HEPARAN SULPHATE PROTEOGLYCANS: THE SWEET SIDE OF DEVELOPMENT

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Abstract | Pattern formation during development is controlled to a great extent by a small number of conserved signal transduction pathways that are activated by extracellular ligands such as Hedgehog, Wingless or Decapentaplegic. Genetic experiments have identified heparan sulphate proteoglycans (HSPGs) as important regulators of the tissue distribution of these extracellular signalling molecules. Several recent reports provide important new insights into the mechanisms by which HSPGs function during development.

DEVELOPMENTAL CELL BIOLOGY

PATTERN FORMATION
The developmental processes by which the complex shape and structure of higher organisms occur.

EXTRACELLULAR MATRIX
The complex, multi-molecular material that surrounds cells. The extracellular matrix comprises a scaffold on which tissues are organized, it provides cellular microenvironments and it regulates many cellular functions.

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In the normal development and function of metazoan tissues, cells have elaborate mechanisms to communicate and exchange signals. They use secreted signalling molecules such as members of the Wingless (Wnt/Wg), Hedgehog (Hh), transforming growth factor- β (TGFβ), bone morphogenetic protein (BMP) and fibroblast growth factor (FGF) families. These molecules bind to receptors on neighbouring cells, where they activate intracellular signal transduction cascades (for recent reviews on these signalling pathways see REFS 1–3). During PATTERN FORMATION, several of these signalling molecules function as morphogens — they form a gradient of extracellular protein, which triggers specific cellular responses in a concentration-dependent fashion. Although intense studies over the past decades have provided a detailed picture of the intracellular signalling cascades that are involved, how these gradients are formed, shaped and maintained continues to be a matter of debate (reviewed in REFS 3,4).

Genetic studies in *Drosophila melanogaster* and *Caenorhabditis elegans* have identified proteoglycans as important extracellular modulators of protein gradient formation and signal transduction (for reviews see REFS 5–7). Mutations that compromise the function of these acidic glycoproteins, which contain more carbohydrate than protein, disrupt the activity of several signalling cascades, including the Wnt/Wg, Hh, TGFβ and FGF pathways[8–14].

Genetic analyses carried out in model organisms have led to reports exploring proteoglycan function during development. Here, we review our current knowledge of the proteoglycans that have been implicated in patterning events, and discuss our understanding of the mechanisms by which these complex molecules modulate the activity of signal transduction pathways.

What are proteoglycans?

Proteoglycans are found in the EXTRACELLULAR MATRIX and attached to the cell membrane. They consist of a core protein to which one or more GLYCOSAMINOGLYCAN (GAG) chains can be attached at specific sites. Several hereditary disorders are causally linked to defects in proteoglycans and in GAG biosynthesis (BOX 1).

GAGs. *GAGs* are unbranched, mostly high-molecular-weight polysaccharides that consist of a backbone of repeating disaccharide units incorporating an AMINO SUGAR and a uronic acid (FIG. 1). Units of *N*-acetylglucosamine and glucuronic acid form heparan sulphate (HS), and units of *N*-acetylgalactosamine and glucuronic acid form a chondroitin sulphate (CS) backbone. EPIMERIZATION of the glucuronic acid in CS to iduronic acid forms dermatan sulphate (DS). Synthesis of the

GLYCOSAMINOGLYCAN
A long, linear, charged polysaccharide that comprises a repeating pair of sugars, of which one is an amino sugar.

AMINO SUGAR
A monosaccharide or its derivative in which an alcoholic hydroxyl group has been replaced by an amino group.

EPIMERIZATION
The process by which an epimer is converted into its diastereoisomer by altering the configuration at the epimeric chiral centre.

WILMS' TUMOUR
A malignant tumour of the kidney that occurs in children.

PROGEROID
Associated with premature ageing.

Box 1 | Heparan sulphate proteoglycans in disease

Mutations affecting the biosynthesis of heparan sulphate proteoglycans (HSPGs) are the cause of several human hereditary diseases. It has been shown, for example, that a member of the glypican family of membrane proteoglycans, GPC3, is mutated in human patients that suffer from Simpson–Golabi–Behmel syndrome (SGBS), an X-linked disorder that is associated with developmental tissue overgrowth and with a high incidence of neuroblastomas and WILMS' TUMOURS[73]. On the basis of an analysis of *GPC3* mutations, it has been proposed that SGBS is probably caused by a lack of functional GPC3 protein, which is supported by the defects shown in GPC3-deficient mice. Although the mechanism by which changes in glypican function lead to cancer is unknown, tumour progression has been found to be associated with changes in the expression of GPC3, and increases in the expression of another member of the glypican family, GPC1, have been observed in cervical and pancreatic cancers[74].

In addition to glypicans, several enzymes involved in the biosynthesis of glycosaminoglycans (GAGs), the polysaccharide chains that are attached to proteoglycan core proteins, have been implicated in disease. *Drosophila melanogaster tout velu* (*ttv*) encodes a heparan sulphate copolymerase that is required to add monosaccharide building blocks to the growing polysaccharide backbone of HS chains. *ttv* is a homologue of the mammalian Ext class of tumour suppressor genes, which are responsible for multiple hereditary exostosis, the most common form of human bone dysplasia[75,76]. Another GAG biosynthetic enzyme, galactosyltransferase I, which is necessary for synthesizing the linkage region (attaching GAGs to the proteoglycan core protein), is mutated in patients afflicted by the PROGEROID form of Ehlers–Danlos syndrome[77].

GAG chain is initiated by the addition of an amino sugar to a tetrasaccharide linker that has the sequence xylose–galactose–galactose–uronic-acid. This tetrasaccharide is itself attached to the core protein by *O*-glycosylation of a serine residue that is N-terminal to a glycine. After synthesis, the polysaccharide chain undergoes various modifications such as *N*-deacetylation–*N*-sulphation, 2-*O*-sulphation, 6-*O*-sulphation and epimerization. These modifications occur only on a subset of sugar residues, which creates an almost endless variety of different GAG chains[15,16].

