respectively. (C) Sequence comparison of Ttv, Sotv and Botv. Identical residues are highlighted (light red) and consensus residues are boxed. A conserved nucleotide sugar-binding motif D*X*D is found at the C-terminal portion of all three proteins and is boxed (blue). The sequence alignment and phylogenetic tree were generated using Lasergene software with the Jotun Hein method.

Ttv, Sotv and Botv are required for Dpp signalling and its gradient distribution

In the wing disc, Dpp functions as a long-range morphogen to control the growth and patterning of cells in the AP axis (Lecuit et al., 1996; Nellen et al., 1996). Dpp signalling is shown to activate its downstream signalling component Mad in a concentration-dependent manner (Tanimoto et al., 2000). Recently, the Dpp morphogen gradient has been visualized directly using GFP-Dpp fusion proteins that retain signalling activity (Entchev et al., 2000; Teleman and Cohen, 2000).

Vein deletions are the most striking defects associated with wing-bearing clones mutant for *ttv*, *sotv* and *botv* (Fig. 1B,C,D). As Dpp signalling is required for vein formation (de Celis et al., 1996; Ray and Wharton, 2001), we investigated whether Dpp signalling and its gradient distribution were defective in clones mutant for *ttv*, *sotv* and *botv*. We first examined Dpp signalling activity by visualizing the activated form of Mad (p-Mad), which is phosphorylated by the activated Dpp receptor Thickveins (Tkv) in response to Dpp signalling (Tanimoto et al., 2000). In the wild-type wing disc (Fig. 5A), p-Mad levels were high in the central region of the wing disc and gradually decline towards the A and P distal cells. p-Mad levels were lower at the AP boundary owing to the reduced expression of *tkv* (Tanimoto et al., 2000). p-Mad

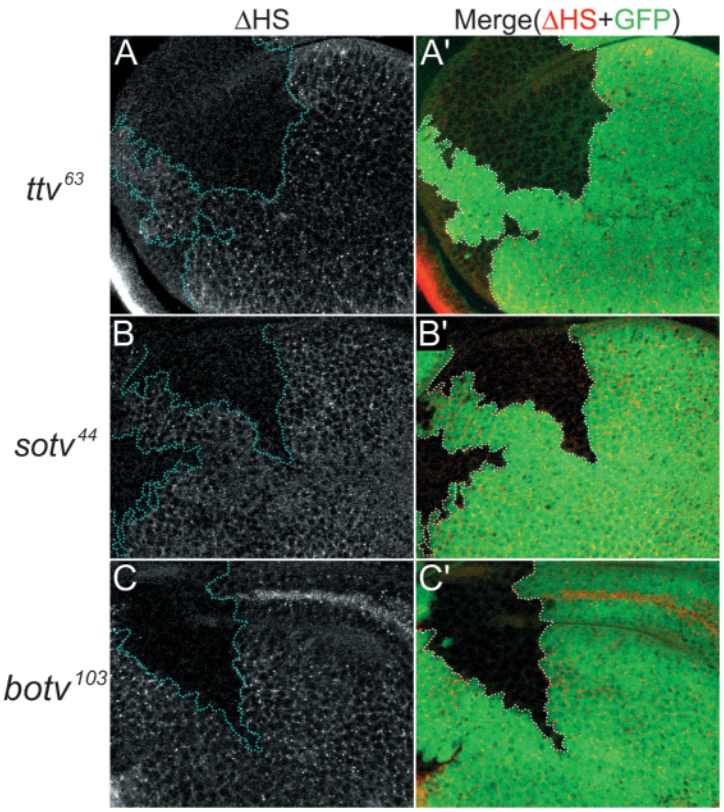


Fig. 3. The *Drosophila* EXT proteins are required for the biosynthesis of HS GAG chains in vivo. Third instar larval wing imaginal discs carrying mutant clones of *ttv*^{63} (A,A′), *sotv*^{44} (B,B′) and *botv*^{103} (C,C′) were fixed, digested with bacterial heparinase III and then stained with mAb 3G10, which recognizes the epitope generated by heparinase III digestion. The mutant clones are marked by the absence of GFP and are outlined with dots. 3G10 staining is absent in mutant clones of *ttv*^{63}, *sotv*^{44} and *botv*^{103}.

levels were strikingly reduced in either A or P cells mutant for *ttv*, *sotv* or *botv* (Fig. 4B-D′), providing evidence that all three *Drosophila* EXT proteins are required for Dpp signalling. We further tested whether the EXT proteins control Dpp signalling by regulating Dpp morphogen distribution. For this purpose, we expressed GFP-Dpp in the endogenous *dpp* expression domain using *Dpp*^{GAL4} and analyzed GFP-Dpp distribution in clones mutant for *ttv*, *sotv* or *botv*. In the wild-type background, GFP-Dpp exhibits a gradient pattern in both the A and P compartment (Fig. 5E). However, levels of GFP-Dpp are reduced in clones mutant for *ttv*, *sotv* or *botv* (Fig. 5F-H′). Together, these results argue that Ttv, Sotv and Botv promote Dpp signalling by modulating its morphogen distribution.

Distinct roles of Botv from those of Ttv and Sotv in Wg signalling in the wing disc

In the wing disc, Wg forms a long-range gradient and acts both at short and long range to regulate the expression of

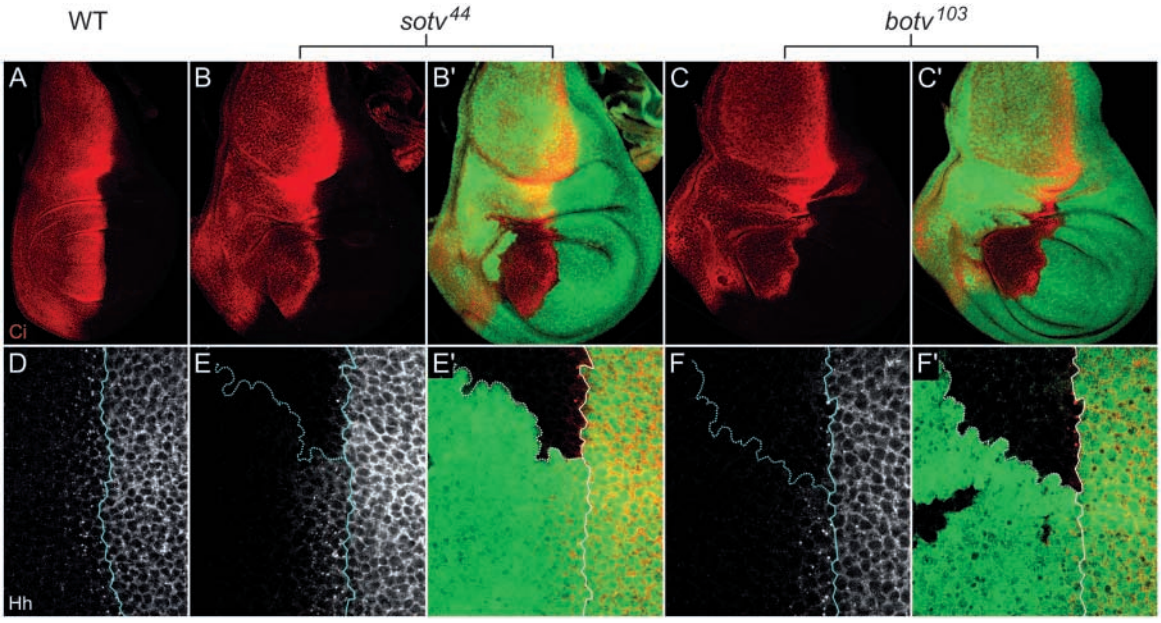


Fig. 4. Involvement of *sotv* and *botv* in Hh signalling. All discs are oriented anterior left, dorsal up. (A-C′) Ci staining (red) in a wild-type wing disc (A), and in discs carrying mutant clones of *sotv*^{44} (B,B′) and *botv*^{103} (C,C′) in the anterior compartment. The mutant clones are marked by the absence of GFP and are outlined with dots. Within the large clones of *sotv*^{44} and *botv*^{103}, accumulated Ci is seen only in a narrow stripe of cells abutting the AP boundary. (D-F′) Hh staining in a wild-type wing disc (D), and in discs carrying mutant clones of *sotv*^{44} (E,E′) or *botv*^{103} (F,F′). The AP boundaries are determined by Ci staining (data not shown) and are marked by lines. Clone boundaries are marked by dotted lines. Hh staining is absent in the clones of *sotv*^{44} and *botv*^{103}, except at a residual level in the posterior-most row of cells adjacent to AP boundary.

