Molecular Cell, Vol. 4, 633–639, October, 1999, Copyright ©1999 by Cell Press

Hedgehog Movement Is Regulated through *tout velu*–Dependent Synthesis of a Heparan Sulfate Proteoglycan

Inge The,* Yohanns Bellaiche,*§ and Norbert Perrimon*†‡
*Department of Genetics
†Howard Hughes Medical Institute
Harvard Medical School
Boston, Massachusetts 02115

Summary

Hedgehog (Hh) molecules play critical roles during development as a morphogen, and therefore their distribution must be regulated. Hh proteins undergo several modifications that tether them to the membrane. We have previously identified *tout velu* (*ttv*), a homolog of the mammalian *EXT* tumor supressor gene family, as a gene required for movement of Hh. In this paper, we present in vivo evidence that *ttv* is involved in heparan sulfate proteoglycan (HSPG) biosynthesis, suggesting that HSPGs control Hh distribution. In contrast to mutants in other HSPG biosynthesis genes, the activity of the HSPG-dependent FGF and Wingless signaling pathways are not affected in *ttv* mutants. This demonstrates an unexpected level of specificity in the regulation of the distribution of extracellular signals by HSPGs.

Introduction

During development, a number of secreted factors have been identified that play critical roles in the patterning of fields of cells. In particular, members of the Wnt and Hedgehog (Hh) families have been shown to act several cell diameters away to instruct cells to adopt specific fates. Unlike other growth factors that can freely diffuse, these molecules are thought to be associated with the membrane and extracellular matrix. One of the major puzzles is how these molecules can reach cells far away from their sites of synthesis and how their concentration gradients can be regulated.

Hh proteins specify cell fates at a distance and can be detected beyond Hh-producing cells (Tabata and Kornberg, 1994). For example, in *Drosophila* Hh induces the expression of its target genes *patched* (*ptc*) and *decapentaplegic* (*dpp*) directly in a domain of 8–10 cell diameters along the anterior–posterior (A/P) boundary of the wing imaginal disc (Mullor et al., 1997; Strigini and Cohen, 1997). Hh is made as a precursor protein that is autocatalytically cleaved to produce an N-terminal (Hh-N) and a C-terminal (Hh-C) fragment (Lee et al., 1994). During this cleavage process, a cholesterol moiety is added to Hh-N (HhNp). HhNp is responsible for all the biological activities of Hh in flies and vertebrates. In the absence of the cholesterol modification, Hh-N molecules act at a longer range and are more potent inducers of Hh target genes than HhNp (Porter et al., 1996b). How HhNp can exert its patterning activity when it is associated to cholesterol, and thus lacks the properties of a diffusible molecule, is not clear.

ttv is a member of the *EXT* gene family, which has been associated with the human multiple exostoses (EXT) syndrome characterized by bone outgrowths and higher incidence of bone tumors (chondrosarcomas and osteosarcomas) (Ahn et al., 1995; Stickens et al., 1996). There are three genetic loci linked to the syndrome, named *EXT1*, *2*, and *3*. The *EXT1* and *2* genes encode homologous proteins, while the *EXT3* gene has not yet been identified. Due to loss of heterozygosity of the *EXT1* and *2* genes in chondrosarcomas, they have been classified as tumor suppressor genes (Hecht et al., 1995; Raskind et al., 1995). This family of *EXT* genes has been extended by the identification of other *EXT-like* (*EXTL*) genes showing a high degree of homology with the *EXT* genes (Wise et al., 1997; Wuyts et al., 1997; Van Hul et al., 1998). Although the sequences of Ext proteins do not provide clues about their functions, recent findings have implicated them in heparan sulfate proteoglycan (HSPG) biosynthesis. In one report, the inability of herpes simplex virus to infect the mouse cell line sog9 was shown to correlate with a defect in heparan sulfate (HS) biosynthesis. This defect can be corrected by introducing an *EXT1* cDNA into the mutant cell line (McCormick et al., 1998). Additionally, an *EXT2* homolog has been biochemically isolated from bovine serum as a glycosyltransferase, which transfers GlcA and GlcNAc sugar residues, required for the biosynthesis of HS (Lind et al., 1998). Altogether, these studies have led to the model that Ext proteins encode HS polymerase enzymes and are involved in HSPG biosynthesis.

HSPGs are large macromolecules, which are found abundantly at the cell surface and are part of the extracellular matrix. They are composed of glycosaminoglycan (GAG) chains linked to a protein core. The HS GAG can be covalently linked to a variety of cell surface proteins but is found consistently on two major families of proteoglycans, Syndecans and Glypicans (David, 1993). In the context of signal transduction, HSPGs have been implicated in a number of functions that include coreceptors for insoluble and soluble ligands, internalization of receptors, or transport of molecules or as soluble paracrine effectors (Salmivirta et al., 1996).

Recently, the identification of a number of mutations in enzymes involved in HSPG biosynthesis has underscored the critical role that HSPGs play in development. Mice lacking the HS 2-O-sulfotransferase exhibit a renal agenesis phenotype (Bullock et al., 1998). In *Drosophila*, mutations in *sugarless* (*sgl*, encoding UDP-glucose dehydrogenase) (Cumberledge and Reichsman, 1997), *sulfateless* (*sfl*, encoding a N-deacetylase/N-sulfotransferase) (Lin and Perrimon, 1999; Lin et al., 1999) (Figure 1), *division abnormally delayed* (*dally*, encoding a Glypican) (Nakato et al., 1995; Lin and Perrimon, 1999; Tsuda et al., 1999), and *pipe* (encoding a putative 2-O-sulfotransferase) (Sen et al., 1998) cause defects in embryonic

‡To whom correspondence should be addressed (e-mail: perrimon@rascal.med.harvard.edu).
§Present address: Equipe ATIPE URA 1857, Ecole Normale Supérieure, 46, rue d'Ulm, 75005 Paris, France.