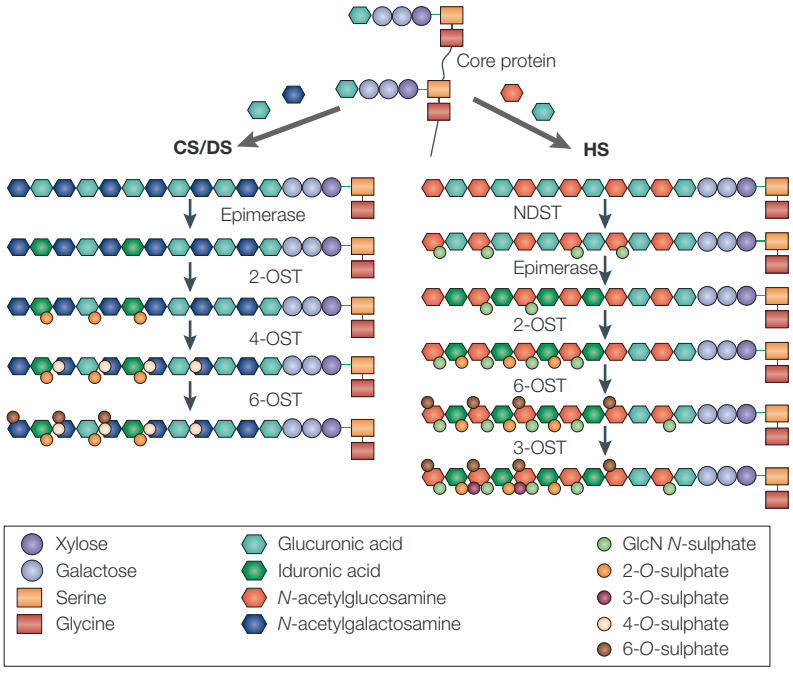


Figure 1 | **Biosynthesis of heparan sulphate, chondroitin sulphate or dermatan sulphate glycosaminoglycans.** Glycosaminoglycans (GAGs) are attached to a serine residue in the core protein by a tetrasaccharide linker (xylose–galactose–galactose–uronic-acid) on which the sugar chain is assembled. Synthesis of *N*-acetylgalactosamine (GalNAc)- and glucuronic acid (GlcA)-containing disaccharide units generates chondroitin sulphate (CS) or dermatan sulphate (DS); synthesis of *N*-acetylglucosamine (GlcNAc)- and GlcA-containing disaccharide units generates heparan sulphate (HS). Successive modification by epimerization of GlcA to iduronic acid, 2-*O*-sulphation, 4-*O*-sulphation and 6-*O*-sulfation in the case of CS or DS, and *N*-deacetylation–*N*-sulphation, epimerization, 2-*O*-sulphation, 6-*O*-sulphation and 3-*O*-sulphation in the case of HS, results in mature GAG chains. Note that not all biosynthesis or modification steps occur in a linear sequence. Some modification steps depend on previous steps to occur first, whereas others are independent. The nature of the modification reactions leads to the generation of an almost infinite diversity of polysaccharide chains. NDST, *N*-deacetylase–*N*-sulphotransferase; OST, *O*-sulphotransferase.

Glypicans and syndecans. Whereas CS chains are found mostly on matrix proteoglycans, membrane proteoglycans such as glypicans and syndecans contain mostly HS side chains. Both of these protein families comprise moderately sized (60–70 kDa and 20–45 kDa, respectively) core proteins that are conserved throughout the animal kingdom and are expressed tissue- and stage-specifically during development.

Glypicans are HS proteoglycans (HSPGs) that are linked to the cell membrane by a glycosylphosphatidylinositol (GPI) ANCHOR (FIG. 2). Six members of the glypican gene family have been found in mammals, two in *D. melanogaster* and at least one in *C. elegans*. Although the different family members have limited sequence homology, all glypicans share 14 conserved cysteine residues and 2–3 GAG attachment sites near the C terminus close to the cell membrane, which implies that the tertiary structures of all glypicans are similar.

Syndecans are TYPE I TRANSMEMBRANE PROTEINS with up to five GAG attachment sites, some of which can carry HS, CS or DS chains (FIG. 1), but they mainly contain HS chains. Although the cytoplasmic and transmembrane domains of different syndecans show similarities, the extracellular domains are divergent and share only the GAG attachment sites. Four syndecan (*sdc*) genes have been identified in mammals, but there is only one in invertebrate genomes. Glypicans and syndecans are of interest in the context of morphogen gradient formation.

HSPGs in Wnt and Hh signalling

Evidence that proteoglycans are important in developmental signalling has emerged from experiments in cell culture systems[17,18] and from studies in animal model

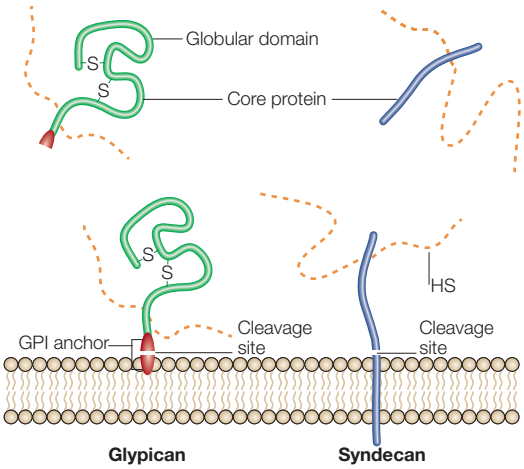


Figure 2 | **Structure of membrane heparan sulphate proteoglycans.** Glypicans (left) contain an N-terminal globular domain that is stabilized by disulphide bonds. Attachment sites for glycosaminoglycans (GAGs) are located near the C terminus, to which a glycosylphosphatidylinositol (GPI) anchor is attached that links the core protein to the cell membrane. The GPI anchor can be cleaved to shed the heparan sulphate (HS) proteoglycan from the cell surface. Syndecans (right) are type I transmembrane proteins with up to five GAG attachment sites. Syndecans can carry HS near the N-terminal tip of the extracellular domain and, in some cases, chondroitin sulphate (CS, not shown) and/or dermatan sulphate (DS, not shown) near the cell surface. Syndecans can also be shed by proteolytic cleavage.

systems such as *D. melanogaster*[19], *Xenopus laevis*[20,21], zebrafish[22,23] and rodents[24,25].

Initial evidence from segment polarity studies. In a genetic screen in *D. melanogaster*, mutations that affected embryonic SEGMENT POLARITY in strikingly similar ways to mutations in components of the Wg or Hh signalling pathways were identified in five genes. All of these genes — *sugarless* (*sgl*)[26–28], *sulfateless* (*sfl*)[8], *tout velu* (*ttv*)[29], *fringe connection* (*frc*)[30,31] and *slalom* (*sll*)[32] — were subsequently found to encode enzymes or nucleotide sugar transporters that are involved in GAG biosynthesis (FIG. 3; TABLE 1).

The Wg and Hh pathways (BOX 2) are linked to each other in a positive-feedback loop during embryonic segmentation, which makes it difficult to determine whether HSPGs are required for Wg or for Hh signalling in the embryonic epidermis. Experiments using the GAL4/UAS SYSTEM have shown that ectopic expression of either Wg or Hh can activate the respective signalling cascade in *sgl*, *sfl*, *frc* or *sll* mutants[28,31,32]. GAGs are therefore not essential components of these signalling pathways, but might instead be accessory factors that modulate activity. The severe embryonic segment polarity phenotypes that are observed in the absence of GAGs might be the result of a successive downregulation of positive feedback between the Wg and Hh pathways, which eventually leads to a complete loss of expression of both factors.

Evidence from studies in wing imaginal discs: Hh. Conclusive evidence regarding the role of GAGs in Hh and signalling has come from studies in *D. melanogaster* WING IMAGINAL DISCS, where Hh and Wg function independently to pattern the anterior–posterior (A–P) and dorsal–ventral (D–V) compartment boundaries, respectively. With respect to Hh, all of the available data consistently show that GAGs function as positive regulators of Hh signalling. In mosaic clones that are mutant for *ttv*, *sfl* or *sll* (F. Lüders and U.H., unpublished observations), expression of the Hh target *patched* is limited to cells that are directly adjacent to the Hh source[11,29], whereas this expression is activated at a distance of 5–6 cell diameters in wild-type imaginal discs.Therefore, it has been proposed that GAGs are required for Hh to travel over distances of more than one cell diameter. It has also been suggested that the *D. melanogaster* homologue of the mammalian Wnt inhibitory factor-1 might be important in the interaction of Hh with HSPGs[33,34].

Conflicting evidence in mouse bone studies: Hh. A recent study that examined the role of GAGs in Hh signalling during skeletal development in mice reached a different conclusion[35]. During CHONDROCYTE differentiation in mice that were homozygous for a HYPOMORPHIC allele of the *ttv* homologue *Ext1*, the mouse Hh homologue Indian Hedgehog (Ihh) was effective over a greater distance than normal. Conversely, the addition of HS to limb explants in culture decreased the distance at which the Ihh-target gene *patched* (*ptc*) could be activated. The authors propose that HS might sequester active Ihh and that increased levels of HS restrict the distribution of Ihh, whereas decreased levels promote long-range transport.

