Available online at www.sciencedirect.com
ScienceDirect
Developmental Biology 300 (2006) 570–582
DEVELOPMENTAL BIOLOGY
www.elsevier.com/locate/ydbio

The function of a *Drosophila* glypican does not depend entirely on heparan sulfate modification

Catherine A. Kirkpatrick [a,1], Sarah M. Knox [a,1], William D. Staatz [b], Bethany Fox [b], Daniel M. Lercher [a], Scott B. Selleck [a,*]

[a] *The Developmental Biology Center, Departments of Pediatrics and Genetics, Cell Biology and Development, 6-160 Jackson Hall, 321 Church Street SE, University of Minnesota, Minneapolis, MN 55455, USA*
[b] *Arizona Cancer Center, 1515 N. Campbell Avenue, Tucson, AZ 85724, USA*

Received for publication 26 January 2006; revised 6 September 2006; accepted 7 September 2006
Available online 15 September 2006

Abstract

Division abnormally delayed (Dally) is one of two glycosylphosphatidylinositol (GPI)-linked heparan sulfate proteoglycans in *Drosophila*. Numerous studies have shown that it influences Decapentaplegic (Dpp) and Wingless signaling. It has been generally assumed that Dally affects signaling by directly interacting with these growth factors, primarily through its heparan sulfate (HS) chains. To understand the functional contributions of HS chains and protein core we have (1) assessed the growth factor binding properties of purified Dally using surface plasmon resonance, (2) generated a form of Dally that is not HS modified and evaluated its signaling capacity in vivo. Purified Dally binds directly to FGF2, FGF10, and the functional Dpp homolog BMP4. FGF binding is abolished by preincubation with HS, but BMP4 association is partially HS-resistant, suggesting the Dally protein core contributes to binding. Cell binding and co-immunoprecipitation studies suggest that non-HS-modified Dally retains some ability to bind Dpp or BMP4. Expression of HS-deficient Dally in vivo showed it does not promote signaling as well as wild-type Dally, yet it can rescue several *dally* mutant phenotypes. These data reveal that heparan sulfate modification of Dally is not required for all in vivo activities and that significant functional capacity resides in the protein core.

Keywords: Heparan sulfate; Proteoglycan; Glypican; Dally; BMP; Decapentaplegic; Wingless; FGF; *Drosophila*

Introduction

A fundamental question in developmental biology is how organisms establish cell identities in order to generate specific tissues. Cell–cell signaling and graded distributions of signaling molecules are required for such developmental patterning. Heparan sulfate proteoglycans (HSPGs) play an essential role in these processes through their ability to regulate the distribution and signaling of many secreted growth factors including members of the fibroblast growth factor (FGF), Hedgehog (Hh), Wnt, and transforming growth factor (TGF-β)/BMP families (De Cat and David, 2001; Esko and Selleck, 2002; Lin, 2004). HSPGs are cell surface and extracellular matrix macromolecules consisting of heparan sulfate (HS) chains – long unbranched polymers of repeating disaccharide units – attached to serine residues of the core protein. These sugar chains, which are differentially modified by a number of enzymes, including sulfotransferases, serve as binding sites for a diverse range of extracellular molecules from growth factors to collagens. The essential contribution of HS for normal development has been demonstrated in genetic studies where defects in HS biosynthetic enzymes abrogated growth factor signaling. For example, mutations in the *Drosophila* genes *brother of tout-velu*, an *N*-acetylglucosamine transferase, and *sulfateless*, the heparan sulfate *N*-deacetylase/*N*-sulfotransferase, compromise the signaling of three major tissue patterning morphogens, Wingless (Wg), Hedgehog (Hh) and Decapentaplegic (Dpp) (Bornemann et al., 2004; Han et al., 2004a; Takei et al., 2004).

It is clear from both genetic and in vitro analyses that HS chains are critical for many signaling processes, but other data suggest that the identity of the core protein also influences the

* Corresponding author.
E-mail address: selle011@umn.edu (S.B. Selleck).
[1] Contributed equally to this work.

0012-1606/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.ydbio.2006.09.011

roles of HSPGs in signaling (Kramer and Yost, 2003). Functional differences have been demonstrated both between members of different proteoglycan families (such as syndecans and glypicans) and within the same family. For example, vertebrate glypican-1 and syndecan-1 play different roles in the response of pancreatic tumor cells to FGF2 (Ding et al., 2005), and *Drosophila* syndecan has distinct activities compared to Dlp both in axon guidance and NMJ development (Rawson et al., 2005; Johnson et al., 2006). Genetic analysis of the two *Drosophila* glypicans, Division abnormally delayed (Dally) and Dally-like protein (Dlp), has shown that they have distinct effects on various signaling pathways. Dally and Dlp have opposing activities in Wg-directed patterning of the wing margin (Baeg et al., 2001; Fujise et al., 2001; Kirkpatrick et al., 2004). Dlp is critical for Hh signaling during anterior–posterior segmental patterning in the embryo (Desbordes and Sanson, 2003; Kirkpatrick et al., 2004) whereas Dally has only a modest effect on embryonic Hh and Wg signaling (Lin and Perrimon, 1999; Tsuda et al., 1999; Desbordes et al., 2005; Franch-Marro et al., 2005). Dally also plays an important role in Dpp signaling (Jackson et al., 1997; Fujise et al., 2003).

The genetic evidence demonstrating the importance of HS for developmental patterning relies largely on the analysis of mutants where HS synthesis is globally disrupted. However, these studies do not address the relative contributions of the HS chains and the core protein to the function of a particular proteoglycan. To evaluate the importance of HS modification for interactions with growth factors, we purified Dally and assessed its direct binding with a number of growth factors and their receptors, as well as the sensitivity of that binding to exogenous HS, using surface plasmon resonance. In order to assess the functional contribution of HS modification in vivo, we mutated the HS attachment sites of Dally and then compared the signaling capacity of the wild-type and mutant proteins in vivo. In vitro binding experiments and in vivo functional assessments indicate that Dally's protein core contributes to growth factor binding and signaling activity.

Materials and methods

Site-directed mutagenesis

The *hs HA dally* plasmid has been described previously (Tsuda et al., 1999). The glycosaminoglycan-deficient *HA dally*$^{\Delta GAG}$ cDNA construct was created in two steps from *hs HA dally* using the QuikChange Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA). The primers 5′-G TAT GGA GGC GCC CAT GGT GCC GGA GAT GGT GCC GGC GAT C-3′ and its complement were used to change Ser569 and Ser573 to Ala and to create a new NcoI restriction site. The primers, 5′-G GGT GAG GGC GCC GGA GGA GGA GAG GGT C-3′ and its complement were used to change Ser549 to Ala and to remove a BanII site. All changes were confirmed by sequencing. *HA dally*$^{\Delta GAG}$ cDNA was then cloned into pF449 under the control of the hsp70 promoter (Tsuda et al., 1999).

DEAE chromatography of dally and Dally$^{\Delta GAG}$

hs HA dally or *hs HA dally*$^{\Delta GAG}$ cDNA constructs were transfected into *Drosophila* S2 cells using Cellfectin (Invitrogen). To induce Dally synthesis, cells were incubated at 37°C for 30 min, then returned to 23°C for 2–4 h before harvesting. Ion exchange chromatography was carried out on DEAE Sepharose (Amersham Pharmacia) according to the method of Herndon and Lander (1990). Column fractions were dialyzed extensively against 40 mM NH_4HCO_3 and then lyophilized. DEAE-purified material was dissolved in 100 μL 0.1 M Tris (pH 7.5) and 5 μL aliquots (approximately 10 μg protein per lane) were subjected to PAGE and Western blot analysis. Immobilon P membranes (Millipore) were probed with anti-HA antibody (HA.11; 1:5000). Secondary detection was with alkaline phosphatase-conjugated goat anti-mouse IgG (BioRad; 1:5000) followed by Immun-Star substrate (BioRad) or peroxidase-conjugated goat anti-mouse IgG followed by ECL (Amersham-Pharmacia) and exposure to BioMax Lite film (Kodak).

Immunohistochemistry

Transformed and control S2 cells were grown for 2 days in Chamber Slides (Nalge Nunc International). HA-Dally synthesis was induced by incubating the slides at 37°C for 30 min then returning them to 23°C for 2 h. The cells were fixed in 3.7% formaldehyde and immunostained using anti-HA antibody (HA.11; 1:1000; Babco), followed by Cy-3 conjugated goat anti-mouse IgG (1:1000; Jackson ImmunoResearch). Cells were photographed with a BioRad MRC 600 laser scanning confocal microscope. For HS detection, wings discs were dissected from third instar larvae and fixed in 3.7% formaldehyde in PBS for 20 min. The discs were washed and blocked as for immunostaining, then treated with 1 mU Heparitinase I (Seikagaku) in 100 mM NaOAc (pH 7.0), 3.3 mM $CaCl_2$ for 1.5 h at 37°C and washed and blocked again. Discs were then stained with 3G10 antibody (1:100; Seikagaku) following standard protocols (Blair, 2000; Kirkpatrick et al., 2004) and imaged on a Nikon C1 confocal microscope. Staining with anti-pMad (1:1000) and anti-Spalt (1:100) was also performed according to standard procedures (Blair, 2000; Fujise et al., 2001). Secondary antibodies were from the AlexaFluor series (1:500; Molecular Probes). All wing discs are shown with the anterior to the left and the ventral compartment down.

SPR analysis

Myc-tagged Dally or Dally$^{\Delta GAG}$ was purified from S2 cells transfected with a pUAST expression plasmid and p-*actin-Gal4*. Cells were lysed in 150 mM NaCl, 10 mM Tris (pH 7.5), 1 mM EDTA, 2% Triton-X114 on ice for 1 h, followed by shaking at 37°C for 10 min. After centrifugation, the detergent phase was washed 3 times with 150 mM NaCl, 10 mM Tris (pH 7.5), 1 mM EDTA and incubated in the same buffer containing 0.05U PI-PLC at 37°C for 1 h, then 4°C overnight. GPI-linked proteins were recovered in the aqueous layer following centrifugation. Myc-tagged Dally or Dally$^{\Delta GAG}$ was isolated by affinity chromatography using anti-Myc agarose beads, eluted with 6 M urea in PBS, concentrated using BIOMAX spin columns and dialyzed against PBS for 24 h. Dally was further purified using a DEAE spin column (Vivascience USA) and dialyzed again against PBS. Dally or Dally$^{\Delta GAG}$ was biotinylated essentially as described by Cole et al. (1987). A ~10 μg/ml solution of Dally in 0.1 M $NaHCO_3$ was incubated with 50× molar excess of LC-NHS–biotin (Pierce) for 3 h at room temperature. Excess biotin was removed by dialysis against PBS for 48 h at 4°C. Ligand binding experiments were performed using a BIAcore 2000 instrument. Biotinylated Dally (10 μg/mL in PBS) was coupled to flow cells of a streptavidin-derivatized sensor chip at a flow rate of 5 μL/min. A change in response of 1000–1800 RU (resonance units) was observed, indicating a change in mass at the surface. Growth factor binding experiments were performed at a flow rate of 20 μL/min in HBS-EP running buffer at 25°C. Mass transport effects were minimal as determined by increasing the flow rate to 60 μL/min. Injected volume for each experiment was 55 μL with a total dissociation time of 150 s. In competition experiments, growth factors were preincubated with their corresponding receptor or 0.5 mg/ml HS for 2 min prior to injection. The Dally surface was regenerated with 2×30-s pulses of 1 M NaCl. Data were analyzed with BIAevaluation 3.0 software.