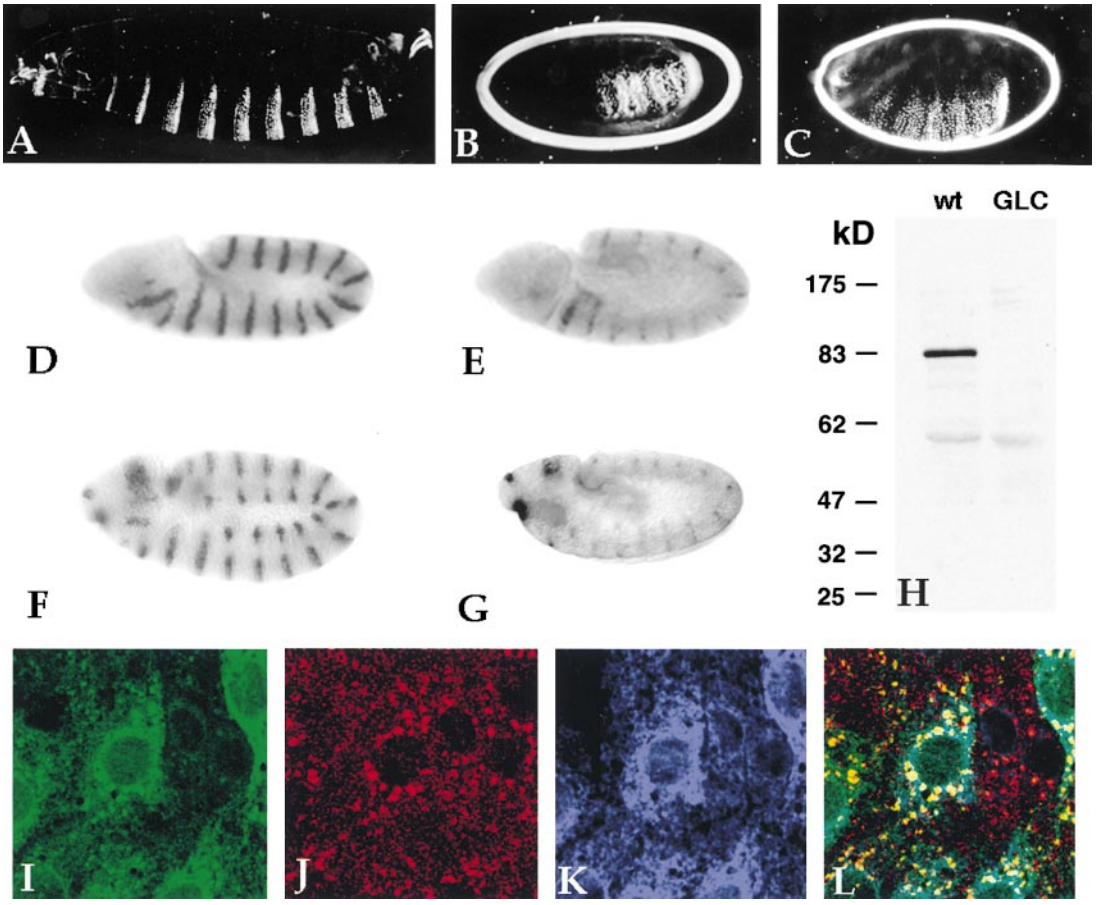

Figure 1. Segment Polarity Phenotype of *ttv* and Localization in the Secretory Apparatus

(A–H) Cuticles of wild type (A), *ttv* embryos (B), and *ttv/Df (2R)Trix* embryos derived from females with *ttv* germline clones (C). En staining in wild-type (D) and *ttv* embryo (E) at stage 10. Wg staining in wild-type (F) and *ttv* embryo (G) at stage 10. Anterior is to the left in all panels. Western blots of unfertilized wild-type (wt) and *ttv* embryos (GLC) probed with rabbit anti-Ttv antibody (H). The predicted molecular weight of Ttv protein is 80 kDa, and a band comigrating with the 83 kDa marker is detected in wild type and not in *ttv* embryos.

(I–L) The *ttv-myc* transgene was expressed in the *engrailed* (*en*) domain by *en-GAL4*. Embryos were stained for Ttv-myc in green (I), the golgi marker β-CopII in red (J), and the endoplasmic reticulum marker Bip in blue (K). Colocalization of Ttv-myc with β-CopII is seen in yellow and with Bip in turquoise (L).

development. These observations suggest that particular HSPGs play a role in specific signaling pathways.

Biochemical analyses have indicated that Wnts (Bradley and Brown, 1990; Reichsman et al., 1996) and Hh are heparin-binding proteins (Lee et al., 1994; Bumcrot et al., 1995). While in the case of Wnt proteins, there is now substantial biochemical and genetic evidence to support the model that they interact with HSPGs in vivo (Cumberledge and Reichsman, 1997), the function of binding to heparin in the case of Hh molecules is unclear. Here, we show that *ttv* is involved in HS GAG biosynthesis in vivo and provide evidence that HSPGs play a critical role in Hh distribution.

Results and Discussion

ttv Is a Segment Polarity Gene Whose Product Is Localized in the Secretory Apparatus

Homozygous *ttv* animals die at pupal stage, but when maternal and zygotic *ttv* activities are removed (see the Experimental Procedures), *ttv* embryos show absence of naked cuticle (Figure 1B) and disappearance of both *wg* and *en* expression. These phenotypes are reminiscent to *hh* or *wg* segment polarity mutants (Figures 1D–1G). This segment polarity phenotype represents the *ttv* null phenotype since the $ttv^{l(2)00681}$ allele behaves as a genetic null (Figures 1B and 1C). In addition, we did not detect any Ttv protein in Western blots prepared from $ttv^{l(2)00681}$ embryos (Figure 1H).

