

# Glypicans regulate JAK/STAT signaling and distribution of the Unpaired morphogen

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## SUMMARY

In *Drosophila*, ligands of the Unpaired (Upd) family activate the Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathway. The JAK/STAT pathway controls many developmental events, including multiple functions in the ovary. These include an early role in the germarium for specification of stalk cells and a later role in the vitellarium to pattern the follicular epithelium surrounding each cyst. In this latter role, graded JAK/STAT activation specifies three distinct anterior follicular cell fates, suggesting that Upd is a morphogen in this system. Consistent with the JAK/STAT activation pattern in the vitellarium, Upd forms a concentration gradient on the apical surface of the follicular epithelium with a peak at its source, the polar cells. Like many morphogens, signaling and distribution of Upd are regulated by the heparan sulfate proteoglycans (HSPGs) Dally and Dally-like. Mutations in these glypican genes and in heparan sulfate biosynthetic genes result in disruption of JAK/STAT signaling, loss or abnormal formation of the stalk and significant reduction in the accumulation of extracellular Upd. Conversely, forced expression of Dally causes ectopic accumulation of Upd in follicular cells. Furthermore, biochemical studies reveal that Upd and Dally bind each other on the surface of the cell membrane. Our findings demonstrate that *Drosophila* glypicans regulate formation of the follicular gradient of the Upd morphogen, Upd. Furthermore, we establish the follicular epithelium as a new model for morphogen signaling in complex organ development.

**KEY WORDS:** *Drosophila*, JAK signaling, Heparan sulfate proteoglycans, Morphogen, Oogenesis

## INTRODUCTION

The JAK/STAT pathway plays vital roles in development and homeostasis in animals. *Drosophila*, with its complete set of JAK/STAT components, provides a powerful genetic system to analyze the molecular functions of this essential pleiotropic pathway (for reviews, see Arbouzova and Zeidler, 2006; Brown and Zeidler, 2008; Müller et al., 2008). In addition to functions in embryonic, larval and imaginal development, JAK/STAT signaling is crucial for several steps of *Drosophila* oogenesis, including two distinct follicle cell specification events (Fig. 1A). Early in oogenesis, in the germarium, JAK/STAT activity stimulates formation of stalk cells that will separate developing egg chambers (Baksa et al., 2002; McGregor et al., 2002). Later in oogenesis, multiple follicular epithelial cell fates are specified by a gradient of JAK/STAT signaling and are altered by changes in JAK/STAT activity levels (Xi et al., 2003). The highest levels of JAK/STAT activity at the anterior and posterior poles coincide with the polar cell expression of the ligand gene *unpaired* (*upd*; *os* – FlyBase) at all stages of oogenesis (McGregor et al., 2002). Moreover, Upd protein is distributed in a gradient in late-stage egg chambers (Ghiglione et al., 2008). These results suggest that Upd acts as a morphogen in the specification of the follicular epithelium.

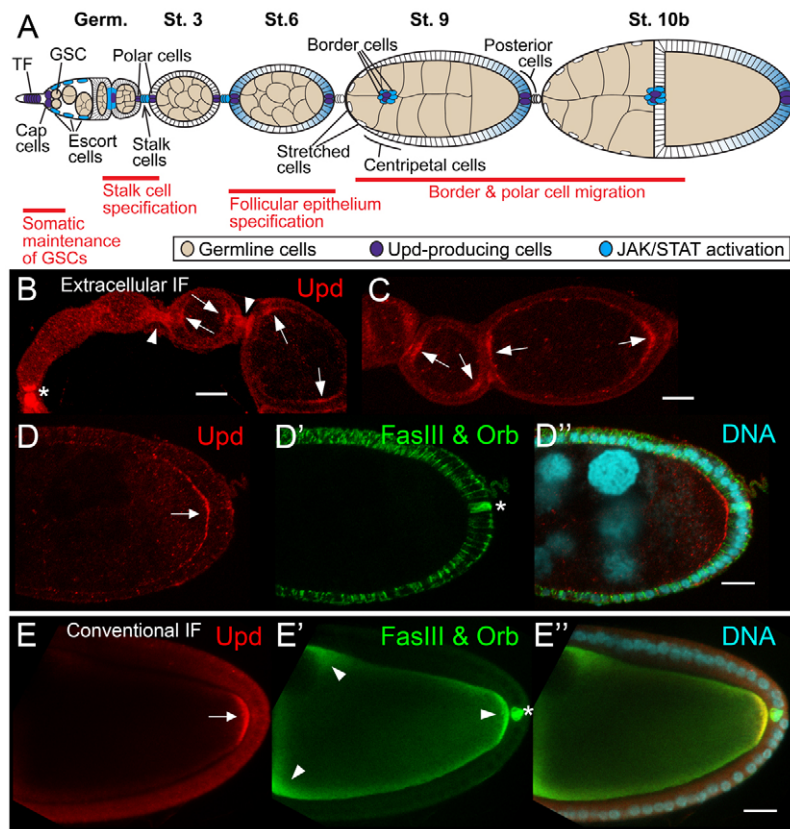
Morphogens are important molecules in development defined by their ability to direct multiple cell fates over a distance in a concentration-dependent manner. It has been well established that extracellular signaling molecules of the Wnt/Wingless (Wg), Hedgehog (Hh) and Bone Morphogenetic Protein (BMP) families act as morphogens during *Drosophila* development. Despite extensive studies on the activities of these morphogens, it is not fully understood how these molecules generate and maintain their gradients in a tissue. One class of molecules that affects the gradient formation and signal transductions of all these morphogen families is heparan sulfate proteoglycans (HSPGs) (Baeg et al., 2001; Fujise et al., 2003; Kirkpatrick and Selleck, 2007; Yan and Lin, 2009). HSPGs are carbohydrate-modified proteins that are abundant in the extracellular matrix and on the cell surface (Esko and Selleck, 2002; Sarrazin et al., 2011). Three families of HSPGs are widely conserved during animal evolution: syndecans, glypicans and perlecanins. In particular, two *Drosophila* HSPGs of the glypican family, *dally* and *dally-like protein* (*dlp*), have been shown to control BMP, Wnt and Hh signaling (Kirkpatrick and Selleck, 2007; Yan and Lin, 2009). A previous study has shown that Upd protein expressed in cultured cells is tightly associated with the extracellular matrix, and the addition of free heparin releases Upd into the medium (Harrison et al., 1998). These observations suggested that Upd associates with HSPGs and thus might be regulated in a mechanism analogous to other morphogens.

