Functions of heparan sulfate proteoglycans in cell signaling during development

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Summary

Heparan sulfate proteoglycans (HSPGs) are cell-surface and extracellular matrix macromolecules that are composed of a core protein decorated with covalently linked glycosaminoglycan (GAG) chains. In vitro studies have demonstrated the roles of these molecules in many cellular functions, and recent in vivo studies have begun to clarify their essential functions in development. In particular, HSPGs play crucial roles in regulating key developmental signaling pathways, such as the Wnt, Hedgehog, transforming growth factor-β, and fibroblast growth factor pathways. This review highlights recent findings regarding the functions of HSPGs in these signaling pathways during development.

Introduction

During metazoan development, the formation of complex body structures and patterns is governed by several secreted signaling molecules, including members of the Wnt/Wingless (Wg), Hedgehog (Hh), transforming growth factor-β (TGFβ) and fibroblast growth factor (FGF) families. Over the past decades, intensive biochemical and genetic studies have elucidated the central components of the signaling pathways that these molecules function in. More recently, attention has shifted to understanding the mechanisms by which the distributions of these signaling molecules are regulated in morphogenetic fields. Studies in *Drosophila* and vertebrates have demonstrated the crucial roles of heparan sulfate proteoglycans (HSPGs) in these signaling pathways during development. This review focuses on recent insights into the functions of HSPGs in regulating the activities and distributions of these signaling molecules. For detailed reviews of previous biochemical and genetic studies on the HSPGs, please see (Belting, 2003; Bernfield et al., 1999; Esko and Selleck, 2002; Lin and Perrimon, 2003; Nybakken and Perrimon, 2002; Princivalle and de Agostini, 2002; Selleck, 2001; Song and Filmus, 2002).

HSPG biochemistry

HSPGs are cell-surface and extracellular matrix (ECM) macromolecules that comprise a core protein to which heparan sulfate (HS) glycosaminoglycan (GAG) chains are attached (Bernfield et al., 1999; Esko and Selleck, 2002). HSPGs are classified into several families based on their core protein structure (Fig. 1). Glypicans and Syndecans are two major cell surface HSPGs, and are linked to the plasma membrane by a glycosylphosphatidylinositol (GPI) linkage or a transmembrane domain, respectively. Perlecans are secreted HSPGs that are mainly distributed in the ECM. Although Glypicans and Perlecans exclusively bear HS GAG chains, Syndecans are decorated with both HS and chondroitin sulfate (CS). All three families of HSPGs are evolutionarily conserved from vertebrates to *Drosophila* and *C. elegans* (Esko and Selleck, 2002; Nybakken and Perrimon, 2002). The *Drosophila* genome encodes four HSPG homologs: a single Syndecan (Sdc) (Johnson et al., 2004; Spring et al., 1994; Steigemann et al., 2004), two Glypicans [Division abnormally delayed (Dally) and Dally-like protein (Dlp) (Baeg et al., 2001; Khare and Baumgartner, 2000; Nakato et al., 1995)], and a Perlecan [Terribly reduced optic lobes (Trol) (Datta, 1995; Voigt et al., 2002)].

To date, most HSPG studies have demonstrated the importance of their HS chains. HS chains are polysaccharides synthesized in the Golgi apparatus and contain repeating disaccharide units of uronic acid linked to glucosamine (Esko and Selleck, 2002). HS chain biosynthesis is initiated at the GAG attachment site(s) of the core protein, which contains two to four Ser-Gly sequences. As depicted in Fig. 2, various glycosyltransferases and modification enzymes are involved in the polymerization and modification processes of HSPG biosynthesis. These enzymes are conserved in *Drosophila* and vertebrates (Esko and Selleck, 2002; Lin and Perrimon, 2003; Nybakken and Perrimon, 2002). In recent years, some of these enzymes have been genetically and biochemically characterized in *Drosophila* (see Table 1).

HSPGs in Wnt/Wg signaling

Wnts are secreted signaling molecules that function in numerous developmental processes in vertebrates and invertebrates (Wodarz and Nusse, 1998). Through the receptors of the Frizzled (Fz) family, Wnts can transduce their signals by a β-catenin/Armadillo-dependent pathway, called the 'canonical' Wnt pathway (Wodarz and Nusse, 1998). Wnts can also relay their signals via the planar cell polarity (PCP) pathway or Wnt/Ca^{2+} pathway, the so-called 'non-canonical' pathways (Veeman et al., 2003). Wnt proteins are lipid-modified, and are tightly associated with the cell surface and

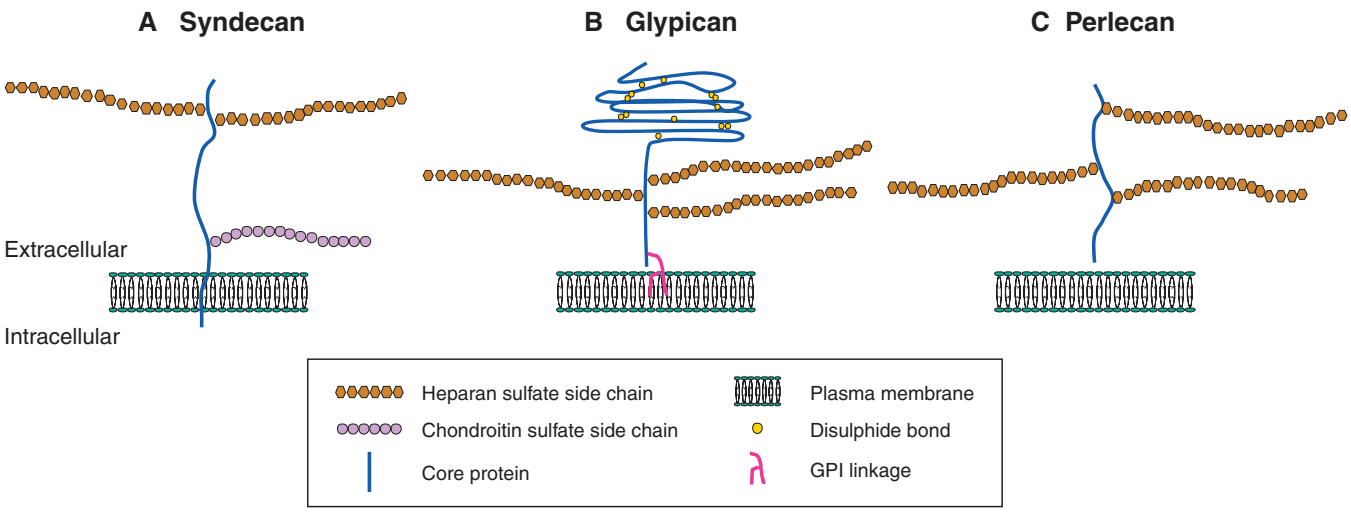


Fig. 1. The three main classes of cell-surface heparan sulfate proteoglycans (HSPGs). (A) Syndecan core proteins are transmembrane proteins that contain a highly conserved C-terminal cytoplasmic domain. Heparan sulfate (HS) chains attach to serine residues distal from the plasma membrane. Some syndecans also contain a chondroitin sulfate (CS) chain(s) that attaches to a serine residue(s) near the membrane. (B) The glypican core proteins are disulphide-stabilized globular core proteins that are linked to the plasma membrane by a glycosylphosphatidylinositol (GPI) linkage. HS chains link to serine residues adjacent to the plasma membrane. (C) Perlecans are secreted HSPGs that carry HS chains.

the ECM (Nusse, 2003). Therefore, how Wnt proteins move from cell to cell to initiate intercellular signaling is of particular interest.

Drosophila wg encodes a homolog of vertebrate Wnt1, and its function has been well explored in the patterning of the fly embryonic epidermis (see Box 1) and of the larval wing imaginal disc (see Box 2), where Wg acts as both a short-range inducer and a long-range morphogen (Tabata and Takei, 2004; Teleman et al., 2001; Vincent and Dubois, 2002).

Roles of HS chains in Wg signaling and distribution

The function of HSPGs in Wg signaling was first revealed by the identification of sugarless (*sgl*) (Binari et al., 1997; Hacker et al., 1997; Haerry et al., 1997) and sulfateless (*sfl*) (Lin and Perrimon, 1999) as segment polarity genes in *Drosophila*. *sgl* and *sfl* mutant embryos develop cuticle defects that are similar to those in Wg or Hh signaling mutants (Box 1), indicating that they function in Wg and/or Hh signaling. Other evidence has also implicated the involvement of HSPGs in Wg signaling. First, several Wg-dependent embryonic developmental processes are abnormal in *sgl* or *sfl* mutant embryos (Hacker et al., 1997; Lin and Perrimon, 1999). Second, Wg target-gene expression and extracellular Wg levels are reduced in *sfl* mutant clones in the wing disc (Baeg et al., 2001; Lin and Perrimon, 1999). Third, in vitro biochemical experiments have supported these findings (Reichsman et al., 1996). Finally, other segment polarity genes, including *fringe connection* (*frc*) and *slalom*, are also involved in HS biosynthesis, and their studies have provided further evidence for the involvement of HS chains in Wg signaling and distribution (Goto et al., 2001; Luders et al., 2003; Selva et al., 2001) (see Table 1 for more).

The *Drosophila* EXT proteins, Tout-velu (Ttv), Sister of ttv (Sotv) and Brother of ttv (Botv), are also involved in Wg signaling and distribution (Bornemann et al., 2004; Han et al., 2004a; Perrimon et al., 2004; Takei et al., 2004) (see Box 3, Fig. 2 and Table 1). Although previous studies had shown that Ttv is required specifically for Hh signaling (The et al., 1999) (as discussed later), the more recent careful analyses of the phenotypes of *ttv*, *sotv* and *botv* mutants have indicated that all three proteins are required for normal Hh, Wg and Dpp functions during wing development (Bornemann et al., 2004; Han et al., 2004a; Takei et al., 2004). Consistent with their expected roles in HS GAG biosynthesis, mutations in any of these genes lead to striking reductions in HS levels (Bornemann et al., 2004; Han et al., 2004a; Takei et al., 2004; Toyoda et al., 2000a; Toyoda et al., 2000b). These mutations also lead to reduced extracellular Wg protein levels and to the reduced expression of *Distal-less* (*Dll*), a Wg long-range target gene (Wodarz and Nusse, 1998). However, these proteins do not function redundantly in HS GAG biosynthesis, as *ttv* expression can only rescue the embryonic cuticle defect that is associated with the *ttv* mutant, and not those of the *sotv* and *botv* mutants (Han et al., 2004a). Together, these data suggest that all three EXT proteins are required for Wg long-range signaling, possibly by modulating extracellular Wg levels (Bornemann et al., 2004; Han et al., 2004a; Takei et al., 2004) (see Box 3).