Ttv encodes a type II transmembrane protein (Bellaiche et al., 1998), which could be localized at the plasma membrane or in the membranes of the secretory apparatus. To investigate the subcellular localization of Ttv, we epitope tagged the Ttv protein (*ttv-myc*) because of the inability of the Ttv polyclonal antibody (Figure 1H) to recognize Ttv in fixed tissues. When expressed under the control of the UAS promoter using the Gal4-UAS system (Brand and Perrimon, 1993), this *ttv-myc* gene is able to rescue the segment polarity phenotype of *ttv* embryos (data not shown), demonstrating that the *myc* epitope–tagged protein is functional.

Staining of embryos or imaginal discs expressing *ttv-*

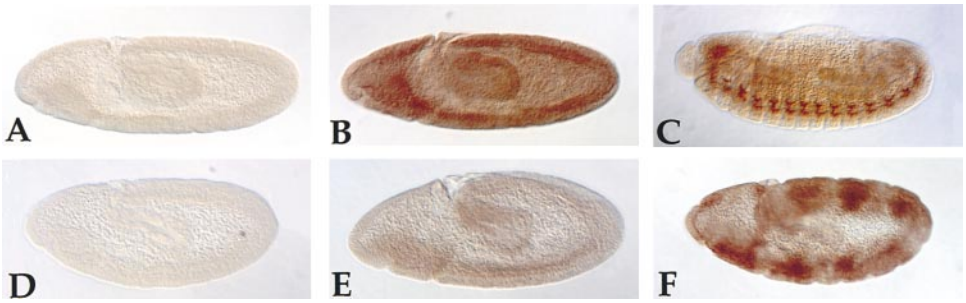

Figure 2. Ttv Is Involved in HSPG Biosynthesis In Vivo

Staining of wild-type (A–C) and *ttv* mutant (D–F) embryos with the 3G10 antibody. No staining was detected by 3G10 in embryos untreated with heparinase III (A and D). In embryos treated with heparinase III, uniform staining is detected at early stages (B) by 3G10. At later stages, stronger staining is observed in the nervous system (C). (B) and (C) are stage 10 and 13 embryos, repectively. In *ttv* embryos (E), the staining detected by 3G10 is strongly reduced. The decreased staining is not due to variability in staining conditions because when *ttv* is ectopically expressed in a *hairy* pattern in *ttv* embryos, the strong 3G10 staining is recovered in the *hairy* striped pattern (F). For expression of *ttv* in the *hairy* domain, *y w FLP^{12}/+; FRT^{G13} ttv /FRT^{G13} P[ovo^{D1}]; hairy-GAL4/+* females were crossed to *ttv/CyO ftz-lacZ; UAS-ttv* males. Anterior is to the left in all panels.

myc construct under control of *en-Gal4* revealed a perinuclear and punctate staining (Figure 1I). To determine in which subcellular compartments the Ttv protein can be found, we tested whether Ttv-myc colocalizes with either the golgi protein β-CopII (Figure 1J) (Ripoche et al., 1994) or the endoplasmic reticulum (ER) protein Bip (Figure 1K) (Kirkpatrick et al., 1995). Interestingly, Ttv-myc mostly colocalizes with Bip and partially with β-CopII (Figure 1L). Further, we did not detect any plasma membrane staining or a colocalization with the membrane markers E-cadherin or Armadillo (data not shown). Altogether, these results indicate that Ttv resides mainly in the ER and in the golgi.

Ttv Is Involved in HSPG Biosynthesis

We have previously shown that, in *ttv* mutant clones induced in the wing imaginal disc, Hh movement and therefore signaling is reduced (Bellaiche et al., 1998). Recently, two reports have implicated Ext proteins in HSPG biosynthesis (Lind et al., 1998; McCormick et al., 1998). Therefore, we investigated whether HS biosynthesis in *Drosophila* embryos was affected in the absence of *ttv* activity. To detect the presence of HSPGs in vivo, we stained embryos with an antibody (3G10) that has been shown to recognize an epitope on mouse tissues following digestion of HSPGs with heparinase III. Following digestion of the HS sugar chains, one desaturated uronate residue per chain will remain linked to the core protein, enabling the 3G10 antibody to recognize it (David et al., 1992). Staining of wild-type embryos with 3G10 reveals a uniform staining during early stages and a more pronounced central nervous system staining at later stages (Figures 2B and 2C). No staining could be detected in embryos that have not been treated with heparinase III (Figures 2A and 2D).

We then analyzed the 3G10 staining in the absence of *ttv* activity. In contrast to wild type, the staining detected by 3G10 in heparinase-treated *ttv* embryos was strongly reduced (Figure 2E). However, this staining was recovered when wild-type Ttv activity is reintroduced in *ttv* embryos (Figure 2F). These results show that the staining in *Drosophila* embryos detected by 3G10 is specific and the reduction of staining is due to absence of *ttv* activity. The reduced staining detected by the 3G10 monoclonal antibody in *ttv* embryos could not reflect residual Ttv activity since the *ttv* allele we used is a null (Figure 1).

It is possible that other *EXT-like* genes in *Drosophila* are responsible for the staining in embryos. We have recently identified another *EXT-like* gene, *DExt2*, with some homology to *ttv*. When the *DExt2* cDNA clone was sequenced, we found that it was more homologous to vertebrate *EXT2* than to *EXT1* (44% and 26% protein identity, respectively; data not shown). Since *DExt2* and *ttv* are maternally expressed and uniformly distributed in early embryos (data not shown), we propose that the residual 3G10 staining present in *ttv* embryos is due to the activity of *DExt2*.