The different effects of mutations in *ttv/Ext1* in *D. melanogaster* wings and mouse limbs might be the result of the nature of the mutant alleles used. The mouse studies show a clear concentration-dependence on HS function. At lower HS levels, as present in the hypomorphic *Ext1* allele used in the studies, Ihh distribution was less restricted and target genes could be activated farther from the source. By contrast, the *ttv* allele used in the *D. melanogaster* studies is probably a null allele. This might abolish all binding of Hh at the cell surface and lead to the loss of tissue transport and signalling activity observed in imaginal discs.

The authors of the mouse study find that changes in HS levels had no significant impact on local Ihh signalling activity, which argues against a role for HSPGs as co-receptors in signal transduction. We suggest that the data are consistent with a role for GAGs as low-affinity binding agents for secreted signalling molecules. The increased range of Hh signalling when levels are reduced is consistent with a passive mode of Hh transport that has been supported by theoretical models[36]. In addition, other processes, such as downregulation of morphogen levels by proteoglycan shedding (see below) or the modulation of morphogen stability by proteoglycan binding, might be important in controlling the range of Ihh movement.

GPI ANCHOR
The function of this post-translational modification is to attach proteins to the exoplasmic leaflet of membranes, possibly to specific domains therein. The anchor is made of one molecule of phosphatidylinositol to which a carbohydrate chain is linked through the C-6 hydroxyl of the inositol, and is linked to the protein through an ethanolamine phosphate moiety.

TYPE I TRANSMEMBRANE PROTEIN
A protein that contains a single membrane-spanning domain, with the C terminus orientated towards the cytoplasm and the N terminus orientated towards the lumen of membrane compartments or in an extracellular direction.

SEGMENT POLARITY
The segmented patterning of the body along the anterior–posterior axis. Segment polarity genes are expressed in a pattern of 14 stripes at the onset of gastrulation and follow the expression of pair-rule genes.

GAL4/UAS SYSTEM
Used in *D. melanogaster* to target the expression of specific genes to specific tissues. UAS stands for the upstream-activating system of the yeast *GAL4* gene.

WING IMAGINAL DISCS
Imaginal discs are the larval precursors of adult structures. The wing imaginal discs give rise to the dorsal thorax and the wing appendages.

CHONDROCYTE
A differentiated cell of cartilage tissue.

HYPOMORPHIC
A mutation that reduces, but does not completely eliminate, the function of a gene.

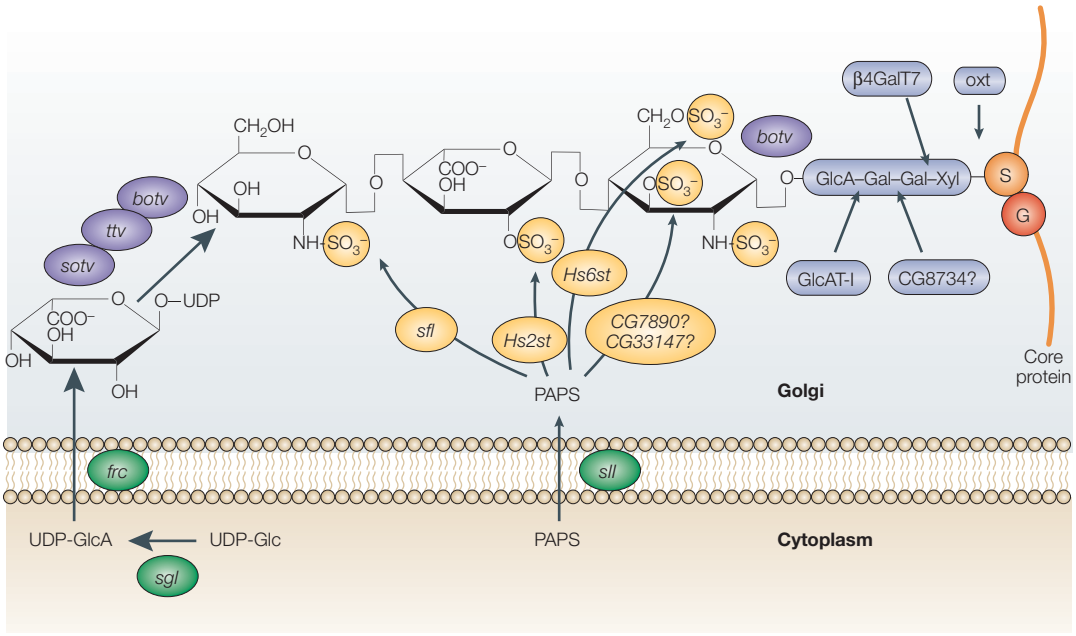


Figure 3 | ***Drosophila melanogaster* genes involved in synthesis and modification of heparan sulphate.** Glycosaminoglycan (GAG) chains are assembled on a serine residue in the core protein that is almost invariably followed by glycine. The tetrasaccharide that links the GAG chain to the core protein is synthesized by the sequential action of peptide-*O*-xylosyltransferase (oxt), two galactosyltransferases (β4GalT7 and CG8734) and a glucuronosyltransferase (GlcAT-I) (all shown in blue). The GAG backbone of alternating disaccharide units is assembled by a set of heparan sulphate (HS) copolymerases encoded by *D. melanogaster* orthologues of the vertebrate Ext family (*tout velu* (*ttv*), *sister of ttv* (*sotv*) and *brother of ttv* (*botv*); shown in purple). *botv* might have another function in HS chain initiation. Sulphate groups are added to the glucuronic acid (GlcA) and *N*-acetylglucosamine residues by the action of *N*-deacetylase–*N*-sulphotransferase, 2-*O*-sulphotransferase, 6-*O*-sulphotransferase and 3-*O*-sulphotransferase (all shown in yellow). Building blocks for HS synthesis are generated in the cytoplasm by enzymes such as UDP-glucosedehydrogenase (encoded by *sgl*) and transported into the Golgi apparatus by a GlcA transporter (encoded by *frc*) or the adenosine 3′-phosphate 5′-phosphosulphate (PAPS) transporter (encoded by *sll*) (all shown in green). A question mark beside a gene name indicates that gene function has been inferred on the basis of homology. See TABLE 1 for genes involved in *D. melanogaster* HS proteoglycan synthesis. Gal, galactose; Xyl, Xylose.

Evidence from studies in wing imaginal discs: Wg. In mosaic clones that are adjacent to the D–V boundary and that are mutant for *sfl*, *sll* or the Ext-class genes *ttv*, *brother of ttv* (*botv*) and *sister of ttv* (*sotv*), levels of extracellular Wg protein are decreased and the expression of genes that require a high input of Wg signalling is abrogated[32,37–39]. The activation of long-range Wg-target genes such as *distalless* (*dll*) is less strongly affected and so *ttv* was initially reported to be specifically involved in Hh signalling[29]. This notion has been revised recently on the basis of reports that *ttv*, *botv* or *sotv* mutant mosaic clones do disrupt the Wg morphogen gradient as well as *dll* activation.

More evidence for an involvement of HSPGs in Wg signalling has emerged from studies on the glypicans Dally and Dally-like (Dlp). *dally* genetically interacts with components of the Wg signalling pathway, and homozygous *dally* adults occasionally show defects at the wing margin that are reminiscent of those resulting from a loss of Wg signalling[8]. In addition, overexpression of *dally* leads to a mild accumulation of Wg in the tissue[40].

