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Biochimica et Biophysica Acta 1573 (2002) 280–291

BIOCHIMICA ET BIOPHYSICA ACTA
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Review

Heparan sulfate proteoglycan modulation of developmental signaling in *Drosophila*

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Received 23 October 2001; received in revised form 22 August 2002; accepted 22 August 2002

Abstract

Heparan sulphate proteoglycans (HSPG's) are cell surface proteins to which long, unbranched chains of modified sugars called heparan sulphate glycosaminoglycans have been covalently attached. Cell culture studies have demonstrated that HSPG's are required for optimal signal transduction by many secreted cell signaling molecules. Now, genetic studies in both *Drosophila* and vertebrates have illustrated that HSPG's play important roles in signal transduction in vivo and have also begun to reveal new roles for HSPG's in signaling events. In particular, HSPG's have been shown to be important in ligand sequestration of wingless, for the transport of the Hedgehog ligand, and for modulation of the Dpp morphogenetic gradient.

Keywords: Heparan sulfate; Developmental signaling; *Drosophila*; Hedgehog signaling; FGF signaling; Dpp signaling

1. Introduction

Signaling molecules, including the members of the Hedgehog (Hh), Wingless/Int (Wnt), transforming growth factor (TGF) beta, and fibroblast growth factor (FGF) families, regulate a large number of patterning events in developing animals. The growth and differentiation of most cells and tissues are coordinated by the secretion, distribution, activation, and downstream signaling events of these molecules. Furthermore, many cancers and genetic diseases are associated with mutations in or changes in the expression and signaling activities of these ligands. Consequently, understanding how these molecules actually carry out their signaling roles is of primary importance in the investigation of development and disease.

Upon release from the cell they are expressed in, secreted signaling molecules act by binding to and activating cell surface receptors. The activated receptors then transduce the signal through the cell membrane to initiate a cascade of cytoplasmic signaling molecules which eventually cause transcriptional changes within the nucleus. For many years, the prevailing dogma with respect to the extracellular requirements for signal transduction was that only ligands and their cognate receptors were required. However, research on the FGF signaling pathway in cultured cells revealed that another class of cell surface molecules, the proteoglycans, is also required for optimal signaling by FGF ligands [1,2]. Subsequently, other signaling molecules were shown to require proteoglycans, including Wg/Wnts [3], TGF beta [4], hepatocyte growth factor [5], Hh [6], and one of the EGF family members [7]. Proteoglycans are proteins to which chains of modified sugar residues are attached (see below). The same studies that identified the requirement for proteoglycans also demonstrated that one class of proteoglycans, the heparan sulfate proteoglycans (HSPGs), was the principal type necessary for FGF, EGF, Hh, Wnt, and TGF-beta signaling.

Biochemical studies in cell culture systems have provided a wealth of data concerning which signaling molecules bind to which HSPGs and the effect that HSPG binding can have on downstream signaling events within the cell. But can all the functions of HSPGs be ascertained from studies in cell culture? For instance, does a single type of HSPG affect only one signaling pathway in vivo? Could HSPGs be required to mediate aspects of signaling only identifiable at the level of tissues? Only recently has genetic data from *Drosophila* and vertebrates demonstrated the importance of HSPGs to signal trans-

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PII: S0304-4165(02)00395-1

duction in vivo. Moreover, these results have also begun to hint at new functions for proteoglycans in signal transduction. In this review, we will summarize and discuss recent studies in *Drosophila* that examine the role of HSPGs in developmentally important signaling events. We will also discuss relevant data from vertebrate systems if there is no similar *Drosophila* data, and finally, we will present some models for how HSPGs may function in signal transduction.

2. Structure and biosynthesis of HSPG glycosaminoglycan (GAG) chains

Proteoglycans are glycoproteins that bear long, unbranched polymers of modified sugar residues called glycosaminoglycans (as opposed to other types of glycoproteins, which predominantly bear unmodified sugar chains that are often branched). GAG chains are attached to serine residues in the proteoglycan core proteins by a tetrasaccharide linkage, which is then followed by a long, linear polymer of repeating, modified disaccharide units (schematically illustrated in Fig. 1). Differences in the composition of the modified disaccharide units define the different types of GAG chains. Heparan, for example, consists of disaccharide repeats of glucuronic acid (GlcA) and *N*-acetylglucosamine (GlcNAc), whereas chondroitin consists of *N*-acetylgalactosamine and GlcA disaccharides. Subsequent to polymerization of the GAG chains, the individual modified sugar residues undergo further modification, including N-deacetylation/N-sulfation (removal of the acetyl group from the N atom of GlcNAc followed by sulfate addition to the same N atom), epimerization (change in the ring conformation of the sugar core), and O-sulfation (addition of a sulfate group to one of the hydroxyl groups of the sugar ring) (Fig. 1) (see Refs. [8–10] for review). All heparan GAG chains undergo some degree of N-deacetylation/N-sulfation and O-sulfation and so GAG chains consisting of heparan are usually referred to as heparan sulfate (HS).

Production of all proteoglycans requires nucleotide sugar building blocks which are synthesized in the cytoplasm by a series of enzymes including isomerases, kinases, and dehydrogenases. The nucleotide sugars are then transported from the cytoplasm into the endoplasmic reticulum by a family of nucleotide sugar transporters. HS GAG chain synthesis is then initiated by the addition of the tetrasaccharide linker to specific serines in the protein core. Linker addition is catalyzed by several enzymes. First, a xylosyltransferase adds a single xylose residue to the core protein serine. Two galactose residues are then added by the actions of two separate galactosyltransferases, and finally, a single GlcNAc residue is added to finish the linker region [8,9]. After the linker is polymerized, the GlcNAc–GlcA disaccharides are added by the actions of an alpha-GlcNAc transferase and a beta-GlcA transferase (Fig. 1). Both of these transferase activities seem to be encoded by members of the mammalian EXT gene family (see below).

N-deacetylation/N-sulfation of HS is catalyzed by a family of enzymes known as the *N*-deacetylase/*N*-sulfotransferases. Several different NDST isozymes with differing expression patterns have been isolated from vertebrates [11], while only one NDST, *sulfateless* (*sfl*), has been found in flies [12]. The purpose of the four NDST isoforms in mammals is not clear although the different isoforms have been shown to have differing N-deacetylation and N-sulfation activities [97]. Conformational change in the sugar core in HS is limited to the GlcA residues and is catalyzed by a C-5 epimerase enzyme (see review by J. Esko, this volume). C-5 epimerase converts GlcA to IdoA which can then be modified by sulfation.