FGF and Wg Signaling Are Not Affected in *ttv* Embryos

Since HSPGs are affecting many different factors, such as FGF and Wnts, we investigated the specificity of *ttv* for Hh. We first examined whether FGF signaling is decreased in the absence of Ttv activity by analyzing the migration of the mesoderm, a process dependent on the FGF receptor Heartless (Htl) signaling pathway (Gisselbrecht et al., 1996). In wild-type embryos, the ventral mesoderm invaginates at stage 6, and at stage 9 mesoderm cells rearrange and form a monolayer (Figures 3A and 3C). Mutants with defects in Htl signaling show aberrant mesodermal cell migration, and mesodermal cells do not form a monolayer. Mutations in *sgl* and *sfl* exhibit a similar mesoderm migration defect consistent with the role of HSPGs in FGF/Htl signaling (Lin et al., 1999). Surprisingly for a mutation involved in HS biosynthesis, we could not detect any defects in mesoderm migration in *ttv* embryos (Figures 3B and 3D).

ttv embryos display a segment polarity phenotype similar to loss of either Wg or Hh signaling (Figures 1A–1G). However, since Wg and Hh signaling pathways in the embryonic epidermis are dependent on each other, the segment polarity phenotype does not allow us to distinguish whether loss of *ttv* affects Hh as well as Wg signaling. Therefore, we looked at two other Wg-dependent processes during embryogenesis; the

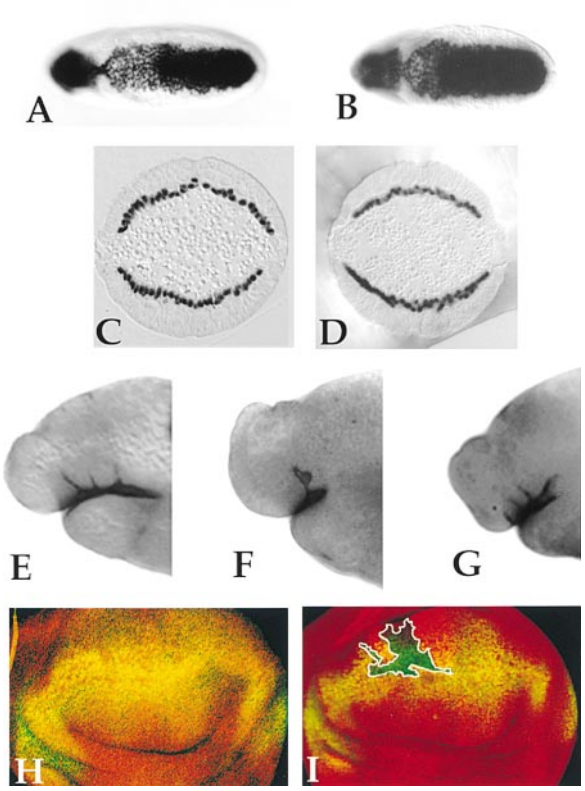

Figure 3. Ttv Activity Is Not Required for FGF and Wg Signaling

Mesoderm migration is visualized by Twi antibody staining in wild-type (A and C) and *ttv* embryos (B and D) at stage 9. (A) and (B) are ventral views of whole embryos; (C) and (D) are transverse sections. In wild-type cells, Twi-stained cells can be seen as a band with smooth edge on the ventral side of stage 9 embryos and cross sections through these embryos show a monolayer of Twi-stained cells. When FGF signaling is impaired and mesodermal migration is affected, Twi-positive cells can be seen as a band with rough edges on the ventral side, and sections through these embryos will show the cells clustered near the ventral midline. This is not detected in *ttv* embryos. The SNS was visualized by staining with anti-Crumbs antibodies in wild type (E), *wg* embryos (F), and *ttv* embryos (G); only the head of the embryos are shown. In *wg* embryos, the SNS is fused to one invagination, while *ttv* embryos have normal SNS invagination (G). Staining of wing imaginal discs with Dll antibody in green shows expression of Dll straddling the D/V boundary in wild type (H); a large clone of *ttv* mutant cells shown as absence of red in the V compartment does not affect Dll expression (I). For generating *ttv* clones, the genotype was *hsFLP; FRT^{G13} $ttv^{l(2)00681}$/ FRT^{G13} ubqGFP*.

formation of the stomatogastric nervous system (SNS) neurons (Gonzalez-Gaitan and Jackle, 1995) and the formation of the RP2 neurons (Patel et al., 1989). In wild-type embryos, the invagination of the three SNS neurons can be visualized by staining with antibodies against the Crumbs protein (Figure 3E). In mutants that decrease Wg signaling, there are less than three SNS invaginations (Figure 3F), while in mutants that increase Wg signaling, more than three invaginations can be found. The SNS phenotype of *ttv* mutant embryos appears wild type (Figure 3G), suggesting that Wg signaling is not affected by loss of Ttv activity. Similarly, we could not detect a requirement for Ttv activity in the formation of the RP2 neurons in the embryonic CNS as detected by an Even-skipped (Eve) antibody (data not shown).

To extend the conclusion that *ttv* is not required for Wg signaling, we examined another developmental stage: the wing imaginal discs. Wg expressed at the wing margin controls patterning along the D/V axis in a concentration-dependent manner. Expression of the proneural genes at the margin and *vestigial* (*vg*) and *distalless* (*dll*) at a farther distance are controlled by Wg (Zecca et al., 1995; Neumann and Cohen, 1997). In *ttv* mutant clones at the margin, the expression of the proneural marker A101 is not affected (data not shown). Further, we find that the expression of *dll* is not affected (Figures 3H and 3I). Thus, Wg signaling in wing imaginal discs does not require Ttv activity.

Specificity of Ttv for Hh Signaling

In the absence of *sgl* or *sfl* activities, which are also involved in HSPG biosynthesis, both Wg and FGF signaling pathways are reduced (Cumberledge and Reichsman, 1997; Lin et al., 1999). Surprisingly, our analysis of *ttv* indicates that Hh was specifically affected while both Wg and FGF dependent processes are not altered. This raised the question of whether all HS GAG chains are involved in Hh movement. Therefore, we investigated whether Hh signaling is affected in clones of *sfl* mutant cells in wing imaginal discs. Our analysis suggests that Hh signaling is affected as well in the *sfl* clones (data not shown).