Ectopic expression of *dlp* in wing imaginal discs leads to a strong accumulation of Wg at the cell surface and results in *wg*-like phenotypes at the wing margin that can be rescued by further overexpression of *wg* or its downstream signal transducer *armadillo* (*arm*)[37]. Conversely, *dlp*-mutant SOMATIC CLONES in the wing show ectopic activation of Wg signalling[11,41–43]. Two recent studies show that in clones that are mutant for null alleles of *dally* or *dlp* the concentration of extracellular Wg is reduced[41,44]. This reduction is enhanced in *dally*, *dlp*-double mutant clones[44]. Previously, it had been reported that Wg accumulates at the boundary of *dlp*-mutant clones but that Wg levels inside clones remained unchanged[42]. Dally and Dlp are distributed in distinct, but partially overlapping, domains in the wing pouch and the regions where defects in distribution are greatest in the respective mutant clones correlate with the specific expression domains of *dally* and *dlp*[44]. Together, these observations imply that *dally* and *dlp* have distinct, but partially redundant, functions in shaping the Wg gradient.

Roles for HSPGs in segmentation. Although the most conclusive evidence for a role of GAGs in Hh and Wg signalling has come from studies in wing discs, HS undoubtedly has a function during segmentation. Investigations addressing the nature of the core proteins that mediate this function have focused on *D. melanogaster dally*[8,9] and *dlp*[37], as both genes are expressed in a segmentally repeated

SOMATIC CLONES
A group of non-germline cells produced by proliferation of a single common ancestor cell.

fashion in partially overlapping sets of cells in the ventral epidermis[45]. Mutations in either gene were initially unavailable and functional analyses relied on RNA-MEDIATED INTERFERENCE (RNAi). RNAi against *dlp* results in a segment polarity phenotype[37] and this has been confirmed by the analysis of *dlp*-null mutants[11]. It has been reported that ectopic expression of *wg*, but not *hh*, in the embryonic epidermis can activate the expression of target genes after RNAi-mediated *dlp* downregulation[10]. So *dlp* might be essential for Hh, but not Wg, signalling during segment polarity determination. This finding contrasts with experiments in *sgl*, *sfl*, *ttv*, *frc* and *sll* mutants showing that ectopic expression of *wg* or *hh* can compensate for the loss of GAGs, resulting in activation of their respective pathways[28,31,32], and that the Dlp core protein might have a specific function in Hh signalling that cannot be mediated by GAGs. The role of Dlp in Hh signalling is corroborated by experiments showing that RNAi against *dlp* blocks Hh signalling in cultured cells when Hh is provided in the growth medium[46]. Interestingly, the same cells responded when *hh* was expressed in these cells. Because mature Hh is anchored to the plasma membrane by lipid modification, this experiment indicates a potential role for Dlp in concentrating Hh at the cell surface, rather than an essential role in signal transduction. RNAi against *dlp* does not stop activation of the Wg pathway induced by ectopic expression of *wg* in the embryo. However, this does not preclude an accessory function for Dlp in Wg signalling during segmentation (see REF. 47 for a more extensive discussion). By measuring the distinct expression of *rhomboid* in *wg* or *hh* mutants and comparing it to *rhomboid* expression observed in *dlp* or *dlp*, *dally*-double mutants, respectively[41], a recent study shows that *dlp* and *dally* both have non-essential, but significant, roles in Wg signalling during segment polarity determination.

dally was initially reported to be essential for segment polarity determination[8,9]. Although this observation could not be confirmed in later studies[10], the participation of *dally* in Wg and Hh signalling in the embryo has recently been corroborated[41]. Taken together with the data obtained in wing imaginal discs (see above), these observations indicate that *dally* might have a partially redundant role in Wg signalling. Similarly, data supporting essential roles of *dally* in Hh signalling are not available. However, clones that are mutant for both *dally* and *dlp* abrogate activation of the Hh target gene *ptc*, which implies that *dally* and *dlp* are required for Hh signalling in a redundant fashion[11].

In summary, the data support a role for *D. melanogaster* glypicans in cell-surface binding and in the tissue distribution of Wg and Hh throughout development. The sensitivity of individual signalling molecules to proteoglycan function varies between tissues and might reflect the specific mode of action of each morphogen.

A role for HSPGs in other signalling pathways

A careful analysis of mutants in glypicans or in GAG biosynthetic enzymes has revealed that HSPGs might have similar roles in several extracellular-signal-regulated cell communication pathways.

Decapentaplegic signalling. The *D. melanogaster* homologue of BMPs, *decapentaplegic* (*dpp*; see also BOX 3), functions in a concentration-dependent

Table 1 | ***Drosophila melanogaster*** **genes involved in HS biosynthesis and modification**

Gene (CG number)	Gene name	Enzyme	Size (aa)	References
CG10072	*sugarless* (*sgl*)	UDP-GlcA-dehydrogenase	476	26–28
CG3974	*fringe connection* (*frc*)	NTP-sugar transporter	373	30,31
CG10117	*tout velu* (*ttv*)	HS polymerase/EXT	760	29
CG33038	*sister of tout velu* (*sotv*)	HS polymerase/EXT	717	38,39
CG15110	*brother of tout velu* (*botv*)	HS polymerase/EXT	972	38,39
CG7623	*slalom* (*sll*)	PAPS-transporter	465	32
CG8339	*sulfateless* (*sfl*)	*N*-deacetylase–*N*-sulphotransferase	1,048	14
CG7890		3-*O*-sulphotransferase	384	
CG33147		3-*O*-sulphotransferase	605	
CG10234	Hs2st	2-*O*-sulphotransferase	349	
CG9614	*pipe* (*pip*)	2-*O*-sulphotransferase	407	86
CG4451	Hs6st	6-*O*-sulphotransferase	462	64,87
CG6725	*sulfated* (*Sulf1*)	6-*O*-sulphatase	1,114	
CG3194		C5 epimerase	614	
CG13076	*notum*	α/β-hydrolase	671	43,71

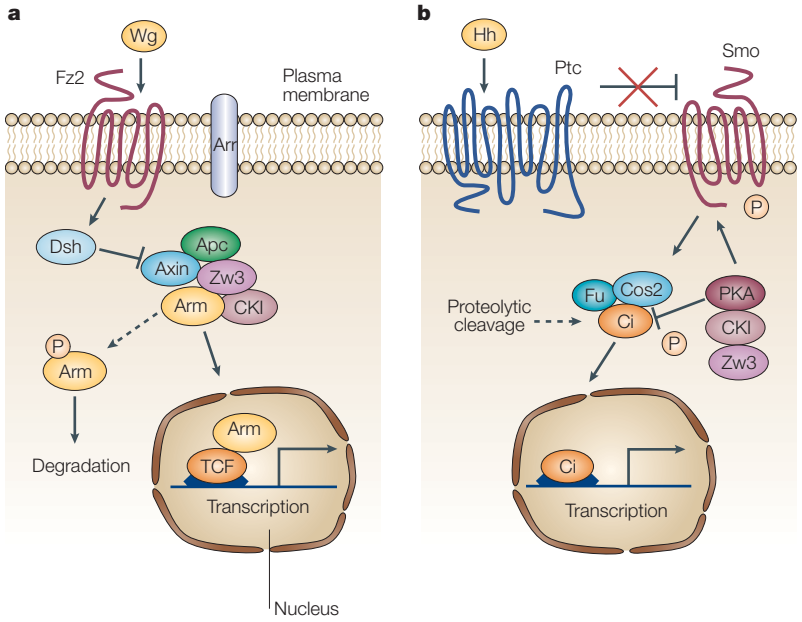
aa, amino acid; HS, heparan sulphate; NTP, nuclear triphosphate; PAPS, 3′-phosphoadenosine 5′-phosphosulphate.

