Glycoconjugate Journal 19, 295–304, 2003

Unlocking the secrets of syndecans: Transgenic organisms as a potential key

Robert Bellin[1,*,†], Ishan Capila[2,*,†], John Lincecum[3], Pyong Woo Park[4], Ofer Reizes[5], and Merton R. Bernfield

Division of Newborn Medicine, Department of Pediatrics, Children's Hospital, Harvard Medical School, 300 Longwood Ave., Enders-950, Boston, MA 02115, USA

Heparan sulfate proteoglycans are known to modulate the activity of a large number of extracellular ligands thereby having the potential to regulate a great diversity of biological processes. The long-term studies in our laboratory have focused on the syndecans, one of the major cell surface heparan sulfate proteoglycan families. Most early work on syndecans involved biochemical studies that provided initial information on their structure and putative biological roles. In recent years, the development of transgenic organisms has allowed a more complete understanding of syndecan function. Studies with transgenic syndecan-1 and syndecan-3 mice have demonstrated an unforeseen role for syndecans in the regulation of feeding behavior. Syndecan-1 knockout mice display a reduced susceptibility to both Wnt-induced tumorigenesis and microbial pathogenesis. Experiments with *Drosophila* show that syndecan is first expressed upon cellularization in the early embryo, and may play a role in the early developmental stages of the fly. This review focuses on these diverse functions of the syndecans that have been elucidated by the use of transgenic mice and *Drosophila* as model systems. *Published in 2003*.

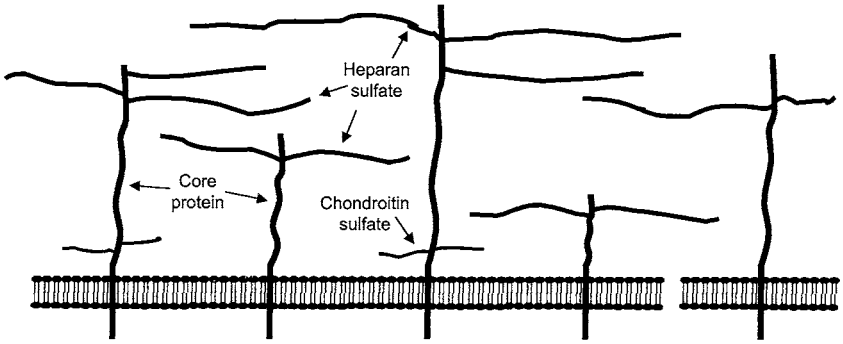
Keywords: **heparan sulfate, syndecan, transgenic, proteoglycan**

Introduction

The communication between cells and the extracellular matrix represents a crucial process in the development and maintenance of multicellular organisms. This process involves elaborate mechanisms of receptor—ligand interaction and signal transduction. Many studies have shown that heparan sulfate (HS), a member of the glycosaminoglycan (GAG) family, plays an essential role in these pathways. HS at the cell surface is involved in diverse biological processes such as cell adhesion, proliferation and differentiation, blood coagulation, lipid metabolism, and microbial pathogenesis [1]. While HS is synthesized on a variety of cell surface core proteins, a major part is found on members of two membrane-bound heparan sulfate proteoglycan (HSPG) families, the syndecans (upon which this review will focus) and the glypicans. These core proteins are fairly conserved in evolution and are expressed at multiple sites, at different times during growth and development, and in pathophysiologic processes. The cell surface HSPGs regulate the actions of a wide variety of HS-binding proteins, which recognize and bind distinct oligosaccharide sequences in the HS chains [2]. The HS chains display an ensemble of binding sites and this structural diversity is by virtue of their biosynthesis [3].

The syndecans are a family of type I transmembrane proteins (four distinct gene products in mammals) that carry HS chains near the tips of their extracellular domains and sometimes also have chondroitin sulfate chains attached to the extracellular domains near the cell surface (Figure 1) [4]. The syndecan core protein can be broadly divided into three domains: an extracellular domain (ectodomain), a transmembrane domain and a cytoplasmic domain. The high proline content of the ectodomain suggests that this portion of the syndecans is likely to have an extended structure. The ectodomain region contains conserved

*These authors have contributed equally to the preparation of this manuscript.
†To whom correspondence should be addressed.
Present addresses:
[1]College of the Holy Cross, Department of Biology, 1 College St., Worcester, MA 01610, USA. E-mail: rbellin@holycross.edu
[2]Massachusetts Institute of Technology, Division of Biological Engineering, 77 Massachusetts Avenue 16-561, Cambridge, MA 02139, USA. E-mail: icapila@mit.edu
[3]Biogen Inc., Cambridge, MA 02142, USA. E-mail: john_lincecum@biogen.com
[4]Baylor College of Medicine, Section of Infectious Diseases, Houston, TX 77030, USA. E-mail: pwpark@bcm.tmc.edu
[5]Procter and Gamble Pharmaceuticals Inc., Division of Muscle/Obesity, Health Care Research Center, 8700 Mason Montgomery Road, Mason, OH 45040, USA. E-mail: reizes.o@pg.com



	Syndecan-1	Syndecan-2	Syndecan-3	Syndecan-4	D-Syndecan
Core protein mol. weight	30.6 (80) kDa	20.2 (48) kDa	44.6 (120) kDa	19.5 (35) kDa	39.0 (100) kDa
Tissue distribution	mammalian epithelia & plasma cells	mammalian endothelia & fibroblasts	mammalian neuronal	most mammalian tissues	most *drosophila* tissues
Reported GAG attachments	heparan sulfate chondroitin sulfate	heparan sulfate	heparan sulfate chondroitin sulfate	heparan sulfate	heparan sulfate

Figure 1. Syndecan family of transmembrane proteoglycans in mammalian and *Drosophila* tissues. Schematically depicted are the four mammalian and the single Drosophila syndecans, and their GAG chain attachments. Listed for each is their reported tissue distribution, the predicted molecular mass of the core protein from the cDNA sequence, and (in parenthesis) the molecular weight based upon SDS-PAGE migration of the core protein without GAG chains.

sites for GAG attachment, cell interaction, and oligomerization. Additionally, all syndecans can be shed from the cell surface by proteolytic cleavage at a conserved site in the ectodomain near the cell membrane [5]. This shedding is highly regulated and can be accelerated by various biological effectors, thereby making it an extremely important process in the regulation of syndecan function [6].

The syndecan transmembrane domain is highly evolutionarily conserved and is believed to be important for localizing the syndecans to distinct membrane compartments [7]. The cytoplasmic domain has two invariant regions separated by a region of variable length and composition. Although quite short, the cytoplasmic domain is postulated to play a role in binding cytoskeletal elements and possibly the formation of cytoplasmic signaling complexes [7].

