

Drosophila Glypicans Regulate Follicle Stem Cell Maintenance and
Niche Competition

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Dedication

For MaGiFra

Abstract

Several types of stem cells are regularly replaced by progenitor cells through competition to ensure the maintenance of a healthy stem cell population in the niche. However, the molecular basis underlying this maintenance and competition for niche occupancy is poorly understood. Here, it is demonstrated that two members of the heparan sulfate proteoglycan proteins in *Drosophila*, Dally and Dally-like (Dlp), differentially regulate follicle stem cell (FSC) maintenance and FSC competitiveness for niche occupancy. We find that *dally* is essential for normal FSC maintenance while *dlp* mutant FSC progenitors outcompete normal FSCs and eventually occupy the entire epithelial sheet. In addition, Dally and Dlp have both partially redundant and distinct roles in regulating JAK/STAT, Wg and Hh signaling in FSCs. This suggests that glypicans regulate the HS-dependent signaling pathway, and therefore influence FSC competitive behavior that was observed.

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List of Abbreviations

BMP	Bone morphogenetic protein
Dally	Division abnormally delayed
Dlp	Dally-like protein
Dph	Days post heat shock
FSC	Follicle stem cell
Fz3	Frizzled 3
GSC	Germline stem cell
Hh	Hedgehog
HS	Heparan sulfate
HSPG	Heparan sulfate proteoglycan
MARCM	Mosaic analysis with a repressible cell marker
Ptc	Patched
Sfl	Sulfateless
Upd	Unpaired
Wg	Wingless

Introduction

Adult Stem Cell and Their Niches

Adult stem cells are tissue specific (Stine and Matunis, 2014). They self-renew to replace old and/or damaged cells to ensure the overall fitness of the cell population (Jiang *et al.*, 2009). In order to replace damaged or aged cells, they migrate and differentiate into the site of injury and gradually replace the cells (Xie *et al.*, 2010; López-Lázaro, 2015a). To ensure the properties of a stem cell, the adult stem cells have to be carefully monitored and maintained in a microenvironment called the stem cell niche.

A niche is a specialized microenvironment that adult stem cells reside in. It is supported by niche cells, which provide self-renewal signals, and extracellular components such as the basement membrane. Though niches vary in different tissues and do not necessarily share the same structures, these components maintain a suitable environment to support the stem cells (Losick *et al.*, 2011). Any defects in the niche could also contribute to subsequent damages to the stem cell (Ferraro *et al.*, 2010). Both the stem cells and their niches influence each other: a defective niche influences stem cell phenotype, while mutant stem cells could prevent normal stem cells from localizing into the niche (Colmone *et al.*, 2008).

Although stem cell activities and the stem cell pool are maintained throughout the lifetime of the organism, individual stem cells undergo regular turnover to ensure that the niche is always occupied by healthy and functional

stem cells (Ryu *et al.*, 2003). To ensure this, cells are regularly replaced by their daughters, or are outcompeted by a healthier stem cell (Jin *et al.*, 2008; Xie and Spradling, 2000). There are two possible ways in which competition can occur in the stem cell niche. First, the niche is occupied by healthy stem cells for homeostasis and later tissue regeneration. Second, the niche could be occupied by stem cells that have acquired spontaneous mutations that boosted their competitiveness for niche occupancy (López-Lázaro, 2015b). The latter simulates early stages of cancer where the mutations will enable mutant stem cells and their progenitors to outcompete other stem cells from their niches and expand aggressively in the tissue (Nystul and Spradling, 2007; Visvader, 2011).

Healthy stem cells are necessary to ensure that the fitness of a cell population in a tissue is maintained when there is an injury or damage, or when cells within a population age or die. When adult stem cells are not properly maintained, they can often overproliferate, resulting in tumor formation. Therefore, to study the complicated relationship between stem cells with their niches and stem cell behavior, we will utilize the *Drosophila* follicle stem cells (FSCs) located in the *Drosophila* ovary.

***Drosophila* Ovary and Follicle Stem Cells as a Model System**

Drosophila melanogaster has been utilized as a model organism system for decades, hence, the tools used to study *Drosophila* have become more sophisticated, along with the advancement of technology. *Drosophila* ovarian follicle stem cell (FSC) has been a widely adopted and useful model to study

epithelial stem cell behavior (Losick *et al.*, 2011; Sahai-Hernandez *et al.*, 2012).

The *Drosophila* ovary consists of 16 to 20 discrete structures called ovarioles. Each ovariole is a string of developing egg chambers in progressive developmental stages. The developmental stages start from the anteriormost region of the ovariole, the germarium, to the most posterior end of the ovariole, stage 14 (Jia *et al.*, 2016).

The stem cells are located in the germarium, including the germline stem cells (GSCs), FSCs, and their niches. The GSCs give rise to the germline cells in the ovary, which will eventually become the oocyte. Each GSC undergoes four divisions, which in turn produces 16 germline cells that will form a germline cyst in region 2a of the germarium. The cysts migrate posteriorly and form a single line when they contact the FSCs located at the 2a/2b border; they remain in a single file after the 2b border (Nystul and Spradling, 2010). One of the germline cells in the cyst will become the future oocyte while the rest of the germline cells become nurse cells. The oocyte will maintain in contact with the posterior polar cells throughout development (Nystul and Spradling, 2010). Conversely, FSCs differentiate into three somatic cell types: prefollicle cells that surround the egg chambers, polar cells, and stalk cells, which connect each egg chambers in the ovariole. These three main cell types make up the epithelial sheet of the ovariole. As the germline cyst slowly moves posteriorly, the prefollicle cells will surround the cyst and mature as the cyst exits the germarium, eventually becoming an independent egg chamber.

The somatic cells are generated by the two FSCs found in the germarium and located within a specialized position. Each FSC is located at opposite ends along the 2a/2b border (Fig. 1A) (Margolis and Spradling, 1995). Furthermore, the FSCs have distinct triangular shapes and have a low Fasciclin III (FasIII) level (Nystul and Spradling, 2007).

The two FSCs cycle between each other to differentiate as each germline cyst enters the 2b region. When each FSC differentiates, the daughters can migrate along two different routes. They could either migrate laterally across the 2a/2b border to the other FSC niche, or migrate posteriorly in the 2b region. When FSC daughters migrate laterally, or cross migrate, they will reach the other FSC niche and can replace the original FSC in that niche. This suggests the capability of the daughter cell to replace or outcompete a host FSC from its niche (Nystul and Spradling, 2007). Previous studies have shown that FSCs have a slow turnover rate (Song and Xie, 2003; Johnston *et al.*, 2016). Cells can migrate along the 2a/2b border (cross migrating) or more posteriorly into the 2b region (posterior migration). It was demonstrated that FSC progenitors cross migrate or posteriorly migrate in roughly equal incidences (Nystul and Spradling, 2010).

The FSC behavior and maintenance in the niche should be tightly regulated for the proper development of the ovary. The presence of multiple signaling pathways are necessary to maintain the FSC activities and behaviors, including Wingless (Wg)/Wnt signaling, Hedgehog (Hh) signaling, Bone Morphogenetic Protein (BMP) signaling, and JAK/STAT signaling pathways (Fujise *et al.*, 2003;

Kirilly and Selleck, 2007; Morrison and Spradling, 2008; Dejima *et al.*, 2011; Hayashi *et al.*, 2012).

Wg, Hh, and JAK/STAT Signaling in the FSC and FSC niches

Several signaling pathways have been established to be crucial during development. Of those, there are three pathways of our interest that play a role in FSC niche maintenance, namely Wg/Wnt signaling, Hh signaling, and JAK/STAT signaling pathways.

The Wingless (Wg)/Wnt signaling pathway has been known to be a major player in carcinogenesis, tissue repair and regeneration (Oshima *et al.*, 2001; Beachy *et al.*, 2004). In the *Drosophila* germarium, the Wg ligand is secreted by the escort cells and received in the FSCs (Sahai-Hernandez and Nystul, 2013). Wg signaling regulates FSC maintenance in the niche area and the proliferation of the follicle cells in the germarium (Sahai-Hernandez and Nystul, 2013). This proper FSC maintenance and behavior is disrupted when Wg signaling is not regulated at a precise level. Overactivation of Wg signaling results in the overproliferation of FSCs and their lineage. This mimics tumor formation which has been found to cause several cancers (Chan *et al.*, 1999). Reduction in the amount of Wg signaling results in a decrease in number of FSC progenitors (Song and Xie, 2003) and causes degeneration of the epithelial structure (Pinto *et al.*, 2003). However, constitutively active Wg signaling leads to rapid loss of the FSCs from their niche (Song and Xie, 2003). Hence, precise regulation of Wg signaling is essential to ensure normal FSC maintenance and behavior.

Hh signaling is another pathway known to maintain progenitor cells and acts as a stem cell factor and promotes regeneration (Zhang and Kalderon, 2000; Machold *et al.*, 2003; Beachy *et al.*, 2004). The Hh ligand is produced and secreted by the terminal filament, cap cells, and escort cells in the *Drosophila* germarium and is required for the maintenance of FSCs. In addition, it is necessary for the proper proliferation and differentiation of the FSC progenitor cells (Forbes *et al.*, 1996; Sahai-Hernandez and Nystul, 2013). It was observed that low Hh signaling reduces the lifespan of FSCs and results in a lower proliferation rate (Zhang and Kalderon, 2000; Hartman *et al.*, 2010). On the other hand, when there is overactivation of Hh signaling, there is an increase in potential of cancer and metastatic tumors (Karhadkar *et al.*, 2004; Beachy *et al.*, 2004). When the Hh negative regulator is mutated in the *Drosophila* ovary, there is excessive proliferation of follicle cells (Zhang and Kalderon, 2000).