Cell binding assays and co-immunoprecipitation

S2 cells were transfected with a plasmid encoding *actin-Gal4*, alone or together with *pUAST* plasmids to express Myc-Dally, Myc-Dally$^{\Delta GAG}$ and/or HA-Dpp. For cell binding assays, cells were harvested after 4–5 days, resuspended in growth medium containing 100 nM BMP4 and incubated at 4°C

for 3 h. Cells were washed 3 times for 10 min each with cold PBS, then resuspended in PBS and lysed by boiling in SDS sample buffer. For co-immunoprecipitation, 10× PBS and 10% BSA were added to the cultures before harvesting to give final concentrations of 1× PBS and 1% BSA. Cells and supernatants were separated, and cells were lysed by incubation in PBS containing 2% TritonX-100 at 4°C for 1 h. After centrifugation, protein G–sepharose beads (Zymed) bearing 3F10 rat anti-HA antibody (Roche) were incubated in the resulting supernatants, or in the earlier culture supernatants, overnight at 4°C. The beads were washed 6 times with Tris-buffered saline, then boiled in SDS sample buffer. Western blots were probed with 9E10 mouse anti-myc (Roche), A14 rabbit anti-myc (Santa Cruz), 12CA5 mouse anti-HA (Roche) and/or mouse anti-BMP4 (R and D Systems). When necessary, band intensities were quantified by using IR-700 and IR-800 fluorescent secondary antibodies (Rockland Immunochemicals) and scanning on an Odyssey infrared imaging system.

Drosophila strains

All flies were maintained at 25°C. The wild-type strain used was Oregon R. *dally* alleles included $dally^{gem}$ and $dally^{\Delta P527}$ (Fujise et al., 2001) and the reported null allele $dally^{80}$ (Han et al., 2004b). *Df(3L)scf-R6* (Bloomington stock center) bears a deletion that removes the *dally* locus. Other fly strains are described in Flybase. Clones of cells overexpressing Dally or $Dally^{\Delta GAG}$ were created using the *FLP-FRT* system as previously described (Struhl and Basler, 1993), where expression of FLP recombinase from a heatshock promoter induces recombination between *FRT* sites flanking a transcription termination signal, allowing expression of an *actin-Gal4* transgene. Cells expressing *actin-Gal4*, and hence *UAS-dally*$^{+}$ or *UAS-dally*$^{\Delta GAG}$, were detected by GFP expression from a *UAS-GFP* transgene. Ectopic expression of *UAS-dally* or *UAS-dally*$^{\Delta GAG}$ was driven by *hh-Gal4, en-Gal4, ey-Gal4* and *ap-Gal4* (Brand and Perrimon, 1993). For rescue experiments, *dally* mutant animals expressing *UAS-myc-dally*, *UAS-dally*$^{\Delta GAG}$ or *UAS-myc-dally*$^{\Delta GAG}$ under *A9-Gal4* control were generated from crosses such as *w UAS-dally*$^{\Delta GAG}$; *dally*$^{\Delta P527}$/*TM6B Tb*×*A9-Gal4; dally*^{gem}/*TM6B Tb*; wing and notum phenotypes were scored in female progeny of the relevant genotype.

Scanning electron microscopy and eye sections

Scanning electron microscopy, thin sectioning of the adult eye and toluidine blue staining were performed according to standard protocols (Wolf, 2000).

Wing preparations

Adult flies were immersed in 75% ethanol overnight, then equilibrated in 50% ethanol for 1 h, 25% ethanol for 1 h and twice in water for 30 min. The wings were dissected from the body, mounted on slides with AquaMount and imaged using a Nikon Eclipse 800 microscope with a DXM 1200 digital camera at 4× or 20×.

Adult cuticle preparations

Dissected adult thoraces were boiled in 2.5N NaOH for 10 min, then washed three times in distilled water and mounted in AquaMount. Preparations were imaged as above at 10 × or 20 ×.

Results

Dally$^{\Delta GAG}$ *is a form of dally without HS modification*

Dally, as a member of the glypican family of HSPGs, shares several features with other glypicans: an N-terminal signal sequence, a set of 14 conserved cysteine residues that are thought to form a disulfide-bonded globular domain, and a C-terminal hydrophobic sequence that is removed upon addition of the glycosylphosphatidylinositol (GPI) anchor that links the protein to the extracellular plasma membrane (De Cat and David, 2001; Kramer and Yost, 2003). Dally also has three predicted HS attachment sites, serine residues that are part of a SGXG sequence with an adjacent group of acidic residues (Fig. 1A), between the globular domain and the GPI anchor. These serine residues correspond closely to two serine residues in human glypican-3 (GPC3) known to serve as HS attachment sites (Gonzalez et al., 1998). To generate a form of Dally without HS modification ($Dally^{\Delta GAG}$, lacking glycosaminoglycan chains), we mutated all three of these serine residues to alanines.

To confirm that $Dally^{\Delta GAG}$ does not carry HS chains, we expressed an epitope-tagged form of the protein in *Drosophila* S2 cells. Wild-type Dally expressed in this manner can be partially purified by ion-exchange chromatography on DEAE-Sepharose, where it elutes in 1 M NaCl (Fig. 1B). Wild-type Dally runs as a smear between 85 and 120 kDa because of its extensive heparan sulfate modification. In contrast, $Dally^{\Delta GAG}$ elutes from DEAE-Sepharose at much lower salt concentrations (150–250 mM NaCl) and runs as a discrete band at approximately 85 kDa. By immunostaining we see no evidence for higher molecular weight forms of $Dally^{\Delta GAG}$, suggesting that the protein is devoid of HS modification. Our analysis of $Dally^{\Delta GAG}$ parallels that of $GPC3^{\Delta GAG}$ (Gonzalez et al., 1998) and another mutant form of Dally (Kreuger et al., 2004), where point mutations altering the normal HS attachment sites do not result in HS addition at alternative cryptic sites in the protein.

To verify that HA-$Dally^{\Delta GAG}$ is appropriately expressed at the cell surface, we stained S2 cells expressing either HA-Dally or HA-$Dally^{\Delta GAG}$ with anti-HA antibody. We observed similar staining patterns in both cases: the proteins appeared to localize in a punctate pattern on the surface of cells (Fig. 1C). No such pattern was seen in cells that did not express either protein. These results suggest that $Dally^{\Delta GAG}$ is delivered to the cell surface comparably to wild-type Dally, and that its lack of HS modification does not result in misrouting during passage through the secretory pathway.

To evaluate potential HS modification of $Dally^{\Delta GAG}$ in a tissue in vivo, we expressed the protein in wing discs and probed with 3G10, a monoclonal antibody that recognizes an HS-epitope (desaturated uronic acid) produced by enzymatic digestion with heparitinase (David et al., 1992). Overexpression of wild-type Dally in the posterior portion of the disc led to a marked increase in 3G10 immunoreactivity (Fig. 1D). In contrast, overexpression of $Dally^{\Delta GAG}$ did not increase 3G10 staining, but rather reduced it below the level in the anterior domain where ectopic $Dally^{\Delta GAG}$ was not expressed. This result suggests that $Dally^{\Delta GAG}$ not only lacks HS modification itself, but that its overexpression interferes in some way with the HS modification of other proteins in vivo.

Binding properties of Dally

HSPGs play a critical role in FGF signaling by promoting the interaction of FGFs with their receptors. HS chains have been shown to bind FGF and are absolutely required for FGF signaling (Rapraeger et al., 1991; Yayon et al., 1991). The