Interestingly, Ttv, Sotv and Botv modulate Wg functions to varying degrees in the wing disc (Han et al., 2004a). Extracellular Wg levels are more greatly reduced in *botv* mutant clones than in either *ttv* or *sotv* clones. Moreover, the levels of *senseless* (*sens*), a Wg short-range target gene, are strikingly reduced in *botv* mutant clones and *ttv-sotv* double mutant clones, but not in *ttv* or *sotv* mutant clones. These data suggest that Ttv and Sotv act redundantly in Wg signaling, but that both are required for Wg distribution (Han et al., 2004a). Based on their observations, Han et al. have proposed that HSPGs have distinct roles in Wg distribution and signaling (Han et al., 2004a) (see Box 3 for more).

However, another study has shown that both Ttv and Sotv are required for Wg short-range signaling (Bornemann et al., 2004). This study showed that the expression of *achaete* (*ac*),

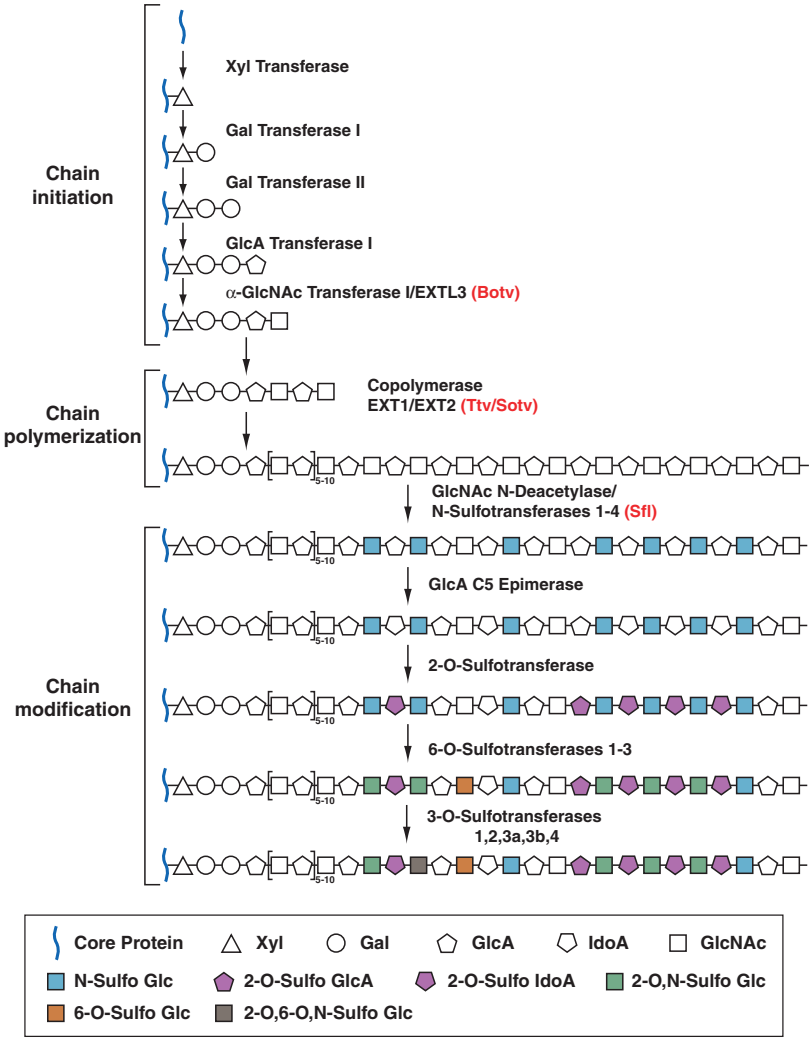


Fig. 2. Heparan sulfate chain biosynthesis. Heparan sulfate (HS) glycosaminoglycan (GAG) chains are synthesized on a core protein by the sequential action of individual glycosyltransferases and modification enzymes, in a three-step process involving chain initiation, polymerization and modification. HS chain synthesis begins with the assembly of a linkage tetrasaccharide on serine residues in the core polypeptide. This process is catalyzed by four enzymes (Xyl transferase, Gal transferase I-II and GlcA transferase I), which add individual sugar residues sequentially to the non-reducing end of the growing chain. After the assembly of the linkage region, one or more α-GlcNAc transferases add a single α1,4-linked GlcNAc unit to the chain, which initiates the HS polymerization process. HS chain polymerization then takes place by the addition of alternating GlcA and GlcNAc residues, which is catalyzed by the EXT family proteins. As the chain polymerizes, it undergoes a series of modifications that include GlcNAc N-deacetylation and N-sulfation, C5 epimerization of GlcA to IdoA, and variable *O*-sulfation at C2 of IdoA and GlcA, at C6 of GlcNAc and GlcNS units, and, occasionally, at C3 of GlcN residues. The HS GAG chains are ~100 or more sugar units long and have numerous structural heterogeneities. Four *Drosophila* enzymes, including Botv, Ttv, Sotv and Sfl, which are homologs of vertebrate EXTL3, EXT1, EXT2 and N-deacetylase/N-sulfotransferase, respectively, are highlighted in red. Gal, galactose; GlcNAc, N-acetylglucosamine; GlcA, glucuronic acid; GlcNS, N-sulfoglucosamine; IdoA, iduronic acid.

another Wg short-range target gene, is reduced in *sotv* or *ttv* mutant cells in the wing disc (Bornemann et al., 2004). The discrepancy between this and the findings of Han et al. could be due to the different sensitivity of *ac* and *sens* to Wg signaling. For example, higher levels of Wg signaling might be needed to activate *ac* expression than to activate *sens* expression, causing *ac* expression to be reduced in *sotv* or *ttv* mutant clones in the wing disc. Nevertheless, the effects of *sotv* and *ttv* mutations on Wg signaling and its distribution are relatively weak compared with those in *ttv-sotv* double or *botv* single mutants (Han et al., 2004a), indicating that some residual HS activity remains in the absence of *ttv* or *sotv* (see Box 3). Analyses of *ttv*, *sotv* and *botv* mutants in other developmental processes will further clarify this issue.

Roles of Dally and Dlp in Wg signaling and distribution

Which HSPG core proteins are involved in Wg signaling? Both Dally and Dlp, two *Drosophila* Glypicans, function in Wg signaling (Baeg et al., 2001; Lin and Perrimon, 1999; Tsuda et al., 1999). Disruption of Dally function by mutations in the *dally* locus or by its RNA interference (RNAi)-mediated knockdown results in weak segment polarity defects, suggesting that Dally may be required for Wg signaling in the embryonic epidermis (Lin and Perrimon, 1999; Tsuda et al., 1999). However, embryonic cuticle defects associated with *dally* mutant or *dally* RNAi embryos are relatively weak compared with those of *sgl* or *sfl* mutants. Subsequent studies have demonstrated that *dlp* RNAi embryonic segment polarity defects are stronger than those of *dally* RNAi knockdown embryos. *dally-dlp* RNAi embryos have more severe segment polarity defects than *dally* RNAi or *dlp* RNAi embryos (Baeg et al., 2001). These data suggest that both Dally and Dlp are required for Wg signaling in the embryonic epidermis (Baeg et al., 2001).

However, a recent study by Desbordes and Sanson suggests that Dally and Dlp, separately or together, are not necessary for Wg signaling in the embryonic epidermis (Desbordes and Sanson, 2003; Perrimon et al., 2004). Using *dally* RNAi against relatively small regions of *dally*, they showed that in *dally* knockdown embryos only very weak denticle fusion is present, similar to that seen in control embryos injected with buffer. Furthermore, the ectopic expression of *wg* in *dally-dlp* RNAi embryos can rescue *engrailed* (*en*) expression, a Wg target gene, and can generate naked cuticle, whereas ectopic expression of *hh* in *dlp* RNAi embryos fails to rescue *wg* expression, which is dependent on Hh signaling (Desbordes and Sanson, 2003). On the basis of these data, the authors argue that both Dally and Dlp are not required for Wg signalling, and

Table 1. *Drosophila* heparan sulfate (HS) biosynthesis mutants

Mutant (gene)	Protein (mammalian homolog)	Function	References
sugarless (*sgl*)	UDP-glucose dehydrogenase	GAG biosynthesis; effects on Wg, FGF, Dpp signaling	Binari et al., 1997; Hacker et al., 1997; Haerry et al., 1997; Toyoda et al., 2000a
sulfateless (*sfl*)	N-deacetylase/N-sulfotransferase	HS modification (mainly in sulfation); effects on Wg, Hh, and FGF signaling	Baeg et al., 2001; Lin et al., 1999; Lin and Perrimon, 1999; Toyoda et al., 2000a
tout-velu (*ttv*)	EXT1	HS co-polymerase; effects on Hh signaling and movement, Wg distribution, Dpp signaling and distribution	Bellaiche et al., 1998; Bornemann et al., 2004; Gallet et al., 2003; Han et al., 2004a; Takei et al., 2004; The et al., 1999; Toyoda et al., 2000a; Toyoda et al., 2000b
sister of ttv (*sotv*)	EXT2	HS co-polymerase; effects on Hh signaling and movement, Wg distribution, Dpp signaling and distribution	Bornemann et al., 2004; Han et al., 2004a; Takei et al., 2004
brother of ttv (*botv*)	EXTL3	HS polymerase; effects on Hh signaling and movement, Wg signaling and distribution, Dpp signaling and distribution	Han et al., 2004a; Kim et al., 2002; Takei et al., 2004
fringe connection (*frc*)	UDP-sugar transporter	Transfer of UDP-glucuronic acid, UDP-N-acetylglucosamine and possibly UDP-xylose from the cytoplasm into the lumen of the ER/Golgi; effects on Wg, Hh, FGF, and fringe-dependent Notch signaling	Goto et al., 2001; Selva et al., 2001
slalom	Adenosine 3′-phosphate 5′-phosphosulfate transporter	Transporter for adenosine 3′-phosphate 5′-phosphosulfate from the cytosol into the Golgi lumen; effects on Wg, Hh, FGF and DV patterning mediated by Pipe	Kamiyama et al., 2003; Luders et al., 2003

Dpp, Decapentaplegic; ER, endoplasmic reticulum; FGF, fibroblast growth factor; GAG, glycosaminoglycan; HS, heparan sulfate; Hh, hedgehog; UDP, uridine 5′-diphosphate; Wg, Wingless.

that Dlp is strictly required for Hh signaling (Desbordes and Sanson, 2003; Perrimon et al., 2004). Whether Dlp is involved solely in Hh, or in both Hh and Wg, signaling needs to be resolved using *dlp* or *dally-dlp* null embryos. It is possible that Dally and Dlp may only be involved in controlling Wg protein retention or stability, but not in Wg signaling per se in the embryonic epidermis, as suggested by Pfeiffer et al. (Pfeiffer et al., 2002).