Virtually all adhesive cells express at least one syndecan, and most express multiple syndecans [5]. In general, all syndecans are also believed to play an important role in regulating growth factor pathways [8] and lipid metabolism [9], by virtue of their interaction with fibroblast growth factors (FGFs) and lipoproteins via HS chains. Additionally, some specific syndecan family members have been shown to play special roles in specific tissue types. For example, syndecan-1 is important in maintaining the normal epithelial phenotype by affecting the organization of the actin cytoskeleton and the expression of E-cadherin [10].

While there is a lot of experimental evidence indicating that multiple ligands are capable of interacting with heparan sulfate, it is only in the last few years that these interactions have been studied in the context of intact organisms. Transgenic and gene deletion approaches (involving HSPGs and HS biosynthetic enzymes) in mice, and genetic screens in *Drosophila* have provided surprising phenotypes that have advanced our understanding of cell surface HS functions *in vivo* and how they relate to normal physiology and disease [11,12]. This review highlights the important physiological insights we have gained into obesity, tumorigenesis and microbial pathogenesis from studying genetic manipulations of syndecan-1 and syndecan-3 in mice, and from the study of D-syndecan expression in *Drosophila* embryos during development. While similar approaches have been used by other labs (for example, see articles by Goetinck; by Ishiguro et al.; and by Lin and Perrimon in this issue), we do not discuss them in this article. Therefore, we would like to emphasize that this is not a review of the entire field, but a summary of the findings that have emerged from the late Merton Bernfield's laboratory in recent years as a tribute to him, and to the numerous contributions he has made to the field of proteoglycan biology.

Syndecan shedding and obesity

The syndecan ectodomain is released from the cell surface of cultured cells and tissues, a process that can be accelerated by a number of physiological stimuli, including growth factor receptor activation, stress, and wounding [2]. The process of ectodomain shedding reduces the syndecan at the cell surface, a rapid mechanism to inactivate receptor-ligand interaction and signaling [1,6]. Shedding also converts the cell surface HSPG into soluble effectors that may compete for the same ligand or may function in a remote location [1]. Proteolytic release or shedding of the extracellular domain (ectodomain) of a diverse set of cell surface proteins (ca. 1%) occurs under a variety of physiological and pathophysiological conditions. Ectodomain shedding is regulated by multiple

signaling pathways converging on a diverse group of extracellular proteases also referred to as secretases or sheddases. The A Disintegrin and Metalloproteinase (ADAM) family of extracellular proteases comprises the largest group of these sheddases. Shedding is fast, resulting in rapid reduction of membrane receptors/proteins. The released ectodomain may also act as an effector, either as competitive inhibitor of its cell surface counterpart or as positive factor at another site. While the mechanism of ectodomain shedding appears to be regulated similarly, the cleavage sites of different shed membrane proteins share no sequence or structural similarity.

The precise mechanism of syndecan-1 ectodomain shedding has not been elucidated, though diverse signal transduction pathways, involving protein kinase C, protein tyrosine kinases, and mitogen activated protein (MAP) kinases stimulate this process [6]. Both the peptide hydroxamate inhibitors and tissue inhibitor of metalloproteinase-3 (TIMP-3) specifically inhibit this process suggesting that shedding of syndecan-1 ectodomain involves a yet to be identified metalloproteinase(s). This enzyme is cell surface associated, inhibitable by peptide hydroxamates and TIMP-3 but not sensitive to TIMP-1, TIMP-2, or inhibitors of aspartic, cysteine, and serine proteinases [6]. The enzyme is likely a member of the TIMP-3 sensitive ADAM family of cell surface proteinases. Mammalian syndecans contain a conserved dibasic sequence within two amino acids of the putative transmembrane domain, which was proposed to be the cleavage site. However, *Drosophila* syndecan does not contain this dibasic sequence yet it is shed. Furthermore, we have shown in preliminary studies with mammalian syndecan that the cleavage site is nine amino acids from the membrane (Reizes, Wang, and Bernfield, unpublished observation).

Shedding of syndecans occurs in a tissue specific manner. Syndecan-1, in epithelial tissues like the skin, is both at the cell surface, extractable with detergent, and shed as the soluble ectodomain. However, transgenic expression of syndecan-1 in the central nervous system shows that it is solely extracted by detergent and is not soluble. In contrast, the endogenous neural syndecan, syndecan-3, is found both soluble and at the cell surface. Furthermore, when syndecans-2 and -4 are overexpressed in the brain they are found both at the cell surface and shed (Reizes and Bernfield, unpublished observation). This suggests that either the shedding enzymes responsible for proteolytic cleavage of syndecan-1 are not present in the hypothalamus or the syndecan-1 is not recognized by neural sheddases.

Transgenic expression of syndecan-1 leads to hyperphagia and maturity onset obesity. Using the cytomegalovirus promoter/enhancer syndecan-1 was expressed at high levels in multiple somatic tissues and in specific regions of the brain where it is not normally expressed. Specifically, transgenically expressed syndecan-1 was found at the cell surface of the hypothalamic nuclei that control energy balance.

Obesity in these mice is similar to obesity in mice with α-melanocyte stimulating hormone deficiency [13–15]. The obesity syndrome is characterized by hyperphagia, maturity onset obesity, increased linear growth, hyperleptinemia, hyperinsulinemia, and hyperglycemia in males. This observation led to the discovery that syndecan-1 via its HS chains binds to and potentiates the inhibitory action of agouti-related protein (Agrp), a competitive antagonist of the melanocortin receptor [16]. Importantly, we demonstrated that syndecan-3 also potentiates the inhibitory activity of this protein. Since misexpression of syndecan-1 in the hypothalamus led to obesity, additional transgenic mice were generated to evaluate the role of the cell surface and soluble syndecan. A constitutively shed syndecan-1 was made by inserting a stop codon in the juxtamembrane domain at amino acid Q245 and an uncleavable syndecan-1 was generated by swapping the juxtamembrane 15 amino acids (^{238}EGATGASQSLLDTKE^{252}) of syndecan-1 with human CD4, which prevents shedding *in vitro* (Reizes, Wang and Bernfield, unpublished observation). Mice containing the uncleavable construct developed maturity onset obesity, while mice containing the shed construct failed to become obese. Thus, hypothalamic syndecan-1 generates obesity solely when at the cell surface (Figure 2A).