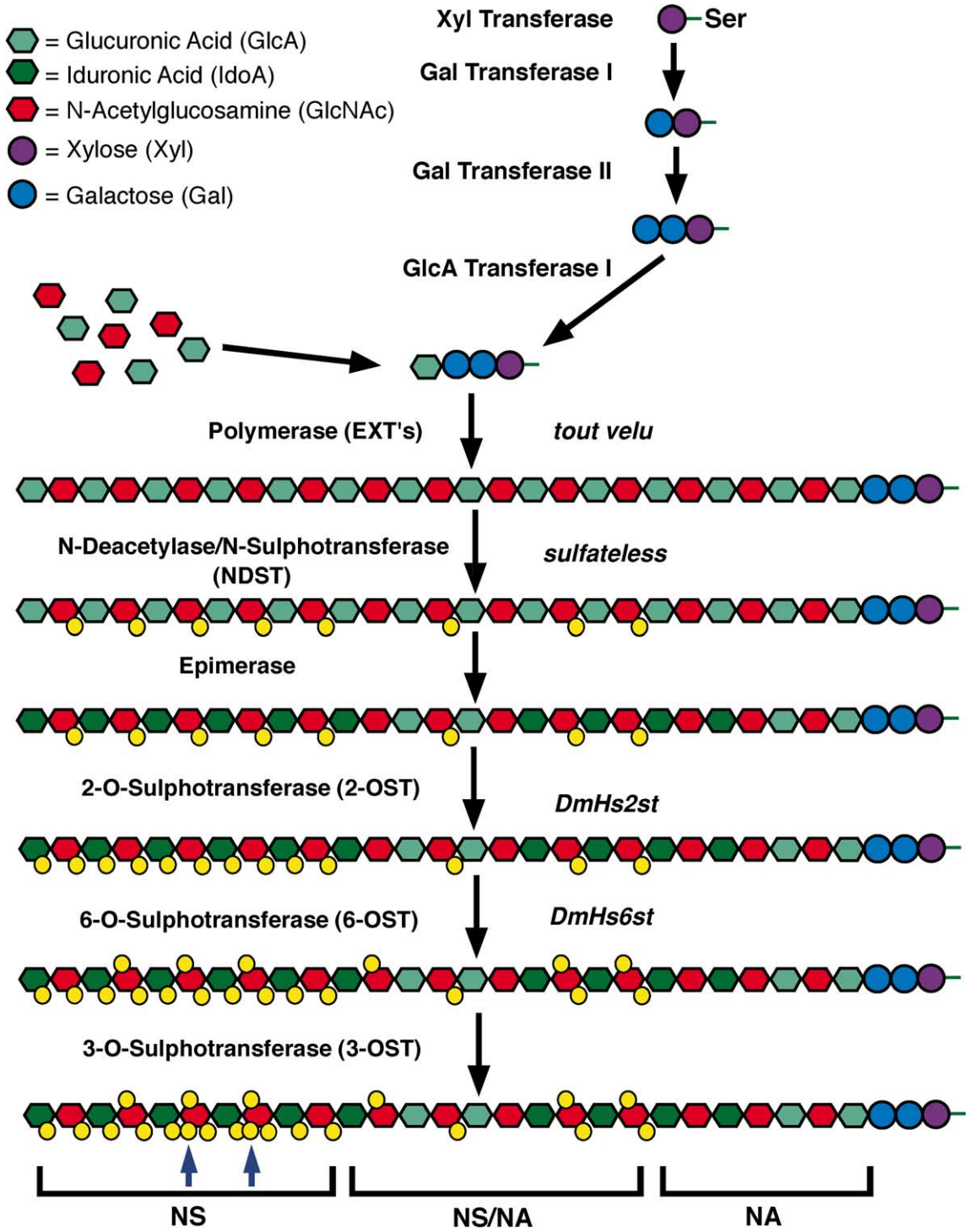
Sulfation is responsible for the majority of the structural diversity found in the GAGs of proteoglycans. Sulfation of heparan occurs at a number of different sites within the repeating disaccharide subunit. N-deacetylation/N-sulfation of the GlcNAc residues adds the most sulfate groups to HS, but does not seem to provide much diversity to HS structure. Once formed by C-5 epimerase, IdoA can be sulfated at the hydroxyl group of the C2 carbon by the actions of a 2-OST. In vertebrates, only one 2-OST gene has been found, while in *Drosophila*, one 2-OST homologous to the vertebrate version [13] and one unique, putative 2-OST, encoded by the *pipe* locus, have been isolated (see below and also review by Stein, this issue). Both *N*-acetyl- or *N*-sulfoglucosamine can also be modified by addition of a sulfate group to their C6 hydroxyl groups by a 6-OST, whereas only *N*-sulfoglucosamine can have a sulfate group added to its C3 hydroxyl group by a 3-OST as outlined in Fig. 1. Multiple 3-OSTs and 6-OSTs that modify HSPGs have been isolated and characterized from mammalian tissues [14–17].

3. Diversity of HS structure

The modification of HS GAG chains is not uniform, and certain contiguous regions contain more modifications than nearby regions, resulting in a heterogeneous distribution of these modifications along the length of a single HS chain [8,10] (Fig 1). Generally, these modifications depend on previous modifications and occur in a hierarchical order. Thus, epimerization and *O*-sulfation generally only occur in regions which have already been N-deacetylated/N-sulfated. In addition, these modifications appear to occur in a regulated fashion, as particular types of HS modifications are found at different frequencies in different mammalian organs. HS from the aorta has a significantly different composition than HS from the cerebral cortex or intestine. The intestine, for instance, has twice as much 6-*O*-sulfoglucosamine as the aorta [18]. Moreover, these proportions vary little between individuals, indicating that these modifications are subject to tight control [19]. Changes in HS

chain composition have also been correlated with normal developmental events and with certain pathological conditions such as cancer and diabetes [20–25], although the cause-and-effect relation of these changes to pathology is not clear.

Sulfation is responsible for the majority of the structural diversity of HS chains. Indeed, where the specific HS sequences required for binding are known, the type and distribution of sulfate groups plays a key role in binding of these ligands. FGF2, for instance, requires three consecutive trisulfated disaccharide units to bind efficiently to HS [26,27], while antithrombin requires a unique pentasaccharide sequence to bind efficiently to heparin, a heavily sulfated form of heparan [28]. HGF, on the other hand, requires two trisulfated disaccharides and two monosulfated disaccharides within a tetrasaccharide sequence for efficient binding [29].