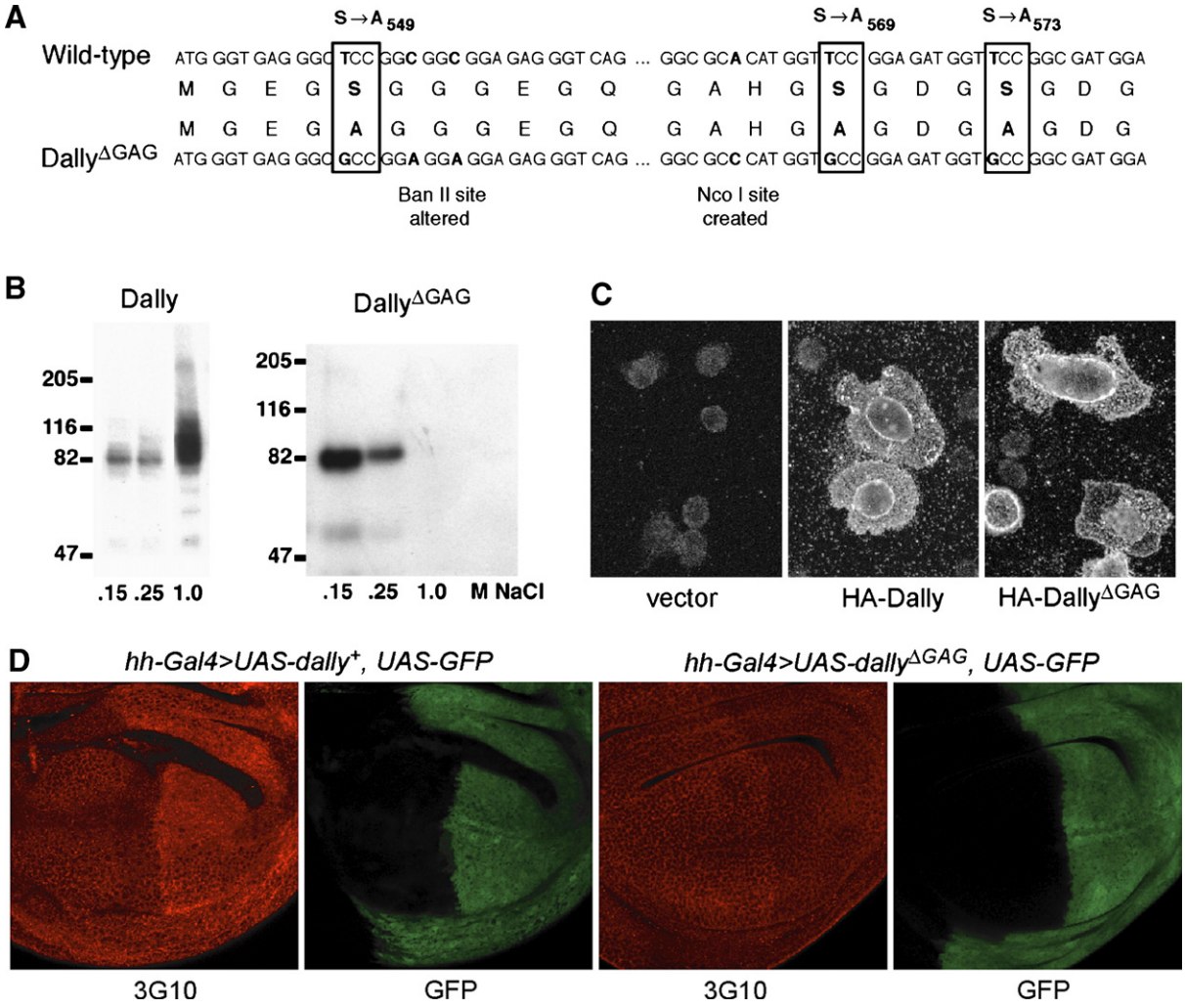


Fig. 1. Dally$^{\Delta GAG}$ is a non-glycanated form of Dally. (A) Partial sequence of *dally* including the putative HS attachment sites: Ser_{549}, Ser_{569} and Ser_{573}. To create Dally$^{\Delta GAG}$, all three Ser residues were changed to Ala by site-directed mutagenesis. Other silent mutations were made to remove or introduce adjacent restriction sites as indicated. (B) HA-tagged forms of Dally and Dally$^{\Delta GAG}$ expressed in S2 cells were isolated from DEAE Sepharose by stepwise elution with increasing concentrations of NaCl and analyzed by Western blotting using HA.11 antibody. Wild-type Dally bound more strongly to DEAE Sepharose, eluting in 1 M NaCl, compared to 0.25 M NaCl for Dally$^{\Delta GAG}$. Also, Dally is heterogeneous in size, as expected for HS-modified proteins, but Dally$^{\Delta GAG}$ is of uniform molecular weight. (C) S2 cells expressing HA-Dally or HA-Dally$^{\Delta GAG}$ were immunostained with HA.11 antibody. Both proteins are distributed in a punctate pattern at the cell surface; this staining is absent from control cells transfected with the empty vector. Both HA-Dally- and HA-Dally$^{\Delta GAG}$-expressing cells spread extensively on culture dishes, whereas control S2 cells are only poorly attached, suggesting that Dally expression influences cytoskeletal architecture. (D) *UAS-dally*$^{+}$ and *UAS*-Dally$^{\Delta GAG}$ were expressed in the posterior wing disc using *hh-Gal4*. HS distribution (red) was visualized by treating the discs with heparitinase, then staining with 3G10 antibody. GFP (green) from *UAS-GFP* marks the posterior compartment of each disc. 3G10 staining is uniform in wild-type discs (data not shown), but is increased or decreased by overexpression of Dally or Dally$^{\Delta GAG}$ respectively.

mechanism by which *Drosophila* Dally enhances BMP and Wg signaling has not been defined as clearly. Dally has not been demonstrated to bind directly to growth factors, and it is not known whether Dally's HS chains are required for it to interact with these proteins or to enhance their signaling. We therefore used Surface Plasmon Resonance (SPR) to investigate the binding capabilities of Dally with potential signaling partners.

For SPR analysis, purified Myc-Dally was immobilized to a biosensor chip; immobilization was confirmed by a change in resonance of approximately 1000 RU, and subsequently by binding of 9E10 anti-Myc antibody. Growth factor association and dissociation were monitored in real time as growth factor solutions were injected across the chip and then washed off by buffer. As expected, vertebrate FGF2 bound directly to Dally (Fig. 2A), as did FGF10, the vertebrate FGF most similar to *Drosophila* Branchless (Fig. 2B). To assess BMP binding, we used vertebrate BMP4, which can functionally substitute for Dpp in *Drosophila* (Padgett et al., 1993). BMP4 also bound directly to immobilized Dally (Fig. 2C). Epidermal growth factor (EGF), which does not bind HSPGs, served as a negative control and showed no interaction with Dally (data not shown).

Although members of both the FGF and BMP families bound directly to Dally, we found substantial differences in the nature of their interactions. Binding of FGF2 was completely abolished by preincubation with excess exogenous heparan sulfate prior to injection, as expected for binding mediated by

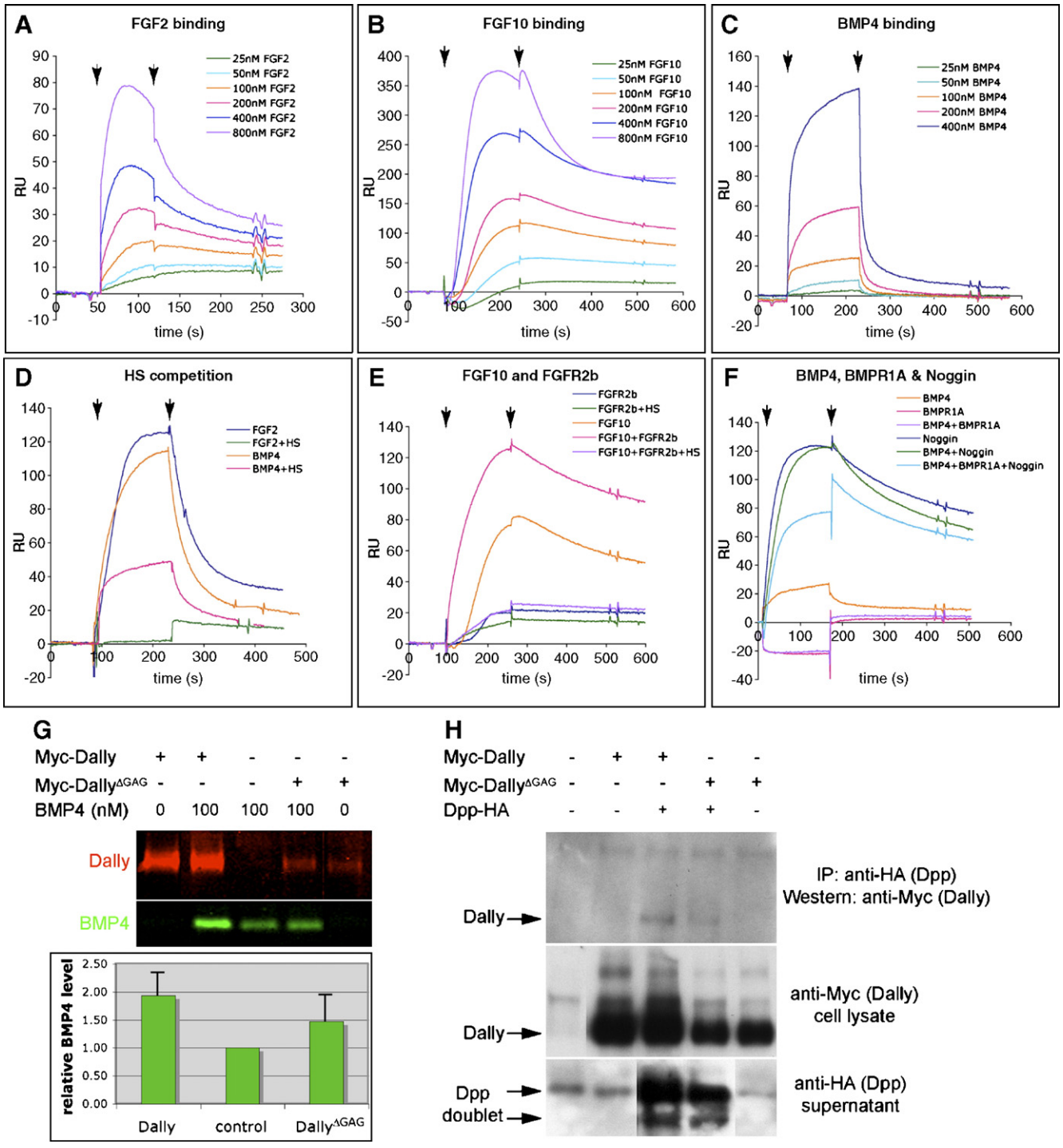


Fig. 2. Binding properties of Dally. (A–F) Surface plasmon resonance. Purified biotinylated Myc-tagged Dally was immobilized on an avidin-coated biosensor chip. Solutions of growth factors (with or without additional binding partners or competitors) were passed over the chip; arrowheads mark the initiation and termination of injections. (A–C) Increasing concentrations of FGF2, FGF10 and BMP4 all bind to immobilized Dally. (D) 0.5 mg/ml Heparan sulfate was preincubated with 400 nM FGF2 or 100 nM BMP4 before injection to determine whether exogenous HS could compete with Dally for growth factor binding. Binding of both proteins to Dally was reduced by addition of HS, but BMP4 retained some ability to bind even in the presence of excess HS. (E) 100 nM FGF10 and 100 nM FGFR2b, alone or preincubated together, were tested for binding to Dally and for sensitivity to exogenous HS. (F) 133 nM BMP4, 133 nM BMPR1A and 133 nM Noggin were injected alone or after preincubation together as indicated in the legend. (G) Binding of BMP4 to cells. S2 cells were incubated in growth medium containing 100 nM BMP4 for 3 h. After washing, cells overexpressing Myc-Dally retained more BMP4 than control cells that did not overexpress Dally. Expression of Myc-Dally$^{\Delta GAG}$ was always considerably lower than Myc-Dally, but caused an average 50% increase in BMP4 binding above the level on control cells. The graph shows average BMP4 binding relative to control cells (arbitrarily set at 1) from 5 different experiments, with 95% confidence intervals. (H) Co-immunoprecipitation of Dpp and Dally. HA-tagged Dpp was isolated from lysates of S2 cells expressing the indicated proteins using anti-HA antibody. Immunoprecipitates and cell lysates were analyzed by Western blotting with anti-Myc antibody. Myc-Dally and Myc-Dally$^{\Delta GAG}$ are present in the immunoprecipitates when co-expressed with Dpp-HA. Western blotting of cell culture supernatants with anti-HA antibody shows Dpp-HA expression levels for these cells. Anti-HA antibody cross-reacts with a protein in Dpp-HA non-transfected cells to produce a faint band at approximately 25 kDa; Dpp-HA appears as a doublet of strong 25 kDa and 23 kDa bands.