The endogenous syndecan in the hypothalamus is syndecan-3 and is expressed in the paraventricular, dorsomedial, and lateral hypothalamic area nuclei of the brain. In contrast to hypothalamic transgenic syndecan-1, hypothalamic syndecan-3 was partially soluble, presumably the shed ectodomain, and the remainder was cell surface or membrane-bound. Additionally, the size of the syndecan-3 HS chains is indistinguishable from those on hypothalamic syndecan-1 in transgenic syndecan-1 over-expressing mice. Importantly, hypothalamic cell surface syndecan-3 levels change in response to nutritional status similar to various hypothalamic peptides that modify feeding behavior [17]. Thus, hypothalamic cell surface syndecan-3 levels change with food deprivation and refeeding in a manner similar to Agrp and other hypothalamic modifiers of feeding behavior. Finally, syndecan-3 null mice do not increase their food intake following an overnight fast. Thus, feeding is enhanced by increased levels of cell surface syndecan-3, and reduced when syndecan-3 is shed following food intake (Figure 2B). The mechanisms regulating increase and decrease of syndecan-3 remain to be elucidated. It is likely that feeding initiation factors increase syndecan-3 levels, while satiety factors decrease syndecan-3 levels by inducing a sheddase.

Syndecans and microbial pathogenesis

Microbial pathogenesis involves various interactions between microbial and host components. Pathogens incur damage to the host not only through their virulence factors but also by taking advantage of host molecules and their activities. The host components targeted by microbes are ubiquitous and they can be on the cell surface, in extracellular compartments and also circulating in the bloodstream. Among the cell surface host molecules, heparan sulfate proteoglycans have been shown to be exploited by a wide spectrum of microbial pathogens, including viruses,

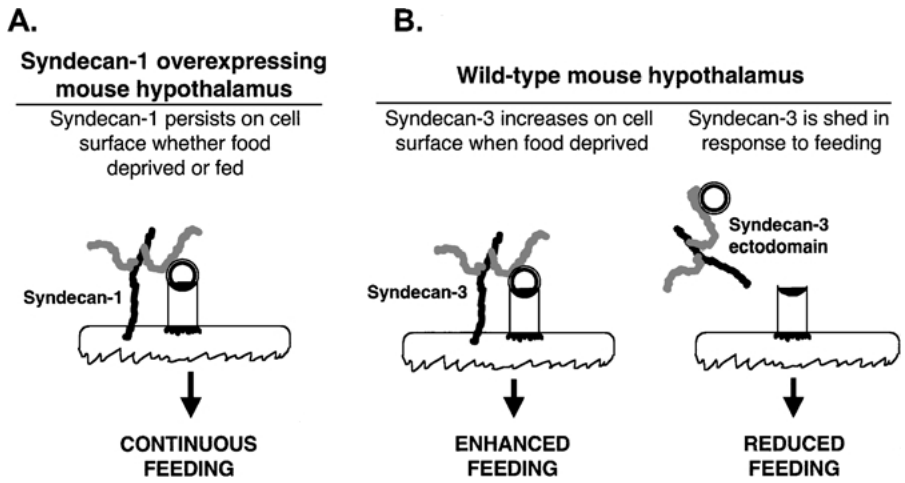


Figure 2. Regulation of feeding behavior based upon syndecan ectodomain shedding. (A) In transgenic syndecan-1 overexpressing mice, the syndecan-1 protein exists on the surface of hypothalamic cells regardless of the fed state of the animal, resulting in continuous feeding by the animal and abnormally increased weight gain. (B) In wild-type mice, syndecan-3 is shed from the surface of hypothalamic cells in response to feeding, thus resulting in the reduced feeding, and a normal rate of weight gain.

bacteria and parasites, for their colonization [2,18]. The list of microbes binding to cell surface HSPGs includes both extracellular and intracellular pathogens, such as herpes simplex virus, *Neisseria gonorrhoeae*, *Bordetella pertussis* and *Plasmodium falciparum*, among others. Cell surface HSPGs not only promote the initial attachment of microbial pathogens, but can also facilitate the invasion of host cells. For many intracellular pathogens, cell surface HSPGs can function as co-receptors by serving as a scaffold for the initial attachment, such that pathogens can interact more efficiently with their second internalization receptors. In some cases, cell surface HSPGs can serve as direct entry receptors. Evidence that these HSPG interactions may be biologically significant is provided by the finding that pathogens that bind cell surface HSPGs can no longer colonize the host cells *in vitro* when the expression of HS has been abrogated by chemical or mutagenic methods. Exogenous addition of HS or its pharmaceutical functional mimic, heparin, also inhibits colonization of cultured host cells by HSPG-binding microbial pathogens. Interestingly, our recent results indicate that cell surface HSPGs can also promote pathogenesis when shed into the extracellular environment as soluble, intact HSPG ectodomains. This section will review the mechanisms of how cell surface HSPGs promote microbial pathogenesis, with a particular emphasis on the syndecans.

Among the HSPGs, syndecan-1 is thought to be the major microbial target because most pathogens target the host epithelia early in their pathogenesis and because syndecan-1 is the predominant HSPG of epithelia. However, specific HSPG receptors have not been experimentally identified for the majority of microbial pathogens that bind HS. One exception is *N. gonorrhoeae*. Binding of *N. gonorrhoeae* to cell surface HSPG triggers a signaling cascade in the host cell involving generation of phosphatidylcholine-specific phospholipase C, diacylglycerol, acidic sphingomyelinase and membrane sphingomyelin-derived ceramide [19]. It has been shown that over-expression of syndecan-1 or -4 in HeLa cells increases the ability of *N. gonorrhoeae* to invade these cells [20], suggesting that syndecan-1 and -4 may signal to mediate entry. Support for this hypothesis is provided by the finding that HeLa cells expressing mutant syndecan-1 or -4 constructs lacking the cytoplasmic domain cannot support invasion. The role of the conserved Tyr and Ser residues in the syndecan cytoplasmic domain has not been studied, but deletion of the C-terminal EFYA motif has been shown to result in loss of *N. gonorrhoeae* invasion. Deletion of the cytoplasmic domain in syndecan-4 that interacts with protein kinase C and phosphatidylinositol 4,5-bisphosphate also results in ablation of invasion. Thus, syndecan-1 or -4 binding by *N. gonorrhoeae* may induce various interactions between the syndecan cytoplasmic domain and intracellular molecules that can activate the signaling cascade essential for *N. gonorrhoeae* invasion.