In this study, we demonstrate that an extracellular gradient of Upd activates JAK/STAT in a concentration-dependent manner. As is the case for other secreted morphogens, Upd signaling is regulated by glypicans. Mutations in *dally* and *dlp* or in the heparan sulfate (HS) biosynthetic enzymes *sulfateless* (*sfl*) and *HS 2-O sulfotransferase* (*Hs2st*) lead to aberrant JAK/STAT pathway activation and disruption of follicle cell specification. These alterations in JAK/STAT signaling and cell differentiation can be

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**Fig. 1. Upd forms a concentration gradient in *Drosophila* follicular epithelium.** (A) Ovariole schematic illustrates stages of oogenesis, including four distinct roles for JAK/STAT signaling (red), with Upd expression (purple) and levels of JAK/STAT activation (blue) shown. See text for details. (B–E'') Extracellular (B–D'') or conventional (E–E'') immunostaining of wild-type ovaries detects a gradient (arrows) of endogenous Upd (red) from the polar cells (asterisks), marked with anti-FasIII (cell membrane staining in epithelial follicle cells, green at asterisk). Orb protein is detected within the oocyte by conventional staining (also green in E' and E'', but in oocyte cytoplasm at interior of egg chamber, marked by arrowheads), but not the extracellular staining protocol (D–D''). Graded Upd is observed on the apical follicular epithelium (arrows) from approximately stage 4 of the vitellarium (see B for early, C for mid, and D, E for later) and Upd can be observed on the stalks (B, arrowheads) and the terminal filament and cap cells at the tip of the germarium (B, asterisk). Scale bars: 20  $\mu$ m.

attributed to effects on the normal extracellular gradient of Upd by loss of glypicans or changes in their modification. Biochemical and histochemical studies show that Dally and Upd physically interact and colocalize on the surface of *Drosophila* S2 cells. In vivo, Upd accumulation on cells lacking glypicans is dramatically reduced and, reciprocally, is enhanced upon ectopic expression of Dally. These results suggest that *Drosophila* glypicans serve to stabilize the Upd morphogen at the cell surface during oogenesis.

## MATERIALS AND METHODS

### Fly strains

Detailed information for most strains used is found in FlyBase (Tweedie et al., 2009). Flies were raised at 25°C. Oregon R and Canton S were used as wild types. Mutations used were: *dally<sup>gem</sup>*, *dally<sup>D527</sup>*, *dally<sup>80</sup>*, *dlp<sup>1</sup>*, *dlp<sup>A187</sup>*, *sf<sup>9B4</sup>*, *sdc<sup>10608</sup>* and *tro<sup>1SD</sup>*. FRT2A, FRTG13 and FRT101 were used for mosaic analyses and were marked with histone2A-GFP or histone2A-RFP, referred to as 'hisFP'. *Ay-Gal4* (Ito et al., 1997) and *UAS-dally* and *UAS-dlp* were used to ectopically express glypicans. *pnt-lacZ* is an enhancer-trap marker for posterior cells (Roth et al., 1995). *dally<sup>P2</sup>*, *dally-Gal4*, *dome<sup>G0367</sup>* (*dome-lacZ*) (Brown et al., 2001), *Gal-E132* (*upd-Gal4*) and *PD* (*upd-lacZ*) (Tsai and Sun, 2004) are enhancer traps in the respective loci.

### Mutant and misexpression clones

Mutant clones were generated by hs-FLP-mediated recombination (Chou and Perrimon, 1992) induced by incubating animals for 2 hours at 37°C. Tissue-directed misexpression using the Gal4-UAS system (Brand and Perrimon, 1993) and 'Flip-out' clonal ectopic expression (Ito et al., 1997; Neufeld et al., 1998) was performed using standard methods.

### Immunological staining and in situ RNA hybridization

Conventional immunostaining of ovaries was performed as previously described (Fujise et al., 2001; Xi et al., 2003; Hayashi et al., 2009). Extracellular staining was adapted to ovaries from an established protocol (Strigini and Cohen, 2000). Ovaries were dissected in ice-cold Schneider's media plus 2.5% fly extract and 5% fetal bovine serum (FBS) and

incubated with primary antibody overnight on ice, fixed for 20 minutes in ice-cold 3.7% formaldehyde, blocked, and incubated with secondary antibodies.

Antibodies used were: mouse anti-Dlp [1:50, Developmental Studies Hybridoma Bank (DSHB)], mouse anti-FasIII (1:50, DSHB), rabbit anti- $\beta$ -galactosidase (1:500, Cappel), rat anti-E-Cadherin (1:10, DSHB), rabbit anti-GFP (1:500, Molecular Probes), mouse anti-GFP (1:500, MAB3580, Chemicon), anti-phosphoSTAT92E (1:200, Cell Signaling Technology), rabbit anti-Upd (1:800) (Harrison et al., 1998) and mouse anti-Orb (1:30, 4H8, DSHB), rabbit and rat anti-Vasa (1:1000 and 1:2000, respectively, gifts from A. Nakamura, RIKEN Center for Developmental Biology, Kobe, Japan), rat anti-HA (1:500, 3F10, Roche) and mouse anti-Myc (1:500, 9E10, Sigma). Can Get Signal Solution B (TOYOBO) was used for staining with anti-pSTAT92E. Stained ovaries were imaged by confocal microscopy (Nikon C1, Leica TCS SP1 and Leica TCS SP5). Quantification of fluorescence intensity was performed using Nikon Elements software.

In situ hybridizations were performed as previously described (Sato et al., 2007). RNA probes were synthesized by DIG-RNA Labeling Kit (Roche) with PCR-amplified DNA template (primers shown in supplementary material Table S1). Detection of mRNA was performed using AP-linked anti-DIG (*dally* and *dlp*; Roche) or TSA Biotin System (Perkin Elmer) for fluorescence in situ hybridization (FISH) (*upd*).

### Biochemical characterization of Upd and Dally

*Drosophila* S2 cells were grown in Schneider's medium (Invitrogen) supplemented with 10% FBS, 100 U/ml penicillin and 100  $\mu$ g/ml streptomycin. Transient transfections were performed using Effectene (Qiagen) as per manufacturer's instructions. After 72 hours, S2 cells transfected with pAW-Upd-HA in combination with pAc5.1 empty vector (control) or pAW-sec-Dally-Myc were harvested. Cell and supernatant fractions were separated by centrifugation and analyzed by immunoblotting.

For co-immunoprecipitation experiments, 1 ml of each cell supernatant was incubated with anti-cMyc monoclonal antibody-agarose beads (Sigma) overnight at 4°C, washed, eluted by urea and analyzed by immunoblotting.

For cellular immuno-colocalization analysis, S2 cells expressing Dally-Myc and Upd-HA were incubated with anti-HA and anti-Myc in slide chambers for 30 minutes at 25°C, washed, and fixed with 3.7% formaldehyde. Subsequent procedures were performed as described elsewhere (Kleinschmit et al., 2010).