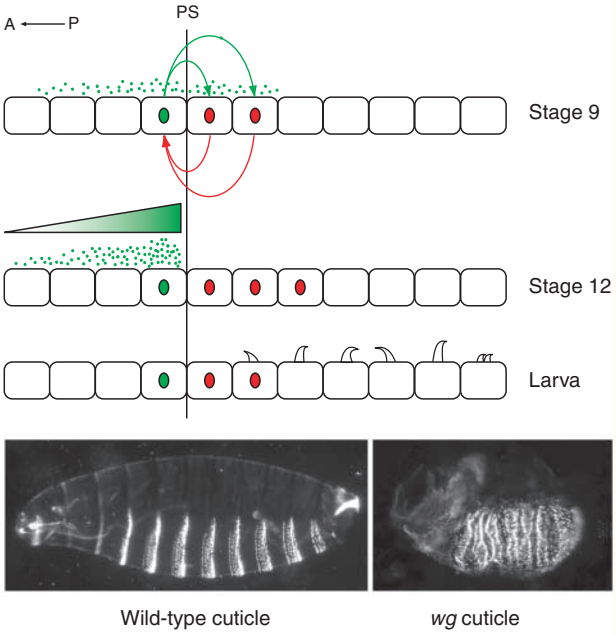
Relatively compelling evidence for the functioning of Dally and Dlp in Wg signaling has been demonstrated in the fly wing disc, where Hh and Wg signaling function independently from each other. *Dally* mutants exhibit wing margin defects and show genetic interactions with Wg signaling components, suggesting that Dally is required for Wg signaling in the wing disc (Lin and Perrimon, 1999). Interestingly, overexpressing *dlp*, but not *dally*, can strikingly increase extracellular Wg protein levels, suggesting that Dlp might bind to Wg with a higher affinity than Dally does (Baeg et al., 2001; Strigini and Cohen, 2000). However, overexpressing Dlp reduces, rather than enhances, Wg signaling. This could be because an excess amount of Dlp may compete with a limiting amount of Wg protein for its receptors Fz and Dfz2 (Fz2 – FlyBase), thereby inhibiting Wg signaling (Baeg et al., 2001). Whether Dlp plays a positive or a negative role in Wg signaling remains to be determined by clonal analysis in available *dlp* null mutants (Han et al., 2004b). Notum, which encodes a member of the alpha/beta-hydrolase superfamily, can also repress Wg signaling, possibly by modifying Dlp and/or Dally (Gerlitz and Basler, 2002; Giraldez et al., 2002), thus providing further evidence for the involvement of Dally and Dlp in Wg signaling.

HSPGs and Wnt signaling: lessons from vertebrate studies

Several recent studies have demonstrated the essential role of HSPGs in Wnt signaling during vertebrate development (De Cat et al., 2003; Ohkawara et al., 2003; Topczewski et al., 2001). In Zebrafish, *knypek* encodes a glypican that is essential for convergent extension movement during gastrulation (Topczewski et al., 2001). Genetic analyses have demonstrated that Knypek is required for the signaling activity of Wnt11, which acts via the non-canonical PCP pathway during vertebrate gastrulation (Topczewski et al., 2001). Similarly, in *Xenopus*, reducing glypican 4 (Xgly4) disrupt cell movements during gastrulation (Ohkawara et al., 2003). Xgly4 physically interacts with Wnt11, and might function in the Wnt/PCP pathway (Ohkawara et al., 2003). Interestingly, vertebrate glypican 3 can influence both canonical and non-canonical Wnt signaling in in vitro cell culture assays (De Cat et al., 2003), and blocking glypican 3 with antisense morpholino oligonucleotides disrupts gastrulation movements in zebrafish (De Cat et al., 2003). Glypican 3 is processed by a furin-like convertase (an enzyme involved in endoproteolytic processing), which is required for glypican 3 modulation of Wnt signaling both in vitro and in vivo (De Cat et al., 2003). Collectively, these data demonstrate that glypicans are required for Wnt signaling during vertebrate development. Whether other glypicans also require processing by a convertase(s), and whether a similar process exists in *Drosophila*, remains to be resolved.

One important finding from HSPG studies in vertebrates has been the role of sulfatases in Wnt signaling. Sulfatases modulate the sulfation states of HS chains by removing sulfates from them. As previously discussed, the *sfl*-encoded sulfotransferase in *Drosophila* is crucially required for proper Wg signaling (Lin and Perrimon, 1999). It is therefore surprising that QSulf1, the avian homolog of the HS-specific, *N*-acetyl glucosamine sulfatases, can promote Wnt signaling in myoblasts (Dhoot et al., 2001). In vertebrates, both Sulf1 and its highly related protein Sulf2 are secreted, HS-specific, 6-*O* endosulfatases (Ai et al., 2003; Morimoto-Tomita et al., 2002;

Box 1. Wg and Hh roles in patterning the *Drosophila* embryonic epidermis



During embryogenesis, Wg and Hh signaling have essential roles in patterning the embryonic epidermis. *wg* (shown in green) and *hh* (shown in red) are expressed in stripes within each segment of the epidermis. The position of the compartmental boundary that separates each segment, called the parasegmental (PS) boundary, is marked by the juxtaposition of Hh- and Wg-expressing cells, with the Wg stripe present at its anterior (A) and the Hh stripe at its posterior (P) edge. At stage 9 of embryonic development, Wg is distributed on both sides of the boundary (as shown by the green dots) and acts over a short-range posteriorly to maintain *hh* expression. Hh acts, in turn, anteriorly to maintain *wg* expression (as shown by the red arrows). At stage 12, Wg (shown by the green dots) is mainly distributed anteriorly, and forms a morphogen gradient that is required to produce the naked cuticle in larvae. In a wild-type embryo, as shown, the cuticle has an alternating pattern of naked cuticle and cuticle covered with small hairs called denticles. The *wg* mutant embryo is devoid of naked cuticle. Mutations in genes that affect either Wg and/or Hh signaling, such as in *sfl*, generate similar cuticle patterning defects, known as segment polarity defects.

Ohto et al., 2002). A recent study has shown that QSulf1 can function cell autonomously to remodel the sulfation state of cell-surface HS chains and can promote Wnt signaling when localized either at the cell surface or in the Golgi apparatus (Ai et al., 2003). Ai et al. propose that the HS 6-*O* desulfation activity of QSulf1 might convert cell-surface HSPGs to a low-affinity Wnt-binding state, thereby promoting the formation of low-affinity HS-Wnt complexes that can functionally interact with Frizzled receptors to initiate Wnt signal transduction (Ai et al., 2003; Ohto et al., 2002). As both Sulf1 and Sulf2 are secreted enzymes and are expressed in a variety of developmental contexts, they may participate in the signaling and distribution of Wnts, as well as that of other signaling molecules, such as the FGFs (see FGF signaling section). As *Drosophila* contains one QSulf1 homolog, it will be interesting to determine whether this protein is also required for Wg signaling.

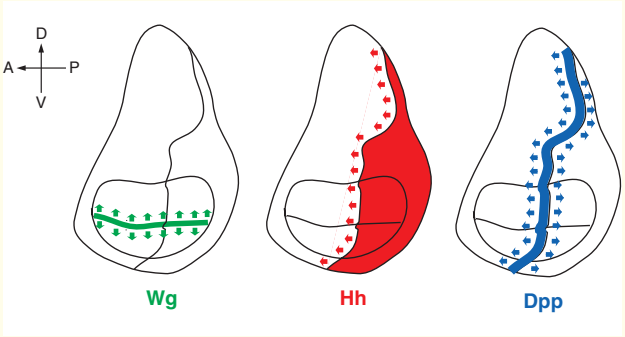
Mechanisms by which HSPGs modulate Wnt/Wg signaling

Two models have been proposed to explain how HSPGs modulate Wg signaling. In the first model, HSPGs can either stabilize the Wg protein from being degraded or reduce the dimensionality of Wg ligand diffusion from three to two dimensions, thereby maintaining the local concentration of Wg ligand that is available for its receptor (Hacker et al., 1997; Pfeiffer et al., 2002) (Fig. 3C). This model is supported by data from several studies. For example, in the fly embryonic epidermis, Wg protein is tightly associated with the surface of the *wg*-expressing cells and is undetectable in *sgl* null embryos (Hacker et al., 1997; Pfeiffer et al., 2002). Similarly, in the wing imaginal disc, extracellular Wg protein levels are strikingly reduced in *sfl* mutants (Baeg et al., 2001), and in the *ttv*, *sotv* and *botv* single mutants (Bornemann et al., 2004; Han et al., 2004a; Takei et al., 2004). Finally, the overexpression of Wg protein can partially rescue the segmentation defects associated with *sfl* and *sgl*, thereby compensating for the loss of Wg signaling (Hacker et al., 1997). In the second model, HSPGs are proposed to act as co-receptors that directly facilitate the formation of Wnt/Wg-Fz signaling complexes (Lin and Perrimon, 1999). Although most data support the first model, there is some evidence that HSPGs may directly regulate Wg signaling, possibly by acting as a co-receptor(s). The overexpression of *Notum*, for example, can extinguish the signaling activity of membrane-tethered Wg that does not move through tissue (Gerlitz and Basler, 2002). And, as already discussed, analyses of EXT proteins in Wg signaling suggest that HSPGs might have two distinct roles in Wg distribution and signaling (Han et al., 2004a) (see also Box 3).