Despite the plethora of available *in vitro* data showing that various microbial pathogens bind specifically to cell surface HSPGs, it is still not clear whether this activity is important *in vivo*. However, in cases studied, available data indicate that microbial binding to cell surface HSPGs is an important step in pathogenesis. For example, insertional mutagenesis of the mycobacterial heparin binding hemagglutinin adhesin (HBHA) gene has been shown to reduce extrapulmonary dissemination of both *Mycobacterium tuberculosis* and *M. bovis* Bacillus Calmette-Guerin (BCG) in a murine lung infection model [21]. The ability to colonize mouse lungs is not affected, but the ability to disseminate into the bloodstream is inhibited by null mutation of this adhesin gene. Function-perturbing antibodies to HBHA also inhibit extrapulmonary dissemination, but not lung colonization, of wild type mycobacterial species, indicating that HBHA function is specifically related to the ability to disseminate. Interestingly, the adhesin is required to bind and

invade epithelial cells, but not phagocytic cells. These results suggest that syndecan-1, the major HSPG on epithelial cells, may be a potential HBHA receptor that promotes transepithelial dissemination of mycobacterial species.

We sought to determine directly the role of syndecan-1 in microbial pathogenesis by comparing the virulence of bacterial pathogens in *syndecan-1* null (*synd-1*$^{-/-}$) and wild type (*synd-1*$^{+/+}$) mice [22]. The *synd-1*$^{-/-}$ mice are phenotypically similar to wild type *synd-1*$^{+/+}$ mice under normal laboratory housing conditions. Thus, because of the lack of background pathology, we were able to assess directly the role of syndecan-1 using these mice. Our results demonstrated that *synd-1*$^{-/-}$ mice significantly resist lung infection and subsequent bacteremia relative to wild type *synd-1*$^{+/+}$ mice when inoculated intranasally with the Gram negative bacterial pathogen, *Pseudomonas aeruginosa*. The virulence of *P. aeruginosa* was also reduced in the corneas of *synd-1*$^{-/-}$ mice, but it was similar in the *synd-1*$^{-/-}$ and *synd-1*$^{+/+}$ backgrounds when the animals were inoculated intraperitoneally. These results indicate that the synd-1$^{-/-}$ mice resist *P. aeruginosa* infection only where the bacteria first encounter the host's epithelia barrier.

Surprisingly, subsequent experiments revealed that the *synd-1*$^{-/-}$ mice resist *P. aeruginosa* infection because they lack the shed syndecan-1 ectodomains and not cell surface syndecan-1. *P. aeruginosa* does not bind to syndecan-1 and *P. aeruginosa* adhesion onto cultured lung epithelia is not syndecan-1 dependent. Importantly, the resistant *synd-1*$^{-/-}$ mice become susceptible to *P. aeruginosa* infection when intranasally given purified syndecan-1 ectodomains or heparin, but not when given other GAGs or HS-free syndecan-1 ectodomain. The virulence-enhancing activity of purified ectodomain and heparin can be abrogated by protamine, a pharmaceutical agent used to neutralize the anticoagulant effects of heparin and HS, and also by the HS-digesting enzyme heparinase II. The specificity of *P. aeruginosa*'s pathogenic mechanism that exploits syndecan-1 shedding to enhance virulence appears to be controlled by the bacterium's ability to specifically activate syndecan-1 shedding by host cells via LasA [23], a secreted virulence factor of *P. aeruginosa* for its lung infection. Because Las A activates syndecan-1 shedding by stimulating the host's mechanism, this shedding can be inhibited by inhibitors of the host cell's shedding mechanism, such as hydroxamate metalloproteinase inhibitors (e.g., BB1101) and protein tyrosine kinase inhibitors (e.g., genistein) [23]. The shedding inhibitor BB1101 also reduces virulence in the murine lung infection model [22]. These results indicate that in contrast to pathogens that bind HSPGs, *P. aeruginosa* exploits syndecan-1 as a shed ectodomain and not as a cell surface attachment site.

Exactly how syndecan-1 ectodomains enhance *P. aeruginosa* virulence remains to be delineated. However, available data indicate that the effect of syndecan-1 ectodomains is on the host and not on the bacteria and that syndecan-1 ectodomains can inhibit several distinct host defense mechanisms [22]. Purified syndecan-1 ectodomains bind and inhibit cationic antimicrobial peptides (e.g., cathelicidins). Syndecan-1 ectodomains also bind to other host defense factors, such as collectins and neutrophil elastase. Furthermore, because soluble HS has been shown to inhibit the activity of various cytokines (e.g., MCP, IL-8) [24], HS chains of syndecan-1 may also interfere with cytokines that bind HS, thereby promoting infection. Taken together, these findings suggest that once shed and solubilized, cell surface HSPGs can inhibit host defenses to promote microbial pathogenesis.

There is now compelling evidence that cell surface HSPGs affect many processes central to microbial pathogenesis. At the cell surface, these complex glycoconjugates can promote attachment and subsequent invasion of a wide variety of intracellular pathogens. As shed, soluble HSPG ectodomains, they can inhibit host defense factors to generate a host environment that favors pathogenesis over eradication of microbes. Because inherent properties of HS dictate these pro-pathogenic activities of HSPGs, strucure:function analyses of HS chains should provide molecular details as to how HSPGs regulate microbial pathogenesis. With recent technological advances in sequencing HS [25,26] and in synthesizing specific HS sequences [27], it should be possible to address these issues directly in the near future.

Syndecans and Wnt-1 tumorigenesis

Heparan sulfate proteoglycans play critical roles in major signaling pathways including the fibroblast growth factor (FGF) and transforming growth factor-β (TGF-β) pathways. The HSPGs act as co-receptors and facilitate the interaction between specific signaling molecules and their signal-transducing receptors. Interaction of HSPGs with these extracellular ligands thus represents an important step in the initiation and regulation of growth and development [2,12].

The Wnt pathway is involved in cell proliferation and differentiation during development and disease [28]. Wnt proteins comprise a highly conserved family of secreted glycoproteins, and they activate a signaling pathway that includes the transducer molecule β-catenin that is crucial to the development of all multicellular organisms [29]. Accumulating evidence implicates a key role for the Wnt pathway in the development of many types of human tumors [30,31]. There is also biochemical evidence suggesting a role for HSPGs in Wnt signaling and the activity of several Wnt proteins is shown to be modulated by the pharmaceutical product heparin [32]. Our studies suggest that syndecan-1 can modulate Wnt signaling and is required for Wnt-1 induced tumorigenesis of the mouse mammary gland [33].

In mice, ectopic expression of *Wnt-1* in the mammary gland induces generalized mammary hyperplasia followed by solid tumor formation [34]. The mice expressing *Wnt-1* were crossed with syndecan-1 knockout mice (*synd-1*$^{-/-}$) giving rise to females that were hemizygous for the *Wnt-1* transgene and either

synd-1$^{+/+}$, *synd-1*$^{+/-}$, or *synd-1*$^{-/-}$. While the presence or absence of syndecan-1 did not affect the expression of the *Wnt-1* transgene, Wnt-1 induced hyperplasia was reduced by 70% in the *synd-1*$^{-/-}$ mice compared to the *synd-1*$^{+/+}$ mice [33]. The results indicate that the absence of syndecan-1 suppresses tumor formation induced by Wnt-1, and implicates syndecan-1 in the response of mammary gland to Wnt-1.