4. Identified HSPGs in *Drosophila*

There are many identified HSPGs in vertebrates, including glypicans, syndecans, betaglycans, agrins, collagens, and perlecans. Syndecans and glypicans are the only known cell surface proteoglycans, while the rest are found in the extracellular matrix [20]. In *Drosophila*, one syndecan homolog, Dsyndecan, and two glypican homologs, Dally and Dally-like (Dly), have been identified [30–32] and are discussed in detail in this volume (S. Selleck, this issue). The roles of these molecules in signaling will be discussed below. An extracellular matrix proteoglycan called Papilin has also been isolated and shown to contain sulfated GAG chains [33]. Papilin has recently been cloned and shown to be important for muscle development in the *Drosophila* embryo [34]. Perlecan is an extracellular matrix HSPG and partial sequence for a *Drosophila* perlecan homolog has been published and the complete gene identified in Flybase [35] (http://flybase.bio.indiana.edu). A HSPG called DROP-1 has been described [36,37], and the *Drosophila* genome project has identified several other putative HSPGs. Of the HSPGs mentioned above, only two, Dally and Dly, have been analyzed for their effects on specific signaling pathways. Both Dally and Dly have been shown to affect Wg signaling, while Dally also affects other signaling pathways.

5. Two *Drosophila* glypican homologs affect the Wg signaling pathway

Drosophila Wg is one of the five members of the Wnt family of signaling molecules found in *Drosophila*. Wnts are secreted glycoproteins that are involved in numerous developmental patterning events and in control of cell proliferation. Wg in the fly regulates patterning of the embryonic cuticle (reviewed in Ref. [38]), the wing margin, the gut [39], the Malpighian tubules [40], subsets of neuroblasts, and the stomatogastric nervous system (SNS) which innervates the gut [41]. The Frizzled (Fz) family of proteins constitutes the receptors for Wnts, along with the newly described *arrow* gene, which encodes a LDL receptor-like protein. Inside the cell, the Dishevelled (Dsh), Zeste-white3/Shaggy/GSK3beta, APC, Axin, and Armadillo (Arm)/Beta-catenin proteins transduce the signal which activates the Tcf/LEF transcription factors [42,43]. GSK3 beta is a kinase whose activity is reduced during activation of Wg signaling, while Arm protein is stabilized and accumulates cytoplasmically when Wg signaling is activated.

Elimination of *dally* function via RNA interference or mutations in the *dally* locus results in mutant embryonic cuticlar structures which phenocopy an embryonic *wg* mutant at low penetrance, suggesting that both *dally* and *wg* may act in the same or a parallel signaling pathway. The penetrance of the *dally* mutant phenotype can be increased by reducing the dosage of *sfl* or *wg* [12,44], while overexpression of *dally* using a heat-shock inducible construct can partially rescue a temperature-sensitive *wg* phenotype. Overexpression of *dally* also seems to expand the domain of *wg* activation as determined by Arm stabilization and cytoplasmic accumulation. Finally, *dally* mutants enhance the phenotype of a DFz2 dominant negative and suppress the phenotype caused by ectopic expression of a wild-type DFz2, which ectopically activates the Wg signaling pathway [12,44]. All of these results indicate that *dally* and its associated HS chains are positive regulators of Wg signaling.

Although no mutations are available in *dly*, several experiments indicate that it also functions in Wg signaling. First, elimination of *dly* by RNA interference generates a partial segment polarity phenotype, as does *dally*. RNA interference-mediated disruption of both *dally* and *dly* results in a more severe disruption of embryonic segmental structure than either alone, suggesting that these two glypicans have partially redundant effects on Wg signaling. Overexpression of *dly* in the wing mimics a *wg* loss of function phenotype and substantially reduces the expression of two genes induced by *wg*, *distalless* (*dll*) and *achaete* (*ac*). Lastly, ectopic *dly* expression in certain regions of the wing using specific drivers and the Gal4-UAS system induces ectopic expression of *wg* and traps most Wg outside of the cell [30]. Why this extracellular trapping of Wg attenuates Wg signaling instead of amplifying it is not clear. It is possible that both Wg and the Fz family Wg receptors have stoichiometric interactions with Dally or Dly, such that excess amounts of Dally or Dly would titrate the limiting amounts of Wg and Fz and prevent formation of productive, trimeric signaling complexes made up of Wg-Fz-Dally/Dly.

One *Drosophila* developmental process in which proteoglycans have not been implicated is gastrulation. Interest-

Fig. 1. Synthesis of HS chains. HS chains are attached to selected Serine residues in the core protein. Heparan chain synthesis is initiated by the attachment of a Xylose residue to the core protein serine by the actions of a xylosyltransferase. Next, two galactose residues are added by the sequential actions of galactosyltransferases I and II, and then a Glucuronic acid (GlcA) Transferase adds a single GlcA to the last galactose. This tetrasaccharide forms the linker region common to both chondroitin sulfate (CS) and HS. In HS synthesis, GlcA/GlcNAc dimers are polymerized to the linker by the actions of beta-GlcA and alpha-GlcNAc transferases, both of which seem to be encoded by the EXT family of enzymes. Once the backbone of the heparan chain is polymerized, it undergoes a series of modifications. First, most of the GlcNAcs are N-deacetylated and N-sulfated by an *N*-deacetylase/*N*-sulfotransferase (NDST). Then an epimerase converts most of the GlcAs to iduronic acid (IdoA). The IdoA can then be O-sulfated by a 2-*O*-sulfotransferase (2-OST). Finally, the GlcNS can be sulfated on the 3 or 6 carbon positions of the sugar ring by the actions of 3-*O*-sulfotransferases (3-OSTs) and 6-*O*-sulfotransferases (6-OSTs), respectively. Different regions of the HS chain are modified to different extents. Thus, some regions are heavily sulfated—the so-called NS regions—while the NS/NA regions are only slightly modified, and the NA regions have very little modification. Precursor sugar nucleotides are imported into the endoplasmic reticulum by the actions of nucleotide sugar transporters (not depicted), which supply the modified sugar residues used for all proteoglycan synthesis. The figure shows the steps of HS synthesis with enzymes responsible for a particular step identified to the left of the arrows, and the homologous *Drosophila* genes, where known, identified to the right of the arrows.

ingly, a glypican named *knypek* has recently been shown to be involved in convergent extension movements during gastrulation in zebrafish [98]. Cells mutant for *knypek* do not undergo proper convergent extension movements as a result of abnormal cell polarity. Double mutant and overexpression studies demonstrated that *knypek* mutants primarily affect the Wnt11 signaling pathway [98], which had already been shown to regulate convergent extension. It will be interesting to determine if any proteoglycans affect gastrulation movements in *Drosophila*.