Likewise, Hh is found to promote FSC progenitor cell differentiation and development, especially in the polar and stalk cell lineages. It was proposed that Hh signaling is not specific to the FSC niche, but rather a general signaling that is involved in proliferation and differentiation of FSCs (Sahai-Hernandez and Nystul, 2013). Nevertheless, a recent study has proposed that Hh acts upstream of the Hippo signaling pathway to regulate the FSC niche (Hsu *et al.*, 2017). Therefore, the precise role that Hh signaling plays in FSC behavior and maintenance is yet to be concluded.

Finally, JAK/STAT signaling is another pathway known to regulate FSC

maintenance and behavior (Vied *et al.*, 2012). In the *Drosophila* ovary, it was found that JAK/STAT is necessary for the proper development of stalk cells (Baksa *et al.*, 2002; Hayashi *et al.*, 2012). FSCs that are deficient in either JAK or STAT are lost from the niche prematurely. It is known that overactivation of JAK/STAT signaling results in an increased number of incompletely differentiated stalk cells (McGregor *et al.*, 2002). Whereas a low JAK/STAT signaling results in the loss of stalk cells and fused egg chambers in the later stages (McGregor *et al.*, 2002; Hayashi *et al.*, 2012). The JAK/STAT ligand, unpaired (Upd), is secreted in the polar cells and travels over a range anteriorly to the FSC regions (McGregor *et al.*, 2002).

JAK/STAT also works in collaboration with other signaling pathways to regulate FSC behavior (Vied *et al.*, 2012). It was proposed that there is a cooperative relationship between the signaling pathways, especially between the Hh and JAK/STAT signaling pathway (McGregor *et al.*, 2002; Vied *et al.*, 2012). Hence, it is important to coordinate the distribution of each signaling pathway in the FSC niches to ensure proper regulation of FSC behavior and niche maintenance. All the aforementioned pathways have HS-dependent ligands, which are Wg, Hh and Upd (Li and Kusche-Gullberg, 2016; Nakato and Li, 2016). This makes the heparan sulfate proteoglycans (HSPGs) a highly likely candidate in modulating the amount of signaling ligands in the FSC niches.

Heparan Sulfate Proteoglycans

Heparan sulfate proteoglycans (HSPGs) are a class of carbohydrate

modified proteins that is known to play a role in regulating signaling pathways, cell adhesion, and are critical for stem cell regulation (Lin and Perrimon, 2000; Esko and Selleck, 2002; Levings *et al.*, 2016; Takemura and Nakato, 2017). HSPGs consist of a core protein and long, unbranched HS chains with covalently linked disaccharide units that are sulfated at specific positions. In *Drosophila*, there are three classes of evolutionarily conserved HSPGs: the syndecans, the glypicans, and the perlecans (Kirkpatrick and Selleck, 2007).

Syndecan is one class of the HSPG family, and is found on cell surface membranes. It has a core protein with long HS side chains attached to it, in addition to a transmembrane domain and a short cytoplasmic domain (Fox and Zinn, 2005; Kirkpatrick and Selleck, 2007). Syndecans play a role during axon guidance and are involved in Slit-Robo signaling (Slit is an HS dependent ligand) (Johnston *et al.*, 2004). The glypicans, which is the second class of HSPGs, share similar structure to the syndecans and are also found on the cell surface membrane, but are attached to the membrane by a glycosylphosphatidylinositol anchor (Häcker *et al.*, 2005). They are known to act as co-receptors for signaling pathways and play a role in morphogen diffusion along a gradient (Franch-Marro *et al.*, 2005; Han *et al.*, 2005; Hayashi *et al.*, 2009). Finally, the perlecans are a class of secreted HSPGs found in the extracellular matrix (Häcker *et al.*, 2005). Perlecans play a role in vascular development, and basement membrane integrity maintenance (Costell *et al.*, 1999; Gonzalez-Iriarte *et al.*, 2003).

The most important step in the HSPG synthesis process is sulfation. HS-

chain are non-functional without sulfation, which would impair the HS-dependent signaling pathways (Lin *et al.*, 1999). HSPGs are involved in many different cellular processes, including adhesion, migration, proliferation, differentiation, morphogen distribution, and axon guidance (Kramer and Yost, 2003; Selleck and Nakato, 2004; Johnson *et al.*, 2004; Steigemann *et al.*, 2004).

There are two glypicans in the *Drosophila*, division abnormally delayed (dally) and dally-like protein (dlp). Dally and dlp are known to be engaged in controlling the BMP, Hh and Wg signaling (Guo and Wang, 2009; Lin and Perrimon, 1999; Fujise *et al.*, 2001; Franch-Marro *et al.*, 2005) and dally serves as a co-receptor for the JAK/STAT ligand, Upd (Hayashi *et al.*, 2012). It was demonstrated that HS affects cell competition both autonomously and non-autonomously in the wing disc (Ferreira and Milán, 2015; Enomoto *et al.*, 2015). Hence, this study will focus on the glypicans and how they influence FSC competitive behavior and niche maintenance in *Drosophila* ovary.

Rationale and Statement of Thesis

HS-dependent signaling pathways are required to maintain FSC behavior and niche maintenance. We hypothesize that HSPGs play a role in modulating the HS-dependent ligands in the FSCs, thereby influencing the eventual FSC competitive behavior. In this study, we will show evidences that HS-dependent signaling is affected in the FSCs. This will pave way to further mechanistic studies of how the HS-dependent pathways regulate FSC competitive behavior.

Materials and Methods

Fly Strains

Fly strains information can be found on flybase (<http://flybase.org>) unless otherwise specified. The control fly strains used were either Canton S or *w¹¹⁸*, a white eye strain backcrossed 20 times to Canton S. Other strains that were used in the experiments for our study were: *dally^{CPT1001339}*, a YFP protein trap line inserted in the endogenous *dally* locus (Lowe *et al.*, 2014); *dlp^{CPT1000445}*, a GFP protein trap line inserted in the endogenous *dlp* locus (Lowe *et al.*, 2014); *sfl^{9B4}*, a null allele of *sfl* (Lin and Perrimon, 1999); *dally^{gem}*, a null allele of *dally* (Tsuda *et al.*, 1999); *dally⁸⁰*, a null allele of *dally* (Han *et al.*, 2004); *dally^{MH32}*, a null allele of *dally* (Franch-Marro *et al.*, 2005); *dlp^{A187}*, a null allele of *dlp* (Han *et al.*, 2004); *dlp^{MH20}*, a null allele of *dlp* (Franch-Marro *et al.*, 2005); *109-30-Gal4* (Bach *et al.*, 2007)(Hartman, 2015); *UAS-dally* (Takeo *et al.*, 2005); *UAS-dlp* (Kleinschmit *et al.*, 2010); *UAS-sfl RNAi*, a UAS short-hairpin RNAi strain for *sfl* (Levings *et al.*, 2016); *UAS-dally RNAi*, a UAS short-hairpin RNAi strain for *dally* (Dietzl *et al.*, 2007); *UAS-dlp RNAi*, a UAS short-hairpin RNAi strain for *dlp* (Ni *et al.*, 2011); *10xSTAT-GFP*, a reporter strain for monitoring JAK/STAT signaling (Vied *et al.*, 2012); *frizzled 3 (fz3)-RFP*, a reporter strain for monitoring Wg signaling (Wang and Page-McCaw, 2014); *ptc-pelican-GFP*, a reporter strain for monitoring Hh signaling (Ulmschneider *et al.*, 2016); *robo1^{HA-robo1}*, an endogenously HA-tagged strain for *robo1* (Spitzweck *et al.*, 2010); *robo2^{HA-robo2}*, an endogenously HA-tagged strain for *robo2* (Spitzweck *et al.*, 2010); *robo3^{HA-robo3}*, an endogenously

HA-tagged strain for robo3 (Spitzweck *et al.*, 2010).

FSC Maintenance and Replacement Assay

We used the Mosaic Analysis with a Repressible Cell Marker technique (MARCM) (Lee and Luo, 2001; Takemura and Nakato, 2015) to assay for FSC maintenance and competition. MARCM clones were generated by heat-shock induced FRT mediated mitotic recombination. Cells that have undergone the recombination will be GFP marked.

To carry out the process, *y w hs-FLP tub-Gal4 UAS-GFP[nls];; FRT2A tub-Gal⁸⁰* was crossed to wild-type or mutant chromosomes with FRT2A to obtain *y w hs-FLP tub-Gal4 UAS-GFP[nls]/+;; FRT2A tub-Gal80/FRT2A ** (* denotes respective mutations). Adult females of 2-4 days old were heat-shocked twice a day at 37°C for one hour each, eight hours apart, for two days (4 heat shocks total). After heat shock, they were maintained at 25°C for 7-21 days before dissection. At 7, 14, 21 days post heat shock (dph), flies were dissected and examined.