The observation that Hh signaling, but not FGF and Wg signaling, is affected in the absence of Ttv activity could indicate that Hh signaling is more sensitive to a reduction of HS GAGs than Wg and FGF signaling. According to this "quantitative" model, Wg and FGF signaling pathways would not be affected in *ttv* embryos because HS GAGs synthesized by another *Drosophila* Ext are sufficient to allow these pathways to function. Alternatively, according to a "qualitative" model, the specificity of Ttv to Hh signaling suggests the existence of Hh-specific HSPGs. In *ttv* mutants Wg and FGF, signaling may not be affected because the HSPGs that these factors interact with are present. According to the quantitative model, we would still expect Wg and FGF signaling pathways to be at the least partially affected. However, we found no evidence that the activity of these pathways are reduced. Further, in the absence of Ttv activity, the effect on Hh signaling is similar to the loss of Hh activity. We therefore favor the "qualitative" model (i.e., that Ttv activity is required for the synthesis of an Hh-specific HSPG).

Recently, another *EXT* family member *EXTL2*, has been identified as an α-GlcNAc transferase, which determines that a heparan sulfate instead of a chondroitin sulfate chain will be attached to the linker region of the proteoglycan (Kitagawa et al., 1999). The initiation of the GAG chain on the protein core depends on Ser–Gly/Ala dipeptides that have one or more acidic amino acids in close proximity (Zhang et al., 1995). It has been proposed that the sequences in the core protein surrounding the GAG attachment site are important for the formation of HS chains. Thus, it is possible that different Ext proteins might recognize different sequences on the protein core and thus be specific for certain HSPGs.

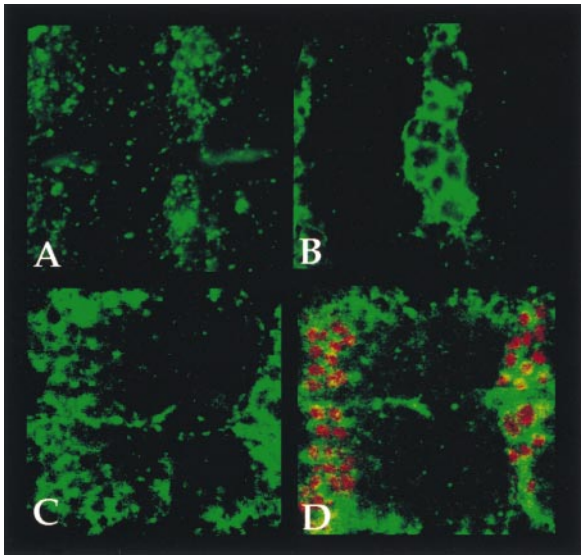

Figure 4. Ttv Is Required for the Movement of HhNp in the Embryo

Hh staining of endogenous Hh in wild-type (A) and *ttv* embryos (B). Hh in wild-type embryos can be seen in vesicle-like structures between the Hh-expressing cells that are not detected in *ttv* embryos. Staining of HhN, without the cholesterol modification, in *ttv* embryos expressing HhN under control of *en* promoter (*en-GAL4; UAS-HhN*) (C). *ttv* embryos expressing *en-HhN* stained for both Hh in green and En in red (D). HhN can be seen in the interstripes of HhN-expressing cells (C and D), and En expression in *ttv* embryos expressing *en-HhN* is wider than two cells as reported in wild-type embryos. For expressing HhN in *ttv* embryos, *y w* FLP^{12}/+; FRT^{G13} *ttv*/FRT^{G13} *P*[ovo^{D1}]; *UAS-HhN*/+ females were crossed to *ttv en-GAL4/CyO ftz-lacZ* males.

According to this model, the protein sequence of the HSPG to which Hh binds would be critical for defining its specificity. Another possibility is that Ext proteins generate specific GAG chains, perhaps in a complex with certain HS-modifying enzymes. This model would explain the specificity of *ttv* on Hh signaling, as *ttv* would generate a GAG chain specific for Hh.

The Movement of HhNp Is Controlled by *ttv*

Previously, we have documented that *ttv* is required for the ability of Hh to reach target cells (Bellaiche et al., 1998). To extend these observations to embryonic stages, we examined Hh expression in *ttv* embryos. Staining wild-type embryos with an Hh antibody shows a strong staining in *hh*-expressing cells and a punctate staining outside of these cells (Figure 4A), as described before (Lee et al., 1994; Tabata and Kornberg, 1994). However, in *ttv* embryos, Hh is only seen in *hh*-expressing cells, indicating that Hh does not move beyond its site of production (Figure 4B). This phenomenon appears specific to Hh because Wg diffusion is not impaired in *ttv* embryos (data not shown).

Hh is produced as a precursor protein, which undergoes autoprocessing (see the Introduction and Lee et al., 1994). During this process, a cholesterol moeity is attached to the N-terminal portion of Hh (HhNp), which contains the signaling domain (Porter et al., 1996a, 1996b). Since HhNp has a cholesterol anchor, it is presumed to remain bound to the membrane. It has been shown that HhN, an N-terminal form of Hh that is not cholesterol modified, can move further in embryos than HhNp and can induce ectopic *wg* expression (Porter et al., 1995). To determine whether the requirement for *ttv* on Hh diffusion depends on the cholesterol modification of Hh, we tested if the diffusion of HhN is reduced in *ttv* embryos by expressing UAS-HhN under the control of *en-Gal4*. Interestingly, HhN diffuses (Figure 4C) and induces ectopic *en* expression (Figure 4D). Thus, *ttv* is required for the proper diffusion of the cholesterol-modified, membrane-associated HhNp but not of unmodified HhN.