HS chains (Fig. 2D). Similarly, FGF10 binding to Dally was completely abolished in the presence of HS (data not shown). FGF receptor 2b did not interact substantially with Dally in our assay (perhaps because FGFRs typically do not associate with HS as strongly as do the FGFs (Harmer, 2006)), but preincubation of FGF10 with FGFR2b led to the formation of ternary FGF10/FGFR2b/Dally complexes (Fig. 2E); like FGF/Dally complexes, these were completely inhibited by addition of HS. In contrast, BMP4 binding to Dally was only partially inhibited by molar excess of exogenous HS (Fig. 2D), suggesting that BMP4 can interact with Dally's protein core as well as its HS chains. Also, preincubation of BMP receptor 1A with BMP4 did not have the same effect as addition of FGFR2b to FGF10: instead of forming a ternary complex, BMPR1A inhibited binding of BMP4 to Dally (Fig. 2F). The BMP antagonist Noggin, which has been previously shown to bind heparin and cell surface heparan sulfate (Paine-Saunders et al., 2002), bound directly to immobilized Dally; addition of BMP4 did not give an additive increase in resonance, but addition of BMP4 and BMPR1A reduced binding of Noggin and/or BMP4 to Dally (Fig. 2F). The SPR analysis indicates that Dally can interact directly with FGFs and BMP4, but that it interacts somewhat differently with these two classes of growth factors. In particular, BMP4 binding to Dally showed a component that could not be competed with excess HS. Also, our results suggest that the nature of the interactions with their receptors and HSPGs are distinct for these different growth factors: BMP4 may not form the same kind of ternary complex with BMPR1A and Dally that has been proposed for FGFs with FGF receptors and HS chains.

To further evaluate the role of HS modification for Dally's interaction with BMP4, we examined binding of BMP4 to S2 cells overexpressing either Dally or Dally$^{\Delta GAG}$. After incubation at 4°C in growth medium containing BMP4, cells were washed to remove unbound growth factor, lysed and analyzed by Western blotting (Fig. 2G). Overexpression of Myc-Dally leads to an approximate 2-fold increase in BMP4 binding to S2 cells (average from 5 independent experiments). Overexpression of a Myc-tagged form of Dally$^{\Delta GAG}$ (with an additional Ser to Ala mutation at residue 597) also increased BMP4 binding, but to a more modest degree. Myc-Dally$^{\Delta GAG}$ was consistently expressed at a lower level than Myc-Dally (approximately 4-fold lower), but expression of Myc-Dally-$^{\Delta GAG}$ increased BMP4 binding by an average of 50% above the level on control cells. These data suggest that Dally$^{\Delta GAG}$ retains some capacity to bind BMP4 on cell surfaces.

To determine if Dally and Dpp form molecular complexes on cell surfaces we performed co-immunoprecipitation assays with *Drosophila* Dpp. For these experiments, Myc-tagged Dally or Dally$^{\Delta GAG}$ was co-expressed in S2 cells with HA-tagged Dpp, and Dpp-bound proteins were recovered by immunoprecipitation with anti-HA antibody. Again Myc-Dally$^{\Delta GAG}$ was not expressed as well as Myc-Dally, yet it was still present in the immunoprecipitates (at varying levels in different experiments; Fig. 2H). This finding corroborates the cell-binding assay and indicates that Dally and Dally$^{\Delta GAG}$ can both form complexes with Dpp or BMP4.

Effects of Dally and Dally$^{\Delta GAG}$ overexpression in vivo

The results of our SPR and other binding experiments suggest that Dally's interactions with BMPs are not mediated entirely through its HS chains, whereas FGF binding is completely dependent on HS modification. These differences in binding properties in vitro may have functional consequences in vivo: in particular, Dally$^{\Delta GAG}$ might retain some function in Dpp or Wg signaling despite its lack of HS modification. As a first test of Dally$^{\Delta GAG}$ activity in vivo, we evaluated the effects of overexpression in the posterior compartment of the wing disc (either *UAS-dally*$^{+}$ or *UAS-dally*$^{\Delta GAG}$ driven by *en-Gal4*). Overexpressing wild-type Dally (Fig. 3B) resulted in enlargement of the posterior portion of the wing by 46%, while the anterior increased only modestly in size (13%) compared to control wings (Fig. 3A). In addition, we observed disruptions in the formation of the anterior and posterior crossveins in wings where Dally was overexpressed. Loss of the posterior crossvein was also observed by Takeo et al. when Dally was overexpressed in the wing disc using a number of different *Gal4*

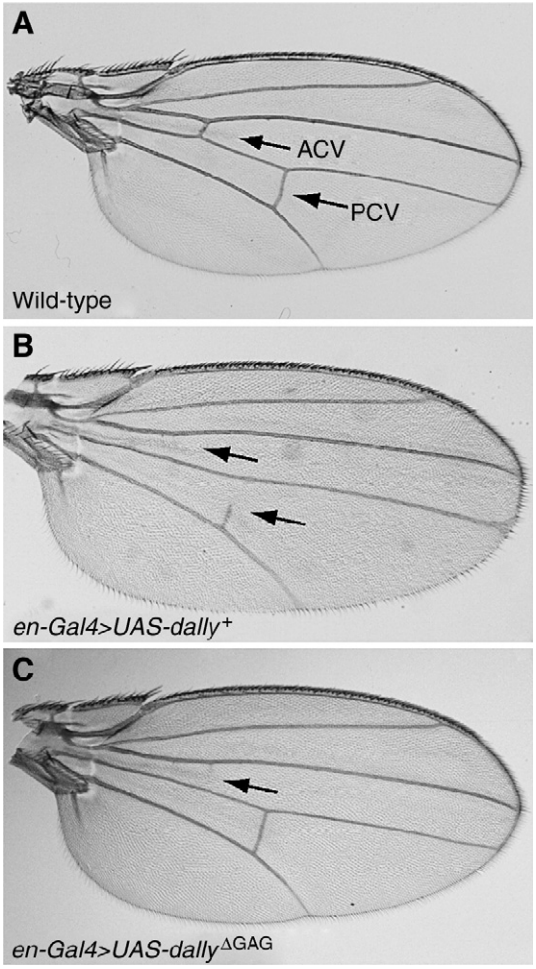


Fig. 3. Overexpression of Dally and Dally$^{\Delta GAG}$ in the wing. (A) Wild-type wing. The anterior and posterior crossveins (ACV and PCV) are indicated. (B) Wing overexpressing wild-type Dally in the posterior compartment under control of *en-Gal4*. The ACV and PCV are disrupted (arrows). (C) Wing overexpressing Dally$^{\Delta GAG}$ in the posterior compartment under control of *en-Gal4*. The ACV is missing (arrow).

drivers (Takeo et al., 2005). A mutation in one copy of the *Mad* gene suppressed the overgrowth phenotype but not the vein defects caused by Dally overexpression (data not shown). Since *Mad* is a component of the Dpp signaling pathway, this result suggests that the overgrowth is due to elevated Dpp signaling. Dally$^{\Delta GAG}$ overexpression (Fig. 3C) had milder effects compared to wild-type Dally: it did not cause enlargement of the posterior wing or loss of the posterior crossvein, but did disrupt anterior crossvein formation. We do not know the precise expression level of Dally$^{\Delta GAG}$ compared to wild-type Dally because there is no reliable Dally antibody available, but we obtained similar results from several different *UAS-dally$^{\Delta GAG}$* transgenic lines. These data suggest that Dally$^{\Delta GAG}$ retains biological activity in vivo, but its activity may be less than that of wild-type Dally.

Further overexpression experiments in another tissue, the eye, confirm and extend this conclusion. Overexpressing wild-type Dally using *ey-Gal4* caused an overgrowth phenotype (Fig. 4B), as it had in the wing. When we examined cross-sections of these eyes, we found disruptions in the organization of the ommatidia. Instead of the highly regular array seen in wild-type eyes (Fig. 4D), the ommatidia were misshapen, with a reduced number of photoreceptors per ommatidial unit (Fig. 4E). The reduction in photoreceptor number was suppressed by a mutation in one copy of the *Mad* gene (Fig. 4F), suggesting that this defect is due to elevated Dpp signaling, like the wing overgrowth described above. Overexpression of Dally$^{\Delta GAG}$ did not cause overgrowth of the eye like wild-type Dally (Fig. 4C) or change the number of photoreceptors (Fig. 4G), but it resulted in more severely disorganized ommatidial structure. Thus, in this