In the beginning of induction, most of the germaria contain 0 or 1 GFP-labelled FSC. We then trace the changes in the number of GFP-labelled cells (either 0 GFP-labelled FSC or 2 GFP-labeled FSCs) at each time point and measure the persistence of marked follicle lineages after recombination to assay for FSC maintenance. As inter-niche FSC replacement occurs overtime, any reduction or increase in the number of GFP-labelled FSC clones relative to day 7 control will be a reflection of the loss or gain of clones corresponding to their

genotype. To assay for FSC competition, any increases or decreases in the percentages of 0, 1, and 2 FSCs were measured at 7 and 21 dph. The ratio between these two time points were calculated.

RNAi Knockdown and Signaling Reporter Assay Quantification

The *109-30-GAL4* line was recombined with individual signaling reporter to allow for the ease of monitoring different signaling pathways. To monitor JAK/STAT signaling, we used a *10xSTAT92E-GFP* reporter (Vied *et al.*, 2012), a *ptc-pelican-GFP* reporter for Hh signaling (Ulmschneider *et al.*, 2016), and a *fz3-RFP* reporter for Wg signaling (Wang and Page-McCaw, 2014).

109-30-GAL4 was used to drive the *UAS-RNAi transgene* in the FSCs and their progenitors. The *109-30-GAL4* strain that was recombined with individual signaling reporters was crossed to either *UAS-sfl RNAi*, *UAS-dally RNAi*, or *UAS-dlp RNAi* to carry out the knockdown experiments. A white-eye strain, *w¹¹⁸*, which served as the control, was also crossed to each GAL4-reporter recombinant strain. After the cross, the flies were cultured at 25°C and females of the right genotype were shifted to 29°C, 1 to 2 days after eclosion. The flies were then dissected 7 days after temperature shift.

To determine the effect of RNAi knockdowns on the different signaling pathways in FSC, we quantified the GFP or RFP intensities in the FSC region. We first identified FSCs along the 2a/2b border, their triangular shape, and their low FasIII expression levels. We isolated the FSCs by using the Polygon selection tool in Fiji. The intensity of the FSCs in both the control and the RNAi

knockdowns for each signaling pathway was then measured using the Measure function. The same microscope setting was used for each signaling pathway such that any differences between the control and the experimental samples could be truly reflected. After measuring the intensities of either GFP or RFP, we quantified the relative intensities of the RNAi knockdowns for each signaling pathway and normalized the values to the control FSC samples by setting the control values to 1.0. A Student's t-test was used to calculate statistical significance between control and RNAi samples.

Stalk Cell Quantification

Stalk cells after stage 1 of the germarium (1st stalk) and after stage 2 of the developing egg chambers (2nd stalk) were quantified for control and each RNAi knockdown experiment. Individual numbers of stalk cells were counted, and a Wilcoxon rank sum test was used to calculate statistical significance between control and RNAi knockdown samples.

Immunohistochemistry and Microscopy

Samples were fixed in 4% formaldehyde for 15 minutes and washed with 0.1% PBST (Triton X-100 in PBS) three times, 20 minutes each. They were then blocked in 0.2% normal goat serum in 0.1% PBST for 30 minutes and incubated overnight at 4°C with primary antibodies. After primary antibody incubation, they were washed three times with 0.1% PBST, 20 minutes each, and incubated overnight at 4°C with AlexaFluor secondary antibodies. After secondary antibody incubation, the samples were again washed three times in PBST, 20 minutes

each, before being mounted in Vectashield and imaged with Zeiss 710 or a Nikon Eclipse E800 laser scanning confocal microscope.

The primary antibodies used were: mouse anti-FasIII [1:100, Developmental Studies Hybridoma Bank (DSHB)], rat anti-Vasa (1:100, DSHB), mouse anti-Dlp (1:50, DSHB), rabbit anti-GFP (1:1000, Invitrogen), rabbit anti-HA (1:1000, Cell Signaling C29F4). Secondary antibodies were conjugated with Alexa-488, Alexa-546, Alexa-633 and used in 1:500 dilutions.

Results

Dally and Dlp expression patterns in the ovary

To determine if Dally and/or Dlp plays any role in FSC niche maintenance and their competitive behavior, we first examined their expression patterns in the *Drosophila* ovary. We used Dally and Dlp protein trap lines (*dally*^{CPT1001339} and *dlp*^{CPT1000445}, respectively) to view their expression patterns. These protein trap lines have a GFP or YFP inserted into an intron of their endogenous loci, and reflects the endogenous protein localization of Dally and Dlp (Morin *et al.*, 2001; Buszczak *et al.*, 2007; Quiñones-Coello *et al.*, 2007). Dally expression is detected in the posterior of the germarium, including the posterior escort cells, FSCs, and the follicle cells (Fig. 1B and B'). In the later egg chambers, Dally expression is restricted exclusively to the somatic cells with higher expression in the apical surface than in the basolateral surface of the follicle cells (Fig. 1C).

Dlp has a broader expression pattern than Dally, and is expressed in both germ cells and follicle cells in the germarium. However, unlike Dally, Dlp expression in the somatic cells significantly decreases after stage 3-4, and its expression is restricted to the germline cells after stage 2 (Fig. 1D and D').

Glypicans regulate FSC maintenance and niche competition

As FSCs express both Dally and Dlp, we next want to determine if they play a role in FSC behavior. To assay for niche maintenance and FSC competition, we used the Mosaic Analysis with a Repressible Cell Marker

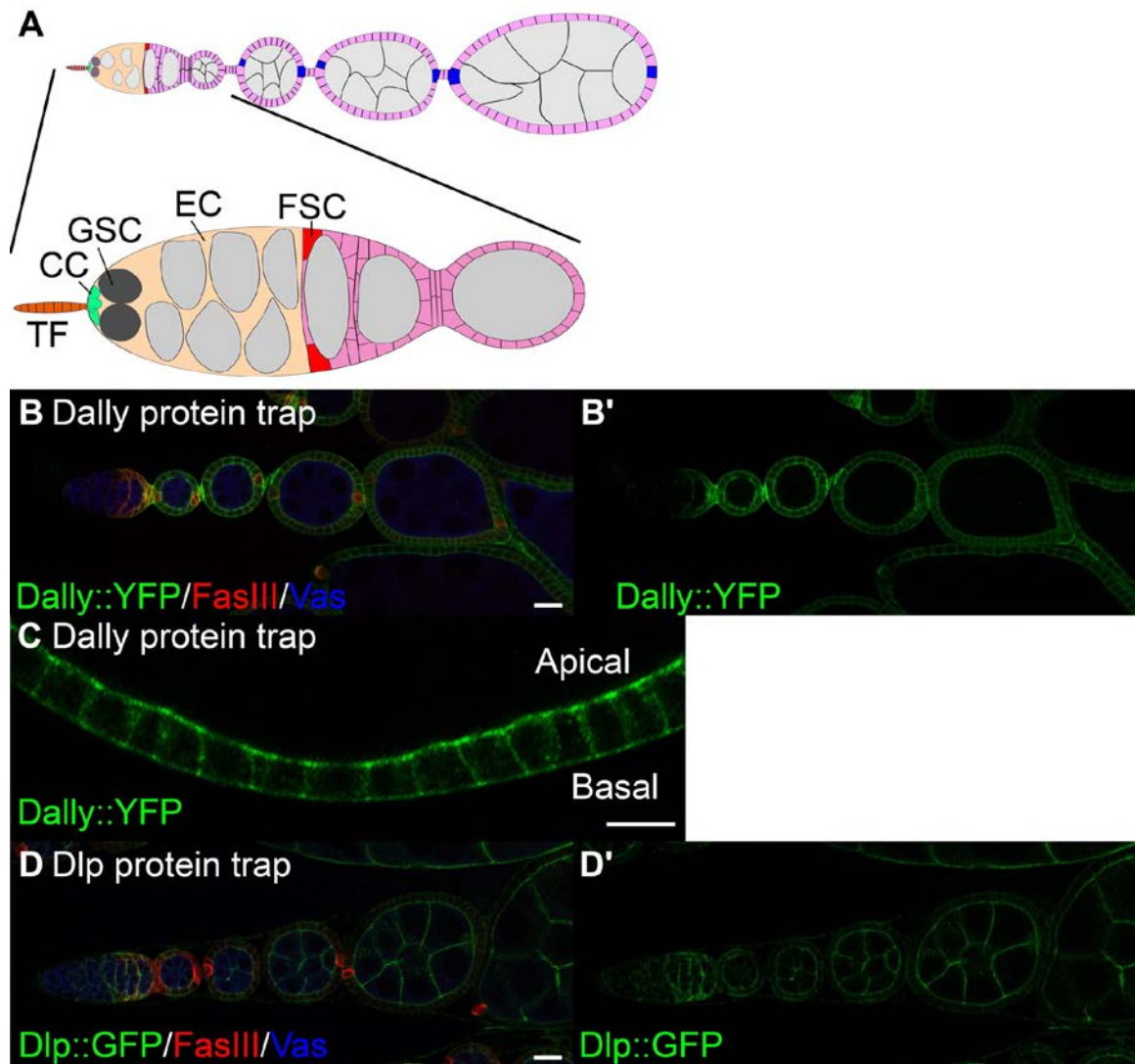


Figure 1. *Drosophila* ovary and expression patterns of Dally and Dlp. (A) The *Drosophila* ovary consists 16-20 discrete structures called the ovariole. At the anterior tip of each ovariole is the germarium. The GSCs resides in the anterior portion of the germarium (grey) while two FSCs (red) resides along the 2a/2b border in the germarium. Somatic cells in the germarium include the terminal filament (orange), cap cells (green), and escort cells (light brown). (B and B') Expression pattern of Dally protein trap. Anti-GFP showed the expression of Dally. It is expressed in the posterior escort cells, FSCs and the FCs. In the later egg chamber stages, it is expressed exclusively in the somatic cells. (C) Dally has a higher expression in the apical surface than in the basolateral surface of the FCs in developing egg chambers. (D and D') Expression of Dlp protein trap. In the germarium, Dlp is expressed in similar regions as Dally, however, expression in somatic cells decrease after stage 3-4. Dlp is expressed in the germline cells after stage 2. Bar: 10 μ m.