HSPGs in Wg morphogen movement

The Wg morphogen can be transported either by transcytosis through dynamin-mediated endocytosis or by passive diffusion through the extracellular space (Tabata and Takei, 2004; Teleman et al., 2001; Vincent and Dubois, 2002). Existing data support the latter mode of Wg movement in the wing disc. As discussed earlier, extracellular Wg protein is lost in clones of cells defective in HS biosynthesis, such as in the *sfl* or *EXT* (*ttv*, *sotv* and *botv*) mutants, suggesting that HSPGs are involved in Wg morphogen distribution (Baeg et al., 2001; Bornemann et al., 2004; Han et al., 2004a; Takei et al., 2004). If this is so, how do HSPGs regulate Wg movement? Several mechanisms have been proposed. Extracellular Wg could move through restricted diffusion by attaching to the GAG chains of HSPGs, as has been proposed for Hh and Dpp movement (Belenkaya et al., 2004; Han et al., 2004b) (Fig. 3A). In support of this view, extracellular Wg accumulates in the wild-type cells next to *ttv-botv* double mutant cells (Takei et al., 2004), while it is strikingly reduced in the mutant cells (Baeg et al., 2001; Bornemann et al., 2004; Han et al., 2004a; Takei et al., 2004). Alternatively, HSPGs could control the stability of Wg morphogen to ensure that it diffuses across a field of cells without being degraded (Bornemann et al., 2004; Han et al., 2004a; Takei et al., 2004). These two processes are not

Box 2. Wg, Hh and Dpp morphogens are essential for patterning the *Drosophila* wing



Drosophila wings arise from wing imaginal discs that are subdivided into anterior (A)/posterior (P) and dorsal (D)/ventral (V) compartments. The wing imaginal disc provides an excellent model system to study morphogen signaling and gradient formation during development. The three morphogens Wg, Hh and Dpp are essential for patterning the wing imaginal disc. In a third instar larvae wing imaginal disc, Wg (green) is expressed at the DV border and acts as a long-range morphogen to signal dorsally (arrows up) and ventrally (arrows down) to organize DV patterning. Hh (red) is expressed in the P compartment and moves into the A compartment of the disc to activate the expression of its target genes, including *dpp* (blue) in a stripe of cells adjacent to the AP compartment boundary. Dpp acts as a long-range morphogen that controls the growth and patterning of wing cells along the AP axis beyond the central domain.

mutually exclusive and are most likely coupled during Wg gradient formation. It is currently unknown how HSPGs control the stability of Wg. HSPGs could prevent extracellular Wg from being degraded by extracellular proteinases (Bornemann et al., 2004), or they might bind to Wg and guide it to a different endocytosis route, thereby preventing it from being degraded by lysosomal-mediated degradation. In support of this, it has been shown that blocking Dynamin-mediated endocytosis can lead to enhanced extracellular Wg levels, suggesting that Wg is normally downregulated through this process (Strigini and Cohen, 2000).

HSPGs might also transport Wg protein through vesicles called argosomes, which are generated from basolateral membranes and can travel from Wg-producing cells to various regions of the wing disc (Greco et al., 2001). Wg-expressing cells produce argosomes that contain high levels of Wg. Following the treatment of these cells with either heparinase I or III, Wg is no longer detected on the plasma membrane of the Wg-expressing cells, nor in the surrounding tissue. These and other data led Greco et al. to propose that argosomes are involved in Wg morphogen transport, and that this process is likely to be mediated by HSPGs (Greco et al., 2001).

HSPGs in Hh distribution and signaling

Like Wnts, Hh signaling molecules act as key mediators in many developmental processes and essentially require HSPGs for their proper distribution and signaling activity (Ingham and McMahon, 2001; Lin and Perrimon, 2003).

Functions of HSPGs in Hh distribution

The first evidence that HSPGs function in Hh distribution came from the genetic analysis of *ttv* (Bellaiche et al., 1998) (see Box 3 and Table 1). In the wing disc, Hh acts as a morphogen that forms a concentration gradient in an anterior strip of cells near the anteroposterior (AP) border of the wing disc (see Box 2). In wing discs containing *ttv* mutant clones, Hh can only be detected in the posterior-most *ttv* mutant cells that lie adjacent to wild-type cells. Furthermore, Hh can diffuse through a *ptc* mutant clone in the wing disc, but not through *ptc-ttv* double mutant cells (Bellaiche et al., 1998). Mainly based on these data, Bellaiche et al. proposed that a Ttv-modified HSPG is required for Hh to move from the cells where it is expressed to the anterior Hh-receiving cells (Bellaiche et al., 1998). Several recent studies have also shown similar defects in Hh distribution in wing clones mutant for *sfl*, *sotv* or *botv* (Han et al., 2004a; Takei et al., 2004). Importantly, Hh protein accumulates abnormally in the posterior compartment when the *ttv-botv* double mutant clones are made in the anterior compartment along the AP boundary, further suggesting that Hh fails to move into the HSPG mutant cells (Takei et al., 2004).

HSPGs are also required for Hh movement in the embryonic epidermis. The mature form of Hh, HhNp (see Box 4) is distributed as large punctate particles between Hh-expressing cells (Gallet et al., 2003). Cholesterol modification is required for HhNp to form these punctate particles (Gallet et al., 2003). In *ttv* mutant embryos, these punctate particles are not distributed between Hh-expressing cells (Gallet et al., 2003; The et al., 1999). Although Ttv is required for HhNp movement, it is not required for that of HhN (see Box 4) (Gallet et al., 2003; The et al., 1999). Together, these studies support the notion that HSPGs are required for the movement of cholesterol-modified HhNp.

The HSPG core proteins Dally and Dlp are also involved in Hh movement in the embryonic epidermis (Han et al., 2004b). *dlp* mutant embryos exhibit virtually identical defects to those of *hh* mutant embryos, and in their epidermis, Hh punctate particles can only be detected in Hh-expressing cells, and not in adjacent cells (Han et al., 2004b). However, in the wing disc, both Dally and Dlp act partially redundantly in Hh movement (Han et al., 2004b).

HSPGs might also control the stability of Hh by protecting it from degradation. Interestingly, Hh levels are reduced in Hh-producing cells mutant for *sotv* or *ttv* (Bornemann et al., 2004). Bornemann et al. argue that, by extension, Hh ligand instability could also contribute to reduced Hh levels and signaling in Hh-receiving cells lacking HSPGs (Bornemann et al., 2004). Although the evidence of Hh accumulation in front of HSPG-defective cells implicates HSPGs in Hh movement (Takei et al., 2004), current data suggest that HSPGs are likely to be involved in both Hh movement and stability.

Mechanism(s) of HSPG-mediated Hh movement

Both vertebrate sonic hedgehog (Shh) and *Drosophila* Hh are secreted from cells as multimeric and monomeric forms (Chen et al., 2004; Zeng et al., 2001). The soluble Shh multimeric form is freely diffusive (Zeng et al., 2001) and is responsible for activating Shh-target genes (Chen et al., 2004). Do secreted Hh proteins freely diffuse to receiving cells through extracellular spaces, and what is the role of HSPGs in Hh

movement? Han et al. have demonstrated that a narrow strip of *sfl* or *ttv* mutant cells in the fly wing disc is sufficient to completely block Hh signaling in the anterior wild-type cells adjacent to mutant cells, suggesting that Hh fails to move across these HSPG-deficient cells (Han et al., 2004a) (Fig. 3). Similar results are observed in clones mutant for both *dally* and *dlp* (Han et al., 2004a). Han et al. further showed that HSPG-mediated Hh movement is independent of dynamin-mediated endocytosis (Han et al., 2004a). On the basis of these and other data, Han et al. proposed that Hh movement is mediated by restricted diffusion involving Dally and Dlp (Han et al., 2004b) (Fig. 3A).

Roles of HSPGs in Hh signaling

HSPGs are also required for Hh signaling. In tissue culture experiments, Dlp, but not Dally, Sdc or Trol, is required for Hh