RNA-MEDIATED INTERFERENCE (RNAi)
A form of post-transcriptional gene silencing in which expression or transfection of double-stranded RNA induces nuclease-mediated degradation of the homologous endogenous transcripts. This mimics the effect of the reduction, or loss, of gene activity.

Box 2 | **The Wingless and Hedgehog signalling pathways**

In the ventral epidermis of the *Drosophila melanogaster* embryo, expression of Hedgehog (Hh) and the Wnt homologue, Wingless (Wg), is established by PAIR-RULE GENES in adjacent rows of cells in each segment. During segment polarity determination, the Wg and Hh pathways are linked in a positive-feedback loop that maintains the expression of each of the other genes. In the absence of Wg, the β-catenin homologue Armadillo (Arm) is phosphorylated by casein kinase I (CKI) and the glycogen synthase kinase-3 (GSK3) homologue Zeste-white 3 (Zw3), both of which reside in a cytoplasmic complex with the cytoskeletal protein Axin and the homologue of the human adenomatous polyposis coli protein, APC. Phosphorylation promotes Arm degradation, which keeps cytoplasmic levels low. In the nucleus, target-gene transcription is repressed by T-cell factor (TCF). Wg stimulation of a cell-surface receptor complex that consists of the lipoprotein receptor-related protein 5/6 (LRP5/6) homologue Arrow (Arr) and the seven-transmembrane-domain protein Frizzled-2 (Fz2) transmits a signal to the cytoplasmic protein Dishevelled (Dsh). Dsh inactivates the Axin complex, leading to stabilization of Arm, which accumulates and associates with TCF to form a transcriptional activator (see figure part **a**).

Hh stimulation regulates the activity of the transcription factor Cubitus interruptus (Ci). The transmembrane receptors Patched (Ptc) and Smoothened (Smo) communicate the Hh signal to an intracellular signalling complex consisting of the kinesin-related protein Costal2 (Cos2), the serine/threonine kinase Fused (Fu) and Ci. In the absence of Hh, Ci is proteolytically cleaved into a transcriptional repressor, which inactivates the transcription of target genes. In response to Hh binding to Ptc, Ci processing is inhibited, resulting in the accumulation of the full-length transcriptional activator. The activity of Ci and Smo is modulated by phosphorylation through protein kinase A (PKA), Zw3 and CKI (see figure part **b**). Several components of the Wg and Hh signalling pathways have been omitted for simplicity. For a more detailed description see recent reviews[2,78–80].



fashion in the patterning of several imaginal tissues, and has been shown to interact genetically with *dally*. A reduction in the dose of *dpp* enhances the phenotypes of *dally* mutants in the eye and antennal discs, but it suppresses phenotypes in the wing disc, which implies that *dally* might have different roles in *dpp* signalling in different tissues[48].

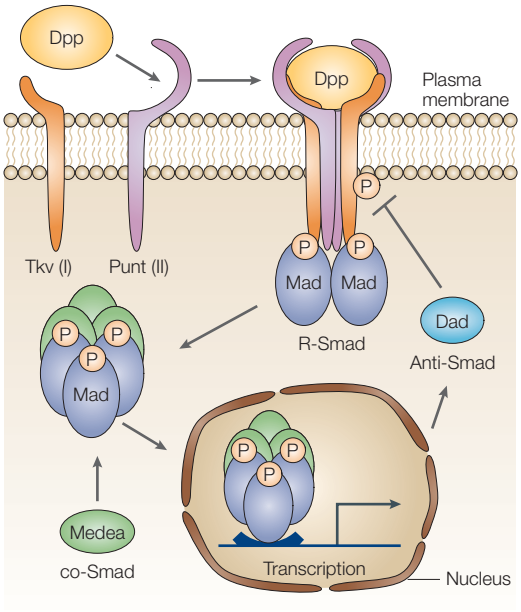
Further studies have shown that expression of *dally* at the A–P compartment boundary in *D. melanogaster* wing imaginal discs is controlled by the same set of regulators that control expression of the gene encoding the Dpp receptor thick veins (*tkv*). In *dally* mutants, the activity gradient of Dpp is limited to the area near the A–P boundary, whereas overexpression of *dally* results in the accumulation of Dpp in the tissue[12]. A study addressing the role of *dally* and *dlp* at the A–P boundary of wing discs shows that Dpp cannot traverse *dally*, *dlp*-double-mutant cell clones, which disrupts the Dpp morphogen gradient inside the clone as well as in wild-type cells that are distal to the clone with respect to the Dpp source. The authors conclude that both genes have partially redundant and NON-CELL-AUTONOMOUS functions in the activation of Dpp signalling as well as in Dpp distribution, with a stronger requirement for *dally* than *dlp*[13]. These observations indicate that glypicans might shape the Dpp morphogen gradient by regulating the stability and tissue distribution of Dpp in a similar way to their role in forming the Wg gradient at the D–V compartment boundary of the wing. The predominant function for *dally* in Dpp gradient formation is in contrast to its function in the determination of segment polarity and in the patterning of the wing margin — here, *dlp*, but not *dally*, seems to be essential. It is possible that the requirement for *dally* or *dlp* in specific signalling pathways might partly be determined by their expression patterns[44] and partly by specific binding affinities of the different core proteins for individual signalling molecules. Core-protein-specific and HS-independent interaction of a glypican with a member of the Wnt family has been described in vertebrates[21,25].

PAIR-RULE GENE
A class of segmentation gene that determines segments along the anterior–posterior axis. The expression of pair-rule genes in a pattern of seven stripes that are perpendicular to the axis is regulated by another class of segmentation genes: the gap genes.

NON-CELL-AUTONOMOUS
A gene functions non-automonously if expression of the gene in one cell influences the phenotype of a different cell.

Box 3 | **The transforming growth factor-β (TGFβ)–Decapentaplegic (Dpp) signalling pathway**

The binding of ligands of the Dpp/bone morphogenetic protein (BMP) subfamily by distantly related transmembrane serine/threonine kinases called type I receptor (Thick veins, Tkv) and type II receptor (Punt) at the cell surface results in the formation of a transient tetrameric complex. The type II receptor is a constitutively active kinase, whereas the type I receptor is activated on phosphorylation by the type II receptor. The type I receptor transduces the signal by phosphorylating the cytoplasmic transcription factor Mothers against Dpp (Mad), which belongs to the family of receptor-regulated Smads (R-Smads). Phosphorylated Mad associates with Medea, a member of the common mediator Smad family (co-Smads). Oligomeric complexes containing R-Smads and co-Smads translocate to the nucleus in a signal-dependent fashion where they can bind to DNA and regulate the transcription of target genes. A third subfamily of Smads, the anti-Smads (such as Daughters against Dpp (Dad)) antagonize signalling (see figure). For a more detailed description see previous reviews[81].



The requirement for HSPGs in Dpp signalling is evident from the analysis of several mutants of GAG biosynthetic enzymes. Mutations in *sgl* have been reported to suppress phenotypes that result from the abnormal activation of *dpp*[26]. In addition, mosaic clones that are homozygous mutant for genes of the Ext family, *sfl* or *sll* in the wing disc show reduced activation of Dpp target genes[13,38,39,49] (F. Lüders and U.H., unpublished observations). It has also been shown that Dpp levels are reduced non-cell-autonomously in *sfl* mutants clones, implying that GAGs are required for tissue distribution of Dpp[13]. Therefore, HSPGs of the glypican family seem to have similar functions in Dpp, Wg and Hh signalling.