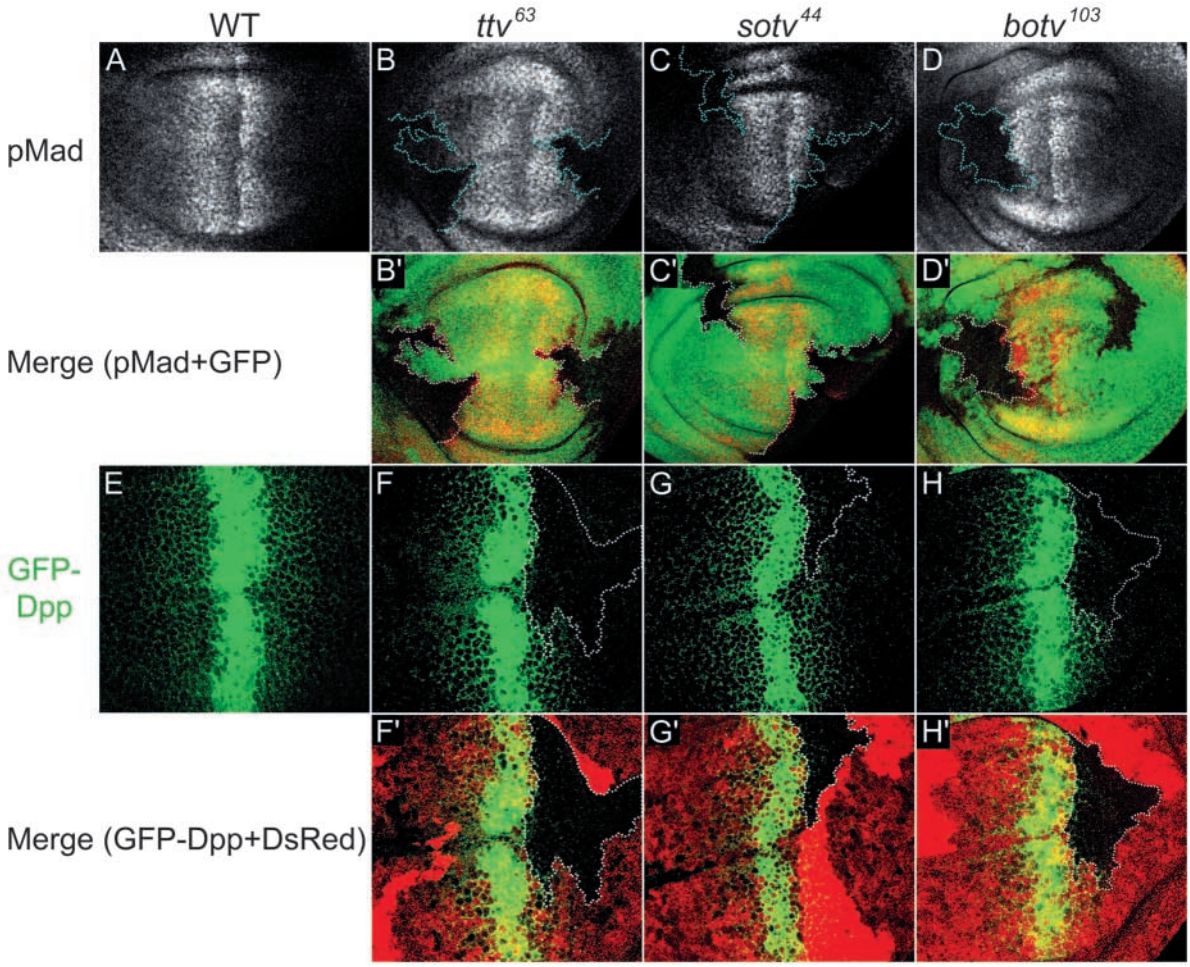


Fig. 5. Involvement of the *Drosophila* EXT genes in Dpp signalling. All discs are oriented anterior left, dorsal up. (A-D′) p-Mad staining in a wild-type wing disc (A), and in discs carrying mutant clones of ttv^{63} (B,B′), $sotv^{44}$ (C,C′) and $botv^{103}$ (D,D′). The mutant clones are marked by the absence of GFP and are outlined with dots. (E-H′) *UAS-GFP-dpp* under the control of dpp^{Gal4} in an otherwise wild-type wing disc (E) and in discs carrying mutant clones of ttv^{63} (F,F′), $sotv^{44}$ (G,G′) and $botv^{103}$ (H,H′). The mutant clones are marked by the absence of DsRed and are outlined with dots. The distribution of GFP-Dpp outside the Dpp expression domain in the wild-type background appears to be a gradient extending towards the A and P compartments. Within the mutant clones of ttv^{63}, $sotv^{44}$ and $botv^{103}$, the ranges of the GFP-Dpp gradient are greatly reduced.

several target genes in different spatial domains (Neumann and Cohen, 1997; Strigini and Cohen, 2000; Zecca et al., 1996). The homeodomain protein Distal-less (Dll) (Neumann and Cohen, 1997; Zecca et al., 1996) and the zinc-finger protein Senseless (Sens) (Nolo et al., 2000; Parker et al., 2002) are the long- and short-range targets of the Wg morphogen, respectively (Fig. 6A,A′). We have previously shown that HSPGs are required for Wg short- and long-range signalling, as well as for its extracellular distribution in the wing disc (Baeg et al., 2001; Lin and Perrimon, 1999). However, a previous study on Ttv demonstrated that Ttv is not involved in Wg signalling during embryogenesis and in the wing disc, suggesting that Ttv selectively participates in morphogen signalling (The et al., 1999). To evaluate the specificity of the three *Drosophila* EXT genes in morphogen signalling in the wing disc, we examined Wg short- and long-range signalling activities, as well as its morphogen distribution in clones mutant for *ttv*, *sotv* and *botv*.