6. HS biosynthetic enzymes and Wg signaling

The first hint that HS GAGs affected Wg signaling came from *Drosophila* cell culture experiments. *Drosophila* S2 cells stably transfected with a Wg expression construct (S2-Wg cells) can produce active Wg as determined by the accumulation of Arm in another cell line, clone-8, treated with the conditioned media from the S2-Wg cells. Most of the Wg in S2-Wg cells is retained on the cell surface or in the extracellular matrix but can be released by treatment of the cells with heparan or chondroitin, indicating that Wg likely binds one of these GAGs. Prior digestion of GAG chains from S2-Wg cells by a heparatinase/chondroitinase mixture eliminated the ability of the conditioned media taken from the treated cells to cause Arm accumulation in naïve clone-8 cells, demonstrating that Wg requires GAGs for signaling as well as binding [3]. Treatment of clone-8 cells with sodium perchlorate, which competitively inhibits sulfate metabolism also inhibited their ability to respond to Wg stimulation and indicates that the cell receiving the signal also requires GAG's. Finally, treatment of the clone-8 cells with heparin strongly potentiated Wg signaling, while CS did not potentiate Wg signaling, suggesting that it is HS GAGs and not CS GAGs which are required for Wg signaling [3].

Subsequently, the *sugarless* (*sgl*) (also called *suppenkasper* or *kiwi*) gene was shown to encode *Drosophila* homolog of uridine diphosphoglucose (UDP) dehydrogenase, an enzyme required for conversion of UDP glucose to UDP-GlcA and hence required for the synthesis of both CS and HS. Loss of both maternal and zygotic copies of *sgl* in embryos using germline clone (GLC) techniques results in a phenocopy of the *wg* segment polarity phenotype in these embryos [45,46]. Loss of function *sgl* alleles also result in enhancement of the weak adult phenotype associated with mutations in *dsh* [47]. Dsh is a downstream effector of the Wg signaling pathway. Enhancement of the *dsh* phenotype and the mutant phenotype of embryos derived from GLCs indicates that *sgl* is a positive effector of the Wg signaling pathway. Initially, it was not clear whether the Wg or Hh signaling pathways were being affected in *sgl* mutants since both pathways are required to maintain the other's expression in development of the embryonic cuticle. *sgl* mutants also perturb SNS development by eliminating two of the three presumptive SNS invaginations and the formation of the second gut constriction, both of which require Wg but not Hh signaling [46]. Together, these results demonstrate that Wg signaling is perturbed in *sgl* mutants [46,47].

GLC mutations in the *sfl* NDST gene also mimic the *wg* mutant embryonic cuticle phenotype and perturb SNS and gut development in a similar fashion to *sgl* mutants. Additionally, expression of the Wg target genes *neuralized* and *distalless* (*dll*) is disrupted in *sfl* mutants, indicating that *sfl* mutants affect Wg signaling [12]. Wg production is normal in *sfl* mutants, but the extracellular accumulation of Wg is greatly reduced in *sfl* mutants [30]. However, Hh signaling is also affected in *sfl* mutant clones, indicating that *sfl* is required for both Wg and Hh signaling (cited as data not shown in Ref. [48]).

Another gene, called *fringe connection* (*frc*), which was isolated in the same screen that identified the *sgl* and *sfl* mutants, has recently been cloned and shown to encode a nucleotide sugar transporter [49,50]. The Frc nucleotide sugar transporter appears to preferentially transport UDP-GlcA and UDP-GlcNAc, although it may transport an even broader spectrum of substrates, including UDP-xylose, UDP-galactose, and UDP-*N*-acetylgalactosamine [49,50]. Hence, *frc* could be required for synthesis of all GAGs. *frc* mutants also give a *wg*-like embryonic cuticle phenotype in GLC analysis, but it is not clear if this is due to perturbation in the Wg or Hh signaling pathways since overexpression of either *wg* or *hh* partially restored the wild-type cuticular phenotype. It is perhaps more likely, as suggested by Selva et al. [50], that *frc* potentiates signaling by both of these pathways in the cuticle but is specific to neither. A general requirement for *frc* activity in supplying sugars for glycoconjugates is further supported by the finding that mutants in *frc* also disrupt Notch signaling [49,50]. Notch is modified by a non-HS-related glycoconjugate that contains both GlcNAc and galactose [51,52] and is not known to be modified by GAGs (although no studies have yet addressed this question), suggesting that Notch utilizes the sugars transported by *frc* to assemble its glycoconjugate.

The results described above demonstrate that proper synthesis of HS is required for Wg/Wnt signaling. Recent data from an avian system have demonstrated that remodeling of HS chains may also play a vital role in HS modulation of Wg signaling. The *QSulf1* gene was isolated as a Shh responsive gene involved in somite induction in chick and encodes a protein with strong similarity to GlcNAc sulfatases that catalyse the removal of 6-*O*-sulfate groups from HS [53]. *QSulf1*, while induced by *Shh*, does not affect Shh signaling as judged by antisense elimination of *QSulf1* transcripts. Instead, *QSulf1* seems to be required for a Wnt signaling pathway downstream of Shh. Wild-type *Qsulf1* expression enhances Wnt1 signaling while expression of *Qsulf1* harboring a mutant which should render the sulfatase catalytically inactive severely reduces Wnt1 activation in cultured cells using a luciferase reporter assay [53]. Hence,

QSulf1 probably remodels HS chains by removal of some or all of the 6-*O*-sulfate groups which then renders the HS chains better able to mediate Wnt signaling. It will be interesting to ascertain if sulfatases in flies also affect Wg signaling.