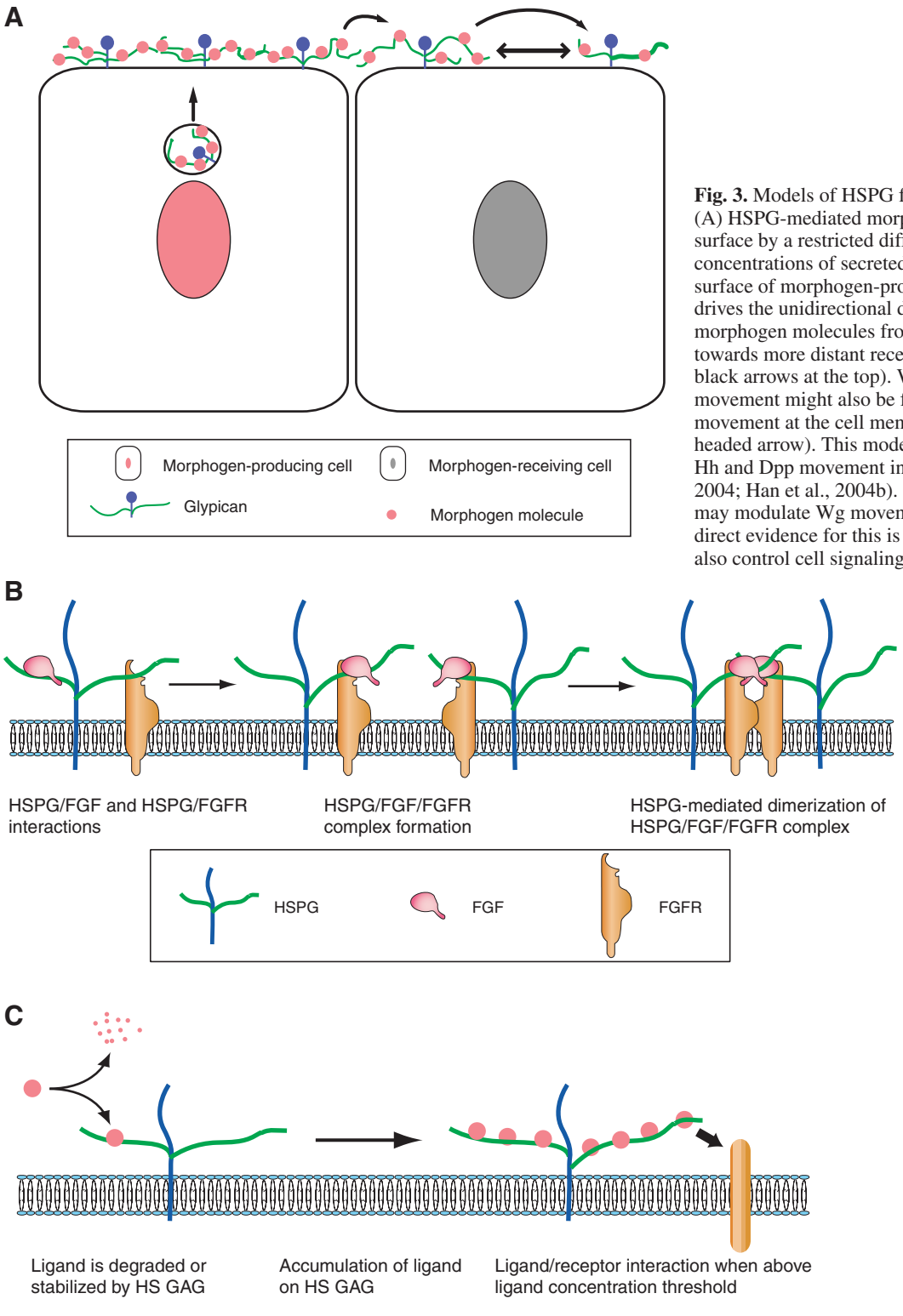


Fig. 3. Models of HSPG function in cell signaling. (A) HSPG-mediated morphogen movement along the cell surface by a restricted diffusion mechanism. The different concentrations of secreted morphogen molecules on the surface of morphogen-producing and -receiving cells drives the unidirectional displacement of secreted morphogen molecules from one HS GAG chain to another, towards more distant receiving cells (indicated by the thin black arrows at the top). Within the same cell, ligand movement might also be facilitated by lateral HSPG movement at the cell membrane (indicated by a double-headed arrow). This model fits well for HSPG-mediated Hh and Dpp movement in the wing disc (Belenkaya et al., 2004; Han et al., 2004b). It is also possible that HSPGs may modulate Wg movement in a similar way, although direct evidence for this is still lacking. (B) HSPGs might also control cell signaling by facilitating the dimerization or oligomerization of ligands with their receptors to initiate cell signaling, as in FGF signalling, where HSPGs facilitate the formation of the HSPG/FGF/FGFR signaling complexes (Ornitz, 2000; Pellegrini, 2001). Dlp may regulate Hh signaling in a similar way by facilitating Hh-Ptc receptor interactions in the embryonic epidermis and in cultured cells (Desbordes and Sanson, 2003; Lum et al., 2003). (C) Rather than being required for the formation of an active ligand-receptor complex(es), HSPGs might alternatively control ligand stability or retention at the cell surface, through the binding of ligands to HS GAG chains. Accumulated ligands might thus promote maximal signaling through their receptors. This model is supported by data on the role of HSPGs in Wg signaling in the embryonic epidermis (Hacker et al., 1997; Lin and Perrimon, 2003; Pfeiffer et al., 2002).

Box 3. A model for EXT protein function in morphogen signaling and distribution

The *Drosophila* genome contains three EXT family members, *tout-velu* (*ttv*), *sister of ttv* (*sotv*) and *brother of ttv* (*botv*), which encode *Drosophila* homologs of vertebrate EXT1, EXT2 and EXT-like 3 (EXTL3), respectively. These genes belong to the hereditary multiple exostoses (EXT) family of glycosyltransferases (Esko and Selleck, 2002; Zak et al., 2002), which function in HS chain biosynthesis, and are so-called because in humans *EXT1* and *EXT2* mutations cause hereditary multiple exostoses (HME), a benign bone tumor characterized by multiple cartilage-capped bone outgrowths (Zak et al., 2002). On the basis of genetic and biochemical data, as well as from previous biochemical data on the EXT proteins (Zak et al., 2002), Han et al. (Han et al., 2004a) have proposed that Botv might have different functions from Ttv and Sotv in HS-GAG biosynthesis; whereas Ttv and Sotv function as co-polymerases in HS GAG polymerization, Botv probably functions in HS-GAG initiation and possibly in HS GAG polymerization as well. According to this theory, no HS GAG chains would be initiated in the absence of Botv; thus *Botv* mutations would disrupt all HS GAG chain functions. However, HS GAG initiation would still occur in the absence of either Ttv or Sotv. In this instance, the residual activity of the remaining HS GAG polymerase(s), together with Botv, might result in the synthesis of abnormally short HS GAG chains. While these might be able to function in Wg signaling as a Wg co-receptor, they might be insufficiently active to maintain the proper levels of secreted Wg, Hh and Dpp proteins. When both Ttv and Sotv are absent, HS GAG polymerization would not occur, as in the botv mutant. Alternatively, fewer intact HS GAG chains might be synthesized in the absence of either Ttv or Sotv.

signaling (Lum et al., 2003). As mentioned earlier, Desbordes and Sanson have also argued that Dlp is specifically required for Hh signaling in the embryonic epidermis (Desbordes and Sanson, 2003). Although there are some arguments regarding whether Dlp is also involved in Wg signaling (Perrimon et al., 2004), the reduced *bagpipe* expression, which is normally activated by Hh signalling but is inhibited by Wg signalling in mesodermal cells of *dlp* null embryos, resemble that of *hh* mutant embryos, providing further evidence that Hh signaling requires Dlp during fly embryogenesis (Han et al., 2004a). Importantly, although previous studies have shown that ectopic expression of Hh can rescue the cuticle defects associated with HS GAG mutants, such as those seen in *sgl*, *sfl*, *frc* and *slalom* mutants (Luders et al., 2003; Perrimon et al., 2004; Selva et al., 2001), ectopic expression of Hh fails to restore Hh signaling activity, as assayed by *wg* expression, in *dly* RNAi embryos (Desbordes and Sanson, 2003). These results suggest that the core protein of Dly is crucially required for Hh signaling, whereas the attached HS chains are required for optimal Hh signaling activity. Alternatively, the Dly core protein may be required for Hh processing in the embryonic epidermis. It is also important to note that although Dlp is required for Hh signaling during embryogenesis, Dlp is functionally redundant with Dally in Hh signaling in the wing disc (Han et al., 2004b), suggesting that the specificity of HSPG involvement in Hh signaling depends on the developmental context.

Dlp might regulate Hh signaling in several ways. It might modulate Hh levels at the cell surface and indirectly control the interaction of Hh with its receptor Ptc. Alternatively, it might act as a co-receptor, perhaps by transferring Hh to its receptor Ptc, or by forming a Hh-Dlp-Ptc ternary complex in which Dlp may function to facilitate the Hh-Ptc interaction or to stabilize a Hh-Ptc complex, as in the case of FGF signaling (Ornitz, 2000; Pellegrini, 2001). On the one hand, in tissue culture experiments, Lum et al. showed that Dlp acts cell autonomously upstream or at the level of Ptc to activate the expression of an Hh responsive-reporter, indicating that Dlp might deliver Hh to its receptor. However, the knockdown of Dlp did not block Hh signaling when Hh was expressed in responding cells (Lum et al., 2003). These results suggest that Dlp is not absolutely required for Hh signaling in the presence of relatively large amounts of Hh. On the other hand, ectopic expression of Hh or HhN fails to rescue Hh signaling defects in *dlp*-RNAi embryos (Desbordes and Sanson, 2003), and thus these data suggest that Dlp acts as a co-receptor for Hh signaling (Desbordes and Sanson, 2003). Studies in the *dlp* null mutant should help to resolve this issue.

Hh signaling might also be regulated by other HSPG core proteins in different tissues or developmental processes. For example, mutations in the gene encoding Trol, the *Drosophila* Perlecan that forms a complex with Hh, causes neuroblasts to undergo cell cycle arrest in the larval brain (Datta, 1995; Park et al., 2003; Voigt et al., 2002). Genetic interaction experiments also indicate that *trol* is required for Hh signaling during neuroblast division (Park et al., 2003).

HSPGs in BMP signaling and morphogen gradient formation

The TGFβ superfamily of secreted proteins, such as the bone morphogenetic proteins (BMPs), act as morphogens in many developmental contexts to pattern tissues. *Drosophila* Decapentaplegic (Dpp), the functional ortholog of vertebrate BMP2 and BMP4, provides a paradigm for studying the mechanisms of BMP signaling (Raftery and Sutherland, 1999). Dpp acts as a long-range morphogen during wing development (Box 2), and its activity and ligand gradients can be assayed by monitoring the activated form of Mothers against Dpp (pMAD), a cytoplasmic transducer of Dpp signaling (Tanimoto et al., 2000), and by GFP-Dpp fusion proteins that retain signaling activity (Entchev et al., 2000; Teleman and Cohen, 2000).

Roles of HSPGs in Dpp signaling

dally mutants exhibit various *dpp*-like patterning defects and these defects show genetic interactions with the *dpp* signaling pathway (Jackson et al., 1997; Nakato et al., 1995). Ectopic expression of Dally results in enhanced Dpp signaling in the fly wing disc (Fujise et al., 2003). These data suggest a positive role of Dally in Dpp signaling. Recent data suggest that Dally and Dlp are partially redundant in Dpp signaling, as the removal of both Dally and Dlp causes stronger Dpp signaling defects than those seen in either *dally* or *dlp* mutants (Belenkaya et al., 2004). Consistent with the involvement of Dally and Dlp in Dpp signaling, Dpp signaling is strikingly reduced in cells defective in HS biosynthesis such as in *EXT* mutants (Bornemann et al., 2004; Han et al., 2004a; Takei et al., 2004) or in the *sfl* mutant (Belenkaya et al., 2004).

Box 4. Hh modifications and trafficking during development

Hh proteins undergo several post-translational modifications to become fully active (Ingham and McMahon, 2001; Jeong and McMahon, 2002). The precursor of Hh is first autocatalytically cleaved to produce an N-terminal (HhN) and a C-terminal (HhC) fragment. A cholesterol moiety is then covalently attached to the last amino acid of HhN to create HhNp (p stands for processed), which is responsible for the biological activities of all Hh proteins (Ingham and McMahon, 2001; Jeong and McMahon, 2002). This hydrophobic cholesterol moeity is thought to bind Hh to cell membranes. HhNp is further modified by the addition of palmitate, which is essential for its signaling activity (Jeong and McMahon, 2002; Nusse, 2003). In *Drosophila* and vertebrates, Hh trafficking is controlled by two transmembrane proteins, patched (Ptc) and dispatched (Disp) (Ingham and McMahon, 2001). Disp is required for the release of Hh from Hh-producing cells. Ptc is a receptor for Hh in its receiving cells and limits the range of Hh. There are three vertebrate Hh members, sonic (Shh), Indian (Ihh) and desert (Dhh) Hh, which transduce their signals through a conserved Hh signaling pathway (Ingham and McMahon, 2001).

What is the role of HSPGs in Dpp signaling? Elevated levels of Dally increase the sensitivity of cells to Dpp in a cell-autonomous fashion in the wing disc, leading the authors to propose that Dally might serve as a co-receptor for Dpp (Fujise et al., 2003). However, Dally overexpression might lead to enhanced Dpp signaling activity by increasing the levels of Dpp on Dally-expressing cells. Moreover, Dpp signaling is not defective in the first row of *sfl* single or *dally-dlp* double mutant cells if they are adjacent to wild-type cells that face Dpp-producing cells (Belenkaya et al., 2004). Finally, extracellular Dpp levels are strikingly reduced in *sfl* or *dally-dlp* mutant cells (Belenkaya et al., 2004). These data suggest that the main function of HSPGs in Dpp signaling in the wing disc is to modulate levels of Dpp (Fig. 3C) (Han et al., 2004a; Takei et al., 2004; Belenkaya et al., 2004).