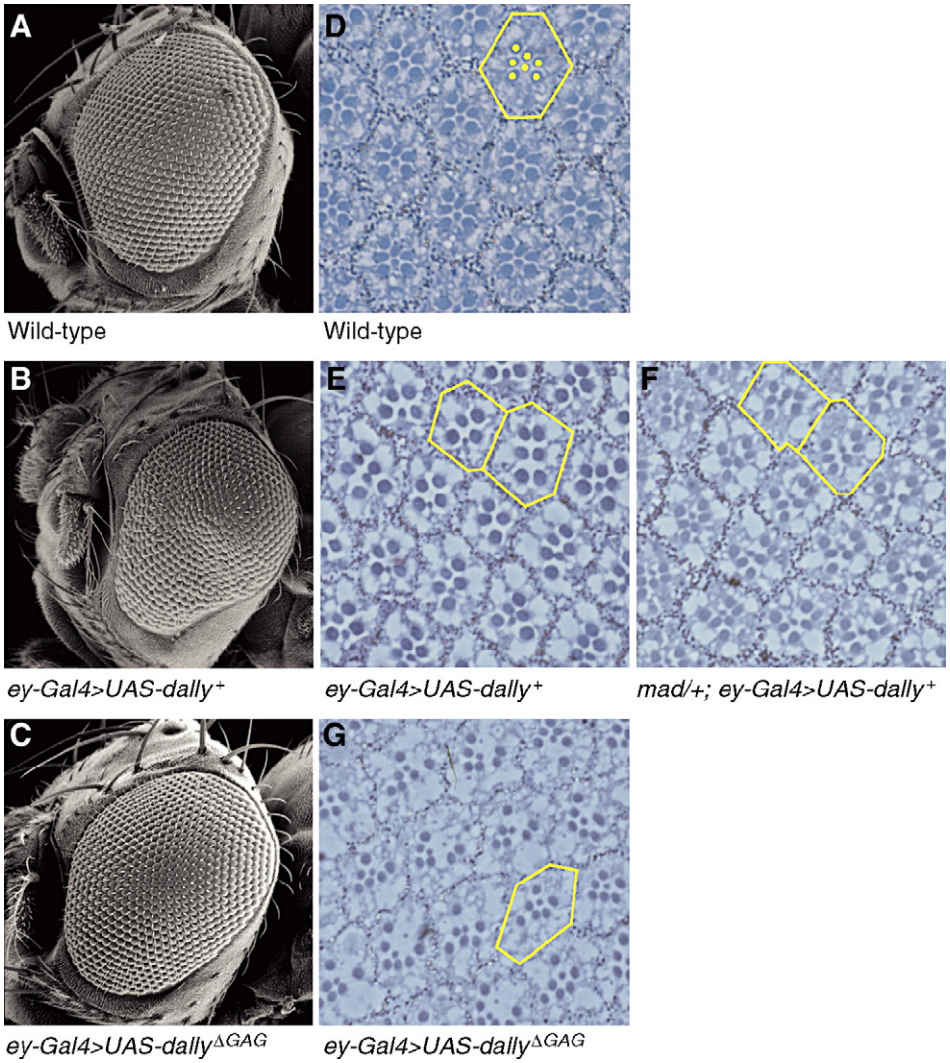


Fig. 4. Overexpression of Dally and Dally$^{\Delta GAG}$ in the eye. (A–C) Scanning electron micrographs of wild-type, Dally- and Dally$^{\Delta GAG}$-overexpressing eyes. Overexpression of Dally, but not Dally$^{\Delta GAG}$, resulted in eye overgrowth. (D–G) Thin sections of adult eyes stained with toluidine blue. The yellow hexagon in panel (D) outlines one ommatidium; yellow dots mark the rhabdomeres (light-sensing structures) of the photoreceptor cells. Seven photoreceptor cells in a stereotypical pattern are visible in wild-type eye sections. Overexpression of Dally reduces the number of photoreceptors per ommatidium; those outlined in panel (E) have only five or six photoreceptor cells. Dally overexpression also disrupts the regular hexagonal structure of the ommatidia. Photoreceptor numbers are restored to normal in *Mad*/+ heterozygous animals (F), but ommatidial shape is still affected. Dally$^{\Delta GAG}$ overexpression does not reduce photoreceptor numbers, but results in smaller rhabdomeres and more severely disrupted ommatidial patterning (G).

context Dally$^{\Delta GAG}$ displayed activity distinct from that of wild-type Dally, rather than a simple reduction in activity.

As described above, overexpression of Dally$^{\Delta GAG}$ with *hh-Gal4* in the wing disc led to a reduction in the level of endogenous HS (Fig. 1D). Therefore, the etiology of the defects observed upon overexpression of Dally$^{\Delta GAG}$ may be complex: some might reflect a portion of normal Dally activity remaining in the overexpressed protein, but others might be caused by loss of other HSPG functions due to reduced levels of HS modification. The ability of Dally$^{\Delta GAG}$ to interfere with HSPG function would likely depend on the level of over-expression and the consequences could vary in different tissues.

Assessing Dally$^{\Delta GAG}$ function in Dpp signaling

Wild-type Dally is known to promote Dpp signaling (Jackson et al., 1997; Fujise et al., 2003); activation of this pathway results in phosphorylation of the downstream effector Mad, as well as activation of Dpp target genes. Therefore, as another measure of in vivo biological activity, we assayed the ability of Dally$^{\Delta GAG}$ to induce accumulation of phosphorylated Mad (pMad). Overexpression of wild-type Dally in clones of cells in the wing disc causes a cell-autonomous increase in pMad levels (Figs. 5B–D), as shown previously by Fujise et al. (2003). When Dally$^{\Delta GAG}$ was overexpressed in the same way, it had no apparent effect on the levels of pMad in the tissue (Figs. 5E–G): the distribution of pMad resembles that in a wild-type wing disc (Fig. 5A). Thus, Dally$^{\Delta GAG}$ does not appear to be able to activate Dpp signaling sufficiently to induce detectable increases in Mad phosphorylation, despite its clear biological activity in overexpression experiments.

In addition to examining pMad accumulation, we also evaluated the effect of Dally$^{\Delta GAG}$ on the expression of the Dpp target gene *spalt*. Normally, *spalt* is expressed in a broad band of cells flanking the anterior–posterior boundary of the wing disc (Fig. 6A); the width of the dorsal and ventral *spalt* domains is about equal in wild-type discs (dorsal/ventral ratio of 1.01 ± 0.05; $n=9$). Overexpression of wild-type Dally in the dorsal compartment resulted in elevated Dpp signaling and a 17% broader band of *spalt* expression in the dorsal compartment compared to the ventral compartment (Fig. 6B; ratio of 1.17 ± 0.09; $n=10$). In contrast, dorsal overexpression of Dally$^{\Delta GAG}$ reduced the domain of *spalt* expression in comparison to the ventral compartment by an average of 23% (Fig. 6C; ratio of 0.77 ± 0.06; $n=13$), although the dorsal/ventral ratio varied considerably between different discs. Therefore, Dally$^{\Delta GAG}$ not only failed to activate *spalt* expression, but appeared to limit Dpp signaling.

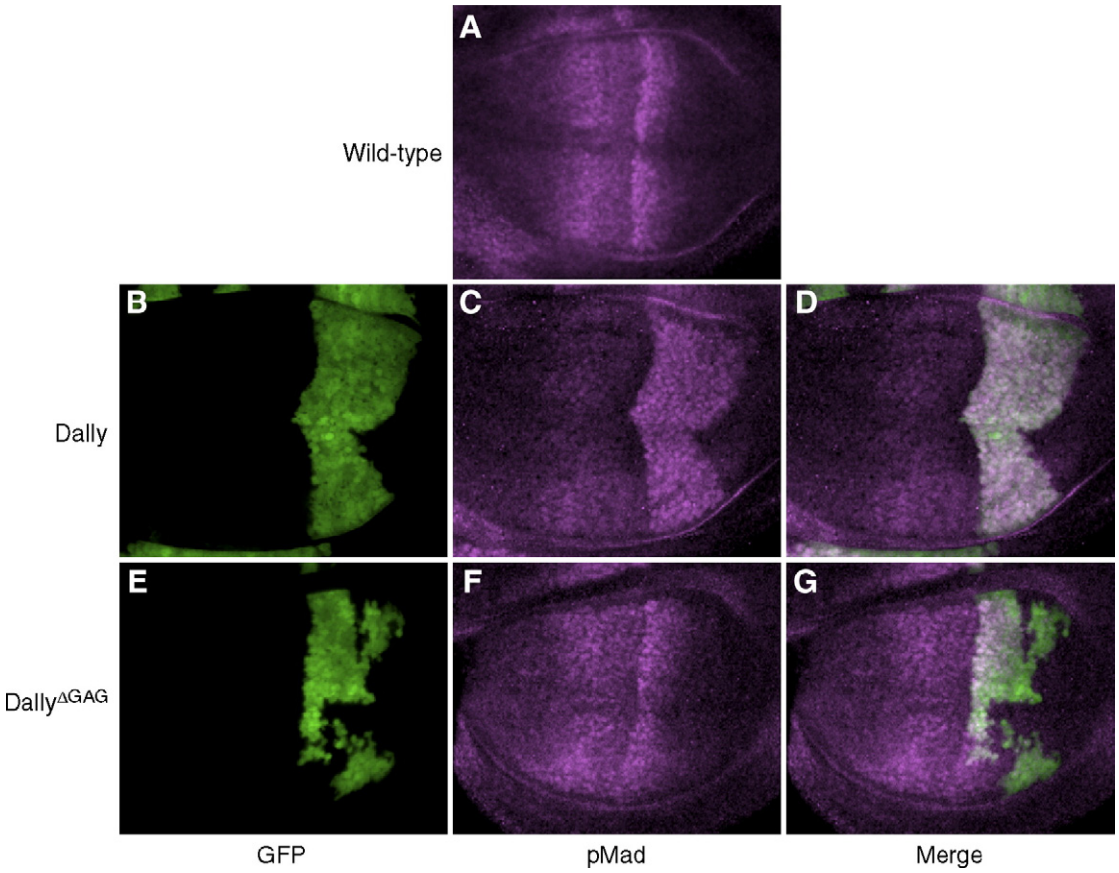


Fig. 5. Effect of Dally and Dally$^{\Delta GAG}$ overexpression on pMad levels. (A) pMad (magenta) is found in graded distribution across the anterior–posterior axis of a wild-type wing disc. Dally- or Dally$^{\Delta GAG}$ was overexpressed in random clones of cells using *the FLP-FRT* system. Expressing cells are marked with GFP (green in B and E respectively). (B–D) Overexpression of Dally leads to increased pMad staining within the clone. (E–G) Overexpression of Dally$^{\Delta GAG}$ does not affect the pMad distribution.

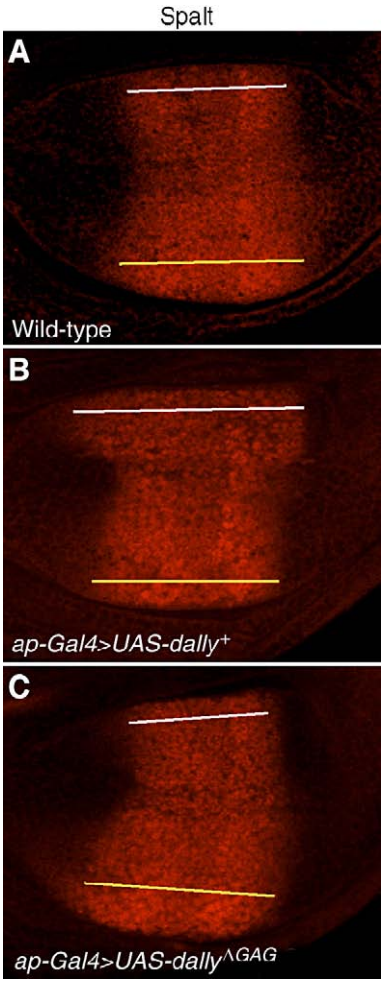


Fig. 6. Effect of Dally and Dally$^{\Delta GAG}$ on *spalt* expression. (A) Spalt protein (red) is normally found in a wide stripe in the central region of the wing disc. The widths of the dorsal and ventral *spalt* domains are indicated by white and yellow lines respectively. (B) Dally overexpression in the dorsal compartment using the *ap-Gal4* driver increases the width of the dorsal *spalt*-expressing domain in comparison to the ventral compartment. The dorsal compartment also frequently appears to be shorter along the dorsal/ventral axis when Dally is overexpressed. (C) Overexpression of Dally$^{\Delta GAG}$ using *ap-Gal4* reduces the width of the dorsal *spalt* expression stripe. (This effect is more variable from disc to disc than the effect of Dally overexpression).