7. HS in *Drosophila* FGF signaling

FGF signaling in *Drosophila* is also impacted when HS synthesis is compromised. *Drosophila* have two FGF receptors (FGFRs): Heartless (Htl), which is required for dorsolateral migration of early mesodermal cells and for development of mesodermal derivatives such as the heart, and Breathless (Btl), which is required for the development and migration of the trachea. Loss of function alleles of both *sfl* and *sgl* induce mesodermal migration defects nearly identical to those found in loss of function alleles of *htl*, as assayed by expression of the mesodermal marker *twist* [54]. In both *sgl* and *sfl* mutant embryos, the *Drosophila* heart does not form. Tracheal development is similarly perturbed in *sfl* and *sgl* mutants with incomplete tracheal branch formation as well as large gaps in the principal tracheal trunks. Htl- and Btl-dependent MAP kinase activation is also dependent on proper *sfl* and *sgl* activities as demonstrated by the lack of staining with diphospho-(activated) MAP kinase antisera in these two mutants. Overexpression of *branchless* (*bnl*), the *Drosophila* FGF ligand for Btl, partially rescues the *sfl* and *sgl* phenotypes, as might be expected if HS is a coreceptor for Bnl [54].

In a separate study, the *Drosophila* homolog of vertebrate HS 6-OST was isolated by homology to vertebrate 6-OSTs. Elimination of this enzyme by RNA-mediated interference severely perturbed tracheal development, indicating that 6-*O*-sulfation of HS is required for FGF signaling in *Drosophila* [55]. This result corresponds well with crystallographic studies of FGF1, FGFR2, and HS cocrystals that show optimal binding of FGF1 and FGFR2 requires a specific HS hexasaccharide containing both 2-*O*- and 6-*O*-sulfate groups [73].

Recently, a UDP-dehydrogenase homolog in zebrafish, *jekyll*, has been identified as a gene required for proper cardiac valve formation [56]. Although the signaling pathway involved in this cardiac valve formation is not known, it is tempting to assume that it is an FGF signaling pathway given the aforementioned data that *sgl* and *sfl* are important for heart formation in *Drosophila*.

8. Hh signaling and HSPGs

Perhaps the most unusual use of HS for signal transduction is found in the Hh signaling pathway. Hh is a developmental morphogen that patterns many different tissues in *Drosophila* and vertebrates. In the embryonic ectoderm and in the wing imaginal disc, *hh* is expressed in the posterior compartment but can act at a distance of up to 8–10 cell diameters away in the anterior compartment to induce expression of target genes such as *patched* (*ptc*) and *decapentaplegic* (*dpp*). Interestingly, Hh would appear to be neither a good candidate for a mobile signaling molecule nor a HS interacting molecule since it is modified by the addition of both cholesterol and palmitoyl moieties which should bind it to cell membranes [57–59].

It was therefore quite surprising when the *tout-velu* (*ttv*) gene's mutant phenotype and molecular identity were described. The embryonic cuticular phenotype of *ttv* mutants is very similar to *hh* and *wg* mutant phenotypes, indicating that it could be involved in the Wg or Hh signaling pathways [48]. Further characterization of the *ttv* mutant phenotype demonstrated that *ttv* does not play a role in mesodermal migration, SNS development, or *dll* expression, demonstrating that lesions in the *ttv* locus do not affect FGF or Wg signaling [48]. However, *ttv* was shown to be required for long-distance signaling by Hh in the wing imaginal disc. *ttv* clones in the posterior compartment of the wing imaginal disc have no effect on Hh signaling. However, clones in the anterior compartment along the anterior/posterior compartment boundary limit Hh signaling to the single row of cells directly adjacent to the compartment border, and therefore directly abutting the *hh*-expressing cells in the posterior compartment, as determined by the seemingly wild-type expression levels of *patched* in this single row of cells [6]. These results suggest that *ttv* is not required for production of active Hh, but rather is necessary for the movement of and/or signaling by Hh away from its source of production.

When *ttv* was cloned, it was shown to encode a member of the EXT protein family [6]. EXTs were originally identified as proteins of unknown function encoded by the locus mutated in the human hereditary multiple exostoses (HME) syndrome [60,61]. HME causes aberrant bone outgrowths and a high incidence of tumors of the bone. These EXT proteins have since been shown to encode the HS polymerases that attach the GlcA–GlcNAc disaccharides to a growing HS chain [62–64]. Several EXT isoforms are found in both vertebrates and invertebrates. Studies in vertebrate cell culture have demonstrated that EXT1 and EXT2 heterodimerize and that this association is required for optimal HS polymerase activity [64]. Knockout mice which are haploinsufficient for EXT1 show reduced levels of HS, but are otherwise normal, while the homozygous knockout mice die at gastrulation due to a lack of mesoderm and extraembryonic tissues [65]. The homozygous knockout mice also have no detectable HS. Furthermore, *Drosophila* embryos homozygous for *ttv* mutations have almost no HS, as determined by immunostaining and biochemical methods [48,66].

The fact that there seems to be very little detectable HS in *Drosophila* embryos homozygous for a *ttv* mutation leads to a conundrum. Why do *ttv* mutants not compromise Wg, FGF, and Dpp signaling, all of which require HS? One

possibility is that HS synthesis is severely compromised but not completely eliminated and the remaining small amounts of HS are sufficient for Wg, FGF, and Dpp signaling but not sufficient for Hh signaling. Another possibility is that significant amounts of HS are escaping detection in *ttv* embryos because they are not isolated in the biochemical purification of HS or are not detected by the HS antisera presently available, perhaps due to unique modification patterns. There are two other EXTs identified in the *Drosophila* genome and perhaps the discovery of mutants in these additional HS polymerase genes will shed light on the role that these HS polymerases play in signaling.

9. Dpp signal modulation by HSPGs

Dpp is the founding member of the *Drosophila* family of TGF-beta signaling molecules. It is critical for cell proliferation and differentiation in numerous developing *Drosophila* tissues, including the legs, wing, trachea, and gut, amongst others. As Dpp can function as a concentration-dependent morphogen, factors that control its distribution and signaling ability are important in development. *dally* and *dpp* have been shown to interact genetically in a number of *Drosophila* tissues. In the fly eye, loss of one copy of *dpp* substantially enhances a homozygous *dally* phenotype. Loss of one copy of *dpp* similarly enhances *dally* phenotypes in the antenna and genitalia. Conversely, in the wing, loss of one *dpp* copy suppresses the phenotype of *dally* mutants, while ectopic *dpp* expression induced by the hh^{Mrt} allele is enhanced by overexpression of wild-type *dally* [67]. These results demonstrate that Dally's role in Dpp signaling will be complicated. Presumably, Dally's HS chains play a key role in its regulation of Dpp signaling, but there is little direct evidence to support this idea at the moment. It has, however, been noted that *sgl* mutations show a mild suppression of an activated Dpp receptor, *thickveins** [47]. It will be important to determine if *sfl* or other HS synthetic enzymes can affect Dpp signaling.