Biochemical evidence to support this observation was obtained using a cell culture assay to measure activity of a highly related *Drosophila* Wnt protein, Wingless (Wg), in the presence and absence of syndecan-1 ectodomain. The cellular accumulation of Armadillo (β-catenin) was monitored as a functional readout of Wg signaling activity. Addition of syndecan-1 ectodomain stimulated Wg activity in a concentration-dependent manner. However, removal of the HS chains from the ectodomain by nitrous acid treatment, abolished the signaling activity. Our results provide both genetic and biochemical evidence that syndecan-1 can stimulate a Wnt signaling pathway and that these interactions are dependent upon the HS chains [33].

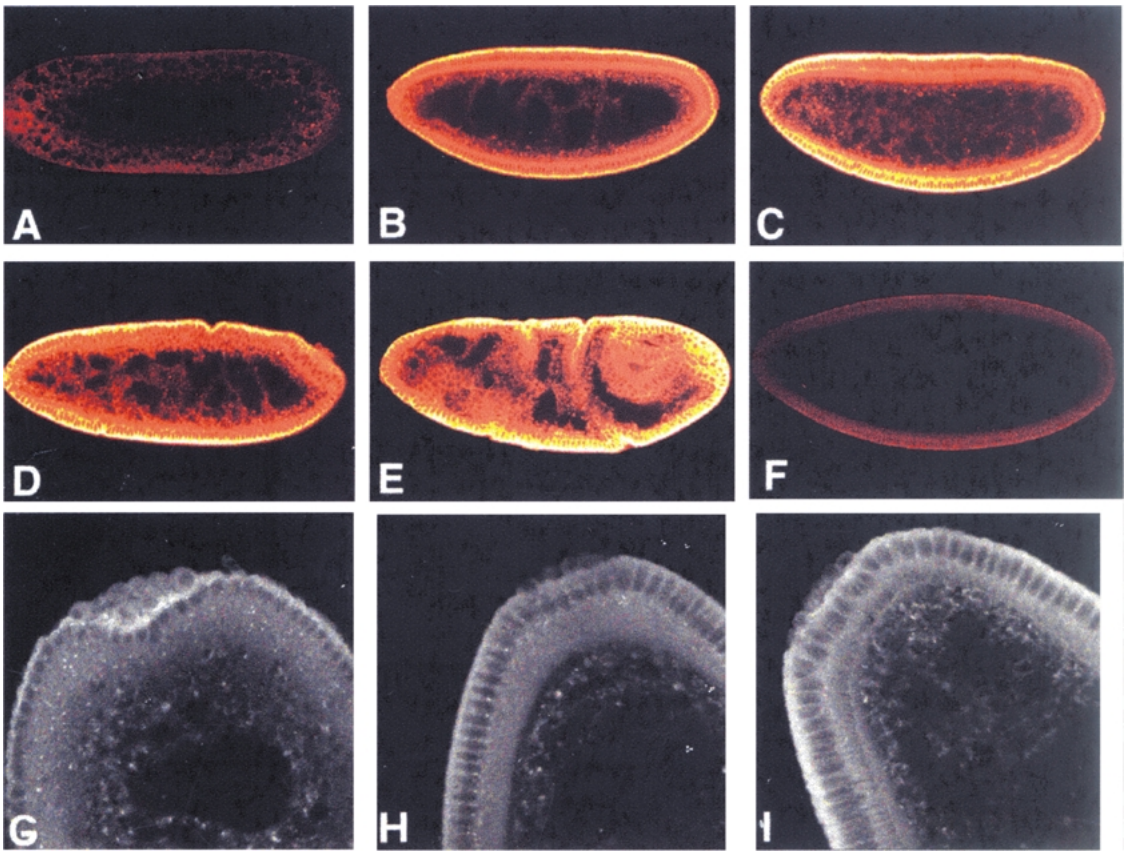


Figure 3. Expression pattern of syndecan protein in the *Drosophila* embryo. (A) Stage 2 precellular blastoderm stained with anti-syndecan ectodomain antibody. No syndecan staining is observed at this stage. (B) Stage 5 embryo stained for syndecan expression shows extensive staining on the apical-lateral faces of the forming epithelium. (C) Late Stage 5 embryo has similar syndecan expression pattern with more pronounced basolateral staining. Also note the cell surface expression on the migrating pole cells. (D) A Stage 6 embryo with syndecan protein expressed at the surface of all cells at the initiation of gastrulation. Expression is strongly apical on the outermost cells with a more uniform expression pattern observed for cells in the interior of the embryo. (E) Stage 8 embryo with syndecan staining on the surface of all cells. A reduction in staining is also observed on the cells actively undergoing gastrulation movements such as those in the cephalic furrow. (F) Stage 5 embryo stained with anti-syndecan ectodomain antibody in the presence of 10 μg/ml GST-syndecan ectodomain fusion protein. The soluble competitor reduces the signal to background (compare panel F and panel B) establishing the specificity of the syndecan antibody. (G) Syndecan protein is localized to the apical plasma membrane of the invaginating furrows of the epithelium. Expression is strongly observed in the forming cell layer under the pole cells and on the surface of the pole cells. (H) Syndecan expression on rapidly invaginating plasma membrane. Strong apical staining is observed as the basolateral sides of the cells are formed by the inward movement of membrane. Additionally punctate staining is seen on the lateral edges of the cells. In contrast to panel A the expression observed beneath and on the surface of the pole cells is reduced. (I) Stage 5 cellularized blastoderm has syndecan staining on the newly "pinched-off" basal face of the epithelial layer. Syndecan staining remains intense on the apical face of the cells but now uniform, non-punctate expression is seen on both the lateral and basal faces of the mature epithelial cells.