We observed reductions in the ranges of Dll expression in *ttv*, *sotv* and *botv* mutant cells (Fig. 6B,C,D). These defects were fully penetrant, suggesting that all three EXT proteins are normally required for Wg long-range activity. Interestingly, we found that Dll levels were not reduced in regions close to the DV boundary within the clones of *ttv* and *sotv* mutant cells; however, they were significantly reduced in the same region within the *botv* mutant clone (Fig. 6B,C,D). These results suggest that both the range of Wg action and its signalling are affected in the *botv* mutant clone; however, only the range of Wg action, and not its signalling per se, is reduced in the *ttv* or *sotv* clones. Consistent with this, we found that Sens expression was diminished in *botv* clones, but not in *ttv* nor *sotv* clones, confirming that Wg signalling is defective only in the *botv* mutant, and not in *ttv* or *sotv* mutant (Fig. 6B′,C′,D′). The observed defects in the ranges of Dll expression in *ttv*, *sotv* and *botv* mutant clones could be due to reduced levels of Wg morphogen. We tested this by staining extracellular Wg. The result was in agreement with the Dll data (Fig. 6F-H′). On one hand, the range of extracellular Wg distribution was reduced

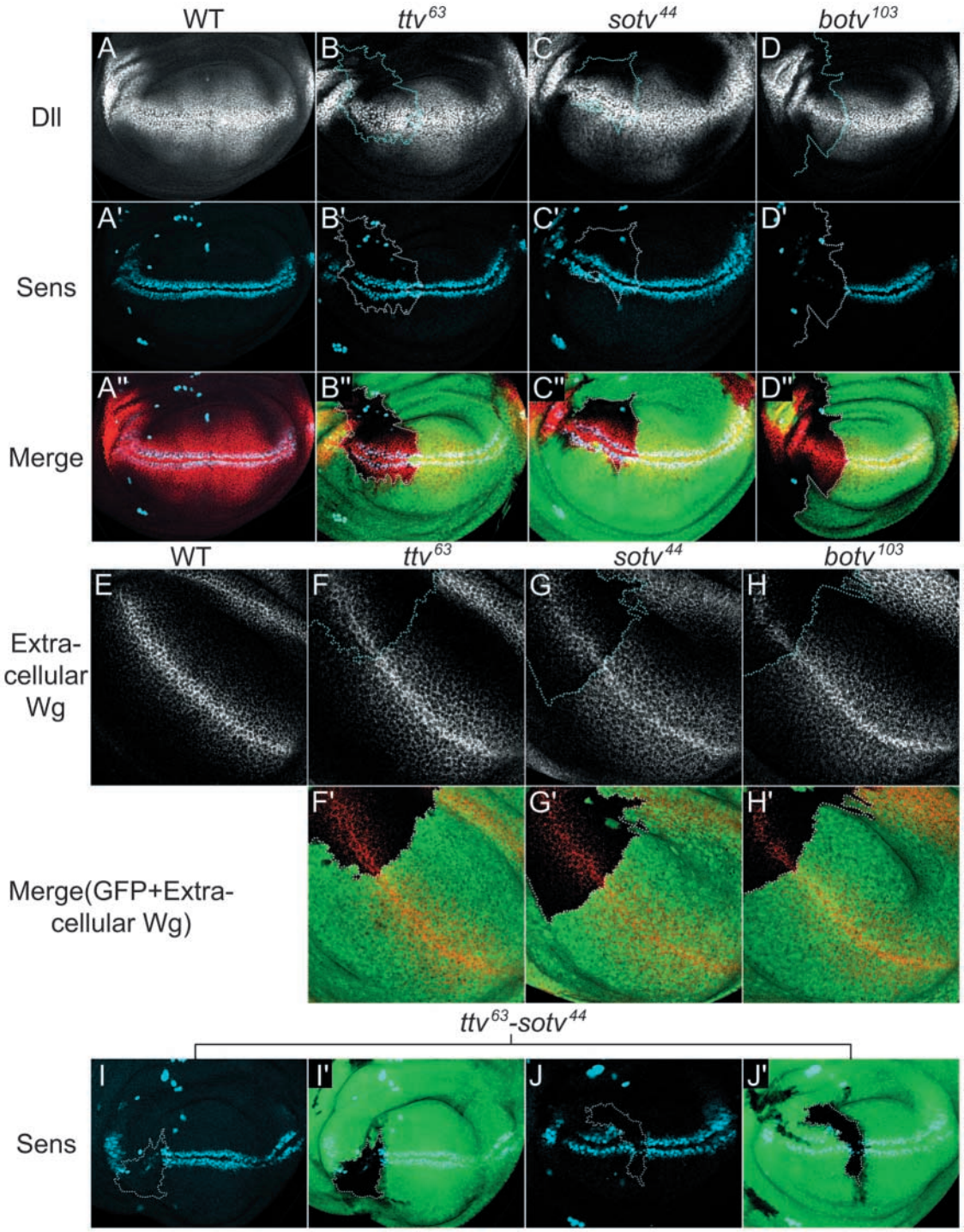


Fig. 6. Involvement of the *Drosophila* EXT genes in Wg signalling. In all discs carrying mutant clones, the clones are marked by the absence of GFP and are outlined with dots. (A-D″) Dll and Sens staining in a wild-type wing disc (A-A″), and in wing discs carrying mutant clones of *ttv*^{63} (B-B″), *sotv*^{44} (C-C″) and *botv*^{103} (D-D″). Dll expression appears to be a gradient with the peak at the DV boundary. The ranges of the Dll gradient in the mutant clones of *ttv*^{63} (B), *sotv*^{44} (C) and *botv*^{103} (D) are reduced. Note that the level of Dll expression close to the DV boundary within the mutant clones of *ttv*^{63} and *sotv*^{44} is comparable with that of wild-type cells; however, it is significantly reduced in the *botv*^{103} mutant clone. Wg-dependent Sens expression in the wing disc is in two narrow stripes abutting Wg expressing cells. This expression is unaffected in the mutant clones of *ttv*^{63} (B′) and *sotv*^{44} (C′), but is diminished in the *botv*^{103} mutant clone (D′). Note that the Wg-independent expression of Sens in *botv*^{103} mutant clone is still maintained. (E-H′) Extracellular Wg distribution is shown in a wild-type wing disc (E), and in discs carrying mutant clones of *ttv*^{63} (F,F′), *sotv*^{44} (G,G′) and *botv*^{103} (H,H′). Extracellular Wg is almost completely lost in the *botv*^{103} clone except at the surface of Wg expressing cells, whereas it is maintained at some level around the DV boundary in the mutant clones of *ttv*^{63} and *sotv*^{44}. (I-J′) Sens staining in discs bearing clones of *ttv*^{63}-*sotv*^{44} double mutant cells. The Wg-dependent Sens expression is diminished in the double mutant clones.

in clones mutant for *ttv*, *sotv* and *botv*. On the other hand, extracellular Wg was maintained at some levels in the regions close to DV boundary in the *ttv* or *sotv* clone, however, it was absent in the same regions within the *botv* clone, except at the surface of Wg-expressing cells. Together, these results suggest that all three *Drosophila* EXT proteins are required for the long-range distribution of Wg protein. However, Wg signalling is only defective in the *botv* mutant, and not in *ttv* or *sotv* mutant cells.

One apparent explanation for this is that Ttv and Sotv are functionally redundant in Wg signalling. To test this, we examined Sens expression in clones of *ttv-sotv* double mutants and found that, indeed, Sens expression was diminished (Fig. I-J′). A virtually identical result in Sens expression was observed in *ttv-sotv-botv* triple mutant cells (data not shown). Taken together, our findings suggest that all three EXT proteins are required for proper extracellular Wg distribution. However, whereas Botv is independently required for Wg signalling, Ttv and Sotv are redundant in Wg signalling.

Biochemical interactions and subcellular localization of the *Drosophila* EXT proteins

The distinct roles of Botv from those of Ttv and Sotv in Wg signalling suggest that they may have unique functions in the biosynthesis of HS GAG chains. In vertebrates, direct enzymatic assay for vertebrate EXTL3 demonstrates that its GlcNAc transferase activities are involved in both the initiation and polymerization reactions (Kim et al., 2001), whereas several biochemical studies suggested that EXT1 and EXT2 may function as a HS GAG co-polymerase in which both EXT1 and EXT2 serve as subunits essential for the activity (McCormick et al., 2000; McCormick et al., 1998; Senay et al., 2000; Wei et al., 2000; Zak et al., 2002). As Ttv and Sotv are most similar to the vertebrate EXT1 and EXT2, respectively, we anticipated that Ttv and Sotv may function as a HS GAG co-polymerase whose full activity requires both Ttv and Sotv. By contrast, Botv, a homologue of vertebrate EXTL3, may participate in the initiation step of HS GAG biosynthesis, which is distinct from the role of Ttv and Sotv.