FGF signalling. Secreted members of the FGF family bind GAGs and are dependent on HSPGs for efficient signal transduction in cell culture systems[17,50]. However, the function of HSPGs during FGF signalling *in vivo* has not been studied in detail. In *D. melanogaster*, FGFs control the lateral migration of the MESODERM shortly after GASTRULATION, and the formation of the tracheal system. In *sgl*, *sfl* or *frc* mutants, these processes are disrupted in a manner that resembles mutations in components of the FGF signalling pathways, which indicates that HSPGs might have a role in FGF signalling during development[14]. Interestingly, lateral migration of the mesoderm has been reported to be unaffected in mutants of the *D. melanogaster Ext1* homologue *ttv*[51]. However, this might be because of partial redundancy between the three existing *Ext* homologues in *D. melanogaster*. The role of glypicans in FGF signalling has not been addressed in *D. melanogaster*, but silencing the gene *glypican 4* in *X. laevis* using MORPHOLINO oligonucleotides causes patterning defects and the loss of FGF-target-gene expression in the forebrain, which implies that glypicans are also involved in FGF signalling in vertebrates[20].

HSPGs in other pathways? The involvement of HSPGs has so far been shown only for signalling pathways that are activated by secreted diffusible ligands. In the case of the Wg/Wnt pathway there is evidence that in vertebrate systems the activity of the canonical as well as the non-canonical intracellular pathways are modulated by glypicans[21,25]. Signalling cascades such as the Notch pathway, which is activated by cell–cell contact, seem to be activated independently of HSPGs. However, it has been shown that the affinity of Notch for different ligands is regulated by polysaccharide modification of the receptor itself, in an HSPG-independent fashion[52,53]. There is no current evidence that other signalling pathways, such as the Janus kinase (JAK)/signal transducer and activator of transcription (STAT) pathway or the epidermal growth factor (EGF) pathway, are sensitive to HSPGs.

Syndecan and perlecan in development

The second group of membrane HSPGs is the syndecans. Although the role of syndecan (Sdc) in developmental signalling has not been thoroughly explored, several recent studies have shown that *D. melanogaster sdc* has a role in axonal guidance in the central nervous system (CNS) midline, a patterning event that involves the Slit–Robo signalling pathway. Slit is an axonal repellent that is secreted by CNS midline cells and, on binding to the Robo protein, Slit prevents axon pathfinding in the direction of the Slit source. Slit binding to Robo is severely reduced when Robo-expressing cells are treated with heparinase III, an enzyme that degrades HS[54,55]. However, the core protein to which these HS chains are attached was

MESODERM
The third embryonic layer generated during gastrulation, which occupies an intermediate position between the ectoderm and the endoderm. It will develop into the skeleton, muscles and connective tissue.

GASTRULATION
A series of morphogenetic movements observed during the early development of most animals that leads to the formation of a multilayered embryo with an outer cell layer (ectoderm), an inner cell layer (endoderm) and an intermediate cell layer (mesoderm).

MORPHOLINO
A chemically modified oligonucleotide that behaves as an antisense RNA analogue and that is used to interfere with gene function.

Box 4 | **Chondroitin sulphate in development**

In addition to heparan sulphate proteoglycans (HSPGs), chondroitin sulphate proteoglycans (CSPGs) have been implicated in the regulation of patterning events during development[82]. In a screen in *Caenorhabditis elegans* eight *squashed vulva* (*sqv*) mutants were isolated that affect invagination of the vulva, which allows the hermaphrodites to lay eggs and take in sperm[67,68]. The mutated genes encode proteins involved in the synthesis of glycosaminoglycans (GAGs; for review see REF. 83). SQV-5 catalyses the polymerization of glucuronic acid–*N*-acetylgalactosamine dimers onto the tetrasaccharide linker to form chondroitin sulphate (CS), indicating that CS is the relevant GAG that affects vulval development[84]. The protein core that carries the CS chains needed for vulval morphogenesis has not been identified.

A separate study characterized the role of glucuronic acid/*N*-acetylgalactosamine polymerase in the development of *C. elegans*. Called chondroitin synthase (ChSy) by these authors, RNA-interference-mediated reduction of ChSy resulted in early embryonic lethality and defects in egg formation and laying. Time-lapse microscopy experiments revealed that the embryonic lethality was caused by cell division defects, including reversal of cytokinesis[85].

Exactly how CS is required in cell division or vulval morphogenesis is currently unclear. CS might control invagination by several means. Many polysaccharides, including HS and CS, can bind large amounts of water and, in so doing, can exert substantial osmotic pressure on their surrounding tissues. A reduction of extracellular CS could result in the loss of sufficient osmotic pressure normally required to create the vulval space. Another possibility is that loss of CS in the *sqv* mutants increases adhesion between vulval cells, thereby preventing expansion of the vulval space[68]. Although signalling events control the formation of the vulva, a role for CS in the tissue distribution of secreted signalling molecules or in the regulation of signalling activity, as shown in the case of HS, has not been shown.

not identified. As *sdc* has an expression pattern that overlaps with all tissues affected by Slit mutations, its effects on Slit–Robo signalling were examined. Mutants in *sdc* cause abnormal axonal guidance in the CNS midline and interact genetically with *slit* and *robo* mutations, and TRANSHETEROZYGOUS COMBINATIONS of *sdc* and *slit* or *robo* function synergistically to produce *slit–robo*-like phenotypes[56,57]. In *sdc* mutants, Slit is mis-distributed, and the resulting phenotype is similar to the effects of *D. melanogaster* glypican mutations on Wg, Dpp and Hh distribution. Furthermore, Slit and Robo both bind to Sdc, and this binding is reduced in *sdc* mutants, which indicates that Sdc might be directly involved in presenting the Slit ligand to Robo. There might be some genetic redundancy between Sdc and Dlp, as overexpression of *dlp* can largely rescue the *sdc* midline phenotype[56].

The secreted matrix HSPG perlecan has been implicated in the regulation of neural stem cell proliferation in the *D. melanogaster* nervous system[58,59]. Mutations in the *D. melanogaster* perlecan homologue, which is encoded by the *terribly reduced optic lobes* (*trol*) gene, reduce FGF signalling activity and cause cell-cycle arrest of neuroblasts in the larval brain. This phenotype can be rescued by the addition of human FGF2 to *trol*-mutant brains in culture. Hh is also required for the initiation of stem-cell division in the larval brain, and this Hh activity is reduced in *trol* mutants. Mutations in components of the *D. melanogaster* FGF or Hh pathway interact genetically with *trol* mutants. Co-immunoprecipitation studies support the possibility that Trol might bind to Hh and FGF2 (REF. 58). These results imply that Trol modulates the activity of the FGF and Hh signalling pathways during neuroblast proliferation.

TRANSHETEROZYGOUS COMBINATIONS
A combination of alleles of the same or different genes located on different homologous or non-homologous chromosomes.

GENE TRAP
A DNA construct that contains a reporter gene sequence downstream of a splice acceptor site that can integrate into random chromosomal locations in mouse. Integration of the gene trap into an intron allows the expression of a new mRNA containing one or more upstream exons followed by the reporter gene.

Post-polymerization modification of HS

The requirement for HS in development and disease has been shown in several model systems, but fewer studies have focused on how post-polymerization modifications of HS, such as *O*-sulphation and epimerization, might modulate the function of HS. Here, we examine how these post-polymerization modifications affect development.