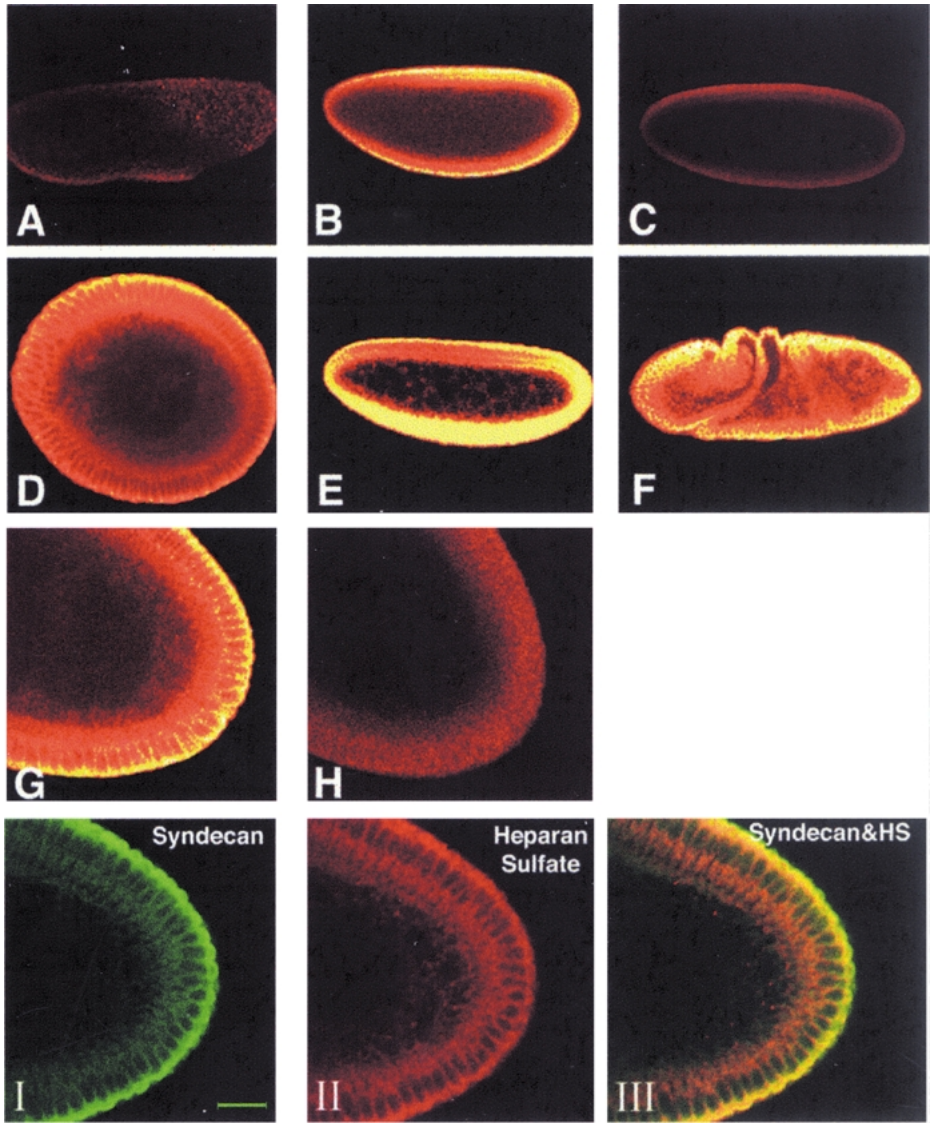


Figure 4. Expression pattern of heparan sulfate in the *Drosophila* embryo. (A) Stage 2 preblastoderm stained with mAb 3G10 following heparinase II digestion. No staining is observed at this stage precluding the presence of a maternal HS contribution. (B) Stage 5 fully cellularized blastoderm, following heparinase II digestion. The cell membranes have reached full depth and extensive staining is observed on the apical-lateral face of the cells. (C) Stage 5 embryo that was not exposed to heparinase II shows no staining and establishes the specificity of the HS staining. (D) Cross section of Stage 5 embryo demonstrating strong apical expression of HS on fully cellularized epithelium. (E) Sagittal section of late Stage 5 embryo. Intense HS staining is observed in the ventral furrow relative to the dorsal side of the embryo. (F) Stage 7 embryo stains strongly for HS with high levels of expression observed in the cephalic furrow and the anterior midgut invagination. (G) High magnification view of early Stage 5 embryo posterior. This optical section was taken above the pole cells and shows strong, uniform apical staining with punctate staining at the basal lateral sides of the inwardly growing plasma membranes. (H) Control section early Stage 5 embryo not digested with heparinase II demonstrating specificity of punctate staining.

Double labeled syndecan/HS stained stage 5 embryo, posterior end. (I) Syndecan staining demonstrating strong apical expression in green. (II) HS staining detected with mAb 3G10 has similar pattern of expression with a more punctate pattern observed on the basal face of the cells. (III) Overlay of panels A and B demonstrates a strong colocalization of syndecan and HS staining as seen by the yellow at the apical face of the cells.

Drosophila syndecan

Recently, large-scale genetic screens in *Drosophila melanogaster* have demonstrated that cell surface HSPGs are components of several morphogen-signaling pathways [35]. These studies have identified *Drosophila* HS biosynthetic enzymes responsible for the post-translational synthesis and sulfation of GAG chains. Each of the secreted morphogens,

Spatzle (Spz), Wg, Decapentaplegic (Dpp), Hedgehog (Hh), and FGF, share the common property of requiring cell surface heparan sulfate for activity [36–40].

Two classes of cell surface HSPGs, the glypican homologues, Division abnormally delayed (Dally), and Dally-like protein (Dlp), as well as syndecan, are present in the fruit fly [41]. Dally is required for eye, antenna, wing and genitalia patterning. It is structurally similar to its vertebrate homologues having a glycosylphosphatidylinositol (GPI) linkage to membrane lipid and covalently attached HS chains [42]. The second glypican gene in the fly, *dlp* acts to modulate *wg* signaling [41,43]. Dally core protein expression in the early embryo has not been extensively characterized, however by segmentation (stage 9) *dally* transcripts are found in segmentally repeating stripes of overlapping cells expressing the *engrailed* (*en*) gene. This pattern of expression is consistent with a role for Dally in the formation of the parasegments of the embryo. *Dlp* has a similar transcript expression pattern as *dally* [43].

Drosophila syndecan has a structure analogous to its vertebrate homologues, in that it is a type I transmembrane core protein with covalently attached HS chains (Figure 1) [2]. Unlike vertebrates, which have 4 syndecan genes, there is one fly syndecan gene (Figure 1) (Lincecum, unpublished observation). Prior studies have focused on the expression of *Drosophila* syndecan core protein in the late stage embryo [44]. Syndecan core protein in the stage 16 embryo is expressed in the lymph glands, the peripheral and central nervous system, and the basal surfaces of the gut epithelium. This late protein expression pattern corresponds to the *in situ* hybridization patterns [45] which localize syndecan transcripts to the ventral furrow, the ventral nerve cord, the abdominal and thoracic segments, the epidermis, and the differentiating central nervous system. Expression of HS in the early embryo has been briefly described in *tout velu* (*ttv*) mutants lacking the HS polymerase [39].