Rescue of dally mutants by expression of UAS-dally^{+} and UAS-dally$^{\Delta GAG}$

In the overexpression experiments described above, Dally$^{\Delta GAG}$ was able to induce some of the same phenotypes as wild-type Dally. However, a more robust test of Dally$^{\Delta GAG}$ function is whether it can effectively substitute for Dally in rescuing a *dally* mutant animal. For these experiments, we used a number of *dally* allelic combinations, including *dally^{gem}* (Fujise et al., 2001) and the *dally^{80}* reported null allele (Han et al., 2004b) over a chromosomal deletion that removes the *dally* locus (*Df(3L)scf-R6*). The severity of the phenotypes in *dally^{gem}* homozygotes and *dally^{gem}/Df(3L)scf-R6* animals was comparable to *dally^{80}/Df(3L)scf-R6*, indicating that *dally^{gem}* is a very strong allele, similar in strength to *dally^{80}*. We expressed Dally and Dally$^{\Delta GAG}$ using the *A9-Gal4* driver in the wing disc of various *dally* mutants, and compared the degree to which several different phenotypes were rescued.

One phenotype of *dally* mutants is a reduction in the number of chemosensory bristles at the anterior margin of the wing (Fujise et al., 2001). Formation of these bristles depends on Wg signaling from the dorsal–ventral boundary of the wing disc and their loss reflects a reduction in Wg signaling. Expression of wild-type Dally using *A9-Gal4* fully rescues the chemosensory bristles in *dally* mutants (Fig. 7A). Expression of Dally$^{\Delta GAG}$ also substantially restores chemosensory bristles at the wing margin, although not to full wild-type numbers. Thus, Dally$^{\Delta GAG}$ apparently retains some ability to promote Wg signaling.

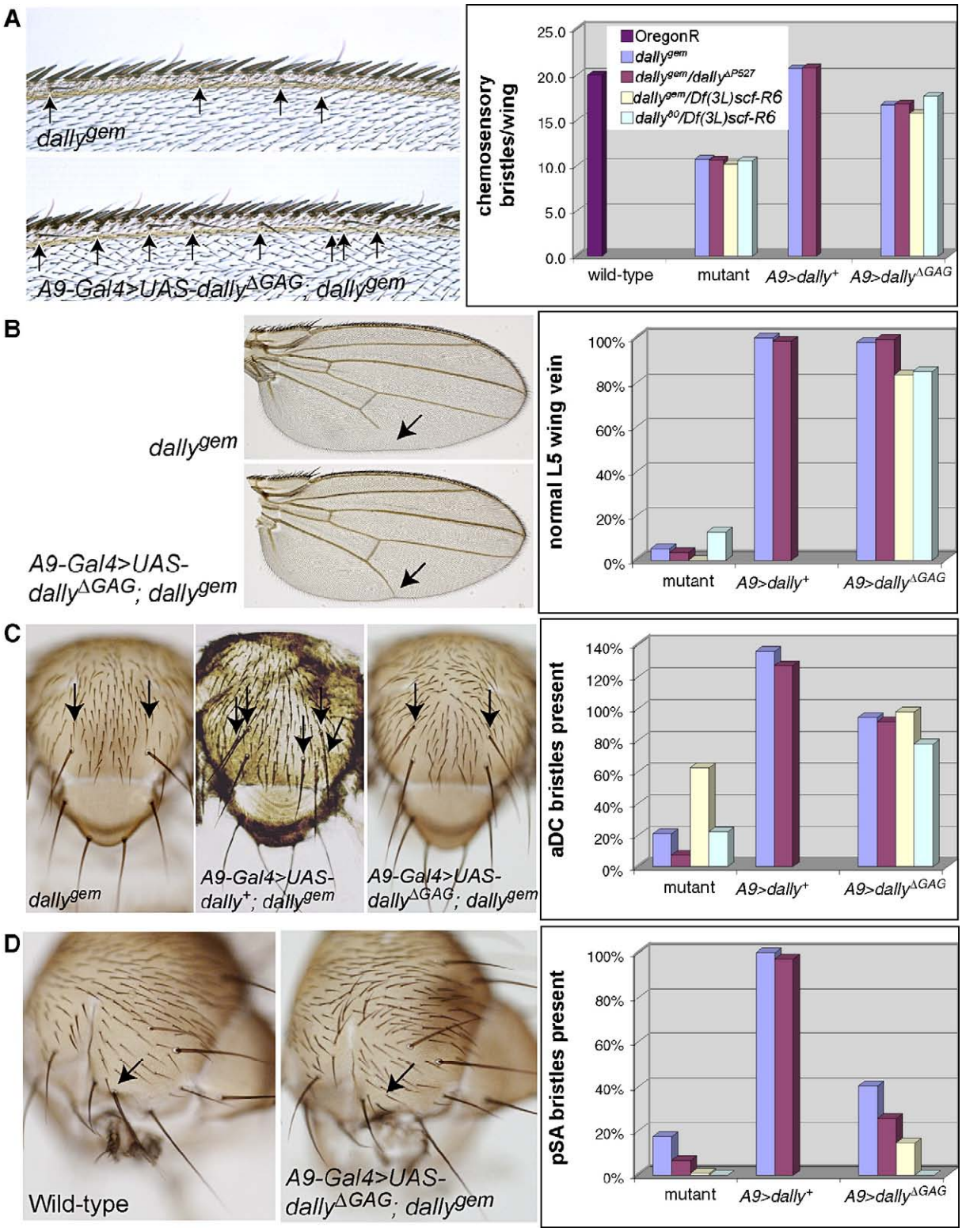
dally mutant wings also show defects in forming the distal L5 wing vein: instead of extending all the way to the wing margin, the vein stops short between the posterior crossvein and the margin. This phenotype is fully or almost fully rescued by expressing either Dally or Dally$^{\Delta GAG}$ with *A9-Gal4* (Fig. 7B). In this context, Dally$^{\Delta GAG}$ seems to provide almost full biological activity, since it can rescue *dally* null mutants.

In addition to wing defects, *dally* mutants show disruptions in the formation of sensory bristles on the notum (Fujise et al., 2001). Since *A9-Gal4* is expressed in the region of the wing imaginal disc that will become the future dorsal notum, we evaluated the ability of wild-type Dally and Dally$^{\Delta GAG}$ to rescue the specification of these sensory bristles. Normally there are four large dorsocentral bristles, and two of these, the anterior dorsocentral (aDC) bristles, are missing in *dally* mutant animals (Fig. 7C). Expressing wild-type Dally with *A9-Gal4* in *dally* mutants rescues the loss of these bristles, and induces the formation of excess dorsocentral bristles (approximately 30% more than wild-type animals), similar to overexpression of *dally^{+}* using other drivers in wild-type animals (Takeo et al., 2005). Expressing Dally$^{\Delta GAG}$ almost fully rescues the aDC bristles without producing any extra ones;

Fig. 7. Rescue of *dally* mutants by Dally and Dally$^{\Delta GAG}$. *UAS-dally^{+}* or *UAS-dally$^{\Delta GAG}$* was expressed in *dally* mutant wing discs using *A9-Gal4*. Up to four *dally* mutant genotypes (*dally^{gem}* homozygotes, *dally^{gem}/dally$^{\Delta P527}$*, *dally^{gem}/Df(3L)scf-R6* and *dally^{80}/Df(3L)scf-R6*) were tested for rescue, with very similar results. Representative photographs of mutant and rescued phenotypes are shown on the left; where only rescue by Dally$^{\Delta GAG}$ is shown, Dally gave similar results. Rescue data are quantified in graphs on the right, where *A9>dally^{+}* and *A9>dally$^{\Delta GAG}$* refer to *dally* mutants expressing the indicated transgene. Note that the *A9-Gal4* driver used in these experiments does not drive expression as strongly as the drivers used for overexpression in Figs. 1 and 3 above. (A) Rescue of chemosensory bristles at the anterior wing margin. Chemosensory bristles (arrows) are less numerous in *dally* mutant animals than in wild type. Expression of Dally or Dally$^{\Delta GAG}$ increases the number of chemosensory bristles to wild-type or intermediate levels. (B) Rescue of the L5 wing vein. The L5 wing vein (arrows) is truncated in *dally* mutants. Expression of Dally or Dally$^{\Delta GAG}$ restores the full-length vein. (C) Rescue of aDC bristles. *dally* mutants typically lack aDC bristles on the notum (arrows). Expression of Dally rescues the aDC bristles and leads to the formation of supernumerary dorsocentral bristles (arrows). Expression of Dally$^{\Delta GAG}$ restores nearly wild-type numbers of aDC bristles. (D) Rescue of pSA bristles. pSA bristles (arrows) are missing in most *dally* mutant animals, but are rescued by expression of Dally using *A9-Gal4*. Similar expression of Dally$^{\Delta GAG}$ does not substantially increase the number of pSA bristles in *dally* mutants.

sometimes the rescued bristles are displaced from their usual positions (Fig. 7C). As well as lacking aDC bristles, *dally* mutants lack the posterior supraalar (pSA) bristles on the more lateral notum. These bristles are rescued by expression of wild-type Dally, but not by Dally$^{\Delta GAG}$ (Fig. 7D). Thus, while Dally$^{\Delta GAG}$ does not provide complete rescue of notum bristles, it retains a considerable level of activity despite its lack of HS modification.