(MARCM) system (Lee and Luo, 2001). MARCM generates FSCs of a defined genotype using heat-shock induced FRT mediated mitotic recombination (Fig. 2A). Cells with our genotype of interest will be marked green after heat shock recombination, which allows us to trace their lineage and persistence in a system. Since FSC daughters take around 5 days to exit the germarium, GFP labeled cells in the germarium after 7 dph are derived from FSCs that have undergone recombination.

To understand FSC behavior in the germarium, we looked at the frequency of GFP-marked FSCs in the germarium at 7, 14 and 21 days post heatshock (dph). By comparing the frequencies of the different time points, we can determine the relative increase or decrease in the frequency of GFP-marked clones of the two latter time points from 7 dph, which reflect the characteristics of the mutant genotypes. Normally in wildtype control ovaries, there is a gradual loss of marked FSC clones due to slow turnover rate. However, if a mutation causes a loss of FSC maintenance in the niche that result in a fast turnover rate, the frequency of the GFP-labelled FSCs will be lost quickly (less competitive phenotype). On the other hand, if a mutation causes the FSCs to be better maintained, stay in their niche longer, and even occupy the other niche by “outcompeting” the host FSC (hypercompetition phenotype), it will eventually result in an epithelial sheet that is being occupied by the marked clones (“all marked” phenotype) (Fig. 2A).

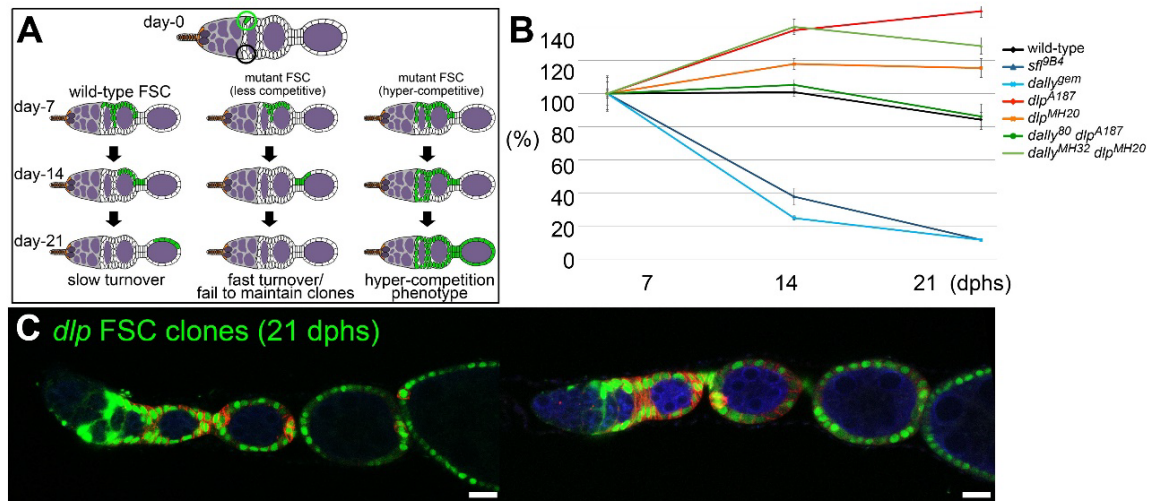


Figure 2. Glypicans are required for FSC niche maintenance. (A) An illustrated version of the MARCM lineage analysis. Green cells represent an FSC that has undergone mitotic recombination, and therefore GFP marked. When there is normal turnover, there is a gradual loss of the GFP marked FSC (slow turnover). When a mutation results in the FSC to be less well maintained in the niche, there GFP marked FSC is rapidly lost from the FSC niche (fast turnover). On the other hand, when a mutation results in the FSC to be better maintained in the niche, the GFP marked FSC will remain in its niche over time, and will expand and occupy the other niche (hypercompetition). Once both niches are occupied, the GFP positive progenitor cells will eventually occupy the entire epithelial sheet. (B) Maintenance assay. Graph showing the percentages of GFP marked FSCs for control (black), *sfl^{9B4}* (dark blue), *dally^{gem}* (light blue), *dlp^{A187}* (red), *dlp^{MH20}* (orange), *dally⁸⁰ dlp^{A187}* (dark green), and *dally^{MH32} dlp^{MH20}* (light green) at 7, 14, 21 dph. *sfl* and *dally^{gem}* mutant GFP marked clones are rapidly lost from the FSC niches, while *dlp^{A187}* GFP marked clones are better maintained and persisted over time. The raw data for the maintenance graph are represented in Table 1. (C) An all-marked phenotype for *dlp* mutant clones. GFP-marked *dlp* clones eventually occupy the entire epithelial sheet. However, there is no overproliferation of the GFP-marked progenitor cells and the gross morphology of the ovarioles remains normal. Bar: 10 μ m.

A previous lab member investigated the role of HSPGs in FSC maintenance and competitive behavior, and first generated FSC mutant clones for *sulfateless* (*sfl*) and compared the clone frequency to wildtype FSC clones for analysis. *sfl* encodes the only *Drosophila* HS *N*-deacetylase/*N*-sulfotransferase (NDST). *N*-sulfation, catalyzed by Sfl, is the first step in a series of HS modifications. It is necessary for subsequent modifications of HS chains. Therefore, *sfl* mutants will have disrupted HS chains activities, and impaired HS-dependent signaling pathways. It was observed that *sfl* null mutant clones are very quickly lost over time from the germarium when compared to wildtype controls. In fact, they were almost completely eliminated from the germarium at 21 dph. This suggests that HS is necessary for FSC maintenance.

Next, *dally* mutant (*dally^{gem}*) FSCs were observed and it was found that these were also lost from the germarium very quickly (Fig. 2B). This phenotype is similar to that of *sfl* null mutants, suggesting that Dally is a primary HSPG regulator of normal FSC maintenance and/or competition. On the other hand, *dlp* mutant (*dlp^{A187}*) FSCs behaved in an opposite manner to that of *dally* and *sfl* mutants. The frequency of *dlp* mutant clones did not decrease over time, rather, they increased in frequency, and eventually occupied the entire epithelial sheet, resulting in an “all marked” phenotype (Fig. 2B). Interestingly, the “all marked” phenotype exhibited by the *dlp* mutant follicle cells show normal morphology and did not show overproliferation and accumulation of follicle cells that simulates a tumorous phenotype (Fig. 2C). This suggests that *dlp* affects FSC replacement

without affecting FSCs and progenitor cell proliferation.

In our study, we first confirmed that we saw a similar trend in *sfl*, *dally*, and *dlp* (Fig. 2B). As the *dlp* mutant behavior was unexpected, we used *dlp^{MH20}*, another *dlp* mutant allele that was generated independently from *dlp^{A187}*, to confirm this phenotype. The *dlp^{MH20}* allele also showed similar frequency and exhibited hypercompetitive behavior as shown by *dlp^{A187}* (Fig. 2B). This result confirmed that the *dlp* mutant FSC behavior is not an allele specific effect, but reflects the *dlp* gene function. In addition, we looked at *dally-dlp* double mutant clones and the double mutants showed similar behaviors to *dlp* single mutant FSCs (Fig. 2B). This suggests that *dlp* is epistatic to *dally*.

Since *dlp* mutants showed an “all marked” phenotype, we next want to confirm that *dlp* mutant FSCs are more competitive than wildtype FSCs, we again utilized the MARCM technique to carry out the competition assay. First, we measured the proportion of germaria with 0, 1, or 2 GFP marked FSCs at 7 dph, and measured the FSC replacement rate at 21 dph (14 days later). There are 2 scenarios that could happen in germaria with 1 marked FSCs at 7 dph after 14 days: first, the GFP marked FSC replace the unmarked FSC in the other niche, resulting in a germarium with 2 marked FSCs; second, the GFP marked FSC is replaced by the unmarked wildtype FSC (Fig. 3A). Either way, the change in ratio of germaria containing 0, 1, or 2 FSCs will reflect the competitiveness of the GFP marked FSC for niche occupancy.