## RESULTS

### Upd forms a concentration gradient during oogenesis

JAK/STAT signaling is essential for at least four separate functions in oogenesis (Fig. 1A). In the anterior germarium, JAK/STAT activity in somatic cells is required for the maintenance of germline stem cells (Decotto and Spradling, 2005; López-Onieva et al., 2008). Subsequently, JAK/STAT activity is essential for specification of multiple follicle cell fates at two distinct stages. In region 3 of the germarium, JAK/STAT activation is needed for the specification of stalk cells, which separate egg chambers. A binary cell fate decision dependent on reaching a threshold level of JAK/STAT activation differentially specifies stalk cells or the default polar cell fate (Baksa et al., 2002; McGregor et al., 2002). Later, at stage 6 in the vitellarium, graded JAK/STAT activation specifies three distinct anterior follicular fates (border, stretched and centripetal cells) and a default main body fate dependent on the level of pathway stimulation (Xi et al., 2003). Because it is the major ligand of the JAK/STAT pathway and is expressed at the poles of developing egg chambers at all stages, it was suggested that Upd acts as a morphogen in this process. Also at that stage, the combination of JAK/STAT and Epidermal growth factor receptor (Egfr) activation at the posterior specifies a single posterior cell fate. Lastly, continued JAK/STAT activation in border cells is necessary to maintain their identity and permit proper migration of the anterior border cell/polar cell cluster (Silver and Montell, 2001; Silver et al., 2005).

To determine the relationship of ligand distribution to specification of follicular fates by the JAK/STAT pathway, anti-Upd was used to examine protein distribution. Both conventional 'fixation-first' and extracellular protein immunostaining protocols (Strigini and Cohen, 2000) were utilized. In the germarium, extracellular staining detected high levels of Upd in the terminal filament and cap cells (Fig. 1B), consistent with the somatic cell function in germline stem cell maintenance. As egg chambers exit the germarium, Upd was also highly enriched in stalk cells and at the apical surface of follicle cells at the anterior and posterior poles, but did not appear to be graded. From approximately stages 4-10, Upd was detected in a gradient along the apical surface of the follicular epithelium (Fig. 1C-D'; supplementary material Fig. S1), consistent with predicted morphogen function in specification of the various epithelial follicle cell fates established by stage 6 (Fig. 1A). Beyond stage 10, Upd persisted in polar cells, but was not clearly graded (not shown), which fits with a requirement for JAK/STAT signaling in border cells. Conventional immunostaining led to similar results (Fig. 1E), but was less sensitive at earlier stages and Upd was not detected as far from the poles. To confirm specificity for extracellular proteins, we simultaneously detected FasIII, an integral membrane protein present at high levels on polar cells, but failed to detect Orb, a cytoplasmic protein in the oocyte (Fig. 1D), whereas both were visible using conventional detection (Fig. 1E). The predominantly apical localization of Upd is consistent with the asymmetrical distribution of *upd* RNA in the follicular epithelium (Van de Bor et al., 2011) and the apicolateral distribution of the Domeless receptor in those cells (Ghigliione et al., 2002). We conclude that Upd is distributed in a gradient during

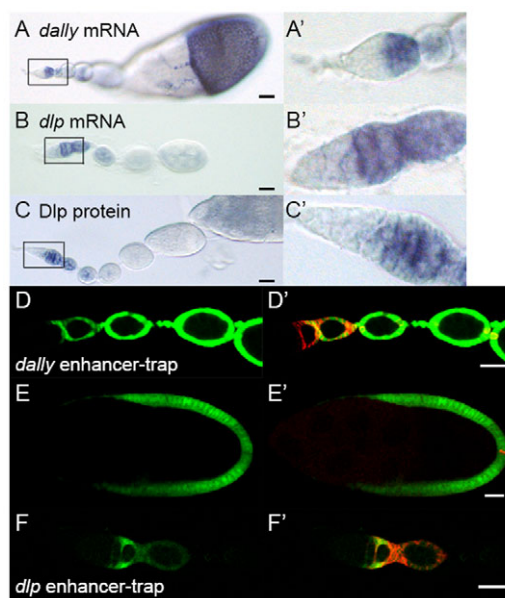
specification of the follicular epithelium and, together with previous observations (Xi et al., 2003), fulfills the criteria of a morphogen in the vitellarium.

### *Drosophila* glypicans are expressed differently in somatic gonadal cells

To determine whether Upd distribution and gradient formation depends on HSPGs, we examined whether HSPG expression in the ovary coincides with JAK/STAT activity. In situ hybridization experiments showed that mRNAs for two glypican genes, *dally* and *dlp*, accumulate in somatic cells at the posterior of the germarium, including precursors of both polar and stalk cells (Fig. 2A-B'). Anti-Dlp antibody staining confirmed Dlp protein accumulation in those cells (Fig. 2C,C'). In addition to expression in the germarium, *dally*, but not *dlp*, was also expressed in the follicular epithelium of egg chambers of the vitellarium older than stage 4 (Fig. 2A-C). Thus, *dally*, and not *dlp*, is expressed at the time and location of specification of the anterior and posterior follicular epithelial cells. GFP enhancer-trap lines in these two loci showed reporter expression patterns consistent with the in situ hybridization data (Fig. 2D-F).

### HS biosynthesis is required for normal differentiation of stalk cells

Expression of *dally* and *dlp* in the polar and stalk cell precursors suggested that HSPGs might be involved in their differentiation. Polar and stalk cells are derived from a common progenitor pool in the germarium and are determined by the combined actions of the Notch and JAK/STAT pathways (Tworoger et al., 1999; Grammont and Irvine, 2001; Baksa et al., 2002; McGregor et al., 2002; Althausen et al., 2005; Assa-Kunik et al., 2007). Notch



**Fig. 2. Expression of *dally* and *dlp* during *Drosophila* oogenesis.** (A-C') In situ hybridization of *dally* (A) and *dlp* (B) mRNA, and anti-Dlp staining (C) show that *dally* and *dlp* are expressed in the posterior of the germarium. Boxed areas in A-C (germaria) are shown at higher magnification in A'-C'. (D-F') Reporter expression (green) for enhancer traps in the *dally* (D-E') and *dally-like* (F,F') loci marks follicle cells from the posterior germarium through late oogenesis for *dally*, but not later than stage 4 for *dlp*. FasIII (red) marks early follicle cells. Scale bars: 20  $\mu$ m.



activation specifies polar cells at the termini of each egg chamber that secrete Upd. This stimulates JAK/STAT activation above a threshold in competent neighbors, specifying them as stalk cells. To examine the role of HSPGs in polar and stalk cell development, we generated mutant clones for *sulfateless* (*sfl*) and analyzed the morphology of developing egg chambers. *sfl* encodes the only *Drosophila* homolog of heparan sulfate *N*-deacetylase/*N*-sulfotransferase (NDST). Because *N*-sulfation of glucosamine residues is essential for subsequent modifications of heparan sulfate chains (Toyoda et al., 2000), *sfl* null mutations are thought to disrupt most, if not all, activities of HS chains and to impair all known HS-dependent pathways (Lin et al., 1999; Lin and Perrimon, 1999) (T. Akiyama and H.N., unpublished results).