Biochemical studies have demonstrated that both Dpp and BMP2 are heparin-binding proteins (Groppe et al., 1998; Ruppert et al., 1996), and that the binding of BMP2 to heparin requires its N-terminal domain (Ruppert et al., 1996). Recently, the crystal structure of BMP2 alone and in complex with its receptor has revealed that the N-terminal domains of both BMP2 monomers are well placed for GAG binding (Kirsch et al., 2000; Scheufler et al., 1999). Moreover, the GAG-binding domain of BMP2 is not located in regions required for BMP2 dimerization and receptor interaction. These data suggest that HSPGs are unlikely to be directly involved in Dpp/BMP signaling as a co-receptor, as they are in FGF signaling (Ornitz, 2000; Schlessinger et al., 1995), nor are they likely to be essential for Dpp signaling in tissues where Dpp is abundant. Indeed, in striking contrast to the requirement for HSPGs for proper Dpp signaling during wing development, in *sgl*, *sfl*, *ttv*, *sotv* and *botv* mutant embryos, embryonic dorsoventral (DV) patterning controlled by Dpp signaling is not defective (Haerry et al., 1997; Han et al., 2004a; Lin and Perrimon, 1999). During early embryogenesis, Dpp is highly expressed in the dorsal half of the embryo, and its activity gradient is regulated by an inverse gradient of its antagonist Short gastrulation (Srinivasan et al., 2002).

HSPGs are required for Dpp morphogen gradient formation

Dpp acts as a morphogen during wing disc development (Tabata and Takei, 2004; Vincent and Dubois, 2002). Do HSPGs control the movement of the Dpp morphogen in the wing disc? Previous findings have suggested that the Dpp morphogen moves across cells by a transcytosis mechanism through Dynamin-mediated endocytosis (Entchev et al., 2000). However, Lander et al. have proposed on theoretical grounds that diffusive mechanisms of Dpp transport are much more plausible than non-diffusive mechanisms (Lander et al., 2002). Several studies implicate the involvement of HSPGs in Dpp distribution. Dpp distribution is defective in wing disc cells mutant for HS biosyntheis, such as in the *EXT* or *sfl* mutants (Belenkaya et al., 2004; Han et al., 2004a; Takei et al., 2004). Both Dally and Dlp are required for Dpp distribution (Belenkaya et al., 2004; Fujise et al., 2003). Importantly, recent data also demonstrate that although Dynamin-mediated endocytosis is required for Dpp signaling it is not essential for Dpp morphogen movement (Belenkaya et al., 2004). Extracellular Dpp can move across endocytosis defective cells, but not across *sfl* or *dally-dly* mutant cells (Belenkaya et al., 2004). These data led Belenkaya et al. to propose that Dpp moves across cells through a restricted diffusion mechanism that involves Dally and Dlp (Belenkaya et al., 2004). Dally and Dlp have been proposed to have a similar role in Hh movement (Han et al., 2004b) (Fig. 3A).

Functions of HSPGs in FGF signaling

FGFs are among the best-studied HSPG-binding proteins (Ornitz, 2000), and play many important roles in regulating cell proliferation, migration and differentiation during development (Coumoul and Deng, 2003; Ornitz and Itoh, 2001). Recent studies have provided new insights into the mechanisms by which HSPGs modulate FGF signaling in development.

HSPGs in FGF signaling

As in the case of Wnt signaling, the first genetic evidence of a role for HSPGs in FGF signaling came from *sgl* and *sfl* mutant embryos, which show defective FGF signaling during *Drosophila* embryogenesis (Lin et al., 1999). In *Drosophila*, two FGF receptors, Heartlless (Htl) and Breathless (Btl), are required for the migration of mesodermal and tracheal cells, respectively. In *sgl* and *sfl* mutant embryos, both Htl and Btl signaling are defective, demonstrating the essential role of HSPGs in FGF signaling in development (Lin et al., 1999; Nybakken and Perrimon, 2002). It is currently unknown which HSPG core proteins are involved in Htl- and Btl-mediated FGF signaling during embryogenesis. However, Trol is not only required for Hh signaling, but is also involved in Btl-mediated FGF signaling during neuroblast division in the larval brain (Park et al., 2003). Study of available null mutants in *sdc*, *dally* and *dly* should reveal which core proteins are involved in FGF signaling in *Drosophila*.

Recent studies have provided in vivo evidence for the requirement of HSPGs in FGF signaling in vertebrates. First, *lazy mesoderm* (*lzme*), a mouse homolog of *Drosophila sgl*, arrests during gastrulation with defects in mesoderm and endoderm migration, which require FGF signaling (Garcia-Garcia and Anderson, 2003). In *lzme* embryos, although *Fgf8* expression is not affected, the expression of several Fgf8

downstream-target genes is defective, resembling the altered expression patterns seen in *Fgf8*$^{-/-}$ embryos. These data strongly suggest that *lzme* is required for FGF8 signaling in mice (Garcia-Garcia and Anderson, 2003). Second, in *Xenopus* embryos, depleting Glypican 4 transcripts with antisense morpholino oligonucleotides causes FGF signaling defects during neural tube closure (Galli et al., 2003).

HS chain structure and FGF signaling

Biochemical studies have demonstrated the importance of 6-*O* sulfation of HS for FGF signaling (Nakato and Kimata, 2002). Crystallographic studies have further suggested that the 6-*O* sulfation of HS is required for FGF1-FGFR2 and FGF2-FGFR1 interactions (Pellegrini et al., 2000; Schlessinger et al., 2000). The 6-*O* sulfation of HS is indeed essential for FGF signaling in development. In *Drosophila*, this 6-*O* sulfation is catalyzed by a single *Heparan sulfate 6-O-sulfotransferase* (*Hs6st*) gene (Kamimura et al., 2001). Interestingly, Hs6st is specifically expressed in mesodermal and tracheal cells. Knocking down this transcript by RNAi severely perturbs tracheal development and diminishes Btl-dependent MAPK activity (Kamimura et al., 2001), suggesting that 6-*O* sulfations in HS chains are essential for FGF signaling.

As mentioned earlier, QSulf1, a HS-specific 6-*O* endosulfatase, and its mammalian orthologs can promote Wnt signaling (Ai et al., 2003; Dhoot et al., 2001). By contrast, two recent studies have shown that Sulf1 inhibits FGF signaling. First, the expression of human *SULF1* resulted in a striking reduction in FGF2 signaling activity in ovarian cancer cell lines (Lai et al., 2003). Second, QSulf1 was shown to block FGF signaling activity in both *Xenopus* and chicken embryos (Wang et al., 2004). These studies provide further evidence of the involvement of 6-*O* sulfation of HS in FGF signalling, and also indicate that Sulf1, and possibly Sulf2, has a dual regulatory function as a positive regulator of Wnt signaling and as a negative regulator of FGF signaling.

How HSPGs modulate FGF signaling

Biochemical studies have provided evidence that HSPGs participate in FGF signaling by directly interacting with FGFs and their receptors in a ternary complex on the cell surface (Ornitz, 2000; Pellegrini, 2001). The crystal structures of FGF-FGFR-heparin complexes have shown that one FGF, one heparin molecule and one FGFR chain constitute the 1:1:1 FGF-FGFR-heparin complex (Pellegrini, 2001; Pellegrini et al., 2000; Schlessinger et al., 2000). One possible role of heparin/HSPGs within the FGF signaling complex is to facilitate the FGF and FGFR interaction (Fig. 3B). Alternatively, heparin/HSPGs may enhance the stability of a binary FGF-FGFR complex. In support of this view, various biochemical studies have demonstrated that heparin can increase the affinity of FGF for its receptor (Ornitz, 2000).

Specificity of HSPGs in developmental signaling pathways

The studies described above have illustrated that HSPGs have essential roles in the Wnt, Hh, TGFβ and FGF developmental signaling pathways. Do HSPGs have any specificity in regulating these pathways? How do HSPGs control these pathways in various tissues? Now studies in *Drosophila* and vertebrates are providing evidence that the specificity of HSPG function in these pathways is controlled by both the HSPG core proteins and their attached HS GAG chains. Furthermore, these HSPG functions are being discovered to be context dependent.

The nature of HSPG core proteins is likely to contribute to the specificity of HSPGs in cell signaling. The distribution of HS GAG chains solely depends on the expression of HSPG core proteins. The expression of HSPG core proteins is, in turn, regulated by Wg, Hh and Dpp signaling, as has been shown for Dally in the wing disc (Fujise et al., 2001; Fujise et al., 2003). HSPG core proteins can also contribute to the modification of HS GAG chains (Chen and Lander, 2001; Esko and Zhang, 1996). The GAG attachment sites and their surrounding amino acid sequence can determine the number of HS chains that attach to a specific HSPG core protein (Esko and Selleck, 2002). Moreover, all of the Glypican core proteins share a unique, highly conserved N-terminal globular domain that contains 14 cysteine residues, which is a potent enhancer of preferential HS glycosylation (Chen and Lander, 2001). Finally, The HSPG core proteins may be directly involved in cell signaling. For example, Glypican proteins contain a GPI anchor. This anchor is enriched in the lipid raft domain, which has been implicated in protein sorting and in signaling complex assembly (Simons and Toomre, 2000). Other parts of the Glypican protein core could be directly involved in cell signaling. Indeed, while overexpression of Hh can rescue Hh signaling defects in *sgl* null embryos (Hacker et al., 1997), it fails to restore Hh signaling activity in *dlp* RNAi embryos (Desbordes and Sanson, 2003). Similarly, in Xenopus, the overexpression of full-length Xgly4 can inhibit Activin-induced elongation (Ohkawara et al., 2003). These data suggest direct roles for Glypican core proteins in cell signaling.