Sulphation, FGF signalling and development. Investigation in cell culture originally showed that sulphation was required for FGF signalling. Artificially inhibiting sulphate modification of HS abolished FGF signalling in cell culture, indicating that sulphation of GAGs is a requirement for high levels of FGF signalling[17,50]. Studies of FGF signalling have subsequently shown that 6-*O*-sulphation of *N*-acetylglucosamine seems to be one of the key structural features in the HS hexasaccharide that determines optimal binding of FGF1 and FGF2 to the FGF receptor[60–62]. Wg signalling in *D. melanogaster* also requires sulphation of HS — inhibiting this process eliminated the ability of a *D. melanogaster* cell line to respond to Wg conditioned media[63].

A *D. melanogaster* 6-*O*-sulphotransferase (6-OST) has been shown to be involved in tracheal development. Tracheae require FGF signalling for their development, and even minor impairments in FGF signalling can cause severe patterning defects. RNAi-mediated inhibition of 6-OST activity severely disrupted tracheal development and partly mimicked FGF signalling pathway mutants[64].

2-*O*-sulphation of the glucuronic/iduronic acid subunit of HS has an important role in the development of the mouse kidney. A GENE-TRAP-mediated mutation in the single mouse 2-*O*-sulphotransferase gene is lethal shortly after birth as the kidney fails to develop properly. Although the affected signalling pathway is not known, it is thought that the loss of 2-*O*-sulphate perturbs the FGF signalling pathway. This gene-trap mutant also causes variable defects in bone mineralization and skeletal development, which implies a broader requirement for 2-*O*-sulphate modification[65].

***Further clues from* C. elegans.** Recent work in *C. elegans* has greatly expanded our understanding of how particular HS modifications can affect different signalling pathways in different tissues. Mutations in genes encoding proteins that synthesize the saccharide precursors for HS and CS (for a brief discussion of the role of CS in development see BOX 4) polymerization, transport them into the ER, modify them in the ER and polymerize the precursors into linear chains affect viability and egg-laying[66–68]. HS post-polymerization modification

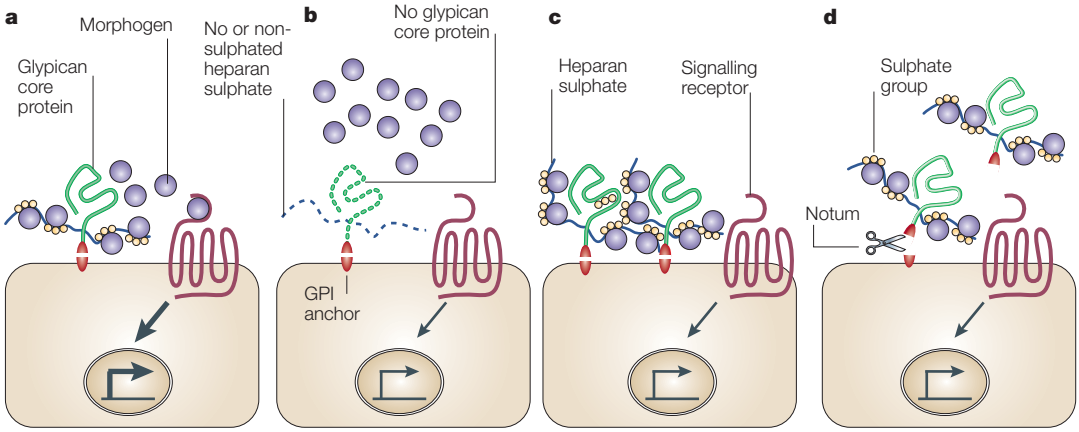


Figure 4 | **Function of glypicans in growth factor signalling. a** | Glypicans function as low-affinity binding proteins for secreted morphogens. Glypicans maintain secreted signalling molecules such as Wnt, Hedgehog (Hh) or transforming growth factor-β (TGFβ) at the cell surface at levels that are sufficient to activate high-affinity signalling receptors and ensure activation of target genes. **b** | In the absence of glypican core protein or sufficiently sulphated glycosaminoglycan (GAG) chains, morphogen levels at the cell surface are reduced and the activity of signal transduction pathways is diminished. **c** | When glypicans are overexpressed, morphogen levels at the cell surface are elevated owing to the increased binding capacity of the additional GAG chains. Signalling activity might be reduced owing to a shift of the equilibrium from signalling receptor activation to GAG binding. **d** | Glypicans can be shed from the cell surface by Notum-mediated cleavage of the glycosylphosphatidylinositol (GPI) anchor. Shedding of glypicans might locally reduce morphogen activity at the cell surface and result in decreased activity of signal transduction pathways.

enzymes, however, are not required for viability in *C. elegans*. Bulow and Hobert[69] isolated mutants of the two 6-OST orthologues and of the single copies of C5 epimerase and 2-OST, and found each to be viable and fertile. Further investigation showed that each enzyme regulates the development of certain classes of neurons in diverse ways.

This is the first study to systematically examine a set of phenotypes caused by post-polymerization modifications of HS. No studies have yet addressed the fundamental questions of HS regulation in cell signalling: what is the nature of the specific post-polymerization-modified HS sequences that contribute to the developmental events, and how do these sequences affect particular signalling pathways? Although optimal binding sequences that regulate FGF and HGF binding to HS have been defined, these studies were carried out using small saccharides that do not necessarily reflect how a ligand might bind to a HS chain that consists of 50–100 dimers. In addition, these studies only showed a preference, and not a requirement, for certain sequences over others. Definitive answers to these questions will have to await the development of robust and highly sensitive sequencing methods for GAGs.

Mechanism of action of HSPGs

Signalling molecules such as Wg, Hh or Dpp are secreted from the cells in which they are expressed and spread to form a morphogen gradient that activates the expression of target genes in a concentration-dependent fashion. The shape of the gradient is determined by the rate of diffusion and stability of the morphogen, and by extracellular factors that affect the amount of morphogen available for signalling receptor activation.

HSPGs are important in the shaping of morphogen gradients. For example, mutations in GAG biosynthetic enzymes or glypicans result in altered tissue distribution of morphogens, which, in turn, can cause loss or gain of target-gene activation[12,32,37–39,44]. Ectopic expression of glypicans leads to the accumulation of morphogen in the tissue[37,40]. This indicates that glypicans bind morphogens and regulate their levels on the surface of the cell in a concentration-dependent manner (FIG. 4a–c). In *D. melanogaster*, secreted signalling molecules such as Hh or Dpp cannot traverse clones that are mutant for GAG biosynthetic enzymes[29,38,39,49], implying that these factors must be bound to HSPGs in order to spread throughout tissues. This contrasts with data obtained in the mouse that show that the range of Hh signalling is increased when GAG levels are reduced[35]. The non-cell-autonomous loss of target-gene activation that is seen in *D. melanogaster* imaginal discs when there is no GAG biosynthesis might therefore reflect the properties of the specific cellular environment of imaginal discs, rather than a requirement for GAGs as co-receptors of morphogen signalling and tissue transport.