In *sfl* clones that include polar cells, both polar and stalk cells differentiated properly (Fig. 3A-A''), indicating that HSPGs are dispensable in polar cells for follicular development. In striking contrast, in *sfl* mutant clones that included presumptive stalk cells, stalk cell differentiation was severely disrupted (Fig. 3B-B''). The number of stalk cells was significantly reduced, often resulting in

the fusion of egg chambers (Fig. 3B, arrow; data not shown). These findings showed that functions of HSPGs in the stalk cells and/or their precursors are critically required for the differentiation of stalk cells but not polar cells. This is consistent with disruption of JAK/STAT signaling (Baksa et al., 2002; McGregor et al., 2002), and suggests a crucial role for HSPGs in JAK/STAT, rather than Notch, signaling in these cells.

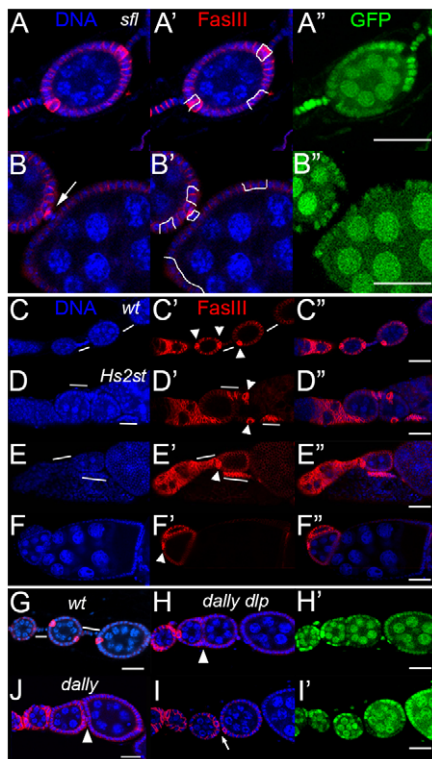
### Normal HS modification is required for stalk cell differentiation

Mutants of *Hs2st* were analyzed to investigate further the requirement for normal HS biosynthesis in polar and stalk cell development. *Hs2st* encodes an HS 2-*O* sulfotransferase responsible for sulfation of the 2-*O* positions of HS. HS in *Hs2st* mutant cells lacks 2-*O* sulfation but has increased levels of 6-*O* sulfation (Kamimura et al., 2006). As a result of this compensation, *Hs2st* mutants are viable, but females are sterile. *Hs2st* mutant ovarioles had normal polar cells, but showed two opposing classes of abnormalities in the number and shape of stalk cells. Some *Hs2st* mutants lacked stalk cells, resulting in fusion of egg chambers (Fig. 3F), but a larger fraction had an increased number of stalk cells that were often thicker than those in wild type (Fig. 3D,E). Furthermore, stalk cells of *Hs2st* mutant ovarioles showed abnormalities in the expression of some molecular markers. Whereas Big brain and  $\alpha$ -Spectrin were always expressed normally (not shown;  $n=39$  and 59, respectively), expression of FasIII persisted abnormally in stalk cells of most egg chambers (41/42) older than stage 4 (Fig. 3D-F). FasIII is normally expressed in the common precursors of polar and stalk cells within the germarium, overlapping the *dally*- and *dlp*-expressing cells (see also Fig. 2). After stage 4 in the vitellarium, FasIII expression becomes restricted to only the polar cells (Fig. 3C). Persistence of FasIII expression in *Hs2st* mutant stalk cells suggests that they might not be fully differentiated. Consistent with this hypothesis, stalk cells of *Hs2st* mutants fail to show the normal stacked discoidal cell shape. Together with the results of the *sfl* clones, these observations show that the differentiation of stalk cells, but not polar cells, requires properly modified HS.

### *dally* and *dlp* are partially redundant in stalk cell differentiation

Stalk cell defects in *sfl* and *Hs2st* mutants and expression of *dally* and *dlp* in the polar/stalk cell precursors suggested that glypicans might be essential to stalk cell differentiation. To test this hypothesis, ovaries mutant for both glypicans were examined. Ovaries bearing *dally/dlp* double-mutant clones in stalk cells showed defects similar to *sfl* clones. All *dally/dlp* clones located in the stalk cell region showed significant reduction of stalk cells (Fig. 3H-I; supplementary material Table S2). Frequently, stalk cell defects were sufficiently severe to result in fused egg chambers (Fig. 3H), yet there were never any defects observed in polar cell differentiation (Fig. 3I). Confocal optical sectioning confirmed that polar cells were not mutant in fused egg chambers (not shown), indicating that loss of glypicans in the Upd-receiving stalk cells, but not in the Upd-expressing polar cells, is responsible for the fused egg chambers. In contrast to egg chambers with stalk cells mutant for *dally* and *dlp*, egg chambers with double-mutant polar cells developed normally (supplementary material Table S2). Thus, glypicans are required for stalk, but not polar cell, development, consistent with the *sfl* phenotype.

To determine whether one or both glypicans are required for stalk cell formation, single mutants were analyzed. Clones of a *dlp* mutant



**Fig. 3. Stalk cell differentiation is disrupted by loss of HSPGs or modifying enzymes.** (A-B'') Mutant clones of *sfl* (A-A'': loss of GFP, green, shown in A'' and outlined in A') in polar cells appeared normal, but clones in stalk cells (B-B'') disrupted proper stalk cell differentiation (arrow). (C-C'') Wild-type egg chambers express FasIII (red) in undifferentiated precursors of the germarium and polar cells (arrowheads), but not stalk cells (lines) of the vitellarium. (D-F'') In many *Hs2st* mutant ovarioles, the stalk cells (white lines) form thick layers and continue to express FasIII. The polar cells develop normally (arrowheads). (G) Wild-type egg chambers contain five to eight stalk cells between chambers in the vitellarium (lines). (H-I'') *dally/dlp* double-mutant clones marked by loss of GFP (green) result in fused egg chambers (arrowhead in H) or significantly reduced stalk cell numbers (I, white arrow). (J) Homozygous mutants for *dally* (*dally<sup>gem</sup>/dally<sup>527</sup>*) also show reduced stalk cell numbers (arrowhead). TO-PRO3 marks DNA (blue). Scale bars: 20  $\mu$ m.

in either stalk cells or polar cells showed no effects on specification of either cell type (supplementary material Table S2). By contrast, ovaries from females carrying a heteroallelic combination of *dally* mutations showed strong impairment of stalk cell specification. Stalk cell numbers were reduced in *dally* mutants relative to wild-type controls (Fig. 3J; supplementary material Table S2). *dally* mutants averaged 3.18 cells per stalk in stage 2-7 egg chambers, versus 6.18 in wild type ( $n=40$  and 39, respectively). However, as measured by the proportion of chambers with fewer than five stalk cells, stalk cell clones of the glypican double-mutant combination were more severely impaired than *dally* mutants (supplementary material Table S2). In conclusion, the observation of defects in *dally* homozygous mutants, but not *dlp* clones, strongly suggests that *dally* has the major role in promoting stalk cell specification. However, these defects are more severe in *dally/dlp* double-mutant clones, suggesting that Dlp contributes to this function; thus, these molecules are partially redundant in the gerarium.