To determine whether Ttv and Sotv function as subunits for the HS copolymerase, we performed a co-immunoprecipitation experiment to examine whether Ttv and Sotv form a complex in cells. Myc-tagged Ttv and V5-tagged Sotv were expressed either individually or in combination in *Drosophila* S2 cells. Upon immunoprecipitation of Myc-tagged Ttv from the cellular lysate of transfected cells, the Sotv protein could be detected by western blotting in the immunoprecipitate (Fig. 7A). Interestingly, we did not observe an interaction between Ttv and Sotv when cellular lysates from individually transfected cells were mixed and immunoprecipitated (Fig. 7A, lane labeled m), indicating that Ttv and Sotv cannot associate ex vivo. We further conducted similar experiments to examine the association of Botv with Ttv or Sotv (Fig. 7B,C). In these cases, no interactions were detected. These data suggest that Ttv and Sotv are physically associated in vivo, but that they do not form complexes with Botv.

We also conducted an experiment to determine the subcellular localization of Ttv, Sotv and Botv in transfected *Drosophila* S2 cells. Epitope-tagged protein constructs were transfected individually or in combination into *Drosophila* S2 cells. Consistent with previous result in embryos (The et al., 1999), Myc-tagged Ttv proteins were present in both endoplasmic reticulum (ER) and Golgi (Fig. 7D-E″). Sotv and Botv were also localized in both ER and Golgi (data not shown) in cells transfected with either V5-tagged Sotv or HA-tagged Botv. Interestingly, in cells transfected with all three EXT proteins (Fig. 7F-F‴), we found that Ttv and Sotv protein staining were virtually identical, and that they were concentrated in certain compartment(s). However, Botv protein staining appeared to be more uniform than that of Ttv or Sotv. This data is consistent with the results obtained from the co-immunoprecipitation experiments, providing further evidence that Ttv and Sotv are present in a complex(es) in vivo.

We further tested whether overexpression of Ttv can replace the function of Sotv and Botv. For this purpose, we ectopically expressed Ttv in the *hairy* expression domain by using $hairy^{Gal4}$ IJ3 in either *sotv* or *botv* null mutant embryos. Ectopic expression of Myc-tagged Ttv fully rescued the cuticle patterning of the *ttv* mutant embryo in the *hairy* domain (Fig. 7H), but was not able to rescue cuticle defects associated with *sotv* or *botv* null mutant embryos (Fig. 7I,J). This result further indicates that individual EXT members play indispensable roles in HS GAG biosynthesis.

Discussion

Biochemical studies have demonstrated essential roles of the EXT family proteins as glycosyltransferases required for HS GAG biosynthesis. However, their respective in vivo roles during development are largely unknown. *Drosophila* contains three EXT family proteins. Although previous studies demonstrated an essential role for Ttv, the *Drosophila* EXT1, in Hh movement and its signalling, the functions of the other two EXT proteins are unclear. Our results demonstrate essential functions of all three *Drosophila* EXT family proteins in signalling events mediated by the Wg, Hh, and Dpp morphogens. We provide strong evidence that all three *Drosophila* EXT proteins are involved in HS biosynthesis and are required for the proper distributions of the morphogen molecules Wg, Hh, and Dpp. Interestingly, we found that Wg signalling is defective only in *botv* mutant or *ttv-sotv* double mutant cells, but not in *ttv* nor *sotv* mutant cells, which suggests partially redundant roles for Ttv and Sotv in Wg signalling. Our results are consistent with a model in which Ttv and Sotv collectively function as a co-polymerase required for the biosynthesis of HS GAG chains, whereas Botv is likely to be involved in distinct step(s), possibly in the initiation of HS GAG biosynthesis. Our results also suggest that HSPGs have two distinct roles in regulating Wg morphogen activity: a function in maintaining the extracellular Wg protein and a co-receptor role in Wg signalling.

Roles of the *Drosophila* EXT family proteins in Hh and Dpp morphogen signalling

Previous studies have demonstrated that Ttv is involved in Hh movement (Bellaiche et al., 1998; Gallet et al., 2003; The et al., 1999). Our results in this work suggest that like Ttv, both Sotv and Botv are also required for Hh movement. Interestingly, we found that Hh is detectable in the first row of mutant cells immediately adjacent to its posterior-producing cells. We propose that specific HSPGs modified by EXT family proteins are required for the movement of Hh from its

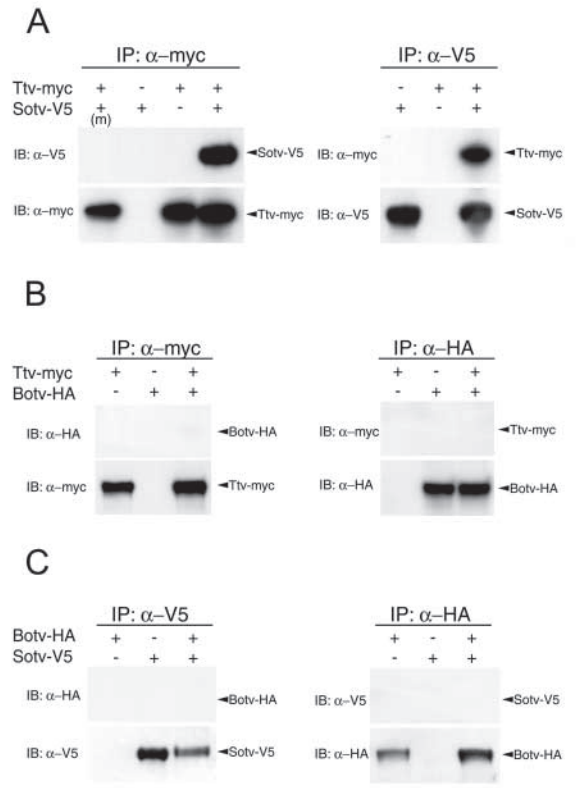
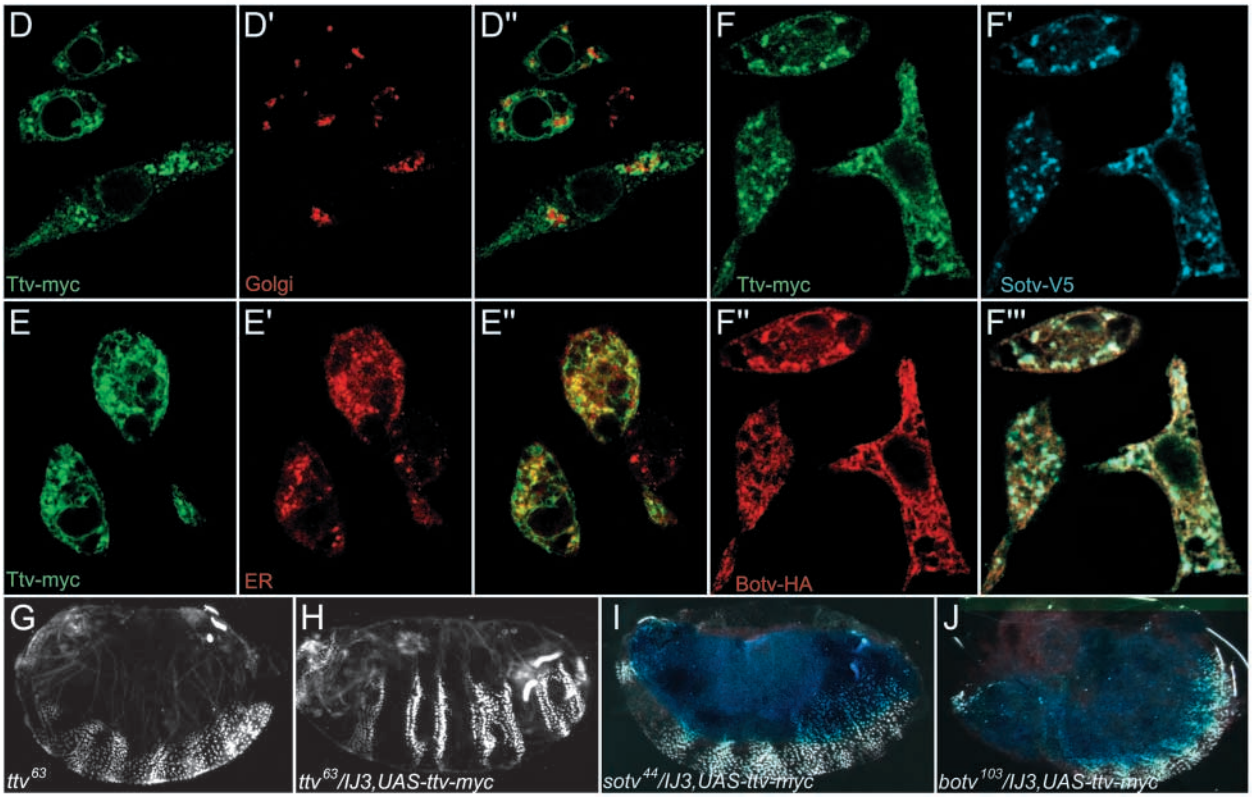