The Role of HSPGs in Hh Movement

Our results indicate that HSPGs are involved in the ability of Hh to reach target cells. Hh can act at a distance and is found only at a very low concentration outside Hh-producing cells. Therefore, either the concentration of Hh required to signal is very low and the low amount of diffusible Hh is sufficient for signaling or the membrane tethered Hh can be transported from cell to cell. One model in which HSPGs could influence Hh distribution is by concentrating Hh and perhaps presenting it to its receptor. Such a function has been proposed for HSPGs in FGF signaling (Schlessinger et al., 1995). This model assumes that HSPGs are not playing a more active role in the extracellular spreading of Hh.

The observation that membrane-targeted Hh requires HSPGs suggests that there is a transport mechanism for Hh that would allow Hh to move from cell to cell. It is possible that HSPGs are required to target Hh to a specific subcellular compartment. Interestingly, Glypicans with reduced GAG chains are sorted differently than fully glycanated ones (Mertens et al., 1996) and therefore might not able to deliver or aid Hh in the right compartment. The transport of Hh might involve so called “lipid rafts,” which are microdomains in the plasma membrane rich in sphingolipids, cholesterol, and GPI-anchored proteins (Simons and Ikonen, 1997). Interestingly, Hh has been reported to localize into the detergent-insoluble fraction, characteristic for proteins found in lipid rafts, after separation of cell extracts (Porter et al., 1996a; Rietveld et al., 1999). Perhaps a GPI-anchored HSPG, such as a Glypican molecule, is required to localize Hh in these rafts. Transfer of GPI-anchored proteins between cells has been observed, and Hh might be transferred from cell to cell in this way (Kooyman et al., 1995). The cholesterol modification on Hh might also facilitate Hh localization into the rafts, after which transport of Hh can occur.

Recently, long cytoplasmic extensions extending toward the A/P boundary have been identified (Ramirez-Weber and Kornberg, 1999). Because these filopodia-like structures or cytonemes may transport molecules such as Hh, these authors have proposed that the mechanism to generate a morphogen gradient is intracellular and that the time and distance of transport along the cytonemes determines the concentration levels of the signal. If this model proves correct, it is possible that HSPGs are facilitating the transport of Hh down the cytonemes of the receiving cells. We envision that the

HSPG could either carry Hh molecules along the membrane or could target Hh to the correct intracellular compartment or vesicles. Interestingly, HhN without cholesterol modification does not require *ttv* and therefore HSPGs to move from the producing cells. HhN is presumably released into the extracellular matrix, is able to diffuse further than HhNp, and behaves like an ectopic Hh. Therefore, the attachment of HhNp to the membrane and its transport represents another level in the regulation of this potent signaling molecule.

Experimental Procedures

Genetics

Females with germline clones (GLCs) were generated as described by Chou and Perrimon (1996). Females with homozygous *ttv* germline clones were mated to *ttv/CyO ftz-lacZ* males, and maternal/zygotic null embryos were identified by the absence of β-galactosidase expression. The *ttv* alleles tested were $ttv^{l(2)00681}$, $ttv^{l(2)k066109}$, and $ttv^{l(2)02055}$. To test if $ttv^{l(2)00681}$ behaves as a genetic null, *ttv* germline clone females were mated to *Df(2R)Trix/Cyo*. Somatic clones in the wing imaginal disc were induced using the FLP/FRT-mediated recombination system (Xu and Rubin, 1993). Third instar larval imaginal discs were fixed for 20 min in 4% formaldehyde in PBS with 0.1% Tween20 (PBT). Mutant clones were identified by the loss of GFP expression.

UAS Constructs and GAL4 Lines

The *UAS-ttv-myc* construct is an SpeI (blunted) and NotI fragment of the *ttv* gene that consists of the whole gene and six *myc* epitope tags cloned into the EcoRI (blunted) and NotI sites of pUAST (Brand and Perrimon, 1993). For ectopic expression, the GAL4 insertions in *hairy-GAL4* (*1J3*) on the third chromosome and *en-GAL4* on the second chromosome were used.

In Situ Hybridization

Antisense *ttv*, *DExt2* probes were generated by the DIG labeling kit from Boehringer Mannheim using wither T3 or T7 RNA polymerase. RNA in situ hybridization was performed as described by Tautz and Pfeifle (1989).

Detection of Ttv

ttv cDNA sequence encoding amino acid 30 to 376 of the Ttv protein was cloned into the pQE10 vector (Qiagen) and expressed in the *E. coli* M15 strain. The purified protein was injected into rabbit and rat. Antisera were affinity purified against GST-Ttv (amino acid 30 to 376) linked on a HiTrap NHS-activated column (Pharmacia).

For Western blot analyses, wild-type and *ttv* unfertilized embryos derived from GLCs were used to avoid contamination by paternally rescued embryos. Embryos were dechorionated in 50% bleach and homogenized in RIPA buffer (50 mM Tris [pH 8], 0.1% SDS, 1% NP40, 150 mM NaCl). Amount of protein was measured using Bio-Rads protein assay (Bio-Rad), and 50 μg of the extract was then separated by SDS PAGE electrophoresis (4%–15% gradient gel) and blotted onto PVDF membrane (Millipore). Affinity-purified rabbit anti-Ttv antibodies and secondary anti-rabbit-IGg (Vectorlabs) were used at 1:1000 and 1:3000, respectively. Detection of Ttv is done by the ECL Western blotting detection method (Amersham).