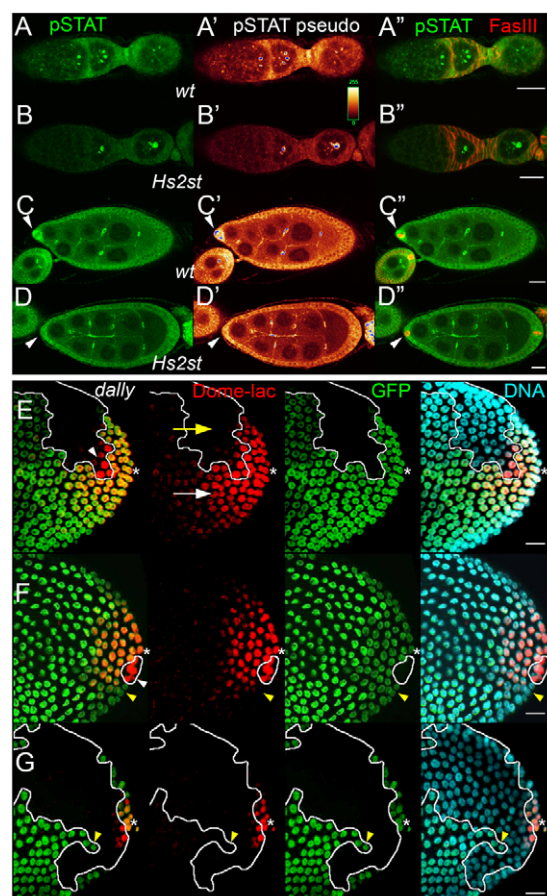
### HS biosynthesis is required for JAK/STAT signaling in the ovary

The specific stalk cell differentiation impairment shown above, together with the previously demonstrated interaction of Upd with heparin (Harrison et al., 1998), suggested that HSPGs might promote JAK/STAT signaling. To test this, we analyzed JAK/STAT signaling in the abnormal stalk cells resulting from *Hs2st* mutants and in the follicular epithelium of late-stage egg chambers with glypican mutant clones. To examine JAK/STAT signaling within defective stalk cells of *Hs2st* mutant ovaries, we used an antibody that is specific for the activated phosphorylated Stat92E (anti-pSTAT) (Li et al., 2003; Wawersik et al., 2005). In wild type, anti-pSTAT was specifically detected in the Upd-receiving cells in the posterior gerarium and stalk cells (Fig. 4A). The staining patterns in the ovary demonstrated that the antibody is useful as a marker for high-threshold JAK/STAT signaling activity. JAK/STAT activity is severely decreased in such cells in *Hs2st* mutant ovaries (Fig. 4B; supplementary material Table S3), without affecting *upd* expression in polar cells (supplementary material Fig. S3). Signal intensity of the mutant stalk cells and precursors was almost indistinguishable from background staining, indicating that JAK/STAT activity does not reach the threshold detectable by this antibody in the mutant cells. This supports the hypothesis that the observed phenotypes of the *Hs2st* mutants are caused by alterations of JAK/STAT signaling.

After stalk cell differentiation, the JAK/STAT pathway regulates the anterior-posterior patterning of the follicular epithelium (Xi et al., 2003) and border cell migration (Beccari et al., 2002). Reduction of pSTAT staining in *Hs2st* mutant cells was also observed in these later stages (supplementary material Table S3) and was illustrated by loss of anti-pSTAT staining in border cells of a *Hs2st* mutant egg chamber compared with wild type (Fig. 4C-D"). Collectively, these observations are consistent with the idea that HS is crucial for JAK/STAT signaling.

### Glypicans promote JAK/STAT signaling in the follicular epithelium

To assess directly the influence of specific HSPGs on the JAK/STAT pathway, analysis of mutant clones for each was performed. Because anti-pSTAT does not robustly reflect graded activation in the vitellarium, we used an enhancer-trap reporter for JAK/STAT activation, *dome-lacZ* (Xi et al., 2003; Hombria et al., 2005), to examine graded JAK/STAT activation in stage 8-10 egg chambers. Though specification of follicular epithelium has



**Fig. 4. JAK/STAT activity is decreased in *dally* mutant cells.**

(A-D") Anti-pSTAT (green) shows greater accumulation of activated STAT in wild-type ovaries (A,C) than in *Hs2st* mutants (B,D). High levels of pSTAT signal were observed in stalk cells as well as their precursors in germaria of wild-type ovaries (A), but not *Hs2st* mutants (B). pSTAT accumulation is much stronger in border cells (arrowhead, adjacent to FasIII-marked polar cells, red) in wild-type egg chambers of the vitellarium (C) than in *Hs2st* mutant chambers (D, arrowhead). Pseudocolored representations of pSTAT staining (A'-D') highlight the quantitative differences in signal intensity. LUT key is shown with dark to white representing weak to strong signal, and blue representing saturation. Ring canal staining (central region of each egg) is non-specific (due to phosphotyrosine) and demonstrates similar detection sensitivity across genotypes. (E-G) JAK/STAT activity, marked by *dome-lacZ* (red) is graded with the highest level adjacent to the polar cells (asterisks). *dome-lacZ* is lost or reduced in cells mutant for *dally* (E-G, lack of GFP outlined in white) compared with wild-type cells at the same distance from the pole (compare yellow and white arrows in E). *dally* mutant cells (white outline) closest to the poles have detectable *dome-lacZ* (E,F, white arrowheads), whereas cells farther from the poles lack expression. In chambers for which *dally* mutant clones lie between the polar cells and wild-type cells within the normal *dome-lacZ* expression domain, the distant wild-type cells show no expression or lower expression than wild-type cells at an equal distance from the pole with no intervening mutant cells (F,G, yellow arrowheads). Scale bars: 20 μm.