Fig. 7. Ttv and Sotv form a complex and are co-localized in vivo. (A-C) Co-immunoprecipitation of Ttv with Sotv (A), of Ttv with Botv (B), and of Sotv with Botv (C). *Drosophila* S2 cells were transfected with plasmids to express Myc-tagged Ttv, V5-tagged Sotv and HA-tagged Botv in various combinations. Cell lysates were immunoprecipitated and then analyzed by western blotting with the antibodies indicated. IP, immunoprecipitation; IB, immunoblot. The 'm' in the first lane of the left panel (A) indicates that *ttv-myc* and *sotv-V5* were individually transfected and the cell lysates mixed in vitro prior to immunoprecipitation. Ttv-Myc and Sotv-V5 can easily precipitate each other (A), whereas Botv-HA cannot precipitate Ttv-myc or Sotv-V5, or vice versa (B,C). (D-D″) Double staining of Ttv-Myc (green) and a Golgi marker (red) in *Drosophila* S2 cells expressing Ttv-Myc. Ttv-Myc is concentrated in the Golgi complex. (E,E′) Double staining of Ttv-Myc (green) and the ER marker Calnexin (red) in *Drosophila* S2 cells expressing Ttv-Myc. Ttv-Myc is co-localized with Calnexin. (F-F‴) Co-staining of Ttv-myc (green), Sotv-V5 (blue) and Botv-HA (red) in S2 cells transfected with all three expression constructs. Although the majority of all three proteins are co-localized, Ttv-myc and Sotv-V5 appear to be more precisely co-localized and concentrated in certain compartments, whereas Botv-HA seems more uniformly distributed. (G-J) All embryos are oriented anterior left, dorsal up. All panels show embryos derived from females with corresponding mutant germline clones. (G) A ttv^{63} null embryo. (H-J) Ectopic expression of *ttv-myc* driven by $hairy^{Gal4}$ (*IJ3*) in null embryos of ttv^{63} (H), $sotv^{44}$ (I) and *botv* (J). X-gal staining was used to identify *sotv* (I) or botv (J) null embryos expressing *UAS-ttv-myc* by $hairy^{Gal4}$ (I,J). Embryos stained blue were selected for cuticle preparation. The genotypes shown are ttv^{63}/ ttv^{63}*UAS-nlacZ*; *IJ3 UAS-ttv-myc*/+ (H), $sotv^{44}$/ $sotv^{44}$ *UAS-nlacZ*; *IJ3 UAS-ttv-myc*/+ (I) and $botv^{103}$/ $botv^{103}$ *UAS-nlacZ*; *IJ3 UAS-ttv-myc*/+ (J). Although ectopic expression of *ttv-myc* can rescue ttv^{63} null with full penetrance, it cannot rescue $sotv^{44}$ and $botv^{103}$ mutant embryos.



expressing cells into the anterior-receiving cells. In the absence of EXT activities, Hh can be carried into the first row of mutant cells, but fails to move further. Our results are consistent with previous work that demonstrated that the first row of *ttv* mutant cells can still transduce Hh signalling and can activate the expression of its downstream target gene *patched* (*ptc*) (Bellaiche et al., 1998). It is important to note that, while HSPGs modified by EXT family members are likely to be required for the movement of Hh, they may also be involved in preventing Hh from being degraded on the cell surface. In

the absence of EXT proteins, Hh may be degraded and therefore it cannot reach the wild-type cells anterior to the clones of EXT mutant cells. It remains to be determined whether both mechanisms are involved in Hh transport.

We provide strong evidence for the involvement of the three EXT proteins in Dpp signalling and its morphogen distribution. Our results suggest that specific HSPG(s) modified by the three EXT proteins may promote Dpp morphogen signalling by modulating levels of Dpp morphogen ligands in Dpp-receiving cells. Previous studies have implicated a role for Dally, a *Drosophila* glypican member of HSPGs, in Dpp signalling in the development of imaginal discs (Fujise et al., 2003; Jackson et al., 1997). Elevated expression of Dally in the wing disc can promote Dpp signalling, as assayed by p-Mad levels (Fujise et al., 2003). It was proposed that Dally may act as a co-receptor for Dpp in activating its signalling. Our results in this work suggest that HS GAG chains control Dpp signalling by modulating levels of Dpp morphogen in its receiving cells. We propose that the three EXT proteins are required for the biosynthesis of HS GAG chains on the Dally protein and that at least one of the roles of Dally in Dpp signalling is to modulate levels of the Dpp morphogen on its receiving cells.

Role of HSPGs in Wg morphogen distribution and its signalling

Perhaps one of the most interesting findings in this work is the differential roles of the three EXT proteins in Wg morphogen signalling. Although mutations in any of the three EXT genes lead to reduced ranges of extracellular Wg distribution, we found that Wg signalling was defective only in *botv* mutant cells, and not in either *ttv* or *sotv* mutant cells. However, *ttv-sotv* double mutants showed virtually identical defects in Wg signalling to those of *botv* mutants. This qualitative difference between Botv and Ttv or Sotv in Wg signalling is unexpected as mutations in all three genes led to striking reductions in Hh and Dpp signalling.