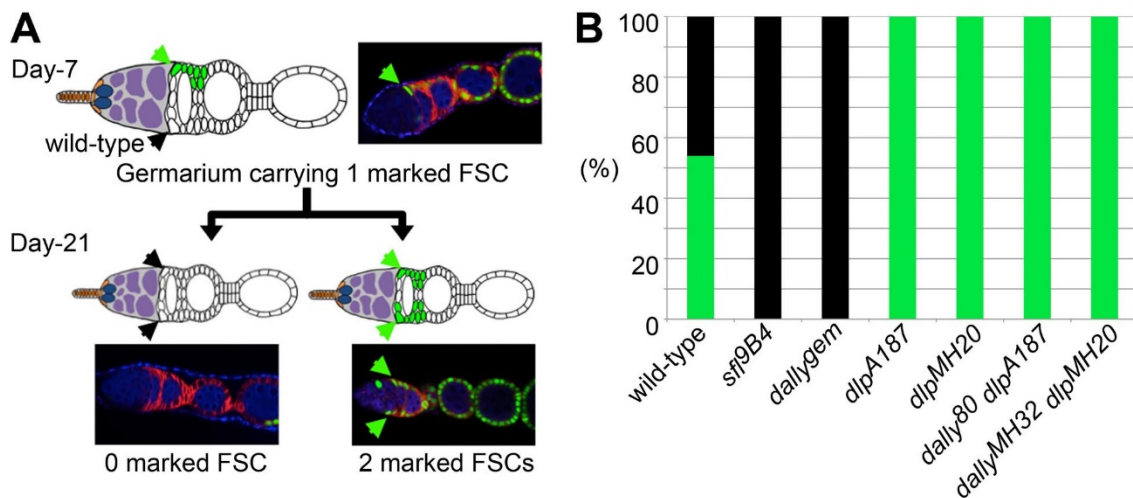


Figure 3. Glypicans regulate FSC competition for niche occupancy. (A) An illustrated version of FSC replacement assay. After heat shock recombination, germaria with 1 GFP labeled FSC can end up with 2 different fate: first, by 21 dph, the wildtype FSC will outcompete the GFP marked FSC, and result in a germarium with 0 GFP marked FSC. Second, the GFP marked FSC will out compete the unmarked wildtype FSC, and by 21 dph, result in a germarium with 2 GFP marked FSCs. **(B)** The ratio of germaria with 2 marked FSCs to 0 marked FSC in each genotype between 7 dph and 21 dph. The black bar represents the ratio of germaria with 0 FSC, while the green bar represents the ratio of germaria with 2 marked FSCs.

When we looked at the niche occupancy rate for our control, the slow turnover rate showed no bias in the FSCs competitiveness (Fig. 3B). In *sfl* and *dally* mutant FSCs, we observed that they are being quickly replaced by the unmarked wildtype FSCs, as depicted by the strong bias to 0 marked clones. On the other hand, *dlp* mutants and *dally-dlp* mutants rapidly replaced the wildtype FSC and showed a strong bias to germaria with both of the niches occupied by GFP marked mutant FSCs. Therefore, this suggested that glypicans affect FSC competitiveness via altering the replacement bias.

Glypicans regulate JAK/STAT signaling

There are studies which have identified and established HS-dependent pathways that regulate FSC maintenance, including JAK/STAT, Wg, and Hh signaling. To investigate how HSPGs affect FSC maintenance and competitive behavior, we looked at how these three pathways will be affected when the glypicans are knocked down by means of RNAi.

JAK/STAT is a recently proposed HS-dependent signaling pathway that plays a role in maintaining FSC behavior. Hence, we wanted to examine if *dally* or *dlp* play any roles in regulating JAK/STAT signaling in the germarium. To carry this out, we used a GAL4-UAS system to knockdown the glypicans specifically in the FSCs and the follicle cell region using *109-30-GAL4*, which is a commonly used GAL4 driver, to express the UAS-RNAi transgenes. We first validated its expression in the FSCs and the follicle cells (Fig. 4A).

To monitor changes in JAK/STAT signaling, we used a *10xSTAT92E-GFP*

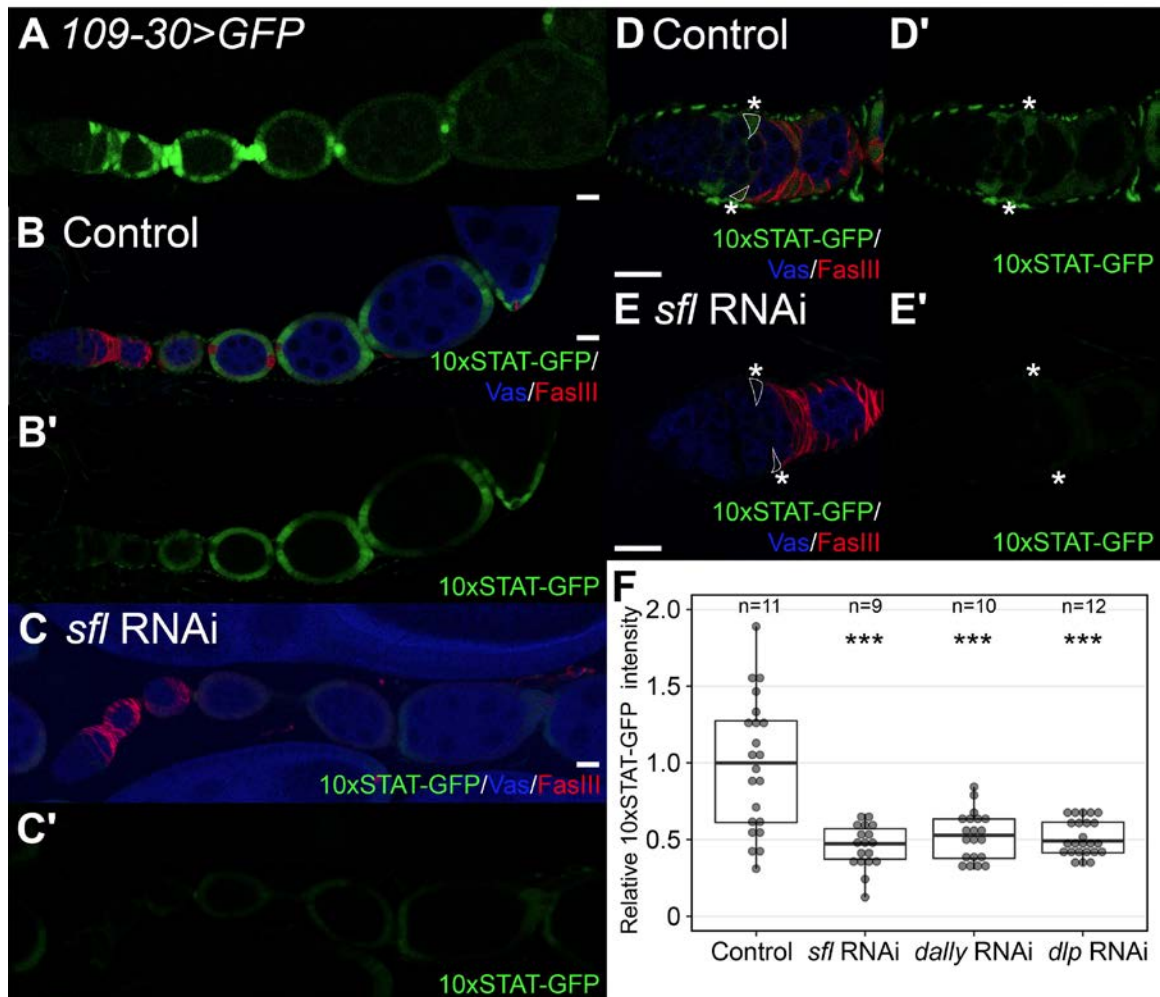


Figure 4. Glypicans regulate JAK/STAT signaling. (A) Expression pattern of *109-30-GAL4* showed that it is expressed in the FSCs and FC populations. (B-C') A *10xSTAT92E-GFP* reporter showed that when *sfl* is knocked down using the *109-30-GAL4* driver, there is an overall and dramatic reduction in the GFP signaling intensity. (D-E') In the germarium, GFP intensity of significantly reduced in the FSC areas with *sfl* RNAi. Asterisks show the FSCs. (F) Quantification of JAK/STAT signaling in the FSCs. GFP signaling intensity was measured over the FSC region of each genotype. The GFP intensity in control was set to 1.0 and the relative intensities in the RNAi knockdowns were calculated. *** $p < 0.001$ (Student's t test). n: number of samples. Bar: $10\mu\text{m}$.

reporter strain as a readout, which has a GFP after 10 copies of stat binding site (Vied *et al.*, 2012). The *10xSTAT92E-GFP* reporter shows a gradient of JAK/STAT signaling starting with the highest at the polar cells to the lowest in the main body follicle cells (Fig. 4B and B'), which was significantly decreased when we knocked down *sfl* using *109-30-GAL4* (Fig. 4C and C'). This decrease in GFP intensity was also observed in the germaria (Fig. 4D-E'). A similar approach was used to knock down *dally* and *dlp*, which also showed that there is a decrease in GFP intensity in the germaria.

After knocking down the glypicans, we quantified the GFP signal intensity in the FSC regions and found out that *sfl*, *dally*, and *dlp* knockdown does impair JAK/STAT signaling in the FSCs (Fig. 4F). This implies that glypicans play a major role in JAK/STAT signaling of FSC behavior.