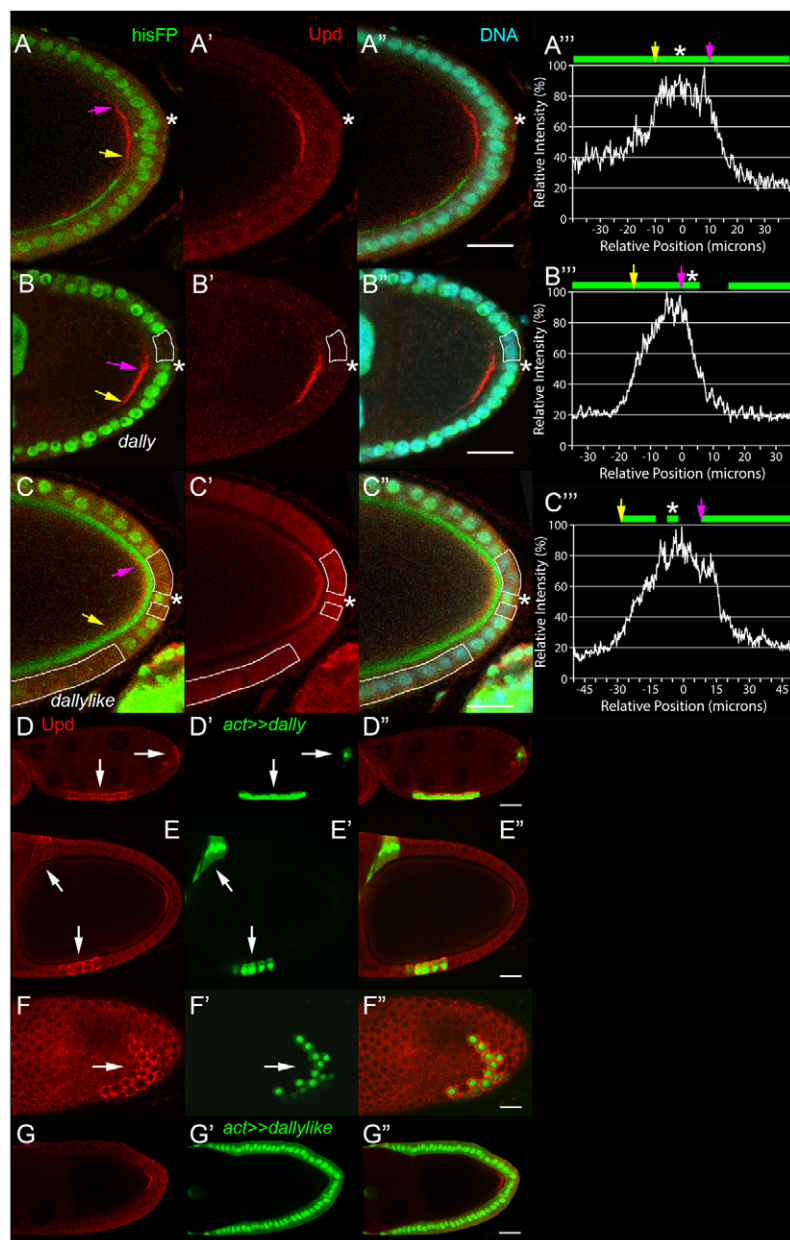
occurred before this stage, JAK/STAT signaling and Upd protein persist in a gradient. Because anterior follicle cells migrate extensively, whereas posterior cells maintain position relative to the pole, we focused on posterior signaling. Clonal analysis of follicle cells homozygous for a *dally* mutation revealed a sharp decline of



the *dome-lacZ* signal compared with wild-type cells (Fig. 4E, arrows), suggesting that *dally* participates in JAK/STAT signaling. Interestingly, reduction of JAK/STAT activity in *dally* clones was non-autonomous, as expression of *dome-lacZ* was seen in *dally* mutant follicle cells at the edge of clones closest to the pole (Fig. 4E,F, white arrowheads). This observation is consistent with the effects of HSPGs on other pathways in the developing wing (Han et al., 2004), and could reflect the activity of this class of molecules as trans co-receptors (Kramer and Yost, 2002; Jakobsson et al., 2006; Hayashi et al., 2009). However, a 'shadow' of reduced activation was observed in wild-type cells separated from the pole by *dally* mutant cells (Fig. 4F,G, yellow arrowheads). The appearance of wild-type (*dally*-positive) cells with reduced JAK/STAT activation adjacent to the distal side of mutant clones relative to the pole suggests that Dally influences the accumulation of the Upd ligand and is not solely a co-factor for activation of the Dome receptor. This is also consistent with effects of HSPGs in the wing disc on other known morphogens (Takei et al., 2004).

### Dally regulates Upd distribution in the follicular epithelium

To determine whether glypicans are required for normal distribution of Upd, localization in mutant follicle cell clones was evaluated using an anti-Upd antibody. Compared with the distribution of Upd in wild-type chambers (Fig. 5A), the concentration of extracellular Upd protein is markedly decreased on cells mutant for *dally* (Fig. 5B, cells lacking green). Fluorescence intensity measurements (Fig. 5B'') show that the drop in Upd protein across mutant cells has a much greater slope than over wild-type cells (Fig. 5B, green cells). By contrast, we detected no marked change in the distribution of Upd near cells mutant for *dlp* in late-stage egg chambers (Fig. 5C, cells lacking green). Thus, Dally regulates the extracellular distribution of Upd protein and plays a major role in Upd gradient formation along the follicular epithelium in these later-stage egg chambers. This is consistent with the observed lack of Dlp accumulation, but continued expression of Dally in the vitellarium (Fig. 2).



**Fig. 5. Dally mediates accumulation of the Upd protein.** (A-C'') In comparison with graded polar accumulation of Upd in late stage wild-type egg chambers (A), in mosaic chambers of a *dally* mutant [B, loss of histone-Fluorescent Protein (hisFP) is outlined], Upd (red) is abruptly reduced on the apical surface of mutant follicle cells (B, not green) compared with wild-type follicle cells (B, green). Upd distribution appears unaffected on *dlp* mutant cells (C, loss of hisFP is outlined). Asterisks mark the positions of the polar cells. Graphs (A''-C'') plot signal intensity of Upd staining along the apical surface of the follicle cells. Colored arrows on images and graphs indicate corresponding positions. Green bars at top of graphs correspond to positions of wild-type (hisFP positive) cells, whereas breaks in bars represent positions of mutant clone cells. (D-F'') In clones (marked green by hisFP, arrows), cells expressing *dally* under the control of an Act5C-GAL4 driver sequester high concentrations of Upd protein (red), but it is not restricted to the apical epithelium. (G-G'') In clones (marked green by hisFP), cells expressing *dlp* under the control of an Act5C-GAL4 driver do not show any alteration in Upd accumulation. Scale bars: 20  $\mu$ m.

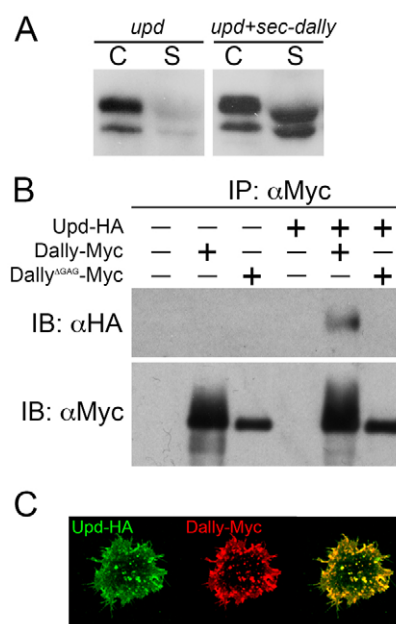
We also examined the possible role of the other types of HSPGs, the syndecan (Sdc) and perlecan (Trol), in Upd function in the ovary. In mutant follicle cell clones of *sdc* or *trol*, there was no observable effect on *dome-lacZ* or *pnt-lacZ* reporter expression, distribution of Upd protein, or follicle cell morphology and migration (supplementary material Fig. S2). Because Trol is secreted, we cannot exclude the possibility of non-autonomous compensation of mutant cells by wild-type neighbors, but even large clones of *trol* mutant cells showed no defects in these assays. We conclude that glypicans play the primary role of HSPGs in regulating the distribution and signaling of Upd during oogenesis.