In support of the recent work describing HS expression in mutant flies, we have conducted a careful examination of wild type flies to establish the normal expression of HS and of the cell surface HSPG syndecan during cellularization and gastrulation in *Drosophila*. Using antibodies directed against the extracellular domain of the syndecan core protein we demonstrate that this protein is first expressed at cellularization (Figure 3). This expression pattern is remarkably similar to the induction of syndecan-1 in mouse blastocysts [46]. Cellularization results from the invagination of the cell membranes as mechanical force is applied by the underlying actin cytoskeleton [47]. As the membrane is drawn inward and sealed off basally a simple epithelial sheet is formed [47]. At this stage (Stage 4) the core protein distributes predominantly apically (Figure 3) but shifts to a more lateral pattern as the plasma membrane invaginates. By late Stage 5, expression has returned to a more apical distribution. This shift from an apical to a lateral distribution within the cellularization furrows mirrors temporally the switch from maternal to zygotic control of the mitotic cycles of nuclear division [48].

Toyoda and Selleck have performed extensive biochemical characterization of HS in broad developmental stages and in the larvae of HS biosynthetic mutants [49]. Our current studies demonstrate that HS is first synthesized upon cellularization (Figure 4) and is not contributed maternally as has been suggested previously [50]. Based on the expression pattern of HS seen at Stages 5–7 (Figure 4) it appears that HS synthesis is developmentally regulated. Moreover, syndecan core protein colocalizes with HS in the cellular blastoderm (Figure 4). Therefore, HS and syndecan core protein are linked as proteoglycan both spatially and temporally during early *Drosophila* embryogenesis.

Conclusion

Ever since the cloning of the first syndecan gene in 1989 in the Bernfield lab [51], studies have been conducted to catalog the abundance of physiological processes that involve these transmembrane proteoglycans. The recent addition of transgenic organism-based approaches to the study of proteoglycans has shown the great power of these methods to reveal previously unknown physiological functions of complex biomolecules. Additional studies of this type hold great promise towards developing an overall understanding of the immense diversity of functions held by the syndecans and by proteoglycans in general.

Acknowledgment

We thank Dr. Caroline Alexander, University of Wisconsin, for critical reading of the manuscript.

References

1 Park PW, Reizes O, Bernfield M, Cell surface heparan sulfate proteoglycans: Selective regulators of ligand-receptor encounters, *J Biol Chem* **275**, 29923–6 (2000).
2 Bernfield M, Gotte M, Park PW, Reizes O, Fitzgerald ML, Lincecum J, Zako M, Functions of cell surface heparan sulfate proteoglycans, *Annu Rev Biochem* **68**, 729–77 (1999).
3 Esko JD, Selleck SB, Order out of chaos: Assembly of ligand binding sites in heparan sulfate, *Annu Rev Biochem* **71**, 435–71 (2002).
4 Bernfield M, Kokenyesi R, Kato M, Hinkes MT, Spring J, Gallo RL, Lose EJ, Biology of the syndecans: A family of transmembrane heparan sulfate proteoglycans, *Annu Rev Cell Biol* **8**, 365–93 (1992).
5 Kim CW, Goldberger OA, Gallo RL, Bernfield M, Members of the syndecan family of heparan sulfate proteoglycans are expressed in distinct cell-, tissue-, and development-specific patterns, *Mol Biol Cell* **5**, 797–805 (1994).
6 Fitzgerald ML, Wang Z, Park PW, Murphy G, Bernfield M, Shedding of syndecan-1 and -4 ectodomains is regulated by multiple signaling pathways and mediated by a TIMP-3-sensitive metalloproteinase, *J Cell Biol* **148**, 811–24 (2000).
7 Rapraeger AC, Molecular interactions of syndecans during development, *Semin Cell Dev Biol* **12**, 107–16 (2001).