Glypicans regulate stalk cell differentiation

Some morphological defects in the ovarioles in addition to the decrease in GFP signaling were noticed while carrying out the knockdown experiments. Ovarioles were missing stalk cells, and also exhibited a fused egg chamber phenotype (Fig. 5A and B). We quantified the number of stalk cells between control and the RNAi knockdowns, and found that *sfl* and *dally* knockdown in the FSCs and follicle cells lead to a complete loss of stalk cells in most ovarioles, with a median of 0 (Fig. 5C). *dlp* also showed a significant decrease in stalk cell number, though not as severe as *sfl* and *dally* RNAi. This result suggests that both Dally and Dlp play significant roles as Upd co-receptor during early

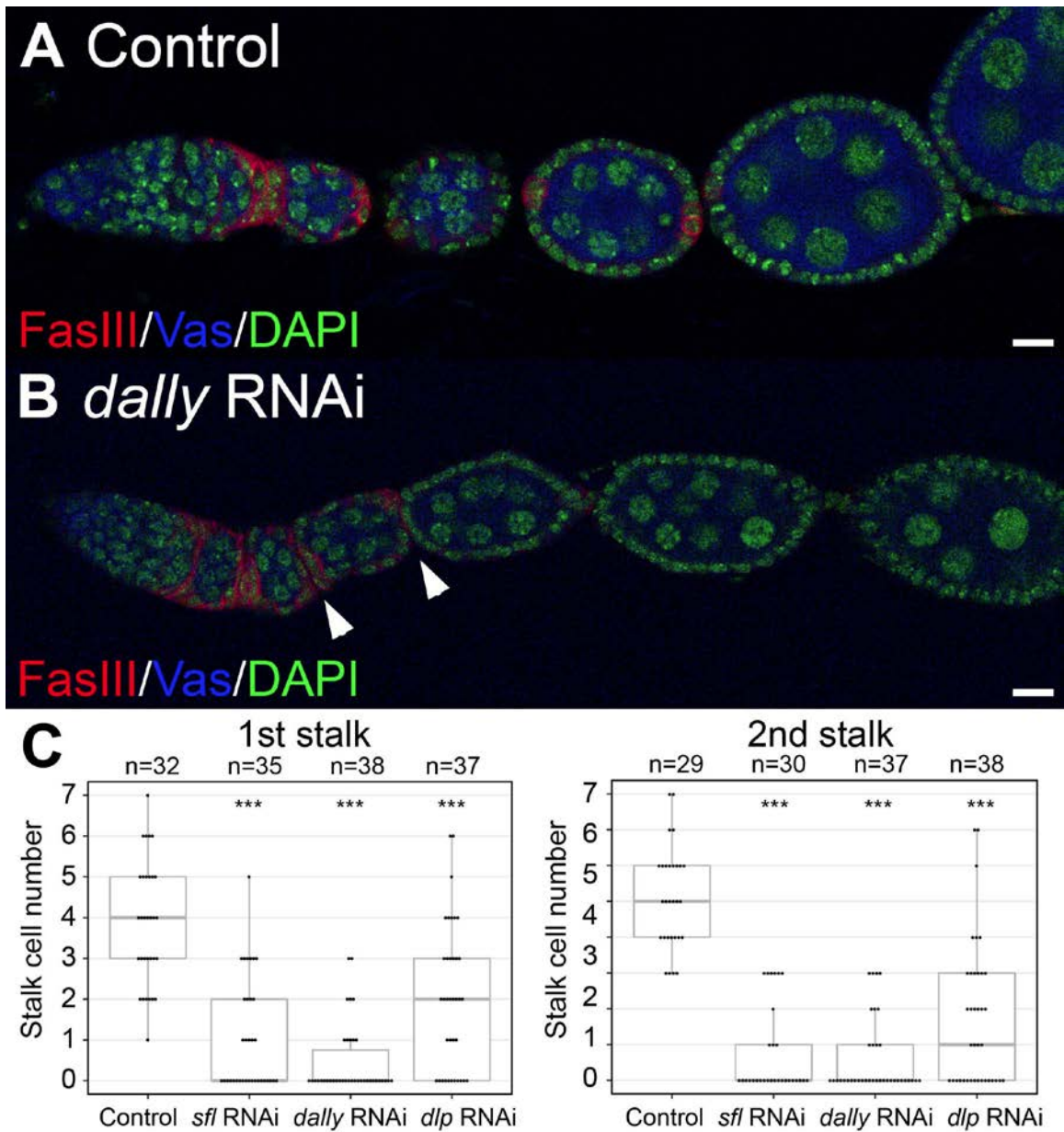


Figure 5. Glypicans regulate stalk cell development. (A) Ovariole of a control (*109-30-GAL4*) and (B) a *dally* RNAi ovariole. Arrowheads show the loss of stalk cells between egg chambers. (C) Stalk cell numbers were quantified and the number for the 1st and 2nd stalk cell were quantified. *** $p < 0.001$ (Wilcoxon rank sum test). n: Number of samples. Bar: 10 μ m.

oogenesis, whereas at later stages, Dlp plays a minor role in the pathway when its expression changes from somatic cells to germline cells.

Dlp regulates Wg signaling in the germarium

Wingless (Wg) is another HS-dependent pathway to regulate FSC, and is a suggested candidate niche factor from the escort cells. Hence, to find out how glypicans affect Wg signaling, we used a similar approach to knockdown *dally* and *dlp* in FSCs and follicle cells using the *109-30-GAL4*, similar to that of the JAK/STAT signaling experiments. In order to monitor Wg signaling, we used a *frizzled 3 (fz3)-RFP* reporter, which fused a *fz3* enhancer to a RFP and then inserted into the genome (Olson *et al.*, 2011; Wang and Page-McCaw, 2014).

Fz3-RFP expression only shows in the anterior region of the germarium, mainly in the escort cells and the FSCs (Fig. 6A). The RFP intensity decreases in the follicle cells located in the posterior region of the germarium. *sfl* knockdown in the FSCs and follicle cells using the *109-30-GAL4* significantly reduced the RFP intensity, which resulted in a drastic decrease in the *fz3-RFP* expression at the 2a/2b border while cells located in the anterior region of the germarium remained unaffected (Fig. 4C).

Again, we quantified the RFP intensity over the FSC region and learned that Wg signaling was significantly disrupted when *sfl* and *dlp* were knocked down (Fig. 4C). Intriguingly, *dally* knockdown did not seem to have any effect on the *fz3-RFP* intensity, suggesting that Dlp acts as a primary co-receptor for Wg signaling of these cells in the ovary.

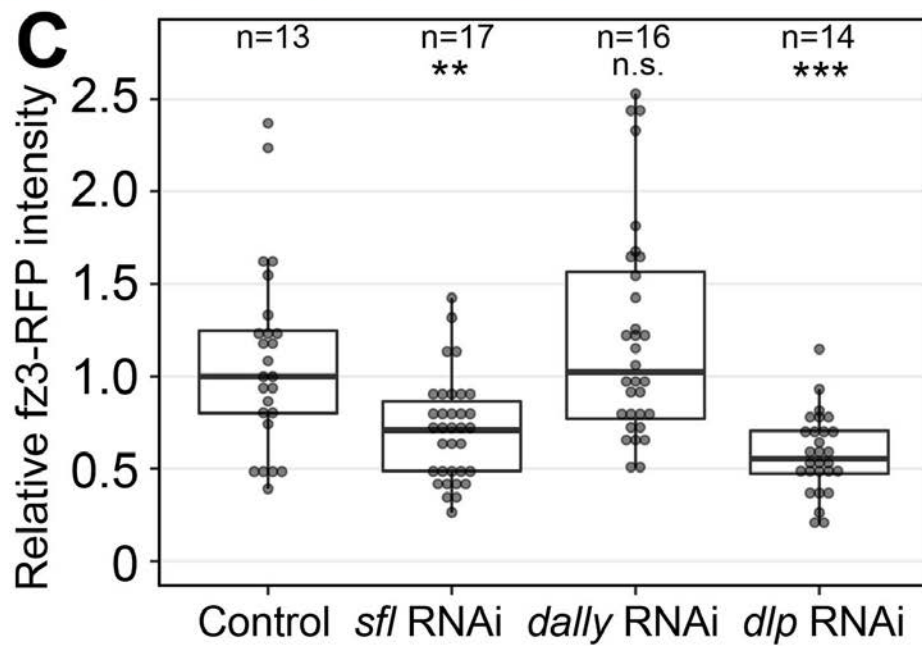
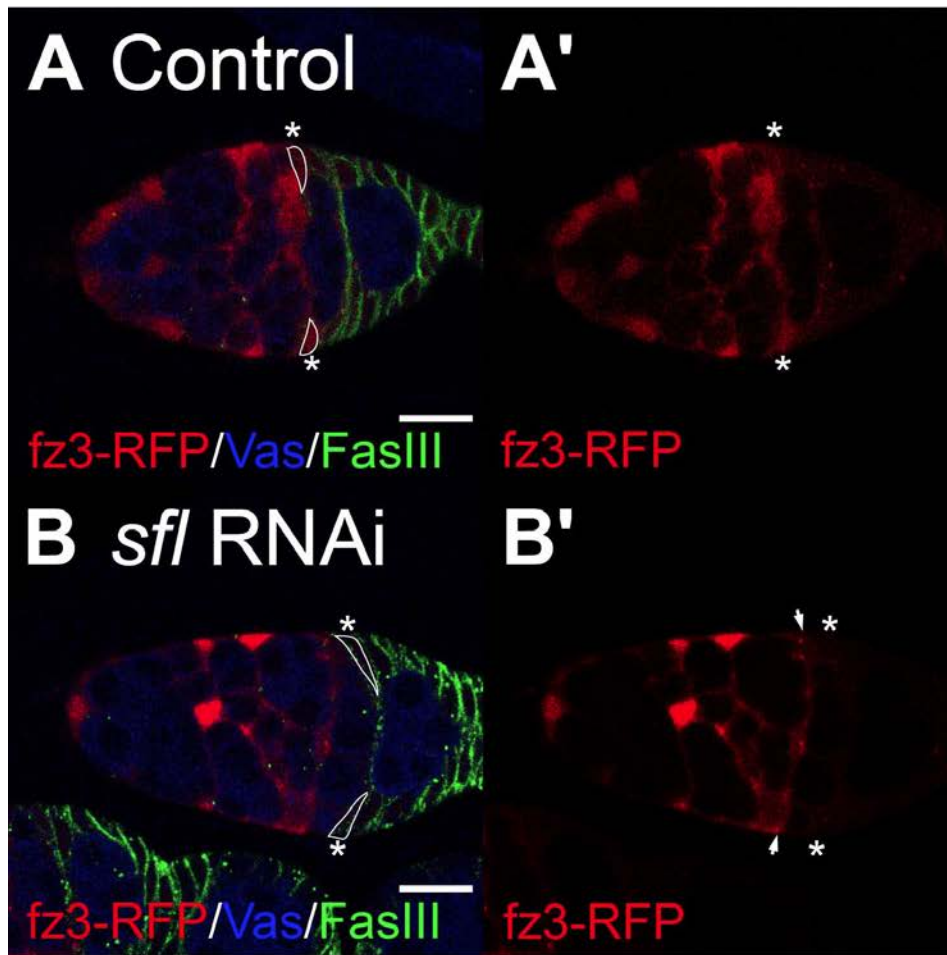