As in the overexpression experiments described above, we do not know the relative level of Dally$^{\Delta GAG}$ expression compared to wild-type Dally in the rescued animals because we do not have a reliable anti-Dally antibody, so we cannot draw firm conclusions



about the relative activity of the two proteins. We attempted to rescue *dally* mutants with the Myc-tagged Dally$^{\Delta GAG}$ that was used for cell binding and co-immunoprecipitation studies and found that this protein also has some capacity for rescue. However, the transgene expressing this tagged form was considerably less active than the *UAS-dally$^{\Delta GAG}$* transgene used in most of our experiments: it restored the L5 wing vein in some animals (76% normal L5 veins in *dally^{gem}/dally$^{\Delta P527}$* flies and 35% in *dally^{gem}* homozygotes, compared to 99% and 98% for untagged Dally$^{\Delta GAG}$ in the same mutant backgrounds); the tagged form had a slight effect on the number of chemosensory bristles and aDC bristles in *dally^{gem}/dally$^{\Delta P527}$* animals and was completely unable to rescue the pSA bristles. When we examined the levels of Myc-tagged Dally and Dally$^{\Delta GAG}$ in vivo by Western blotting, Myc-Dally$^{\Delta GAG}$ was present at less than 2% of the level of Myc-Dally (data not shown). Thus, even when expressed at markedly lower levels than Myc-Dally, Myc-Dally$^{\Delta GAG}$ demonstrated measurable rescue activity, showing that Dally$^{\Delta GAG}$ has significant biological activity.

Discussion

The profound effect of mutations affecting HS biosynthesis on growth factor signaling in vivo indicates that HS modification is critical for HSPG function in development. However, the relative contributions of proteoglycan core proteins and their HS side chains in vivo have not been determined. The significant sequence conservation in the extracellular domain of all glypicans suggests a shared core protein structure vital for their function (De Cat and David, 2001; Kramer and Yost, 2003). To assess the relative importance of HS modification and the protein core for an individual proteoglycan, we investigated the HS dependence of Dally's interactions with growth factors in vitro, and evaluated the function of a form of Dally that lacks HS chains in vivo. In vitro binding of Dally to BMPs and FGFs differed: FGF binding appeared to be mediated by HS chains, but BMP4 and Dpp binding were not entirely HS-dependent. Surprisingly, we found that HS modification of Dally is not essential for all its in vivo functions, showing that the core protein retains significant activity in the absence of direct HS modification.

Using SPR to measure binding of purified Dally, our results demonstrate for the first time that there is a direct interaction between a *Drosophila* glypican and growth factors in vitro. Similar to other HS growth factor interaction studies, we found that FGF binding can be completely inhibited by excess soluble heparan sulfate, suggesting that binding of FGF to Dally occurs via the HS chains. In contrast, BMP4-Dally association displays an HS-resistant component, suggesting that BMP4 binding is mediated in part by the Dally core protein. In other binding studies, we found that Dally$^{\Delta GAG}$ retains some ability to interact with BMP4 and its *Drosophila* homolog Dpp, despite its lack of HS modification. FGF and BMP4 also differed in their ability to form ternary complexes with Dally and growth factor receptors. Preincubation of FGF10 with its receptor FGFR2b produced a complex that bound to Dally in an HS-dependent manner, whereas addition of BMPR1A eliminated binding of BMP4 to Dally. These findings argue that the interactions of FGFs and BMPs with glypicans can have fundamentally different molecular properties and that HS chains are not essential for all interactions of these cell surface proteins.

The binding we observed between Dally and FGFs in vitro raises the question of whether Dally promotes FGF signaling in vivo. Eliminating all HS modification, by mutation of *sulfateless* for example, reduces FGF signaling (Lin et al., 1999) as well as signaling by other growth factors (Bornemann et al., 2004; Han et al., 2004a; Takei et al., 2004). There are no published data showing whether *dally* mutants have defects in FGF-dependent processes such as embryonic tracheal morphogenesis. However, *dally* enhancer traps are strongly expressed in the developing tracheal system (K. Kamimura and H. Nakato, personal communication), suggesting that Dally could participate in such signaling.

Our other studies show that Dally's core protein possesses substantial biological function in vivo despite its lack of HS chains. Dally$^{\Delta GAG}$ was delivered to the cell surface like wild-type Dally, but showed no evidence of HS modification either in S2 cells or in the wing disc (Fig. 1), suggesting that HS chains were not added at cryptic sites following mutation of the normal attachment sites (similar to a previously described mutant (Kreuger et al., 2004)). Dally$^{\Delta GAG}$ remained partially active in overexpression experiments, showing some effect on crossvein formation in the wing, and displayed a distinct activity from wild-type Dally in disrupting patterning of the retina (Figs. 3 and 4). Significantly, Dally$^{\Delta GAG}$ can rescue many defects of *dally* mutants, although not always to the same extent as wild-type Dally (Fig. 7); its ability to rescue *dally* null alleles indicates that Dally$^{\Delta GAG}$ function does not depend on the presence of some wild-type Dally. A direct comparison of the activity of wild-type and non-HS modified Dally for any given patterning process is difficult since we do not necessarily have equivalent levels of expression, but Dally$^{\Delta GAG}$ clearly has activity in vivo. Non-HS-modified proteoglycans have been found at significant levels in some cells, such as rat NRM2 mesothelial cells and HepG2 hepatoma cells (Gonzalez et al., 1998), suggesting a potential normal biological role for such "naked" proteins.

Our in vivo analysis of Dally$^{\Delta GAG}$ indicates that it can promote signaling by more than one patterning molecule. Rescue of the chemosensory bristles at the anterior wing margin, although not complete, shows that Dally$^{\Delta GAG}$ retains the capacity to promote Wg signaling. Consistent with our results, *Xenopus* glypican 4 core protein inhibits activin-induced elongation of *Xenopus* animal caps like the wild-type protein, suggesting it still functions in this Wnt-dependent process (Ohkawara et al., 2003). Similarly, non-glycanated vertebrate GPC3 immunoprecipitates with various Wnts, and stimulates Wnt signaling and cell proliferation in hepatocellular carcinoma cells, although not always as well as wild-type GPC3 (Capurro et al., 2005). Dally$^{\Delta GAG}$ rescued other *dally* mutant phenotypes to varying degrees, suggesting that it may promote some Dpp-dependent signaling events, but not others.

Dally$^{\Delta GAG}$ restored the distal L5 wing vein, which is lost in *dally* mutants due to reduced levels of signaling by Dpp and/or Gbb, another member of the BMP family (Ray and Wharton, 2001). The specification of the aDC sensory bristles is effectively rescued by Dally$^{\Delta GAG}$ but another notum bristle, pSA, is only weakly rescued by Dally$^{\Delta GAG}$ compared to Dally. The formation of both these sensory structures depends on Dpp signaling, in addition to other pathways (Phillips and Whittle, 1993; Tomoyasu et al., 1998; Garcia-Garcia et al., 1999; Fujise et al., 2001; Gomez-Skarmeta et al., 2003). Our findings indicate that some patterning events are more critically dependent on an HS-modified version of Dally.

By what mechanism is Dally$^{\Delta GAG}$ able to promote signaling? Clearly HS chains contribute to normal Dally function, but our in vivo analyses suggest that the glypican core protein provides significant function independent of its ability to deliver HS chains. One hypothesis is that Dally$^{\Delta GAG}$ participates in the formation of multimeric signaling complexes that include other HSPGs present on the same cell or adjacent cells; the HS chains of these other molecules might co-operate with Dally$^{\Delta GAG}$ to promote signaling. In support of such a mechanism, Jakobsson et al. have shown, using cultured embryonic stem cells, that HSPGs on adjacent cells are able to promote VEGF signaling in endothelial cells in trans (Jakobsson et al., 2006). Since growth factors, especially those with a lipid modification such as Wg, may diffuse through tissues as part of a lipoprotein complex (Panakova et al., 2005), Dally$^{\Delta GAG}$ may interact with some component of the complex while separate HS chains mediate other critical interactions. It is also possible that some interactions of Dally with growth factors or their receptors are entirely independent of HS chains.

Our findings suggest that Dally's HS chains are more important for some aspects of Dally function than others. Dally exhibits some HS-independent binding to BMP4 and Dpp in vitro (Fig. 2) and Dally$^{\Delta GAG}$ shows some capacity to rescue Dpp-dependent *dally* mutant phenotypes (Fig. 7), but Dally$^{\Delta GAG}$ does not cause Dpp-mediated overgrowth like wild-type Dally (Figs. 3 and 4) or induce high-level Dpp signaling, as demonstrated by its effects on the levels of pMad or the range of the Dpp target *spalt* (Figs. 5 and 6). It is possible that Dally$^{\Delta GAG}$ is essentially hypomorphic for Dpp signaling, with partial activity sufficient to exceed certain developmental thresholds but not others. Also, the reduction in HS modification of other proteins caused by expression of Dally$^{\Delta GAG}$ (Fig. 1D) may contribute to some effects we observed. However, another potential explanation for Dally$^{\Delta GAG}$'s distinct activity is that Dally's HS chains are required in cis to form high-threshold, but not low-threshold, BMP signaling complexes. In the early embryo, high-level BMP signaling (detected by anti-pMad antibody staining) occurs only at the dorsal midline, where receptor complexes containing two different Type I receptors are activated in response to a BMP heterodimer (reviewed in O'Connor et al., 2006); in more lateral regions of the embryo, distinct receptor complexes specify lower levels of signaling. Dally$^{\Delta GAG}$ may only support formation of low-threshold signaling complexes sufficient to rescue some phenotypes such as the L5 wing vein. However, without direct HS modification Dally$^{\Delta GAG}$ may be incapable of forming high-threshold signaling complexes, and therefore completely inactive in some biological contexts. It is remarkable that high- and low-threshold Hh signaling are also differentially dependent on HSPG function: *ttv* activity is required for Hh to activate high-threshold but not low-threshold target genes (Callejo et al., 2006), suggesting that HSPG core proteins could serve distinct functions in low- versus high-threshold morphogen signaling. The roles of HSPGs in signaling are complex and still not completely understood; clearly, further experiments will be needed to clarify the molecular and cell biological mechanisms of their action and the roles for both their protein and glycosaminoglycan components.

Acknowledgments

We are grateful to P. ten Dijke, H. Nakato, the Developmental Studies Hybridoma Bank and the Bloomington Stock Center for fly stocks and reagents, and to B. Dimitroff, R. Peterson and T. Metzger for their assistance with the experiments. We especially thank T. Akiyama and H. Nakato for providing the Myc-tagged Dally and Dally$^{\Delta GAG}$, for the analysis of their relative expression levels and for helpful discussions. We also thank T. Akiyama and M. Serpe for their advice on binding assays, and H. Nakato and members of the Selleck laboratory for the discussions and comments on the manuscript. This work was supported by the National Institutes of Health (GM54832-09).