The loss-of-function experiments described above demonstrate that glypicans are necessary for proper follicular distribution of Upd, but not whether they are sufficient to alter Upd distribution. To test this, flip-out clones were generated that express *dally* under the control of a strong promoter (*act>>dally*) and Upd protein accumulation was analyzed in these ovaries. Interestingly, in cells that express high levels of *dally* (Fig. 5D-F, green), there is a much greater accumulation of Upd (red) than in surrounding cells with normal *dally* expression. Furthermore, Upd on *act>>dally* cells is not restricted to the apical surface, but is also found on the lateral and basal surfaces (Fig. 5D-F, arrows). This suggests that ectopic Dally is not localized with normal polarity or that those cells have lost proper apico-basal polarity. Increased accumulation of Upd is not due to transcriptional activation caused by *dally* expression, as there was no effect on either an enhancer-trap reporter for *upd* (supplementary material Fig. S4) or on in situ hybridization to *upd* (not shown). Interestingly, similar ectopic Dlp did not alter accumulation of Upd (Fig. 5G) nor expression of *upd* (not shown). These results indicate that Dally is sufficient to direct sequestration of Upd, but Dlp is not.

### Upd and Dally form a complex in vitro

HSPGs serve as co-receptors for growth factor signaling by binding to ligands. This is thought to facilitate formation of the signaling complex or affect its stability on the cell surface to modulate signal strength. To establish that HSPGs function as a cell surface component of the JAK/STAT pathway, we investigated whether Dally physically interacts with Upd. As previously shown (Harrison et al., 1998), C-terminally HA-epitope tagged Upd (Upd-HA) expressed in S2 cells was detected as two forms that migrate at 54 and 62 kDa (Fig. 6A). The smaller form is presumed to be the mature, processed peptide, whereas the larger form matches the predicted size of the unprocessed protein, retaining the signal sequence. Most Upd protein was associated with the cell fraction, with very little free in the supernatant (Fig. 6A, left panel). When Upd was co-expressed with a secreted form of Dally, the amount of Upd in the soluble fraction was substantially increased, consistent with the idea that Upd is normally associated with HSPGs on the surface of S2 cells (Fig. 6A, right panel).

To confirm physical interaction by co-immunoprecipitation (co-IP), *upd-HA* was expressed with or without the secreted form of Dally-Myc in S2 cells. Dally-Myc was immunoprecipitated from conditioned medium with anti-Myc and precipitates were analyzed by western blotting. Upd-HA was precipitated only when Dally and Upd were co-expressed, indicating that Upd forms a complex with Dally (Fig. 6B). Binding was abolished when we used an HS-deficient form of Dally (Dally<sup>AGAG</sup>), in which all HS-attachment serine residues are substituted to alanine (Kirkpatrick et al., 2006). The binding of Upd to Dally but not Dally<sup>AGAG</sup> indicates that interaction is mainly mediated through HS chains on Dally.



**Fig. 6. Binding of Upd to Dally.** (A) Upd-HA was expressed in S2 cells with or without a secreted form of Dally-Myc. Fractions from the cell pellet (c) and supernatant (s) were probed with anti-HA antibody. (B) Upd-HA was expressed in S2 cells with or without a secreted form of Dally-Myc or Dally<sup>AGAG</sup>-Myc. Protein was recovered from conditioned media using an anti-Myc antibody and precipitates were blotted and probed with anti-HA and anti-Myc antibodies. Upd was recovered in the precipitate when co-expressed with Dally-Myc, but not Dally<sup>AGAG</sup>-Myc. Cells expressing only Myc-Dally or Upd proteins were used as controls. (C) An S2 cell expressing Upd-HA and Dally-Myc shows extensive colocalization on the surface (anti-HA and anti-Myc antibodies).

The cellular localization of Upd and Dally was examined by expressing *upd-HA* and *dally-Myc* in S2 cells. Using an extracellular staining protocol with anti-HA and anti-Myc antibodies, both Upd and Dally were detected as dots on the cell surface with extensively overlapping signals (Fig. 6C). We conclude that Upd and Dally colocalize on the S2 cell surface. Collectively, our studies strongly suggest that *Drosophila* glypicans serve as co-receptors of Upd to regulate its gradient formation and signal transduction.

### DISCUSSION

Quantitative spatial regulation of JAK/STAT signaling is crucial for proper specification of *Drosophila* follicle cells, particularly in the proposed morphogen-directed patterning of the follicular epithelium. We demonstrated here that the Upd ligand forms an extracellular concentration gradient along the apical surface of the epithelium within chambers of the vitellarium, consistent with JAK/STAT activation. Together with prior observations, these data provide strong evidence that Upd acts as a morphogen to organize the *Drosophila* follicular epithelium. Upd is the first JAK/STAT ligand shown to act as a morphogen in any animal. Upd is expressed in tissues besides the ovary, including eye and wing discs, embryonic epidermis, gut and many others. In some tissues, Upd distribution is graded, although it is not known whether it acts as a morphogen elsewhere. Moreover, two other JAK/STAT ligands, Unpaired2 and Unpaired3, are expressed in patterns that partially overlap with Upd (Gilbert et al., 2005; Hombria et al.,

2005) including *upd3* in the polar cells (L. Wang and D.A.H., unpublished data). Protein similarities raise the possibility that these ligands might also serve as morphogens.

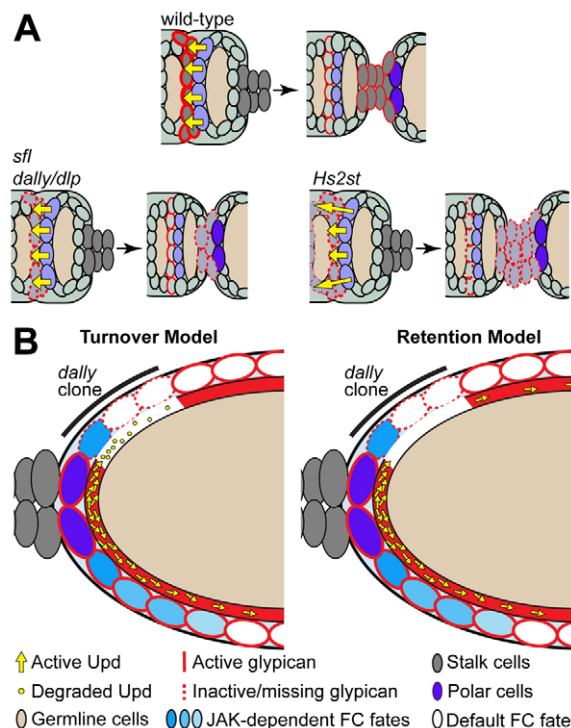
Like other established morphogens (Strigini, 2005; Kirkpatrick and Selleck, 2007; Yan and Lin, 2009; Sarrazin et al., 2011), the formation of an Upd gradient is affected by HSPGs. We demonstrated here that glypicans with intact HS chains are essential in the ovaries for proper Upd distribution, JAK/STAT signaling, and stalk cells specification. Furthermore, co-IP and immunolocalization experiments showed that Dally forms a complex with Upd on the cell surface. These data suggest that Dally serves as a Upd co-receptor, stabilizing Upd on the surface of receiving cells. However, the purpose of this cell surface stabilization cannot yet be determined. HSPGs have the potential to alter retention of ligand at the cell surface, to reduce the turnover of ligand, or both (Fig. 7B) (Sperinde and Nugent, 2000;