Figure 6. Dlp regulates Wg signaling in the germarium. (A-A') Control *fz3-RFP* signaling reporter. (B-B') When *sfl* is knocked down using the *109-30-GAL4* driver, there is an overall and dramatic reduction in the RFP signaling intensity. However, *fz3-RFP* intensity in escort cells that are located anterior to the FSCs (not expressing *109-30-GAL4*) are not affected by the *sfl* knockdown. Asterisks show the FSCs. Arrows show the beginning of *109-30-GAL4* expression and a drastic decrease in RFP signaling. (C) Quantification of *fz3-RFP* signaling in the FSCs. RFP signaling intensity was measured over the FSC region of each genotype. The RFP intensity in control was set to 1.0 and the relative intensities in the RNAi knockdowns were calculated. ** $p < 0.01$; *** $p < 0.001$; n.s. not significant (Student's t test). n: Number of samples. Bar: $10\mu\text{m}$.

Hh signaling is not affected in Dally and Dlp knockdown

Hh signaling is another pathway that is necessary for normal FSC maintenance. Therefore, to find out how glypicans affect Hh signaling, we used a similar approach to that of JAK/STAT and Wg signaling. To monitor for Hh signaling, we used a *ptc-pelican-GFP* reporter (Ulmschneider *et al.*, 2016), which is a reporter that has a GFP located downstream of multiple Cubitus interruptus binding sites, and activates a nuclear localized GFP with active Hh signaling (Sahai-Hernandez and Nystul, 2013).

We expressed the UAS-RNAi transgenes using *109-30-GAL4*, and found out that *sfl* RNAi in the FSCs and the follicle cells decreases Hh signaling (Fig. 7A-B'). However, there seemed to be no effect when either *dally* or *dlp* is knocked down in the FSCs and follicle cells (Fig. 7C). Therefore, though it is unmistakable that HS is required for Hh signaling, it is still undetermined which specific molecule is responsible as the Hh signaling co-receptor in the FSCs. This suggests that glypicans play a functionally redundant role in Hh signaling.

To summarize, we have shown that HS biosynthesis and glypicans are required for FSC maintenance and FSC niche competition behavior. We have also demonstrated that Dally and Dlp play specific and redundant roles in regulating different signaling pathways in the FSCs. Their involvement in different pathways imply Dally and Dlp's role in orchestrating and modulating signaling dosages of different pathways to control FSC maintenance/replacement and niche competition.

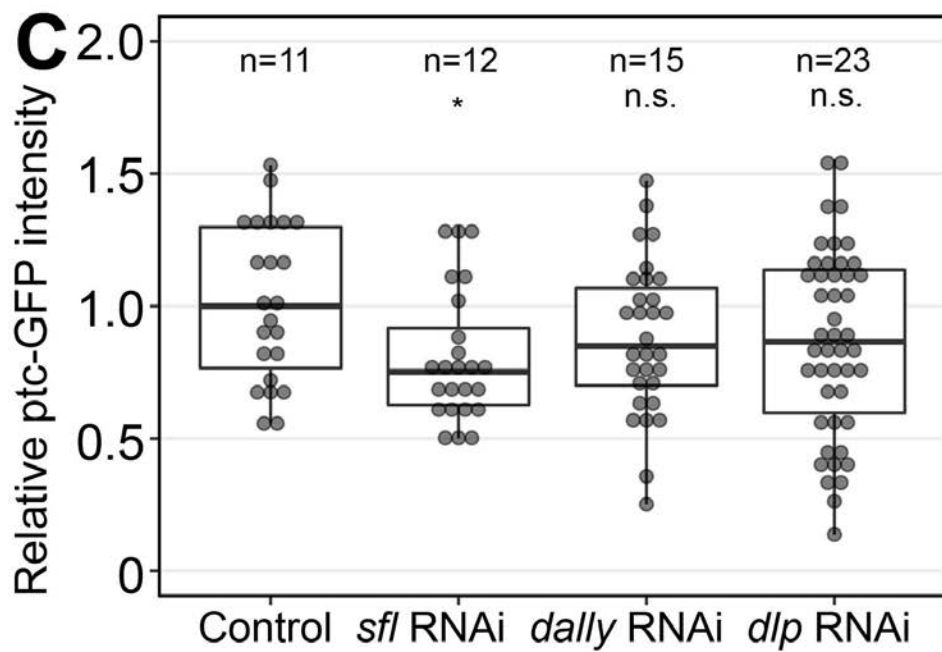
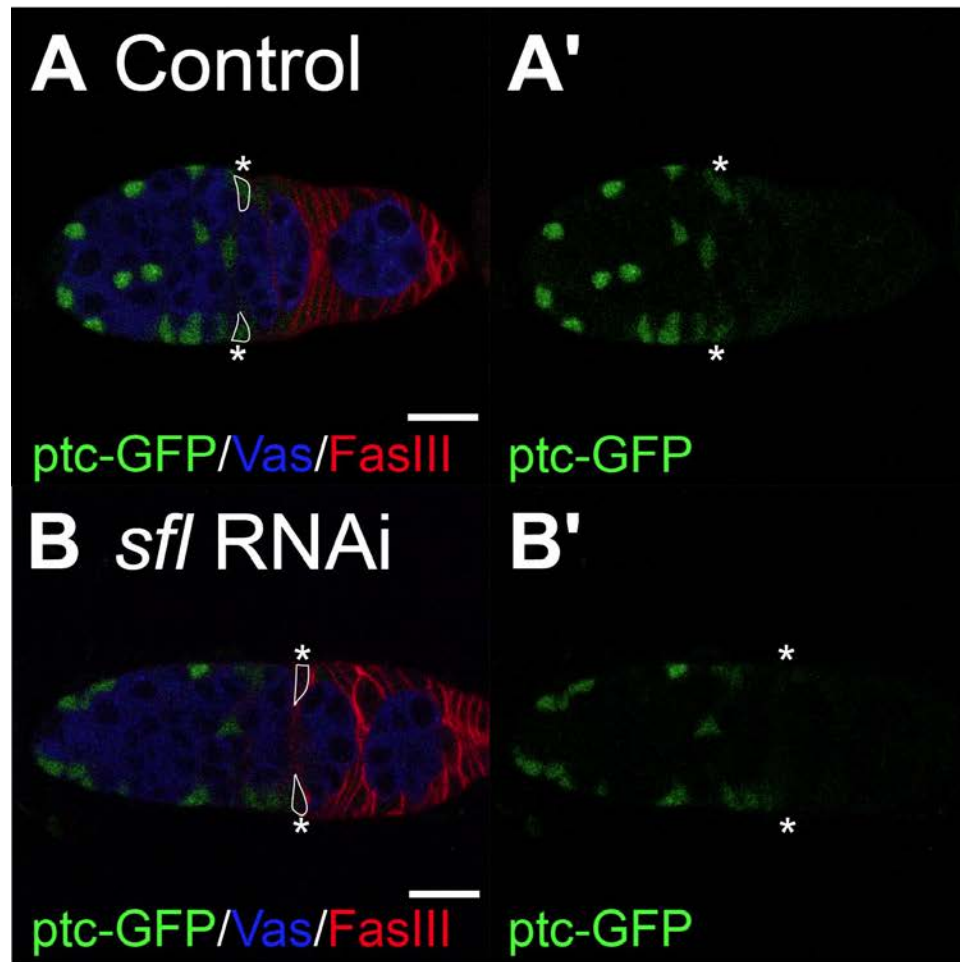


Figure 7. Hh signaling is not affected by glypican knockdown. (A-A') Control *ptc-pelican-GFP* expression in the germarium. (B-B') *sfl* RNAi using the *109-30-GAL4* showed a decrease in GFP signaling intensity in the FSC. Signaling intensities of escort cells located anterior to the FSCs were not affected by the knockdown. Asterisks show the FSCs. (C) Quantification of *ptc-pelican-GFP* expression signaling in the FSCs. GFP signaling intensity was measured over the FSC region of each genotype. The GFP intensity in control was set to 1.0 and the relative intensities in the RNAi knockdowns were calculated. * $p < 0.05$; n.s. not significant (Student's t test). n: Number of samples. Bar: 10 μ m.