The specificity of HSPGs is also controlled by the biosynthesis of HS GAG chains. As shown in Fig. 2, several glycosyltransferases and modification enzymes are involved in this process. Genetic studies of the *Drosophila* EXT proteins Ttv, Botv and Sotv, have illustrated the involvement of HS biosynthesis in specific functions of HSPGs. For example, these proteins are required for Hh and Dpp signaling, but neither Wg nor FGF signaling is defective in *ttv* mutant embryos during embryogenesis (The et al., 1999). By contrast, Wg short-range signaling is defective in both *botv* single and *ttv-sotv* double mutant clones in the wing disc (Han et al., 2004a). Specific defects associated with distinct modifications of HS GAG chains have also been reported. *Drosophila Hs6st* is specifically expressed in mesodermal and tracheal cells during fly embryogenesis, and is required for FGF signaling (Kamimura et al., 2001). In the mouse, mutations in HS 2-*O*-sulfotransferase (Hs2st) (Bullock et al., 1998; Merry et al., 2001) and D-glucuronyl C5-epimerase (Li et al., 2003) are associated with renal agenesis and skeletal malformations, but major organ systems, including the brain, liver and gastrointestinal tract appear to be normal. Although specific signaling defects have not yet been identified in these two mouse mutants, the unique patterning defects observed indicate that Hs2st and the C5 epimerase have specific functions in development. Perhaps the disruption of the *Drosophila* homologs of these genes will shed light on the signaling pathways they act in.

The requirement for HSPGs for the activity of certain

signaling pathways also depends on the developmental context. For example, Dpp signaling in DV patterning during embryogenesis is not defective in fly embryos mutant for HS biosynthesis enzymes (*sgl*, *sfl* or Exts) or HSPG core proteins (Dally and Dlp) (Haerry et al., 1997; Han et al., 2004a; Lin and Perrimon, 1999; The et al., 1999). By contrast, clones mutant for these genes show striking defects in Dpp distribution and signaling in the wing disc (Belenkaya et al., 2004; Bornemann et al., 2004; Fujise et al., 2003; Han et al., 2004a; Takei et al., 2004). One explanation for this is that *dpp* is highly expressed in the dorsal half of embryos, where the Dpp activity gradient is regulated by an inverse gradient of its antagonist Short gastrulation (Srinivasan et al., 2002), whereas in the wing disc, Dpp forms concentration gradients to transduce its signaling. As such, the main function of HSPGs in Dpp signaling in the wing disc is likely to be the modulation of extracellular Dpp levels by preventing it from being degraded. A recent study has also suggested that the role of proteoglycans in *Drosophila* Wg and Dpp signaling has not been conserved in vertebrates (Garcia-Garcia and Anderson, 2003), owing to the fact that mouse embryos with mutations in *lzme* are defective in FGF signaling, but not in Wnt3 or Nodal (a TGFβ member) signaling during gastrulation. These authors propose that many of the phenotypes of proteoglycan-related human disorders could be due to disrupted FGF signaling. However, in other developmental contexts, such as in developing limbs, HSPGs may play essential roles in Wnt and BMP signaling. For example, loss of mouse glpyican 3 in association with heterozygous *Bmp4* deficiencies results in polydactyly and skeletal abnormalities that are not seen in either *Gpc3* null or *Bmp4* heterozygous mice, suggesting a role for glypican 3 in BMP4 signaling (Paine-Saunders et al., 2000).

Conclusion

Developmental and biochemical studies in *Drosophila* and vertebrate model systems have revealed essential roles for HSPGs in regulating a number of developmental signaling pathways in a variety of developmental contexts. HSPGs appear to control these signaling pathways in various ways, such as by retaining and stablizing ligands, by transporting ligands and by facilitating ligand-receptor interactions. These roles are likely to be mediated by both the HSPG protein cores and the specific HS GAG modifications that are carried out by a combination of enzymes. Further detailed analyses of animals mutant for individual core proteins and HS GAG chain-modifying enzymes are needed to define the molecular mechanisms by which HSPGs regulate cell signaling in various developmental processes. Cell biology studies of live tissues are also needed to further define the cellular basis of the HSPG-mediated regulation of morphogen gradient formation during development. Finally, we also need to determine the HS structures of specific HSPGs by HS GAG sequencing and advanced mass spectroscopy to elucidate the molecular nature of HSPG-ligand interactions. Given their molecular complexity, a complete understanding of HSPG functions in development requires that we investigate them using combined genetic, cell biological and biochemical approaches.

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Note added in proof

Two recent papers have demonstrated that Dlp promotes low-level Wg activity far from the site of Wg production, but reduces high-level Wg signaling near the Wg source (Kreuger et al., 2004; Kirkpatrick et al., 2004). Kreuger et al. further show that Notum, a previously identified secreted enzyme, can cleave the GPI anchor of Dlp, releasing Dlp from the cell surface. Kirkpatrick et al. show through genetic interaction studies that Dlp and Notum cooperate to restrict high-level Wg signaling. As Notum is transcribed in response to high-level Wg signaling, these findings suggest that feedback confers spatial specificity on proteoglycan function by switching Dlp from a membrane-tethered cofactor to a secreted antagonist.

References

Ai, X., Do, A. T., Lozynska, O., Kusche-Gullberg, M., Lindahl, U. and Emerson, C. P., Jr (2003). QSulf1 remodels the 6-O sulfation states of cell surface heparan sulfate proteoglycans to promote Wnt signaling. *J. Cell Biol.* **162**, 341-351.

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.

Belenkaya, T. Y., Han, C., Yan, D., Opoka, R., Khodoun, M., Liu, H. and Lin, X. (2004). Drosophila Dpp morphogen movement is independent of Dynamin-mediated endocytosis, but regulated by the glypican members of heparan sulfate proteoglycans. *Cell* **119**, 231-244.

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.

Belting, M. (2003). Heparan sulfate proteoglycan as a plasma membrane carrier. *Trends Biochem. Sci.* **28**, 145-151.

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 signaling. *Development* **124**, 2623-2632.

Bornemann, D. J., Duncan, J. E., Staatz, W., Selleck, S. and Warrior, R. (2004). Abrogation of heparan sulfate synthesis in Drosophila disrupts the Wingless, Hedgehog and Decapentaplegic signaling pathways. *Development* **131**, 1927-1938.

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.

Chen, M. H., Li, Y. J., Kawakami, T., Xu, S. M. and Chuang, P. T. (2004). Palmitoylation is required for the production of a soluble multimeric Hedgehog protein complex and long-range signaling in vertebrates. *Genes Dev.* **18**, 641-659.

Chen, R. L. and Lander, A. D. (2001). Mechanisms underlying preferential assembly of heparan sulfate on glypican-1. *J. Biol. Chem.* **276**, 7507-7517.

Coumoul, X. and Deng, C. X. (2003). Roles of FGF receptors in mammalian development and congenital diseases. *Birth Defects Res. Part C Embryo Today* **69**, 286-304.

Datta, S. (1995). Control of proliferation activation in quiescent neuroblasts of the Drosophila central nervous system. *Development* **121**, 1173-1182.

De Cat, B., Muyldermans, S. Y., Coomans, C., Degeest, G., Vanderschueren, B., Creemers, J., Biemar, F., Peers, B. and David, G. (2003). Processing by proprotein convertases is required for glypican-3 modulation of cell survival, Wnt signaling, and gastrulation movements. *J. Cell Biol.* **163**, 625-635.

Desbordes, S. C. and Sanson, B. (2003). The glypican Dally-like is required for Hedgehog signalling in the embryonic epidermis of Drosophila. *Development* **130**, 6245-6255.

Dhoot, G. K., Gustafsson, M. K., Ai, X., Sun, W., Standiford, D. M. and Emerson, C. P., Jr (2001). Regulation of Wnt signaling and embryo patterning by an extracellular sulfatase. *Science* **293**, 1663-1666.

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.

Esko, J. D. and Zhang, L. (1996). Influence of core protein sequence on glycosaminoglycan assembly. *Curr. Opin. Struct. Biol.* **6**, 663-670.

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.

Galli, A., Roure, A., Zeller, R. and Dono, R. (2003). Glypican 4 modulates FGF signalling and regulates dorsoventral forebrain patterning in Xenopus embryos. *Development* **130**, 4919-4929.

Garcia-Garcia, M. J. and Anderson, K. V. (2003). Essential role of glycosaminoglycans in Fgf signaling during mouse gastrulation. *Cell* **114**, 727-737.

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.

Goto, S., Taniguchi, M., Muraoka, M., Toyoda, H., Sado, Y., Kawakita, M. and Hayashi, S. (2001). UDP-sugar transporter implicated in glycosylation and processing of Notch. *Nat. Cell Biol.* **3**, 816-822.

Greco, V., Hannus, M. and Eaton, S. (2001). Argosomes: a potential vehicle for the spread of morphogens through epithelia. *Cell* **106**, 633-645.

Groppe, J., Rumpel, K., Economides, A. N., Stahl, N., Sebald, W. and Affolter, M. (1998). Biochemical and biophysical characterization of refolded Drosophila DPP, a homolog of bone morphogenetic proteins 2 and 4. *J. Biol. Chem.* **273**, 29052-29065.

Hacker, U., Lin, X. and Perrimon, N. (1997). The Drosophila sugarless gene modulates Wingless signaling 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 signaling in Drosophila. *Development* **124**, 3055-3064.

Han, C., Belenkaya, T. Y., Khodoun, M., Tauchi, M. and Lin, X. (2004a). Distinct and collaborative roles of Drosophila EXT family proteins in morphogen signalling and gradient formation. *Development* **131**, 1563-1575.

Han, C., Belenkaya, T. Y., Wang, B. and Lin, X. (2004b). Drosophila glypicans control the cell-to-cell movement of Hedgehog by a dynamin-independent process. *Development* **131**, 601-611.

Ingham, P. W. and McMahon, A. P. (2001). Hedgehog signaling in animal development: paradigms and principles. *Genes Dev.* **15**, 3059-3087.

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.

Jeong, J. and McMahon, A. P. (2002). Cholesterol modification of Hedgehog family proteins. *J. Clin. Invest.* **110**, 591-596.

Johnson, K. G., Ghose, A., Epstein, E., Lincecum, J., O'Connor, M. B. and van Vactor, D. (2004). Axonal heparan sulfate proteoglycans regulate the distribution and efficiency of the repellent slit during midline axon guidance. *Curr. Biol.* **14**, 499-504.

Kamimura, K., Fujise, M., Villa, F., Izumi, S., Habuchi, H., Kimata, K. and Nakato, H. (2001). Drosophila heparan sulfate 6-O-sulfotransferase (dHS6ST) gene. Structure, expression, and function in the formation of the tracheal system. *J. Biol. Chem.* **276**, 17014-17021.

Kamiyama, S., Suda, T., Ueda, R., Suzuki, M., Okubo, R., Kikuchi, N., Chiba, Y., Goto, S., Toyoda, H., Saigo, K. et al. (2003). Molecular cloning and identification of 3′-phosphoadenosine 5′-phosphosulfate transporter. *J. Biol. Chem.* **278**, 25958-25963.