Immunohistochemistry

Embryonic cuticles were prepared by the Hoyer's method (van der Meer, 1977). Treatment of embryos to expose the epitope recognized by the 3G10 monoclonal antibody (Seikagaku) was done by incubating fixed embryos with 500 mU/ml heparinase III (Sigma) in 50 mM Tris-HCL (pH 7.2), 100 mM NaCl, 1 mM Ca Cl_2, 0.1% Triton-X100, 5 μg/ml BSA. Embryos were incubated for 16 hr at 37°C and stained with 3G10 antibody, 1:100 in PBT. Embryo staining was done as described before (Patel, 1994).

Other antibodies diluted in PBT include mouse anti-myc, 1:200 (Calbiochem); rabbit anti-β-CopII, 1:200; rat anti-Bip, 1:5; anti-E-cadherin, 1:20; mouse anti-Armadillo, 1:200; rabbit anti-Twist, 1:5000; mouse anti-Engrailed, 1:10; rabbit anti-Wingless, 1:200; mouse anti-Crumbs, 1:5; mouse anti-Evenskipped, 1:10; rabbit anti-β-galactosidase, 1:1000 (Capel); mouse anti-Distalless, 1:10; rabbit anti-Hedgehog, 1:2000 with TSA enhancement (TSA Renaissance kit, NEN). Secondary antibodies for histochemical staining were from Vectorlabs, and fluorescent secondary antibodies were from Jackson Immunoresearch. Embryos sections were performed as described in Gisselbrecht et al. (1996).

Acknowledgments

We thank P. Beachy, S. Cohen, S. Cumberledge, E. Knust, M. Rosenberg, I. Guerrero, P. Ingham, V. Malhotra, and S. Roth for reagents; the Bloomington *Drosophila* Stock Center for stocks; S. Eaton for discussion about lipid rafts; C. Arnold for injections; and E. Bach, D. Bilder, X. Lin, B. Mathey-Prevot, and S. Noselli for comments on the manuscript. I. T. is a Fellow of the Cancer Research Fund of the Damon Runyon Walter Winchell Foundation (DR 1461), and Y. B. is supported by a predoctoral fellowship from the Boehringer Ingelheim Funds. N. P. is an Investigator of the Howard Hughes Medical Institute.

Received July 7, 1999; revised August 11, 1999.

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.

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.

Bradley, R.S., and Brown, A.M. (1990). The proto-oncogene int-1 encodes a secreted protein associated with the extracellular matrix. EMBO J. *9*, 1569–1575.

Brand, A.H., and Perrimon, N. (1993). Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. Development *118*, 401–415.

Bullock, S.L., Fletcher, J.M., Beddington, R.S., and Wilson, V.A. (1998). Renal agenesis in mice homozygous for a gene trap mutation in the gene encoding heparan sulfate 2-sulfotransferase. Genes Dev. *12*, 1894–1906.

Bumcrot, D.A., Takada, R., and McMahon, A.P. (1995). Proteolytic processing yields two secreted forms of sonic hedgehog. Mol. Cell. Biol. *15*, 2294–2303.

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

Cumberledge, S., and Reichsman, F. (1997). Glycosaminoglycans and WNTs: just a spoonful of sugar helps the signal go down. Trends Genet. *13*, 421–423.

David, G. (1993). Integral membrane heparan sulfate proteoglycans. FASEB J. *7*, 1023–1030.

David, G., Bai, X.M., Van der Schueren, B., Cassiman, J.J., and Van den Berghe, H. (1992). Developmental changes in heparan sulfate expression: in situ detection with mAbs. J. Cell Biol. *119*, 961–975.

Gisselbrecht, S., Skeath, J.B., Doe, C.Q., and Michelson, A.M. (1996). *heartless* encodes a fibroblast growth factor receptor (DFR1/DFGF-R2) involved in the directional migration of early mesodermal cells in the *Drosophila* embryo. Genes Dev. *10*, 3003–3017.

Gonzalez-Gaitan, M., and Jackle, H. (1995). Invagination centers within the *Drosophila* stomatogastric nervous system anlage are positioned by Notch-mediated signaling which is spatially controlled through *wingless*. Development *121*, 2313–2325.

Hecht, J.T., Hogue, D., Strong, L.C., Hansen, M.F., Blanton, S.H., and Wagner, M. (1995). Hereditary multiple exostosis and chondrosarcoma: linkage to chromosome II and loss of heterozygosity for *EXT*-linked markers on chromosomes II and 8. Am. J. Hum. Genet. *56*, 1125–1131.

Kirkpatrick, R.B., Ganguly, S., Angelichio, M., Griego, S., Shatzman, A., Silverman, C., and Rosenberg, M. (1995). Heavy chain dimers as well as complete antibodies are efficiently formed and secreted from *Drosophila* via a BiP-mediated pathway. J. Biol. Chem. *270*, 19800–19805.

Kitagawa, H., Shimakawa, H., and Sugahara, K. (1999). The tumor suppressor *EXT-like* gene *EXTL2* encodes an α1, 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.

Kooyman, D.L., Byrne, G.W., McClellan, S., Nielsen, D., Tone, M., Waldmann, H., Coffman, T.M., McCurry, K.R., Platt, J.L., and Logan, J.S. (1995). In vivo transfer of GPI-linked complement restriction factors from erythrocytes to the endothelium. Science *269*, 89–92.

Lee, J.J., Ekker, S.C., von Kessler, D.P., Porter, J.A., Sun, B.I., and Beachy, P.A. (1994). Autoproteolysis in hedgehog protein biogenesis. Science *266*, 1528–1537.

Lin, X., and Perrimon, N. (1999). Dally cooperates with *Drosophila* Frizzled2 to transduce Wingless signaling. Nature *400*, 281–284.

Lin, X.H., Buff, E.M., Perrimon, N.P., and Michelson, A.M. (1999). Heparan sulfate proteoglycans are essential for FGF receptor signaling during *Drosophila* embryonic development. Development *126*, 3715–3723.

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.