8 Rapraeger AC, Heparan sulfate-growth factor interactions, *Methods Cell Biol* **69**, 83–109 (2002).
9 Williams KJ, Interactions of lipoproteins with proteoglycans, *Methods Mol Biol* **171**, 457–77 (2001).
10 Kato M, Saunders S, Nguyen H, Bernfield M, Loss of cell surface syndecan-1 causes epithelia to transform into anchorage-independent mesenchyme-like cells, *Mol Biol Cell* **6**, 559–76 (1995).
11 Principalle M, de Agostini A, Developmental roles of heparan sulfate proteoglycans: A comparative review in Drosophila, mouse and human, *Int J Dev Biol* **46**, 267–78 (2002).
12 Perrimon N, Bernfield M, Specificities of heparan sulphate proteoglycans in developmental processes, *Nature* **404**, 725–28 (2000).
13 Cone RD, The central melanocortin system and its role in energy homeostasis, *Ann Endocrinol (Paris)* **60**, 3–9 (1999).
14 Cone RD, Cowley MA, Butler AA, Fan W, Marks DL, Low MJ, The arcuate nucleus as a conduit for diverse signals relevant to energy homeostasis, *Int J Obes Relat Metab Disord* **25** (Suppl 5), S63–7 (2001).
15 Cone RD, Lu D, Koppula S, Vage DI, Klungland H, Boston B, Chen W, Orth DN, Pouton C, Kesterson RA, The melanocortin receptors: Agonists, antagonists, and the hormonal control of pigmentation, *Recent Prog Horm Res* **51**, 287–317; discussion 318 (1996).
16 Reizes O, Lincecum J, Wang Z, Goldberger O, Huang L, Kaksonen M, Ahima R, Hinkes MT, Barsh GS, Rauvala H, Bernfield M, Transgenic expression of syndecan-1 uncovers a physiological control of feeding behavior by syndecan-3, *Cell* **106**, 105–16 (2001).
17 Schwartz GJ, The role of gastrointestinal vagal afferents in the control of food intake: Current prospects, *Nutrition* **16**, 866–73 (2000).
18 Rostand KS, Esko JD, Microbial adherence to and invasion through proteoglycans, *Infect Immun* **65**, 1–8 (1997).
19 Grassme H, Gulbins E, Brenner B, Ferlinz K, Sandhoff K, Harzer K, Lang F, Meyer TF, Acidic sphingomyelinase mediates entry of N. Gonorrhoeae into nonphagocytic cells, *Cell* **91**, 605–15 (1997).
20 Freissler E, Meyer auf der Heyde A, David G, Meyer TF, Dehio C, Syndecan-1 and syndecan-4 can mediate the invasion of OpaHSPG-expressing Neisseria gonorrhoeae into epithelial cells, *Cell Microbiol* **2**, 69–82 (2000).
21 Pethe K, Alonso S, Biet F, Delogu G, Brennan MJ, Locht C, Menozzi FD, The heparin-binding haemagglutinin of M. tuberculosis is required for extrapulmonary dissemination, *Nature* **412**, 190–4 (2001).
22 Park PW, Pier GB, Hinkes MT, Bernfield M, Exploitation of syndecan-1 shedding by Pseudomonas aeruginosa enhances virulence, *Nature* **411**, 98–102 (2001).
23 Park PW, Pier GB, Preston MJ, Goldberger O, Fitzgerald ML, Bernfield M, Syndecan-1 shedding is enhanced by LasA, a secreted virulence factor of Pseudomonas aeruginosa, *J Biol Chem* **275**, 3057–64 (2000).
24 Kuschert GS, Coulin F, Power CA, Proudfoot AE, Hubbard RE, Hoogewerf AJ, Wells TN, Glycosaminoglycans interact selectively with chemokines and modulate receptor binding and cellular responses, *Biochemistry* **38**, 12959–68 (1999).
25 Turnbull JE, Hopwood JJ, Gallagher JT, A strategy for rapid sequencing of heparan sulfate and heparin saccharides, *Proc Natl Acad Sci USA* **96**, 2698–703 (1999).
26 Venkataraman G, Shriver Z, Raman R, Sasisekharan R, Sequencing complex polysaccharides, *Science* **286**, 537–42 (1999).
27 Plante OJ, Palmacci ER, Seeberger PH, Automated solid-phase synthesis of oligosaccharides, *Science* **291**, 1523–7 (2001).
28 Huelsken J, Birchmeier W, New aspects of wnt signaling pathways in higher vertebrates, *Curr Opin Genet Dev* **11**, 547–53 (2001).
29 Sharpe C, Lawrence N, Martinez Arias A, Wnt signalling: A theme with nuclear variations, *Bioessays* **23**, 311–8 (2001).
30 Taipale J, Beachy PA, The hedgehog and wnt signalling pathways in cancer, *Nature* **411**, 349–54 (2001).
31 Polakis P, Wnt signaling and cancer, *Genes Dev* **14**, 1837–51 (2000).
32 Reichsman F, Smith L, Cumberledge S, Glycosaminoglycans can modulate extracellular localization of the wingless protein and promote signal transduction, *J Cell Biol* **135**, 819–27 (1996).
33 Alexander CM, Reichsman F, Hinkes MT, Lincecum J, Becker KA, Cumberledge S, Bernfield M, Syndecan-1 is required for wnt-1-induced mammary tumorigenesis in mice, *Nat Genet* **25**, 329–32 (2000).
34 Shackleford GM, MacArthur CA, Kwan HC, Varmus HE, Mouse mammary tumor virus infection accelerates mammary carcinogenesis in wnt-1 transgenic mice by insertional activation of int-2/fgf-3 and hst/fgf-4, *Proc Natl Acad Sci USA* **90**, 740–4 (1993).
35 Baeg GH, Perrimon N, Functional binding of secreted molecules to heparan sulfate proteoglycans in Drosophila, *Curr Opin Cell Biol* **12**, 575–80 (2000).
36 Lin X, Buff EM, Perrimon N, Michelson AM, Heparan sulfate proteoglycans are essential for fgf receptor signaling during Drosophila embryonic development, *Development* **126**, 3715–23 (1999).
37 Lin X, Perrimon N, Dally cooperates with Drosophila frizzled 2 to transduce wingless signalling, *Nature* **400**, 281–4 (1999).
38 Sen J, Goltz JS, Stevens L, Stein D, Spatially restricted expression of pipe in the Drosophila egg chamber defines embryonic dorsal-ventral polarity, *Cell* **95**, 471–81 (1998).
39 The I, Bellaiche Y, Perrimon N, Hedgehog movement is regulated through tout velu-dependent synthesis of a heparan sulfate proteoglycan, *Mol Cell* **4**, 633–9 (1999).
40 Tsuda M, Kamimura K, Nakato H, Archer M, Staatz W, Fox B, Humphrey M, Olson S, Futch T, Kaluza V, Siegfried E, Stam L, Selleck SB, The cell-surface proteoglycan dally regulates wingless signalling in Drosophila, *Nature* **400**, 276–80 (1999).
41 Baeg GH, Lin X, Khare N, Baumgartner S, Perrimon N, Heparan sulfate proteoglycans are critical for the organization of the extracellular distribution of wingless, *Development* **128**, 87–94 (2001).
42 Selleck S, Genetic analysis of functions for cell surface proteoglycans, *Matrix Biol* **17**, 473–6 (1998).
43 Khare N, Baumgartner S, Dally-like protein, a new Drosophila glypican with expression overlapping with wingless, *Mech Dev* **99**, 199–202 (2000).
44 Spring J, Paine-Saunders SE, Hynes RO, Bernfield M, Drosophila syndecan: Conservation of a cell-surface heparan sulfate proteoglycan, *Proc Natl Acad Sci USA* **91**, 3334–8 (1994).
45 Kopczynski CC, Noordermeer JN, Serano TL, Chen WY, Pendleton JD, Lewis S, Goodman CS, Rubin GM, A high throughput screen to identify secreted and transmembrane proteins involved in Drosophila embryogenesis, *Proc Natl Acad Sci USA* **95**, 9973–8 (1998).

46 Sutherland AE, Sanderson RD, Mayes M, Seibert M, Calarco PG, Bernfield M, Damsky CH, Expression of syndecan, a putative low affinity fibroblast growth factor receptor, in the early mouse embryo, *Development* **113**, 339–51 (1991).
47 Miller KG, Role of the actin cytoskeleton in early Drosophila development, *Curr Top Dev Biol* **31**, 167–96 (1995).
48 Foe VE, Odell GM, Edgar BA, Mitosis and morphogenesis in the Drosophilia embryo: Point and counterpoint. In *The Development of Drosophila melanogaster*, edited by Bate M, Arias AM (Cold Spring Harbor Press, Plainview, NY, 1993) pp. 149–300.
49 Toyoda H, Kinoshita-Toyoda A, Selleck SB, Structural analysis of glycosaminoglycans in Drosophila and Caenorhabditis elegans and demonstration that tout-velu, a Drosophila gene related to ext tumor suppressors, affects heparan sulfate *in vivo*, *J Biol Chem* **275**, 2269–75 (2000).
50 Binari RC, Staveley BE, Johnson WA, Godavarti R, Sasisekharan R, Manoukian AS, Genetic evidence that heparin-like glycosaminoglycans are involved in wingless signaling, *Development* **124**, 2623–32 (1997).
51 Saunders S, Jalkanen M, O'Farrell S, Bernfield M, Molecular cloning of syndecan, an integral membrane proteoglycan, *J Cell Biol* **108**, 1547–56 (1989).