Khare, N. and Baumgartner, S. (2000). Dally-like protein, a new Drosophila glypican with expression overlapping with wingless. *Mech. Dev.* **99**, 199-202.

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.

Kirkpatrick, C. A., Dimitroff, B. D., Rawson, J. M. and Selleck, S. B. (2004). Opposing activities of Dally-like Glypican at high and low levels of wingless morphogen activity spatial regulation of wingless morphogen distribution and signaling by Dally-like Protein. *Dev. Cell* **7**, 513-523.

Kirsch, T., Sebald, W. and Dreyer, M. K. (2000). Crystal structure of the BMP-2-BRIA ectodomain complex. *Nat. Struct. Biol.* **7**, 492-496.

Kreuger, J., Perez, L., Giraldez, A. J. and Cohen, S. M. (2004). Opposing activities of Dally-like Glypican at high and low levels of wingless morphogen activity. *Dev. Cell* **7**, 503-512.

Lai, J., Chien, J., Staub, J., Avula, R., Greene, E. L., Matthews, T. A., Smith, D. I., Kaufmann, S. H., Roberts, L. R. and Shridhar, V. (2003). Loss of HSulf-1 up-regulates heparin-binding growth factor signaling in cancer. *J. Biol. Chem.* **278**, 23107-23117.

Lander, A. D., Nie, Q. and Wan, F. Y. (2002). Do morphogen gradients arise by diffusion? *Dev. Cell* **2**, 785-796.

Li, J. P., Gong, F., Hagner-McWhirter, A., Forsberg, E., Abrink, M., Kisilevsky, R., Zhang, X. and Lindahl, U. (2003). Targeted disruption of a murine glucuronyl C5-epimerase gene results in heparan sulfate lacking L-iduronic acid and in neonatal lethality. *J. Biol. Chem.* **278**, 28363-28366.

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. (2003). Developmental roles of heparan sulfate proteoglycans in Drosophila. *Glycoconj. J.* **19**, 363-368.

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

Luders, F., Segawa, H., Stein, D., Selva, E. M., Perrimon, N., Turco, S. J. and Hacker, U. (2003). Slalom encodes an adenosine 3′-phosphate 5′-phosphosulfate transporter essential for development in Drosophila. *EMBO J.* **22**, 3635-3644.

Lum, L., Yao, S., Mozer, B., Rovescalli, A., von Kessler, D., Nirenberg, M. and Beachy, P. A. (2003). Identification of Hedgehog pathway components by RNAi in Drosophila cultured cells. *Science* **299**, 2039-2045.

Merry, C. L., Bullock, S. L., Swan, D. C., Backen, A. C., Lyon, M., Beddington, R. S., Wilson, V. A. and Gallagher, J. T. (2001). The molecular phenotype of heparan sulfate in the Hs2st–/– mutant mouse. *J. Biol. Chem.* **276**, 35429-35434.

Morimoto-Tomita, M., Uchimura, K., Werb, Z., Hemmerich, S. and Rosen, S. D. (2002). Cloning and characterization of two extracellular heparin-degrading endosulfatases in mice and humans. *J. Biol. Chem.* **277**, 49175-49185.

Nakato, H. and Kimata, K. (2002). Heparan sulfate fine structure and specificity of proteoglycan functions. *Biochim. Biophys. Acta* **1573**, 312-318.

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.

Nusse, R. (2003). Wnts and Hedgehogs: lipid-modified proteins and similarities in signaling mechanisms at the cell surface. *Development* **130**, 5297-5305.

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

Ohkawara, B., Yamamoto, T. S., Tada, M. and Ueno, N. (2003). Role of glypican 4 in the regulation of convergent extension movements during gastrulation in Xenopus laevis. *Development* **130**, 2129-2138.

Ohto, T., Uchida, H., Yamazaki, H., Keino-Masu, K., Matsui, A. and Masu, M. (2002). Identification of a novel nonlysosomal sulphatase expressed in the floor plate, choroid plexus and cartilage. *Genes Cells* **7**, 173-185.

Ornitz, D. M. (2000). FGFs, heparan sulfate and FGFRs: complex interactions essential for development. *Bioessays* **22**, 108-112.

Ornitz, D. M. and Itoh, N. (2001). Fibroblast growth factors. *Genome Biol.* **2**, R3005.

Paine-Saunders, S., Viviano, B. L., Zupicich, J., Skarnes, W. C. and Saunders, S. (2000). glypican-3 controls cellular responses to Bmp4 in limb patterning and skeletal development. *Dev. Biol.* **225**, 179-187.

Park, Y., Rangel, C., Reynolds, M. M., Caldwell, M. C., Johns, M., Nayak, M., Welsh, C. J., McDermott, S. and Datt, S. (2003). Drosophila perlecan modulates FGF and hedgehog signals to activate neural stem cell division. *Dev. Biol.* **253**, 247-257.

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

Pellegrini, L., Burke, D. F., von Delft, F., Mulloy, B. and Blundell, T. L. (2000). Crystal structure of fibroblast growth factor receptor ectodomain bound to ligand and heparin. *Nature* **407**, 1029-1034.

Perrimon, N., Hacker, U., Sanson, B. and Tabata, T. (2004). Wingless, hedgehog and heparan sulfate proteoglycans. *Development* **131**, 2509-2513.

Pfeiffer, S., Ricardo, S., Manneville, J. B., Alexandre, C. and Vincent, J. P. (2002). Producing cells retain and recycle Wingless in Drosophila embryos. *Curr. Biol.* **12**, 957-962.

Princivalle, M. and de Agostini, A. (2002). Developmental roles of heparan sulfate proteoglycans: a comparative review in Drosophila, mouse and human. *Int. J. Dev. Biol.* **46**, 267-278.

Raftery, L. A. and Sutherland, D. J. (1999). TGF-beta family signal transduction in Drosophila development: from Mad to Smads. *Dev. Biol.* **210**, 251-268.

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.

Ruppert, R., Hoffmann, E. and Sebald, W. (1996). Human bone morphogenetic protein 2 contains a heparin-binding site which modifies its biological activity. *Eur. J. Biochem.* **237**, 295-302.

Scheufler, C., Sebald, W. and Hulsmeyer, M. (1999). Crystal structure of human bone morphogenetic protein-2 at 2.7 A resolution. *J. Mol. Biol.* **287**, 103-115.

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.

Schlessinger, J., Plotnikov, A. N., Ibrahimi, O. A., Eliseenkova, A. V., Yeh, B. K., Yayon, A., Linhardt, R. J. and Mohammadi, M. (2000). Crystal structure of a ternary FGF-FGFR-heparin complex reveals a dual role for heparin in FGFR binding and dimerization. *Mol. Cell* **6**, 743-750.

Selleck, S. B. (2001). Genetic dissection of proteoglycan function in Drosophila and C. elegans. *Semin. Cell Dev. Biol.* **12**, 127-134.

Selva, E. M., Hong, K., Baeg, G. H., Beverley, S. M., Turco, S. J., Perrimon, N. and Hacker, U. (2001). Dual role of the fringe connection gene in both heparan sulphate and fringe-dependent signalling events. *Nat. Cell Biol.* **3**, 809-815.

Simons, K. and Toomre, D. (2000). Lipid rafts and signal transduction. *Nat. Rev. Mol. Cell Biol.* **1**, 31-39.

Song, H. H. and Filmus, J. (2002). The role of glypicans in mammalian development. *Biochim. Biophys. Acta* **1573**, 241-246.

Spring, J., Paine-Saunders, S. E., Hynes, R. O. and Bernfield, M. (1994). Drosophila syndecan: conservation of a cell-surface heparan sulfate proteoglycan. *Proc. Natl. Acad. Sci. USA* **91**, 3334-3338.

Srinivasan, S., Rashka, K. E. and Bier, E. (2002). Creation of a Sog morphogen gradient in the Drosophila embryo. *Dev. Cell* **2**, 91-101.

Steigemann, P., Molitor, A., Fellert, S., Jackle, H. and Vorbruggen, G. (2004). Heparan sulfate proteoglycan syndecan promotes axonal and myotube guidance by slit/robo signaling. *Curr. Biol.* **14**, 225-230.

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

Tabata, T. and Takei, Y. (2004). Morphogens, their identification and regulation. *Development* **131**, 703-712.

Takei, Y., Ozawa, Y., Sato, M., Watanabe, A. and Tabata, T. (2004). Three Drosophila EXT genes shape morphogen gradients through synthesis of heparan sulfate proteoglycans. *Development* **131**, 73-82.

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.

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.

Topczewski, J., Sepich, D. S., Myers, D. C., Walker, C., Amores, A., Lele, Z., Hammerschmidt, M., Postlethwait, J. and Solnica-Krezel, L. (2001). The zebrafish glypican knypek controls cell polarity during gastrulation movements of convergent extension. *Dev. Cell* **1**, 251-264.

Toyoda, H., Kinoshita-Toyoda, A., Fox, B. and Selleck, S. B. (2000a). 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.

Toyoda, H., Kinoshita-Toyoda, A. and Selleck, S. B. (2000b). Structural analysis of glycosaminoglycans in Drosophila and Caenorhabditis elegans and demonstration that tout-velu, a Drosophila gene related to EXT tumor suppressors, affects heparan sulfate in vivo. *J. Biol. Chem.* **275**, 2269-2275.

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.

Veeman, M. T., Axelrod, J. D. and Moon, R. T. (2003). A second canon. Functions and mechanisms of beta-catenin-independent Wnt signaling. *Dev. Cell* **5**, 367-377.

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

Voigt, A., Pflanz, R., Schafer, U. and Jackle, H. (2002). Perlecan participates in proliferation activation of quiescent Drosophila neuroblasts. *Dev. Dyn.* **224**, 403-412.

Wang, S., Ai, X., Freeman, S. D., Pownall, M. E., Lu, Q., Kessler, D. S. and Emerson, C. P., Jr (2004). QSulf1, a heparan sulfate 6-O-endosulfatase, inhibits fibroblast growth factor signaling in mesoderm induction and angiogenesis. *Proc. Natl. Acad. Sci. USA* **101**, 4833-4838.

Wodarz, A. and Nusse, R. (1998). Mechanisms of Wnt signaling in development. *Annu. Rev. Cell Dev. Biol.* **14**, 59-88.

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

Zeng, X., Goetz, J. A., Suber, L. M., Scott, W. J., Jr, Schreiner, C. M. and Robbins, D. J. (2001). A freely diffusible form of Sonic hedgehog mediates long-range signalling. *Nature* **411**, 716-720.