Mertens, G., Van der Schueren, B., van den Berghe, H., and David, G. (1996). Heparan sulfate expression in polarized epithelial cells: the apical sorting of Glypican (GPI-anchord proteoglycan) is inversely related to its heparan sulfate content. J. Cell Biol. *132*, 487–497.

Mullor, J.L., Calleja, M., Capdevila, J., and Guerrero, I. (1997). Hedgehog activity, independent of decapentaplegic, participates in wing disc patterning. Development *124*, 1227–1237.

Nakato, H., Futch, T.A., and Selleck, S.B. (1995). The division abnormally delayed (dally) gene: a putative integral membrane proteoglycan required for cell division patterning during postembryonic development of the nervous system in *Drosophila*. Development *121*, 3687–3702.

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.

Patel, N.H. (1994). Imaging neuronal subsets and other cell types in whole-mount *Drosophila* embryos and larvae using antibody probes. In Methods in Cell Biology, L.S.B. Goldstein and E.A. Fyrberg, eds. (New York, NY: Academic Press), pp. 445–487.

Patel, N.H., Schafer, B., Goodman, C.S., and Holmgren, R. (1989). The role of segment polarity genes during *Drosophila* neurogenesis. Genes Dev. *3*, 890–904.

Porter, J.A., von Kessler, D.P., Ekker, S.C., Young, K.E., Lee, J.J., Moses, K., and Beachy, P.A. (1995). The product of hedgehog autoproteolytic cleavage active in local and long-range signaling. Nature *374*, 363–366.

Porter, J.A., Ekker, S.C., Park, W.J., von Kessler, D.P., Young, K.E., Chen, C.H., Ma, Y., Woods, A.S., Cotter, R.J., Koonin, E.V., and Beachy, P.A. (1996a). Hedgehog patterning activity: role of a lipophilic modification mediated by the carboxy-terminal autoprocessing domain. Cell *86*, 21–34.

Porter, J.A., Young, K.E., and Beachy, P.A. (1996b). Cholesterol modification of hedgehog signaling proteins in animal development. Science *274*, 255–259.

Ramirez-Weber, F.-A., and Kornberg, T.B. (1999). Cytonemes: cellular processes that project to the principal signaling center in *Drosophila* imaginal discs. Cell *97*, 599–607.

Raskind, W.H., Conrad, E.U., Chansky, H., and Matsushita, M. (1995). Loss of heterozygosity in chondrosarcomas for markers linked to hereditary multiple exostoses loci on chromosomes 8 and 11. Am. J. Hum. Genet. *56*, 1132–1139.

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.

Rietveld, A., Neutz, S., Simons, K., and Eaton, S. (1999). Association of the cholesterol-linked N-terminal fragment of Hedgehog domain with *Drosophila* raft lipid microdomains. J. Biol. Chem. *274*, 12049–12054.

Ripoche, J., Link, B., Yucel, J.K., Tokuyasu, K., and Malhotra, V. (1994). Location of Golgi membranes with reference to dividing nuclei in syncytial *Drosophila* embryos. Proc. Natl. Acad. Sci. USA *91*, 1878–1882.

Salmivirta, M., Lidholt, K., and Lindahl, U. (1996). Heparan sulfate: a piece of information. FASEB J. *10*, 1270–1279.

Schlessinger, J., Lax, I., and Lemmon, M. (1995). Regulation of growth factor activation by proteoglycans: what is the role of the low affinity receptors? Cell *83*, 357–360.

Sen, J., Goltz, J.S., Stevens, L., and Stein, D. (1998). Spatially restricted expression of pipe in the *Drosophila* egg chamber defines embryonic dorsal–ventral polarity. Cell *95*, 471–481.

Simons, K., and Ikonen, E. (1997). Functional rafts in cell membranes. Nature *387*, 569–572.

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. (1997). A Hedgehog activity gradient contributes to AP axial patterning of the *Drosophila* wing. Development *124*, 4697–4705.

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

Tautz, D., and Pfeifle, C. (1989). A non-radioactive in situ hybridization method for the localization of specific RNAs in *Drosophila* embryos reveals translational control of the segmentation gene *hunchback*. Chromosoma *98*, 81–85.

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 signaling in *Drosophila*. Nature *400*, 276–280.

van der Meer, J. (1977). Optical clean and permanent whole mount preparations for phase contrast microscopy of cuticular structures of insect larvae. Dros. Inf. Serv. *52*, 160.

Van Hul, W., Wuyts, W., Hendrickx, J., Speleman, F., Wauters, J., De Boulle, K., Van Roy, N., Bossuyt, P., and Willems, P.J. (1998). Identification of a third *EXT-like* gene (*EXTL3*) belonging to the *EXT* gene family. Genomics *47*, 230–237.

Wise, C.A., Clines, G.A., Massa, H., Trask, B.J., and Lovett, M. (1997). Identification and localization of the gene for *EXTL*, a third member of the multiple exostoses gene family. Genome Res. *7*, 10–16.

Wuyts, W., Van Hul, W., Hendrickx, J., Speleman, F., Wauters, J., De Boulle, K., Van Roy, N., Van Agtmael, T., Bossuyt, P., and Willems, P.J. (1997). Identification and characterization of a novel member of the *EXT* gene family, *EXTL2*. Eur. J. Hum. Genet. *5*, 382–389.

Xu, T., and Rubin, G.M. (1993). Analysis of genetic mosaics in developing and adult *Drosophila* tissues. Development *117*, 1223–1237.

Zecca, M., Basler, K., and Struhl, G. (1995). Sequential organizing activities of *engrailed*, *hedgehog* and *decapentaplegic* in the *Drosophila* wing. Development *121*, 2265–2278.

Zhang, L., David, G., and Esko, J.D. (1995). Repetitive Ser–Gly sequences enhance heparan sulfate assembly in proteoglycans. J. Biol. Chem. *270*, 27127–27135.