10. *Pipe* and generation of oocyte polarity

Generation of dorsal/ventral polarity in the developing *Drosophila* embryo is controlled by a group of maternal effect loci that act within the oocyte and its surrounding follicular epithelium [68,69]. Ventral cell fates in the egg chamber are determined by the actions of the "dorsal" group of maternal genes. Loss of function alleles in 11 of these genes causes the resultant embryos to become dorsalized, while loss of function of one, *cactus*, results in ventralized embryos [70]. The dorsal group genes, Dorsal, Toll, and Cactus, all encode proteins involved in an NF-kappa-B-like signaling pathway in the oocyte. The remaining dorsal group genes, including *windbeutel* (*wind*), *nudel* (*ndl*), *spatzle*, *easter* (*ea*), *snake* (*snk*), *gastrulation defective* (*gd*), and *pipe* (*pip*), are required in the ventral follicle cells to produce the signal required to activate the Toll receptor. *spatzle* encodes the precursor of the ligand for the Toll receptor. Although the exact mechanism remains to be defined, the Spatzle precursor is then thought to be activated in the perivitelline space by the Snk, Gd and Ea proteases.

The *wind*, *ndl*, and *pip* genes are required in the somatic follicle cells and are presumably required for the proper secretion and/or activation of Spatzle and the proteases. When the *pipe* locus was cloned, it was found to encode a heparan 2-OST-like molecule whose expression was restricted to ventral follicle cells and repressed in dorsally fated cells. Uniform overexpression of *pipe* in the follicle cells induced a ventralized phenotype in embryos and targeted expression of *pipe* in the presumptive dorsal areas of the embryo caused ventralization of those areas. These results suggested that the presence of Pipe alone was sufficient to specify ventral cell fates in the follicle cells and, subsequently, in the oocyte (Ref. [71] and D. Stein, this issue). Pipe has not yet been shown to have heparan 2-OST activity, but its close homology to HS 2-OSTs and its requirement for ventral cell fate specification imply that a HSPG is involved in activation or localization of the Spatzle ligand. This target HSPG of *pipe* has not been identified, but it will be of great interest to pinpoint its identity and determine if, as the data suggest so far, its HS chains are 2-*O*-sulfated only in the ventral follicle cells.

Recently, the *pipe* locus has been shown to be much more complex than originally thought due to the discovery of 10 homologous *pipe* exons that encode conserved sulfotransferase domains [72]. Five transcripts have been isolated containing five of these conserved domains and it is possible that more transcripts will be found in the future. Two of the *pipe* transcripts have been localized to the ovary, while two others are found in the salivary glands [71,72]. The ovarian transcripts appear to have a partially redundant function, as ectopic expression of one transcript appears sufficient to specify new ventral poles in the oocyte. A better characterization of the transcripts found in various *pipe* mutants and exon-specific knockouts will better inform us of the function of each of the transcripts. It is tempting to speculate that the 2-OST activity resulting from translation of each particular OST exon could act upon slightly different HS substrates or could place 2-*O*-sulfate groups in different positions on a given HS chain, thereby creating a diversity of 2-*O*-sulfated structures. These diverse structures might then be required for different functions in the developing embryo.

11. Models of HSPG regulation of *Drosophila* signaling

The flood of recent data concerning the role of HSPGs in developmental signaling has raised a number of important questions. Primary amongst these questions is how exactly do HSPGs regulate signaling? In the case of FGF in cell

culture systems, it seems that HSPGs are required for optimal signaling through the FGFR, whereas in the case of Wg and Hh signaling in *Drosophila*, it appears that HSPGs are primarily involved in the transport/diffusion of the signal away from its area of expression. It is of course possible that the role of HSPGs in signaling varies according to ligand and tissue context. Here, we discuss five possible models of HSPG modulation of signaling activity and relate these models to *Drosophila* studies. It is important to note that none of these models are mutually exclusive.

11.1. HSPGs as necessary partners for dimerization

The original studies demonstrating the role of HSPGs in signaling by FGF clearly showed that FGFRs are capable of binding FGF in the absence of HSPGs. However, these same studies also showed that FGF signaling was maximally potentiated when both FGFR's and HS were present. Moreover, two crystallographic studies of FGF bound to its receptor and HS indicated that the two FGF molecules do not contact one another and require HS to form an active complex with the FGFR [73,74]. These studies indicate that HS is required to promote the dimerization of FGF, an event known to be required for signaling by FGFs and other ligands of receptor tyrosine kinases (RTKs) such as PDGF, EGF, and NGF [75]. Low levels of spontaneous FGF dimerization could occur in the absence of HS, generating the low levels of FGFR activation seen in the absence of HS, but HS-induced dimerization, and hence activation, would maximally potentiate FGF activity (Fig. 2A). This model would seem not to apply to ligands such as Hh and Wg, which are not known to oligomerize. However, HS changes the conformation of the clotting factor antithrombin III when bound to it, and so enhances the protein–protein interactions of antithrombin III and thrombin [28,76,77]. HS could similarily change the conformation of signaling molecules such as Wg to allow them to interact with their receptors.

11.2. HSPG stabilization of ligand–receptor complexes

Dimerization of ligands and/or presentation of ligands to receptors might not require HS. Rather, HS could instead be necessary for the stabilization of the ligand–receptor interaction (Fig. 2B). FGF signaling, for example, could occur at a low level in the absence of HS due to transient FGF–FGFR association, but the signaling would not occur at a substantive rate until the FGF–FGFR interaction was stabilized by HS binding to the ligand, the receptor, or both. Several biochemical studies show that FGF dimers appear to form in the absence of HS, and crystallographic studies suggest that FGFs can bind to their receptors (although not dimerize) in the absence of HS. However, maximal signaling in cell culture clearly requires HSPGs [78–83]. These data tend to support such a stabilization model. This model also might be more applicable to ligands which are not thought to dimerize, such as Wg (Fig. 2B). Both the dimerization and stabilization models could explain the suppression of the *sfl* and *sgl* phenotypes by overexpression of *bnl*, the *Drosophila* FGF ligand. In this case, the loss of the potentiating effects of HSPGs on FGF signaling would be overcome by the increase in the Bnl ligand, which could either increase the amounts of spontaneously dimerized ligand, or could increase the occupation and thus activation of the Btl receptor.