Previous studies have implicated HSPGs in both Wg signalling and its morphogen gradient distribution. Embryos null for either *sugarless* or *sulfateless*, exhibit defects in Wg signalling in various tissues (Binari et al., 1997; Hacker et al., 1997; Haerry et al., 1997; Lin and Perrimon, 1999). The *Drosophila* glypican Dally has been shown to be required for Wg signalling (Fujise et al., 2001; Lin and Perrimon, 1999; Tsuda et al., 1999). A reduction of *dally* activity can block the Wg signalling activity induced by overexpression of the Wg receptor *Drosophila frizzled 2* (*fz2*) in the wing disc (Lin and Perrimon, 1999). Interestingly, recent studies have demonstrated that Notum (Wingful), a putative *Drosophila* pectin acetylesterase, can inhibit Wg signalling activity by modulating the activity of the heparin sulfate proteoglycans Dally-like (Dly) and Dally (Gerlitz and Basler, 2002; Giraldez et al., 2002). Overexpression of *Notum* can block the signalling activity of the Wg protein tethered to the cell surface by a transmembrane domain from Neurotactin, suggesting that Notum inhibits the function of proteoglycans that are involved in Wg signalling (Gerlitz and Basler, 2002). The function of HSPGs in Wg morphogen gradient distribution has also been demonstrated. In the wing disc, cells mutant for *sulfateless* showed a reduction in the levels of extracellular Wg (Baeg et al., 2001). It was shown that ectopic expression of Dly leads to the accumulation of extracellular Wg protein (Baeg et al., 2001). Furthermore, loss of Notum function leads to increased Wingless activity by altering the shape of the Wingless protein gradient (Giraldez et al., 2002).

Taken together, our results, along with previous studies, suggest that HSPGs are involved in both Wg signalling reception and in extracellular Wg morphogen distribution in the wing disc. We suggest that HSPGs have at least two distinct functions in the wing disc: (1) in the distribution of the extracellular Wg protein; and (2) as a co-receptor for Wg signalling. In this regard, Botv is required both for Wg signalling and for its morphogen gradient formation, whereas Ttv and Sotv are only required for the distribution of extracellular Wg protein; they are functionally redundant in Wg signalling. Consistent with our observations, a previous report on *ttv* showed that, during embryogenesis, Wg signalling in the stomatogastric nervous system (SNS) is not defective in *ttv* mutant embryos (The et al., 1999). It remains to be determined whether *botv* mutants and *sotv-ttv* double mutants show effected Wg signalling in the SNS and in other tissues as well.

Mechanisms and specificities of Ttv, Sotv and Botv in cell signalling

Previous analysis of Ttv has demonstrated its specificity in cell signalling (The et al., 1999). Although Hh signalling is defective in the *ttv* null mutant, neither Wg nor Fgf signalling is altered (The et al., 1999). It was proposed that Ttv is required for the synthesis of an Hh-specific HSPG. Consistent with the previous report, our results demonstrated that Wg signalling is only defective in the *ttv-sotv* double mutant, and is not altered in either *ttv* or *sotv* single mutants. However, we also observed striking defects in both Dpp signalling and the range of extracllular Wg protein distribution in *ttv* and *sotv* mutants. Therefore, the previous view that Ttv is involved only in Hh signalling should be revised.

To understand the molecular mechanisms by which the *Drosophila* EXT proteins play distinct and collaborative roles in cell signalling, we performed biochemical experiments to analyze their interactions and subcellular localization. We found that Ttv and Sotv physically associate with each other to form a complex, and that they have virtually identical subcellular localizations. However, neither Ttv nor Sotv physically interact with Botv. Botv also has a more diffusive staining in cells than Ttv and Sotv. Consistent with our results, biochemical studies in vertebrates showed that vertebrate EXT1 and EXT2 also physically associate with each other to form a complex. Biochemical studies have further demonstrated that both EXT1 and EXT2 have GlcNAc and GlcA transferase activities when expressed independently, although EXT1 has a more robust activity than does EXT2 (Lind et al., 1998; McCormick et al., 2000; McCormick et al., 1998; Senay et al., 2000; Wei et al., 2000). However, co-expression of EXT1 and EXT2 has a synergistic effect on enzyme activities (McCormick et al., 2000; Senay et al., 2000). These results led to a proposal that the fully active HS co-polymerase may be a complex containing EXT1 and EXT2, in which both subunits are essential for the activity (Zak et al., 2002). The functions of EXT-like proteins have also been investigated. EXTL3 appears to be a bifunctional αGlcNAc transferase that can transfer GlcNAc to both the linkage region and to intermediates during chain polymerization, suggesting that EXTL3 is involved in both the

initiation and polymerization of HS GAG chains (Kim et al., 2001). Importantly, a recent biochemical study demonstrated that Botv has αGlcNAc transferase activities that can transfer GlcNAc to both the linkage region and to intermediates in chain polymerization (Kim et al., 2002).

On the basis of our genetic evidence and previous biochemical studies, we propose that Ttv and Sotv are likely to function as co-polymerases required for HS GAG polymerization, whereas Botv is likely to be involved in the initiation of HS GAG and is possibly involved in HS GAG polymerization as well. In the absence of Botv, no HS GAG chains are initiated and therefore mutations in Botv disrupt all the functions of HS GAG chains. However, in the absence of either Ttv or Sotv, initiation of HS GAG biosynthesis occurs, and the residual activity of HS GAG polymerase(s), carried out by another member (either Sotv or Ttv) together with Botv, may synthesize relatively short HS GAG chains that could act as co-receptors for Wg signalling, but have less capacity for maintaining the levels of secreted Wg, Hh and Dpp morphogen proteins. When the activities of both Ttv and Sotv are removed in the *ttv-sotv* double mutant, HS GAG polymerization may not occur because of the lack of GlcA transferase activity, even though Botv is present. In support of this view, previous biochemical studies demonstrated that short HS GAG oligosaccharides have the capacity to form Fgf-Fgfr-HS complexes and to stimulate Fgf signalling (Pellegrini, 2001). In this regard, in the absence of Ttv, both Wg and Fgf signalling may occur. Consistent with this view, a previous study demonstrated that Fgf signalling is not affected in *ttv* mutant embryos (The et al., 1999). Biochemical studies on the activities of Ttv and Sotv as HS co-polymerases will further validate this view. An alternative model is that fewer intact HS GAG chains are synthesized in the absence of either Ttv or Sotv. Although this is less likely to be the case, our current results can not exclude this possibility.

EXT proteins and HME disease

Human mutations in *EXT1* and *EXT2* are associated with hereditary multiple exostoses (HME), which is an autosomal dominant disorder characterized by the formation of multiple cartilage-capped tumors (exostoses) of various bones. Our results demonstrate the essential functions of both Ttv (EXT1) and Sotv (EXT2) in regulating the activities of the three secreted morphogen molecules Hh, Wg and Dpp. Wg and Dpp are the homologues of human WNT and bone morphogen protein (BMP) molecules, respectively. As both WNT and BMP family proteins have been shown to be essential for bone growth and differentiation, our results suggest possible roles for WNT and BMP signalling in the generation of HME diseases associated with *EXT1* and *EXT2*. Our new findings together with previous work on the role of Ttv in Hh movement may provide new insights into the molecular mechanism(s) associated with HME disease.

We thank H. Bellen, S. Cohen, S. Cumberledge, I. Duncan, J. Duffy, M. González-Gaitán, R. Holmgren, P. Ingham and S. Tabata for reagents; and H. Liu for technical assistance. This work was supported by NIH grant GM63891, a grant from the Ohio Cancer Associates and a start-up fund from the Cincinnati Children's Hospital Research Foundation to X.L. C.H. is an Albert J. Ryan Fellow and is supported by an American Heart Association Pre-doctoral Fellowship from American Heart Association.

References

Ahn, J., Ludecke, H. J., Lindow, S., Horton, W. A., Lee, B., Wagner, M. J., Horsthemke, B. and Wells, D. E. (1995). Cloning of the putative tumour suppressor gene for hereditary multiple exostoses (EXT1). *Nat. Genet.* **11**, 137-143.

Aza-Blanc, P., Ramirez-Weber, F. A., Laget, M. P., Schwartz, C. and Kornberg, T. B. (1997). Proteolysis that is inhibited by hedgehog targets Cubitus interruptus protein to the nucleus and converts it to a repressor. *Cell* **89**, 1043-1053.