Belenkaya et al., 2004; Akiyama et al., 2008). In support of a model in which Dally retains ligand, we observed significant accumulation of Upd on cells ectopically expressing high levels of Dally, even at large distances from the ligand source. By contrast, mosaic loss of Dally does not result in accumulation of Upd on wild-type cells on the distal side of clones, nor is there a visibly shallower gradient of Upd accumulation adjacent to clones. These results are consistent with increased turnover of Upd in the absence of association with Dally. These mechanisms need not be mutually exclusive. Furthermore, a recent study showed that in BMP signaling, HS facilitates a novel mechanism of promoting signaling through the recruitment of type II receptors to BMP-type I receptor complexes (Kuo et al., 2010). This highlights the potential for alternative or multiple mechanisms through which glypicans may facilitate Upd signaling. Moreover, the mechanisms could be distinct between stalk cell and follicular epithelium specification.

Dally and Dlp appear to be partially redundant as co-receptors for Upd during early oogenesis. Stalk cell development was strongly disrupted by *dally/dlp* double mutations, less severely affected by *dally* mutations, and not detectably affected by *dlp* mutations. This indicates that the two glypicans have a similar, overlapping function in JAK/STAT signaling, but *dally* apparently plays a more significant role at this stage. The functional redundancy is similar for morphogens in wing imaginal discs (Belenkaya et al., 2004; Han et al., 2005), although they can also have unique or even opposing roles in some other signaling contexts (Baeg et al., 2004; Kirkpatrick et al., 2004; Williams et al., 2010). In later stages of oogenesis, Upd gradient formation was severely impaired in *dally* mutant clones, but unaffected by loss of *dlp*, suggesting that Dally might be the only glypican used at this stage. This is consistent with the co-expression of the glypicans in the germarium, but exclusive expression of *dally* in later oogenesis. Redundancy may explain why previous studies, including genome-wide RNAi (Baeg et al., 2005; Müller et al., 2005) and genetic interaction screens (Bach et al., 2003; Mukherjee et al., 2006), did not identify glypicans as components of JAK/STAT signaling. By contrast, loss of syndecan or perlecan had no effect, indicating dispensability for JAK/STAT signaling in the ovary, though they might contribute in other tissues.

Loss of *Hs2st* or *sfl* caused aberrant numbers of stalk cells, suggesting that heparan sulfate chains on glypicans are important for their role in JAK/STAT signaling. Intriguingly, *Hs2st* mutants showed opposing effects on the formation of stalk cells: reduction and hyperplasia of the stalk. In fact, it is not uncommon for HSPG-related mutants to show phenotypes resulting from both reduction and increase of signaling. As HSPGs could both facilitate signal reception on the cell surface and restrict ligand diffusion, their disruption may result in both reduced signaling and expansion of ligand distribution. Therefore, our observations of *Hs2st* mutant ovarioles implicate HSPGs in signal reception and ligand distribution in the JAK/STAT pathway during stalk cell differentiation. Furthermore, *Hs2st* ovaries showed normal expression of some stalk cell markers but retained FasIII expression, suggesting that stalk cells are only partially differentiated in these mutants. Interestingly, these phenotypes are similar to those caused by Upd misexpression (McGregor et al., 2002). These results could be explained by distribution of Upd at lower concentration, but over a greater distance in *Hs2st* mutants (Fig. 7A), implicating 2-O sulfation in ligand distribution.

We present here a new molecular model for the study of morphogen signaling, as well as a novel tissue in which to examine morphogen function: the follicular epithelium. This tissue has



**Fig. 7. Models for the functions of HSPGs in stalk cell and follicular epithelial specification.** (A) In the germarium, Upd is secreted by the presumptive polar cells. In wild type (top row), Dally and Dlp (red) expressed in stalk cell precursors regulate JAK/STAT activity by serving as co-receptors for Upd (yellow). In *sfl* and *dally/dlp* mutant clones (second row, left), loss of the co-receptor activity results in failure of stalk cell differentiation. Abnormal HS modification in *Hs2st* mutants (second row, right) may permit Upd distribution beyond the normal target cells, causing inappropriate stalk cell induction in surrounding cells, leading to stalk hyperplasia. (B) In the vitellarium, graded Upd secreted by the polar cells specifies multiple follicular fates, dependent on levels of JAK/STAT activity. In wild-type follicular epithelium (lower half of egg chambers), Dally in the ECM (red) associates with Upd (yellow) on the apical surface of follicle cells to mediate graded Upd distribution. In *dally* mutant clones (dotted lines, upper half), Upd distribution is disrupted, although the mechanism is uncertain. In the turnover model (left), Dally binds Upd to protect it from degradation. On cells lacking Dally, Upd is released and degraded. In the retention model (right), Dally binds Upd to maintain distribution near the poles. On cells lacking Dally, Upd is not retained and diffuses to distant *dally*<sup>+</sup> follicle cells, but is insufficient for cell fate induction.



several distinctions from the other *Drosophila* models, primarily the early embryo and the imaginal discs. In both the embryo and the imaginal discs, signaling occurs within an epithelium adjacent to a fluid-filled space: the perivitelline space in the embryo and the lumen of the imaginal discs. As a consequence, extracellular signals in that environment are potentially subject to rapid diffusion if not tethered to the epithelium. Furthermore, the follicular epithelium develops in direct juxtaposition to the germline cells of a cyst, with well-established communication between these tissues at multiple stages of oogenesis. This arrangement creates a unique environment for morphogen signaling in the follicular epithelium in which the apical signals are contained and potentially influenced by the neighboring germline cells. By contrast, the epithelia of the early embryo and imaginal discs are largely autonomous and develop in the absence of close contact with other tissues, although signals from the follicular epithelium loaded into the perivitelline space prior to completion of egg development and fertilization do mediate morphogen signaling in the embryo. Morphogen signaling by Upd in the follicular epithelium is a better model of such communication in organs and complex tissue development, such as vertebrate urogenital or limb development. The ability to exploit powerful *Drosophila* genetic and developmental tools to study morphogen signaling in this accessible system promises to aid our understanding of development of such complex tissues.

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#### Competing interests statement

The authors declare no competing financial interests.

#### Supplementary material

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