Several recent reports show that glypican activity can be locally regulated[41–43,70] (FIG. 4d). In agreement with earlier results, these reports find that a reduction in Dlp levels in *D. melanogaster* wing discs causes a loss of Wg target-gene activation at greater distances from the D–V boundary where Wg levels are low, which confirms that Dlp functions as a Wg-binding protein and maintains Wg levels at the cell surface. When Dlp levels are reduced, less Wg is bound and available for receptor activation, therefore decreasing target-gene activation. Interestingly, close to the

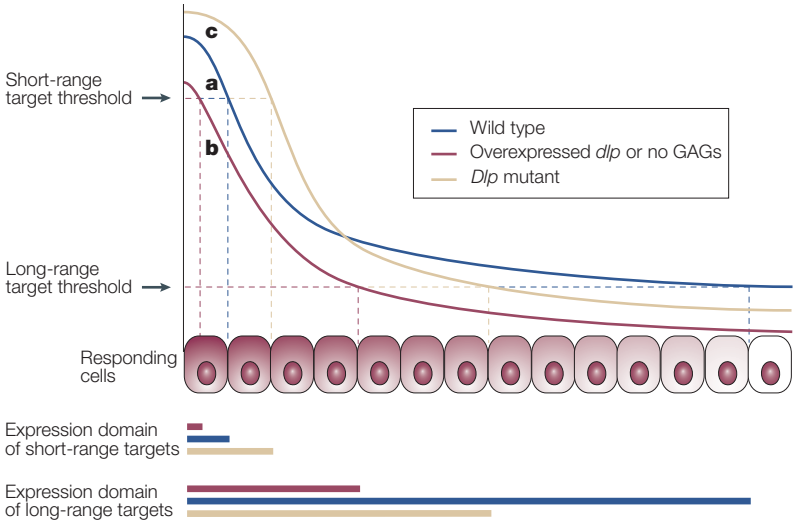


Figure 5 | **Heparan sulphate proteoglycans and Wingless morphogen activity gradients.** **a** | In wild-type *Drosophila melanogaster* wing imaginal discs, glycosaminoglycans (GAGs) regulate morphogen levels at the cell surface. Target genes are activated at Wingless (Wg) activity thresholds that are specific for short-range or long-range targets (blue bars). **b** | In the absence of GAGs, or when *dally-like (dlp)* is overexpressed, levels of active Wg (equal to the area under the curve) are reduced throughout the tissue — owing to lack of binding or sequestration of morphogen, respectively — leading to a downward shift of the activity curve. The expression domains of short-range and long-range targets are reduced (magenta bars). **c** | The Wg activity gradient is differentially affected by *dlp* mutants in different regions of the wing disc. Close to the dorsal–ventral boundary, levels of active Wg are increased owing to reduced morphogen sequestration, which results in increased target-gene activation. At farther distances from the morphogen source, active Wg levels are reduced, leading to decreased long-range target-gene activation (yellow bars). Note that the differential effect of Dlp probably depends on the compensatory function of Dally as it is not seen in *dlp*, *dally* double mutants. Curves represent levels of Wg activity and not levels of extracellular Wg protein, which are increased when glypicans are overexpressed.

D–V boundary the same reduction in Dlp levels leads to the opposite effect — increased activation of short-range Wg target genes. Conversely, expression of *dlp*, which is associated with an increase in morphogen levels at the cell surface, causes loss of target-gene activation[37,41–44] (FIG. 5).

An intriguing explanation for this phenomenon comes from work on the role of the α/β-hydrolase enzyme, which is encoded by *notum*, in shaping the Wg gradient[42,43]. *notum* is expressed at the D–V boundary in a *wg*-dependent manner and has been shown to negatively regulate Wg signalling by modifying Dlp[71]. The similarity between *notum*- and *dlp*-mutant phenotypes prompted Kreuger *et al.*[43] to investigate how Notum affects Dlp and the authors showed that Notum cleaves the GPI anchor that attaches Dlp to the membrane, thereby shedding Dlp from the cell surface. A genetic interaction between *notum* and *dlp* in the modulation of Wg signalling implies that Wg remains bound to Dlp when it is released from the cell surface and that this shedding is a mechanism to locally downregulate the levels of Wg morphogen available for signalling[43]. According to this model, the upregulation of high-level Wg target genes seen in *dlp* mutants close to the D–V boundary could be caused by decreased removal of Wg (which is normally mediated by shedding). Conversely, ectopic expression of Dlp might lead to increased shedding from the cell surface, which would result in lower levels of active Wg and loss of target-gene activation. This situation is phenotypically similar to the effect of mutations that abrogate GAG synthesis (FIG. 5).

It has been suggested that Notum might also shape the Wg gradient by regulating the activity of Dally. The expression of *dally* is positively regulated by Wg signalling and Dally is present at high levels at the D–V boundary, whereas *dlp* is negatively regulated by Wg signalling and Dlp levels are low in a 7–10 cell-wide strip spanning the expression domains of Wg and Notum. The phenotype of *notum*, *dally*-double mutants resembles that of *dally* single mutants, which implies that *dally* might function genetically downstream of *notum*[44]. These data imply a model in which Dally is the main glypican that regulates extracellular Wg levels near the D–V boundary, whereas Dlp functions farther from the morphogen source. Further biochemical characterizations of Dally and Dlp core proteins will be necessary to resolve this issue.

The opposing effects of *dally* and *dlp* mutations on gene activation near the D–V boundary might be the result of sequestration of Wg by Dlp. When *dlp* is expressed near the D–V boundary, the increased amounts of Dlp at the cell surface might lead to more Wg being sequestered, which would make it unavailable for receptor interaction. Conversely, in *dlp*-mutant cell clones, Wg molecules might be retained at the cell surface by binding to Dally and therefore become available for signalling. This model is consistent with the suggestion that Dlp might bind Wg with a higher affinity than Dally does, and that both proteins might have distinct, but overlapping, roles in shaping the Wg gradient, reflected by their expression patterns.

Further evidence for distinct functions of Dlp and Dally comes from a study showing that overexpression of the *dlp* gene in mosaic clones decreases Wg signalling autonomously in the expressing cells while increasing Wg signalling non-autonomously in adjacent tissues. The authors interpret this by suggesting that Dlp might function mainly in the long-range transport of morphogens, whereas Dally might present the signalling molecules to the receptor[41].

The positive effect of *dlp* mutations on Wg target-gene activation near the D–V boundary is in contrast to observations in mutants of GAG biosynthetic enzymes. In mosaic clones that are mutant for members of the *Ext* gene family near the D–V boundary, expression of the high-level *wg* target genes *achaete* (*ac*) or *senseless* (*sens*) is reduced[38,49]. A possible explanation for this is that, in the absence of Dlp, levels of available Wg near the D–V boundary are increased (as outlined above) and GAG chains attached to Dally can maintain Wg at the cell surface at levels that are sufficient to enable ectopic target-gene activation. In the absence of GAGs, Wg levels are reduced throughout the tissue owing to a lack of

binding at the cell surface, and target-gene activation is decreased.

Conclusions and perspectives

Overall, the data indicate that the main role of glypicans in shaping the activity gradients of extracellular signalling molecules is to function as low-affinity binding proteins. They retain morphogens at the cell surface and release them in the presence of high affinity receptors — in so doing, they maintain morphogen levels through the tissue. The data also indicate that, in some tissues, secreted signalling molecules must be bound at the cell surface in order to spread throughout the tissue and that the shedding of these proteins can locally regulate their activity.

Of the remaining questions, the function of the GAG chains that are linked to the core proteins of glypicans and syndecans is yet to be solved. It is not clear whether GAGs provide binding specificity or simply increase the binding affinity of the ligands to the protein core. With the tools now at hand, the answer to this important issue should not be far away. More difficult to answer will be the question of whether signalling molecules interact differentially with specific glycans and whether this interaction is developmentally regulated. Recent advances in the development of oligosaccharide microarrays might provide an entry point to the investigation into the specificity of such glycan–protein interactions in the future[72].

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The authors declare no competing financial interests.

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