Baeg, G. H., Lin, X., Khare, N., Baumgartner, S. and Perrimon, N. (2001). Heparan sulfate proteoglycans are critical for the organization of the extracellular distribution of Wingless. *Development* **128**, 87-94.

Basler, K. and Struhl, G. (1994). Compartment boundaries and the control of Drosophila limb pattern by hedgehog protein. *Nature* **368**, 208-214.

Belenkaya, T. Y., Han, C., Standley, H. J., Lin, X., Houston, D., Heasman, J. and Lin, X. (2002). *pygopus* encodes a nuclear protein essential for Wingless/Wnt signalling. *Development* **129**, 4089-4101.

Bellaiche, Y., The, I. and Perrimon, N. (1998). Tout-velu is a Drosophila homologue of the putative tumour suppressor EXT-1 and is needed for Hh diffusion. *Nature* **394**, 85-88.

Bernfield, M., Gotte, M., Park, P. W., Reizes, O., Fitzgerald, M. L., Lincecum, J. and Zako, M. (1999). Functions of cell surface heparan sulfate proteoglycans. *Annu. Rev. Biochem.* **68**, 729-777.

Binari, R. C., Staveley, B. E., Johnson, W. A., Godavarti, R., Sasisekharan, R. and Manoukian, A. S. (1997). Genetic evidence that heparin-like glycosaminoglycans are involved in wingless signalling. *Development* **124**, 2623-2632.

Brown, N. H. and Kafatos, F. C. (1988). Functional cDNA libraries from Drosophila embryos. *J. Mol. Biol.* **203**, 425-437.

Cadigan, K. M. (2002). Regulating morphogen gradients in the Drosophila wing. *Semin. Cell Dev. Biol.* **13**, 83-90.

Capdevila, J. and Guerrero, I. (1994). Targeted expression of the signalling molecule decapentaplegic induces pattern duplications and growth alterations in Drosophila wings. *EMBO J.* **13**, 4459-4468.

Chou, T. B. and Perrimon, N. (1996). The autosomal FLP-DFS technique for generating germline mosaics in Drosophila melanogaster. *Genetics* **144**, 1673-1679.

de Celis, J. F., Barrio, R. and Kafatos, F. C. (1996). A gene complex acting downstream of dpp in Drosophila wing morphogenesis. *Nature* **381**, 421-424.

Duffy, J. B., Harrison, D. A. and Perrimon, N. (1998). Identifying loci required for follicular patterning using directed mosaics. *Development* **125**, 2263-2271.

Duncan, D. M., Burgess, E. A. and Duncan, I. (1998). Control of distal antennal identity and tarsal development in Drosophila by spineless-aristapedia, a homolog of the mammalian dioxin receptor. *Genes Dev.* **12**, 1290-1303.

Entchev, E. V., Schwabedissen, A. and Gonzalez-Gaitan, M. (2000). Gradient formation of the TGF-beta homolog Dpp. *Cell* **103**, 981-991.

Esko, J. D. and Selleck, S. B. (2002). Order out of chaos: assembly of ligand binding sites in heparan sulfate. *Annu. Rev. Biochem.* **71**, 435-471.

Fujise, M., Izumi, S., Selleck, S. B. and Nakato, H. (2001). Regulation of dally, an integral membrane proteoglycan, and its function during adult sensory organ formation of Drosophila. *Dev. Biol.* **235**, 433-448.

Fujise, M., Takeo, S., Kamimura, K., Matsuo, T., Aigaki, T., Izumi, S. and Nakato, H. (2003). Dally regulates Dpp morphogen gradient formation in the Drosophila wing. *Development* **130**, 1515-1522.

Gallet, A., Rodriguez, R., Ruel, L. and Therond, P. P. (2003). Cholesterol modification of hedgehog is required for trafficking and movement, revealing an asymmetric cellular response to hedgehog. *Dev. Cell* **4**, 191-204.

Gerlitz, O. and Basler, K. (2002). Wingful, an extracellular feedback inhibitor of Wingless. *Genes Dev.* **16**, 1055-1059.

Giraldez, A. J., Copley, R. R. and Cohen, S. M. (2002). HSPG modification by the secreted enzyme Notum shapes the Wingless morphogen gradient. *Dev. Cell* **2**, 667-676.

Golic, K. G. (1991). Site-specific recombination between homologous chromosomes in Drosophila. *Science* **252**, 958-961.

Gurdon, J. B. and Bourillot, P. Y. (2001). Morphogen gradient interpretation. *Nature* **413**, 797-803.

Hacker, U., Lin, X. and Perrimon, N. (1997). The Drosophila sugarless gene modulates Wingless signalling and encodes an enzyme involved in polysaccharide biosynthesis. *Development* **124**, 3565-3573.

Haerry, T. E., Heslip, T. R., Marsh, J. L. and O'Connor, M. B. (1997). Defects in glucuronate biosynthesis disrupt Wingless signalling in Drosophila. *Development* **124**, 3055-3064.

Hatini, V. and DiNardo, S. (2001). Divide and conquer: pattern formation in Drosophila embryonic epidermis. *Trends Genet.* **17**, 574-579.

Jackson, S. M., Nakato, H., Sugiura, M., Jannuzi, A., Oakes, R., Kaluza, V., Golden, C. and Selleck, S. B. (1997). dally, a Drosophila glypican, controls cellular responses to the TGF-beta-related morphogen, Dpp. *Development* **124**, 4113-4120.

Kennerdell, J. R. and Carthew, R. W. (1998). Use of dsRNA-mediated genetic interference to demonstrate that frizzled and frizzled 2 act in the wingless pathway. *Cell* **95**, 1017-1026.

Kim, B. T., Kitagawa, H., Tamura, J., Saito, T., Kusche-Gullberg, M., Lindahl, U. and Sugahara, K. (2001). Human tumor suppressor EXT gene family members EXTL1 and EXTL3 encode alpha 1,4-N-acetylglucosaminyltransferases that likely are involved in heparan sulfate/heparin biosynthesis. *Proc. Natl. Acad. Sci. USA* **98**, 7176-7181.

Kim, B. T., Kitagawa, H., Tamura Ji, J., Kusche-Gullberg, M., Lindahl, U. and Sugahara, K. (2002). Demonstration of a novel gene DEXT3 of Drosophila melanogaster as the essential N-acetylglucosamine transferase in the heparan sulfate biosynthesis: chain initiation and elongation. *J. Biol. Chem.* **277**, 13659-13665.

Kitagawa, H., Shimakawa, H. and Sugahara, K. (1999). The tumor suppressor EXT-like gene EXTL2 encodes an alpha1, 4-N-acetylhexosaminyltransferase that transfers N-acetylgalactosamine and N-acetylglucosamine to the common glycosaminoglycan-protein linkage region. The key enzyme for the chain initiation of heparan sulfate. *J. Biol. Chem.* **274**, 13933-13937.

Klueg, K. M., Alvarado, D., Muskavitch, M. A. and Duffy, J. B. (2002). Creation of a GAL4/UAS-coupled inducible gene expression system for use in Drosophila cultured cell lines. *Genesis* **34**, 119-122.

Lander, A. D. and Selleck, S. B. (2000). The elusive functions of proteoglycans: in vivo veritas. *J. Cell Biol.* **148**, 227-232.

Lawrence, P. A. and Struhl, G. (1996). Morphogens, compartments, and pattern: lessons from Drosophila? *Cell* **85**, 951-961.