References

Baeg, G.H., Lin, X., Khare, N., Baumgartner, S., Perrimon, N., 2001. Heparan sulfate proteoglycans are critical for the organization of the extracellular distribution of Wingless. Development 128 (1), 87–94.

Blair, S.S., 2000. Imaginal Discs. In: Sullivan, W., Ashburner, M., Hawley, R.S. (Eds.), *Drosophila* Protocols. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, pp. 159–173.

Bornemann, D.J., Duncan, J.E., Staatz, W., Selleck, S., Warrior, R., 2004. Abrogation of heparan sulfate synthesis in *Drosophila* disrupts the Wingless, Hedgehog and Decapentaplegic signaling pathways. Development 131 (9), 1927–1938.

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

Callejo, A., Torroja, C., Quijada, L., Guerrero, I., 2006. Hedgehog lipid modifications are required for Hedgehog stabilization in the extracellular matrix. Development 133 (3), 471–483.

Capurro, M.I., Xiang, Y.Y., Lobe, C., Filmus, J., 2005. Glypican-3 promotes the growth of hepatocellular carcinoma by stimulating canonical Wnt signaling. Cancer Res. 65 (14), 6245–6254.

Cole, S.R., Ashman, L.K., Ey, P.L., 1987. Biotinylation: an alternative to radioiodination for the identification of cell surface antigens in immunoprecipitates. Mol. Immunol. 24 (7), 699–705.

David, G., Bai, X.M., Van der Schueren, B., Cassiman, J.J., Van den Berghe, H., 1992. Developmental changes in heparan sulfate expression: in situ detection with mAbs. J. Cell Biol. 119 (4), 961–975.

De Cat, B., David, G., 2001. Developmental roles of the glypicans. Semin. Cell Dev. Biol. 12 (2), 117–125.

Desbordes, S.C., Sanson, B., 2003. The glypican Dally-like is required for Hedgehog signalling in the embryonic epidermis of *Drosophila*. Development 130 (25), 6245–6255.

Desbordes, S.C., Chandraratna, D., Sanson, B., 2005. A screen for genes regulating the wingless gradient in *Drosophila* embryos. Genetics 170 (2), 749–766.

Ding, K., Lopez-Burks, M., Sanchez-Duran, J.A., Korc, M., Lander, A.D., 2005. Growth factor-induced shedding of syndecan-1 confers glypican-1 dependence on mitogenic responses of cancer cells. J. Cell Biol. 171 (4), 729–738.

Esko, J.D., Selleck, S.B., 2002. Order out of chaos: assembly of ligand binding sites in heparan sulfate. Annu. Rev. Biochem. 71.

Franch-Marro, X., Marchand, O., Piddini, E., Ricardo, S., Alexandre, C., Vincent, J.P., 2005. Glypicans shunt the Wingless signal between local signalling and further transport. Development 132 (4), 659–666.

Fujise, M., Izumi, S., Selleck, S.B., Nakato, H., 2001. Regulation of dally, an integral membrane proteoglycan, and its function during adult sensory organ formation of *Drosophila*. Dev. Biol. 235 (2), 433–448.

Fujise, M., Takeo, S., Kamimura, K., Matsuo, T., Aigaki, T., Izumi, S., Nakato, H., 2003. Dally regulates Dpp morphogen gradient formation in the *Drosophila* wing. Development 130 (8), 1515–1522.

Garcia-Garcia, M.J., Ramain, P., Simpson, P., Modolell, J., 1999. Different contributions of pannier and wingless to the patterning of the dorsal mesothorax of *Drosophila*. Development 126 (16), 3523–3532.

Gomez-Skarmeta, J.L., Campuzano, S., Modolell, J., 2003. Half a century of neural prepatterning: the story of a few bristles and many genes. Nat. Rev., Neurosci. 4 (7), 587–598.

Gonzalez, A.D., Kaya, M., Shi, W., Song, H., Testa, J.R., Penn, L.Z., Filmus, J., 1998. OCI-5/GPC3, a glypican encoded by a gene that is mutated in the Simpson–Golabi–Behmel overgrowth syndrome, induces apoptosis in a cell line-specific manner. J. Cell Biol. 141 (6), 1407–1414.

Han, C., Belenkaya, T.Y., Khodoun, M., Tauchi, M., Lin, X., Lin, X., 2004a. Distinct and collaborative roles of *Drosophila* EXT family proteins in morphogen signalling and gradient formation. Development 131 (7), 1563–1575.

Han, C., Belenkaya, T.Y., Wang, B., Lin, X., 2004b. *Drosophila* glypicans control the cell-to-cell movement of Hedgehog by a dynamin-independent process. Development 131 (3), 601–611.

Harmer, N.J., 2006. Insights into the role of heparan sulphate in fibroblast growth factor signalling. Biochem. Soc. Trans. 34 (Pt. 3), 442–445.

Herndon, M.E., Lander, A.D., 1990. A diverse set of developmentally regulated proteoglycans is expressed in the rat central nervous system. Neuron 4, 949–961.

Jackson, S.M., Nakato, H., Sugiura, M., Jannuzi, A., Oakes, R., Kaluza, V., Golden, C., Selleck, S.B., 1997. *dally*, a *Drosophila* glypican, controls cellular responses to the TGF-beta-related morphogen, Dpp. Development 124 (20), 4113–4120.

Jakobsson, L., Kreuger, J., Holmborn, K., Lundin, L., Eriksson, I., Kjellen, L., Claesson-Welsh, L., 2006. Heparan sulfate in trans potentiates VEGFR-mediated angiogenesis. Dev. Cell 10 (5), 625–634.

Johnson, K.G., Tenney, A.P., Ghose, A., Duckworth, A.M., Higashi, M.E., Parfitt, K., Marcu, O., Heslip, T.R., Marsh, J.L., Schwarz, T.L., Flanagan, J.G., Van Vactor, D., 2006. The HSPGs syndecan and Dallylike bind the receptor phosphatase LAR and exert distinct effects on synaptic development. Neuron 49 (4), 517–531.

Kirkpatrick, C.A., Dimitroff, B.D., Rawson, J.M., Selleck, S.B., 2004. Spatial regulation of wingless morphogen distribution and signaling by Dally-like protein. Dev. Cell 7 (4), 513–523.

Kramer, K.L., Yost, H.J., 2003. Heparan sulfate core proteins in cell–cell signaling. Annu. Rev. Genet. 37, 461–484.

Kreuger, J., Perez, L., Giraldez, A.J., Cohen, S.M., 2004. Opposing activities of Dally-like glypican at high and low levels of Wingless morphogen activity. Dev. Cell 7 (4), 503–512.

Lin, X., 2004. Functions of heparan sulfate proteoglycans in cell signaling during development. Development 131 (24), 6009–6021.

Lin, X., Perrimon, N., 1999. Dally cooperates with *Drosophila* Frizzled 2 to transduce Wingless signalling. Nature 400 (6741), 281–284.

Lin, X., Buff, E.M., Perrimon, N., Michelson, A.M., 1999. Heparan sulfate proteoglycans are essential for FGF receptor signaling during *Drosophila* embryonic development. Development 126 (17), 3715–3723.

O'Connor, M.B., Umulis, D., Othmer, H.G., Blair, S.S., 2006. Shaping BMP morphogen gradients in the *Drosophila* embryo and pupal wing. Development 133 (2), 183–193.

Ohkawara, B., Yamamoto, T.S., Tada, M., Ueno, N., 2003. Role of glypican 4 in the regulation of convergent extension movements during gastrulation in *Xenopus laevis*. Development 130 (10), 2129–2138.

Padgett, R.W., Wozney, J.M., Gelbart, W.M., 1993. Human BMP sequences can confer normal dorsal–ventral patterning in the *Drosophila* embryo. Proc. Natl. Acad. Sci. U. S. A. 90 (7), 2905–2909.

Paine-Saunders, S., Viviano, B.L., Economides, A.N., Saunders, S., 2002. Heparan sulfate proteoglycans retain Noggin at the cell surface: a potential mechanism for shaping bone morphogenetic protein gradients. J. Biol. Chem. 277 (3), 2089–2096.

Panakova, D., Sprong, H., Marois, E., Thiele, C., Eaton, S., 2005. Lipoprotein particles are required for Hedgehog and Wingless signalling. Nature 435 (7038), 58–65.

Phillips, R.G., Whittle, J.R., 1993. wingless expression mediates determination of peripheral nervous system elements in late stages of *Drosophila* wing disc development. Development 118, 427–438.

Rapraeger, A.C., Krufka, A., Olwin, B.B., 1991. Requirement of heparan sulfate for bFGF-mediated fibroblast growth and myoblast differentiation. Science 252, 1705–1708.

Rawson, J.M., Dimitroff, B., Johnson, K.G., Ge, X., Van Vactor, D., Selleck, S.B., 2005. The heparan sulfate proteoglycans Dally-like and syndecan have distinct functions in axon guidance and visual-system assembly in *Drosophila*. Curr. Biol. 15 (9), 833–838.

Ray, R.P., Wharton, K.A., 2001. Context-dependent relationships between the BMPs gbb and dpp during development of the *Drosophila* wing imaginal disk. Development 128 (20), 3913–3925.

Struhl, G., Basler, K., 1993. Organizing activity of wingless protein in *Drosophila*. Cell 72 (4), 527–540.

Takei, Y., Ozawa, Y., Sato, M., Watanabe, A., Tabata, T., 2004. Three *Drosophila* EXT genes shape morphogen gradients through synthesis of heparan sulfate proteoglycans. Development 131 (1), 73–82.

Takeo, S., Akiyama, T., Firkus, C., Aigaki, T., Nakato, H., 2005. Expression of a secreted form of Dally, a *Drosophila* glypican, induces overgrowth phenotype by affecting action range of Hedgehog. Dev. Biol. 284 (1), 204–218.

Tomoyasu, Y., Nakamura, M., Ueno, N., 1998. Role of dpp signalling in prepattern formation of the dorsocentral mechanosensory organ in *Drosophila melanogaster*. Development 125 (21), 4215–4224.

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, S.B., 1999. The cell-surface proteoglycan Dally regulates Wingless signalling in *Drosophila*. Nature 400 (6741), 276–280.

Wolf, T., 2000. Histological techniques for the *Drosophila* eye. In: Sullivan, M., Ashburner, M., Hawley, R.S. (Eds.), *Drosophila* Protocols, 2. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 229–243.

Yayon, A., Klagsburn, M., Esko, J.D., Leder, P., Ornitz, D., 1991. Cell surface, heparin-like molecules are required for binding of basic fibroblast growth factor to its high affinity receptor. Cell 64, 841–848.