11.3. Two-dimensional sliding of ligands

Given the weak binding of HSPGs to signaling ligands and the high density of HSPGs on cell surfaces, it has been proposed that HSPGs might limit the diffusion of ligands by essentially trapping them in a two-dimensional surface formed by the overlapping matrix of HS chains. This would then allow HS-associated ligands to diffuse by "sliding" within this two-dimensional surface [84]. In this model, ligands bind weakly to sites along the length of a HS chain and diffuse or "slide" along the chain by releasing from one weak binding site, diffusing, and then binding another weak binding site further along the chain. This "two-dimensional sliding" could allow a ligand to travel further in the same time period by preventing time-consuming movements outside the plane of the cell surface (Fig. 2C). It could also increase the likelihood that the ligand would encounter its receptor, which would also be limited to this two-dimensional surface. Ligand–receptor encounters are still random in this model, but it effectively reduces diffusion to two dimensions. This model is primarily theoretical and there is little supporting evidence. In fact, studies in *Drosophila* have shown that Dly traps Wg outside of cells and seems to retard its movement [30]. Another study has demonstrated that transport of bFGF and interleukin-1-beta is increased if HS is reduced by heparatinase treatment, suggesting that the transport of ligands is reduced by HS [85].

11.4. HSPG involvement in ligand transport

The discovery that *ttv* is required for the transmission of the Hh signal beyond the anterior/posterior compartment boundary of the wing imaginal disc suggested a more general model in which HSPGs are required for transport of signaling molecules to their sites of action. This model is attractive since it was already known that transmembrane HSPGs such as syndecan can be shed from the cell surface [20]. Thus, it would not be surprising that an HSPG and its bound signaling ligands could diffuse away from the cell of origin. More difficult to explain is why small ligands such as FGF and Dpp, or even the larger Hh or Wg molecules, would need a binding partner as large as an HSPG to effectively diffuse and signal at a distance. It would seem that firmly linking a ligand to a HSPG would only further slow that ligand's distribution by diffusion, although it could be argued that the HSPG is required for presentation of the ligand. A more parsimonious alternative might be that