Lecuit, T., Brook, W. J., Ng, M., Calleja, M., Sun, H. and Cohen, S. M. (1996). Two distinct mechanisms for long-range patterning by Decapentaplegic in the Drosophila wing. *Nature* **381**, 387-393.

Lin, X. and Perrimon, N. (1999). Dally cooperates with Drosophila Frizzled 2 to transduce Wingless signalling. *Nature* **400**, 281-284.

Lin, X. and Perrimon, N. (2000). Role of heparan sulfate proteoglycans in cell-cell signalling in Drosophila. *Matrix Biol.* **19**, 303-307.

Lind, T., Tufaro, F., McCormick, C., Lindahl, U. and Lidholt, K. (1998). The putative tumor suppressors EXT1 and EXT2 are glycosyltransferases required for the biosynthesis of heparan sulfate. *J. Biol. Chem.* **273**, 26265-26268.

McCormick, C., Leduc, Y., Martindale, D., Mattison, K., Esford, L. E., Dyer, A. P. and Tufaro, F. (1998). The putative tumour suppressor EXT1 alters the expression of cell-surface heparan sulfate. *Nat. Genet.* **19**, 158-161.

McCormick, C., Duncan, G., Goutsos, K. T. and Tufaro, F. (2000). The putative tumor suppressors EXT1 and EXT2 form a stable complex that accumulates in the Golgi apparatus and catalyzes the synthesis of heparan sulfate. *Proc. Natl. Acad. Sci. USA* **97**, 668-673.

Methot, N. and Basler, K. (1999). Hedgehog controls limb development by regulating the activities of distinct transcriptional activator and repressor forms of Cubitus interruptus. *Cell* **96**, 819-831.

Motzny, C. K. and Holmgren, R. (1995). The Drosophila cubitus interruptus protein and its role in the wingless and hedgehog signal transduction pathways. *Mech. Dev.* **52**, 137-150.

Nellen, D., Burke, R., Struhl, G. and Basler, K. (1996). Direct and long-range action of a DPP morphogen gradient. *Cell* **85**, 357-368.

Neumann, C. J. and Cohen, S. M. (1997). Long-range action of Wingless organizes the dorsal-ventral axis of the Drosophila wing. *Development* **124**, 871-880.

Nolo, R., Abbott, L. A. and Bellen, H. J. (2000). Senseless, a Zn finger transcription factor, is necessary and sufficient for sensory organ development in Drosophila. *Cell* **102**, 349-362.

Nybakken, K. and Perrimon, N. (2002). Heparan sulfate proteoglycan modulation of developmental signalling in Drosophila. *Biochim. Biophys. Acta* **1573**, 280-291.

Parker, D. S., Jemison, J. and Cadigan, K. M. (2002). Pygopus, a nuclear PHD-finger protein required for Wingless signalling in Drosophila. *Development* **129**, 2565-2576.

Pellegrini, L. (2001). Role of heparan sulfate in fibroblast growth factor signalling: a structural view. *Curr. Opin. Struct. Biol.* **11**, 629-634.

Perrimon, N. and Bernfield, M. (2000). Specificities of heparan sulphate proteoglycans in developmental processes. *Nature* **404**, 725-728.

Persson, U., Izumi, H., Souchelnytskyi, S., Itoh, S., Grimsby, S., Engstrom, U., Heldin, C. H., Funa, K. and ten Dijke, P. (1998). The L45 loop in type I receptors for TGF-beta family members is a critical determinant in specifying Smad isoform activation. *FEBS Lett.* **434**, 83-87.

Posakony, L. G., Raftery, L. A. and Gelbart, W. M. (1990). Wing formation in Drosophila melanogaster requires decapentaplegic gene function along the anterior-posterior compartment boundary. *Mech. Dev.* **33**, 69-82.

Ray, R. P. and Wharton, K. A. (2001). Context-dependent relationships between the BMPs gbb and dpp during development of the Drosophila wing imaginal disk. *Development* **128**, 3913-3925.

Reichsman, F., Smith, L. and Cumberledge, S. (1996). Glycosaminoglycans can modulate extracellular localization of the wingless protein and promote signal transduction. *J. Cell Biol.* **135**, 819-827.

Senay, C., Lind, T., Muguruma, K., Tone, Y., Kitagawa, H., Sugahara, K., Lidholt, K., Lindahl, U. and Kusche-Gullberg, M. (2000). The EXT1/EXT2 tumor suppressors: catalytic activities and role in heparan sulfate biosynthesis. *EMBO Rep.* **1**, 282-286.

Stickens, D., Clines, G., Burbee, D., Ramos, P., Thomas, S., Hogue, D., Hecht, J. T., Lovett, M. and Evans, G. A. (1996). The EXT2 multiple exostoses gene defines a family of putative tumour suppressor genes. *Nat. Genet.* **14**, 25-32.

Strigini, M. and Cohen, S. M. (2000). Wingless gradient formation in the Drosophila wing. *Curr. Biol.* **10**, 293-300.

Sullivan, W., Ashburner, M. and Hawley, S. (2000). Drosophila *Protocols*. New York: Cold Spring Harbor Laboratory Press.

Tabata, T. and Kornberg, T. B. (1994). Hedgehog is a signalling protein with a key role in patterning Drosophila imaginal discs. *Cell* **76**, 89-102.

Tanimoto, H., Itoh, S., ten Dijke, P. and Tabata, T. (2000). Hedgehog creates a gradient of DPP activity in Drosophila wing imaginal discs. *Mol. Cell* **5**, 59-71.

Taylor, A. M., Nakano, Y., Mohler, J. and Ingham, P. W. (1993). Contrasting distributions of patched and hedgehog proteins in the Drosophila embryo. *Mech. Dev.* **42**, 89-96.

Teleman, A. A. and Cohen, S. M. (2000). Dpp gradient formation in the Drosophila wing imaginal disc. *Cell* **103**, 971-980.

Teleman, A. A., Strigini, M. and Cohen, S. M. (2001). Shaping morphogen gradients. *Cell* **105**, 559-562.

The, I., Bellaiche, Y. and Perrimon, N. (1999). Hedgehog movement is regulated through tout velu-dependent synthesis of a heparan sulfate proteoglycan. *Mol. Cell* **4**, 633-639.

Toyoda, H., Kinoshita-Toyoda, A., Fox, B. and Selleck, S. B. (2000). Structural analysis of glycosaminoglycans in animals bearing mutations in sugarless, sulfateless, and tout-velu. Drosophila homologues of vertebrate genes encoding glycosaminoglycan biosynthetic enzymes. *J. Biol. Chem.* **275**, 21856-21861.

Tsuda, M., Kamimura, K., Nakato, H., Archer, M., Staatz, W., Fox, B., Humphrey, M., Olson, S., Futch, T., Kaluza, V. et al. (1999). The cell-surface proteoglycan Dally regulates Wingless signalling in Drosophila. *Nature* **400**, 276-280.

Vincent, J. P. and Dubois, L. (2002). Morphogen transport along epithelia, an integrated trafficking problem. *Dev. Cell* **3**, 615-623.

Wei, G., Bai, X., Gabb, M. M., Bame, K. J., Koshy, T. I., Spear, P. G. and Esko, J. D. (2000). Location of the glucuronosyltransferase domain in the heparan sulfate copolymerase EXT1 by analysis of chinese hamster ovary cell mutants. *J. Biol. Chem.* **275**, 27733-27740.

Zak, B. M., Crawford, B. E. and Esko, J. D. (2002). Hereditary multiple exostoses and heparan sulfate polymerization. *Biochim. Biophys. Acta* **1573**, 346-355.

Zecca, M., Basler, K. and Struhl, G. (1996). Direct and long-range action of a wingless morphogen gradient. *Cell* **87**, 833-844.