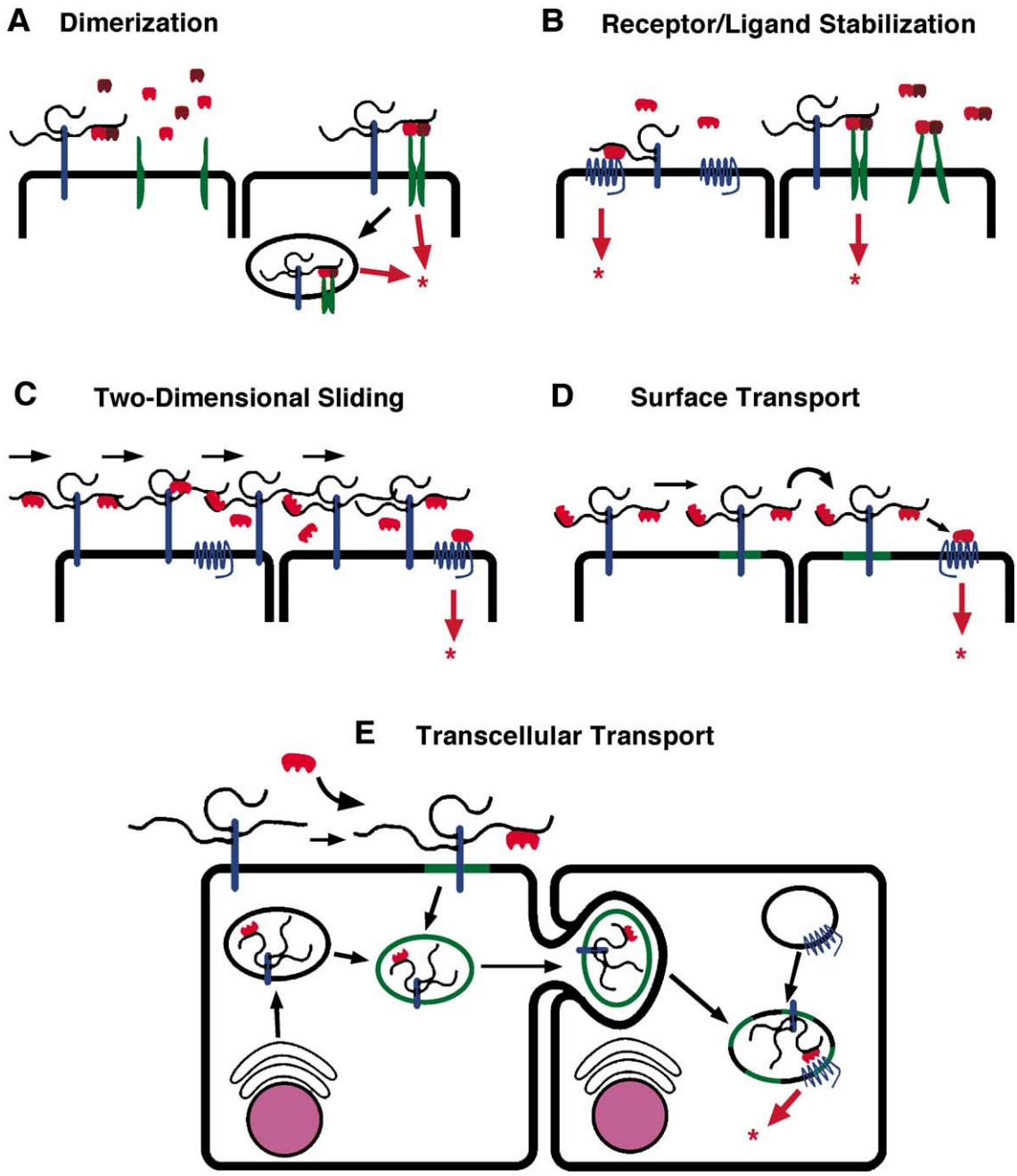


Fig. 2. Models of HSPG regulation of developmental signaling. See text for a detailed discussion of each model. In all models, the red arrows pointing to a red asterisk indicate activation of the signaling pathway. (A) HSPG control of dimerization. Many signaling ligands must dimerize or oligomerize to initiate effective signaling. HSPGs may be required for dimerization or oligomerization of ligands and/or presentation of these oligomers to their appropriate signaling receptors. As represented in the model, this could occur on the surface or in intracellular vesicles. (B) Receptor–ligand stabilization. HSPGs may not be required for ligand dimerization, but rather to stabilize the ligand/receptor signaling complex and promote maximal signaling from the receptors. (C) Two-dimensional sliding. In this model, HSPGs regulate the distribution of ligands by binding the ligands and allowing them to "slide" along the HS chain. This limits the ligand distribution to the plane formed by cell surfaces. (D) Surface transport of ligands by HSPGs. HSPGs might be required for active transport of signaling ligands away for the producing cell. This transport could occur in two different ways. The ligand-bound HSPGs could actually move between cells, perhaps via lipid rafts which can move between cells (represented by the green cell membrane region), or by one HSPG passing its bound ligand to a HSPG on adjacent cell. Once localized to the adjacent cell, the signaling molecule can then bind its receptor and activate signaling. (E) Transcellular transport. This model is similar to the surface transport model, but in this model, the ligand-bound HSPG is placed into a vesicle and this vesicle is then expelled from the ligand-producing cell and taken up by an adjacent cell. The HSPG-ligand-containing vesicle can then fuse with a vesicle containing the ligand's receptor and signaling is activated. HSPG's are represented as blue, single-pass transmembrane molecules with HS chains attached. The green, single-pass transmembrane molecules represent individual, dimerizing receptors such as tyrosine kinase receptors. The blue, multiple pass transmembrane proteins represent non-dimerizing receptors, such as seven pass G-protein receptors. Ligands are depicted in various shades of red.

cell surface HSPGs are actively involved in moving ligands between cells (Fig. 2D). This could be accomplished in two ways. First, the ligand-bound HSPGs could actually move between cells, perhaps via lipid rafts (represented in Fig. 2D and E by the green cell-membrane region) which have been demonstrated to move proteins between cells [86]. Alternatively, HSPGs on one cell surface could "pass" their ligands to HSPGs on adjacent cell surfaces through an as yet undefined mechanism. Once localized to the adjacent cell, the signaling molecule could then bind its receptor and activate signaling (Fig. 2D).

Vesicular transport is another method by which ligands could be moved between cells. HSPGs might carry out their role in ligand transport by controlling distribution and/or secretion of vesicles containing the ligand. Transcellular movement of these vesicles could then carry a ligand through neighboring cells to signal in regions far beyond the cells where the signaling molecule was originally expressed (Fig. 2E). In this model, the ligand would bind to a HSPG which would then be targeted to a transport vesicle. The transport vesicle containing the HSPG-bound ligand would then move into a neighboring cell by a mechanism that could be similar to phagocytosis. Once in a neighboring cell, the ligand-carrying vesicle could then fuse with a vesicle containing the receptor and signaling would be activated in that cell (Fig. 2E). For a signal to move several cell diameters away from the expressing cell, as Hh, Wg, and Dpp are able to do, only a portion of the ligand-containing vesicles that come into a particular cell could fuse with a receptor-containing vesicle. Those ligand vesicles that do not bind with a receptor-containing vesicle in a particular cell would then move through the cell that they are in and into the next cell, where this cycle would repeat itself.

Several recent studies in *Drosophila* have lent credence to the vesicular transport model. Two groups have shown that a Dpp–GFP fusion protein is transported far away from its site of production by ligand aggregates at least partially coincident with endocytic vesicles. They further show that this transport is likely responsible for the formation of the gradient of Dpp along the anterior–posterior axis of the disc [87,88]. Entchev et al. [87] also demonstrated that genes required for endocytosis and for transport to lysosomes affect the shape of the Dpp morphogen gradient. Rab5, a small GTPase required for transport to early endosomes, increases the range of Dpp target gene expression when overexpressed, while a dominant negative form of the same protein greatly reduces the range of Dpp target gene expression. Conversely, expression of a dominant gain of function Rab7, which is required for trafficking proteins to the lysosome, reduces the range of Dpp-induced gene expression [87]. These data strongly suggest that intracellular trafficking of endocytosed signaling molecules is necessary for productive signaling. They further imply that uptake of signal, via endocytosis, is required for activation of Dpp signaling, while destruction of the signal, via lysosomal transport, is important in attenuating Dpp signaling.

While these studies imply that vesicles are the vehicle moving Dpp, they are not definitive proof of this idea. However, another recent study has suggested a link between vesicular transport, Wg, and HSPGs and complements the Dpp studies. In this study, GFP linked to GPI was shown to move far beyond its site of expression in membranous vesicles called argosomes [89]. A portion of the transported GFP–GPI was then shown to colocalize with a portion of the transported Wg, implicating argosomes in Wg movement. Finally, treatment of wing imaginal discs with heparatinases eliminated Wg-containing vesicles but not argosomes. This result suggested that HSPGs may be required for localization of Wg to argosomes. It could, however, also imply that argosomes do not carry Wg and that the partial colocalization of Wg and GFP–GPI is simply due to the fact that they both move through the same intracellular compartment. These possibilities remain to be distinguished, but the model of vesicle-based movement of Wg using argosomes is an intriguing one that merits further investigation.

11.5. Requirement of HSPGs in endocytosis

Several pieces of evidence suggest that HSPGs regulate endocytosis [90], and endocytosis has further been shown to regulate the range of Wg signaling [91–93]. Since recent studies have demonstrated that the multiple signaling functions of many activated cell surface receptors can occur in different intracellular compartments and the cell surface, it could be that HSPGs control a subset of an activated receptor's signaling functions by regulating the distribution of the receptor between intracellular compartments and the cell surface (Fig. 2E). Perhaps signaling molecules such as Dpp can diffuse freely long distances, but cannot relay the proper signal unless the receptor–ligand complex is taken to the proper compartment of the cell. Examining the endocytosis and subcellular localization of signaling ligands and their receptors in HSPG mutants might be one way to investigate this possibility.

12. Conclusion

In conclusion, it is now apparent that HSPGs play critical roles in a number of developmentally important signaling pathways in *Drosophila* as well as in other organisms. It is also apparent that HSPGs regulate these signaling pathways in more than one way, affecting ligand dimerization, ligand binding to receptors, and transport of the ligands. Thus, HSPGs may control the activity of a particular signaling molecule on several levels. To determine in detail how a particular proteoglycan core protein affects signaling by a particular ligand, it will ultimately be necessary to determine the exact structure of that proteoglycan's HS chains. The advent of HS sequencing strategies and mass spectroscopy of HS may soon allow us to address these questions [94–

96]. This combination of genetics and detailed structural analysis should further elucidate this burgeoning field.

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