Genotype	7 dph (%)					14 dph (%)					21 dph (%)				
	0	1	2	1+2	1+2 Normalized	0	1	2	1+2	1+2 Normalized	0	1	2	1+2	1+2 Normalized
FRT2A	60.5	26.8	12.7	39.5	100.0	60.1	22.5	17.4	39.9	100.9	66.7	13.3	20.0	33.3	84.3
<i>sf^{9B4}</i>	83.1	16.0	0.9	16.9	100.0	93.6	6.4	0.0	6.4	37.7	98.1	1.94	0.0	1.94	11.5
<i>dally^{gem}</i>	92.2	7.8	0.0	7.8	100.0	98.1	1.9	0.0	1.9	24.8	99.1	0.9	0.0	0.9	11.5
<i>dlp^{A187}</i>	56.7	23.2	20.2	43.3	100.0	40.0	19.6	40.4	60.0	138.4	35.0	23.9	41.0	65.0	149.9
<i>dlp^{MH20}</i>	62.9	30.4	6.7	37.1	100.0	56.2	27.2	16.6	43.8	118.0	57.1	23.5	19.4	43.9	115.5
<i>dally⁸⁰dlp^{A187}</i>	62.6	22.6	14.8	37.4	100.0	60.6	18.1	21.3	39.4	105.3	67.8	16.1	16.1	32.2	86.1
<i>dally^{MH32}dlp^{MH20}</i>	67.8	19.0	13.2	32.2	100.0	54.8	23.7	21.5	45.2	140.4	58.5	18.3	23.3	41.5	128.9

Table 1. Maintenance assay showing the percentages of individual genotype at each time point.

Genotype	7 dph (%)			14 dph (%)			21 dph (%)		
	0 FSC	1 FSC	2 FSC	0 FSC	1 FSC	2 FSC	0 FSC	1 FSC	2 FSC
FRT2A	60.5	26.8	12.7	60.1	22.5	17.4	66.7	13.3	20.0
<i>sf^{9B4}</i>	83.1	16.0	1.0	93.6	6.4	0.0	98.1	1.9	0.0
<i>dally^{gem}</i>	92.2	7.8	0.0	98.1	1.9	0.0	99.1	0.9	0.0
<i>dlp^{A187}</i>	56.7	23.2	20.2	40.0	19.6	40.4	35.0	23.9	41.0
<i>dlp^{MH20}</i>	62.9	30.4	6.7	56.0	27.4	16.6	57.5	22.1	20.4
<i>dally⁸⁰dlp^{A187}</i>	62.6	22.6	14.8	60.6	18.1	21.3	55.5	22.3	22.3
<i>dally^{MH32}dlp^{MH20}</i>	67.8	19.0	13.2	54.8	23.7	21.5	58.5	18.3	23.3

Table 2. Replacement assay showing the percentages of individual genotype at each time point.

Conclusion and Discussion

In this study, we have established that, i) glypicans regulate FSC maintenance and niche competitive behavior; ii) glypicans play a partially specific, sometimes redundant role in regulating HS-dependent signaling pathways that are known to maintain FSCs, thereby influencing FSC maintenance and competitive behavior. However, more assessments are needed to tease apart the role for each individual glypican. Our working model (Fig. 8) proposes that glypicans regulate the HS-dependent signaling pathways, which would affect the cellular behaviors that ultimately affect how competitive the FSCs are and how well they are maintained in the niche.

Upon examining the expression patterns of Dally and Dlp, we saw a higher expression of Dally on the apical surface of follicle cells in developing egg chambers than on the basolateral surface (Fig. 1C). Previous studies by Xi *et al.* (2003) and Hayashi *et al.* (2012) have shown that the JAK/STAT signaling ligand, Upd, forms a gradient along the follicle cells in the egg chambers after diffusing from the Upd secreting polar cell source. In addition, Hayashi *et al.* (2012) have shown that Dally is a co-receptor for JAK/STAT signaling. Therefore, it is likely that the higher expression observed on the apical surface of the follicle cells aids Upd diffusion along the follicle cells.

In our FSC maintenance and competition assays, we saw that Dally and Dlp influence FSC maintenance and competition in an opposite fashion. FSCs mutant for Dally are less competitive than wildtype FSCs and are less well

maintained in the niche. On the other hand, FSCs mutant for Dlp are more competitive than wildtype FSCs and stay in the niche longer. It is still uncertain whether cell proliferation or follicle cell survival are altered, or what happens to a cross migrating FSC if it is unable to outcompete the other resident FSC from its niche. More experiments are rendered necessary to answer these questions. However, this phenomenon corresponds to previous studies which suggested that some human glypicans are oncogenic while others are tumor suppressors. Of the six mammalian glypicans, GPC3 mutation in humans lead to overgrowth during the pre- and post-natal period by inhibiting Hh signaling (Pilia *et al.*, 1996; Cano-Gauci *et al.*, 1999; Capurro *et al.*, 2008). On the other hand, GPC5 has been found to be significantly upregulated in rhabdomyosarcoma, a soft tissue sarcoma (Williamson *et al.*, 2007; Li *et al.*, 2011).

In addition to the three HS-dependent pathways analyzed in this study, another potential pathway that could be playing a role is the Slit-Robo signaling pathway. Our data (Fig. S1), along with Reich and Papoulas' study (2012), showed that both Slit and the three Robo receptors are expressed in the FSC region. This suggests a potential functional role for Slit-Robo signaling in mediating FSC behavior. Slit-Robo signaling, which could be influenced by HSPGs to affect FSC behavior, is known to be involved in axon guidance and tracheal formation. Slit is a known HS-dependent ligand and requires HSPGs on the target cell membrane (Inatani *et al.*, 2003; Hohenester, 2008).

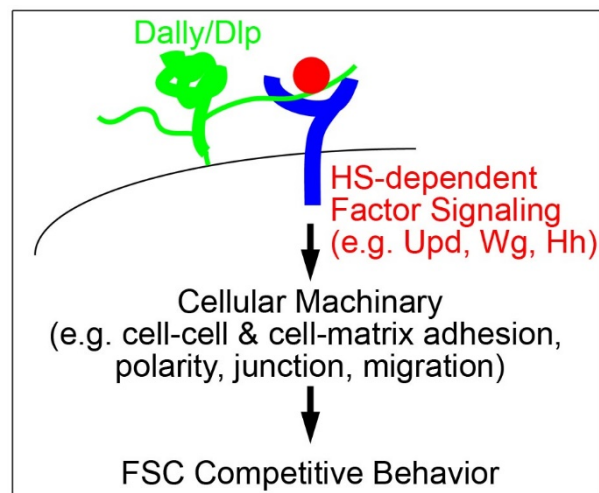


Figure 8. Model of FSC regulation by glypicans. Our model propose that glypicans regulate multiple HS-dependent signaling pathways including Upd, Wg, and Hh on the FSC surface. This fine regulation of multiple signaling pathways could regulate cellular machineries (e.g. cell-cell and cell-matrix adhesion, cell polarity complexes), which will in turn affect FSC behavior and niche maintenance.

Slit-Robo influences cell polarity and adhesion in non-neuronal tissues, and was also shown to be involved in regulating the intestinal cells (Ypsilanto *et al.*, 2010; Biteau and Jasper, 2014), tracheal cell migration (Schulz *et al.*, 2011), and is crucial in the male GSC niche (Stine *et al.*, 2014).

Because of the possible involvement of Slit-Robo signaling in FSC behavior, it would be interesting to understand further if and how Slit-Robo signaling affects FSC behavior in the germarium and explore what other effects it plays in *Drosophila* ovary development. However, more analyses and studies have to be done on the Slit-Robo pathway to show that it does indeed play a role in FSC maintenance and/or competition.

In addition, multiple studies have shown and implied that different signaling pathways play crucial roles in maintaining the apical-basal polarity complexes, which were proposed to regulate FSC behaviors (Song and Xie, 2002; Humbert *et al.*, 2008; Constanieto *et al.*, 2014). Therefore, it is also important to investigate if apical-basal polarity complexes are affected in glypican mutant FSC progenitors in the future. Observing the apical-basal polarity complexes would provide a stronger support to our model. Should we observe any defects in the complexes, it will imply that the signaling pathways influence FSC competitive behavior and maintenance via alteration of the complexes.

More studies should be done to fully uncover the mechanisms that explain how glypicans affect FSC niche competition and maintenance. The *Drosophila* FSC remains to be an excellent model system for this study due to its simplicity

and the number of available techniques to carry out studies in the ovary. This will be useful for studies related to cancer as FSC behaviors show similarity to how cancer cells migrate, proliferate, and metastasize. Output from studying the *Drosophila* FSC model can be used to further understand how adult stem cells behave, and how stem cells in the niches are maintained.

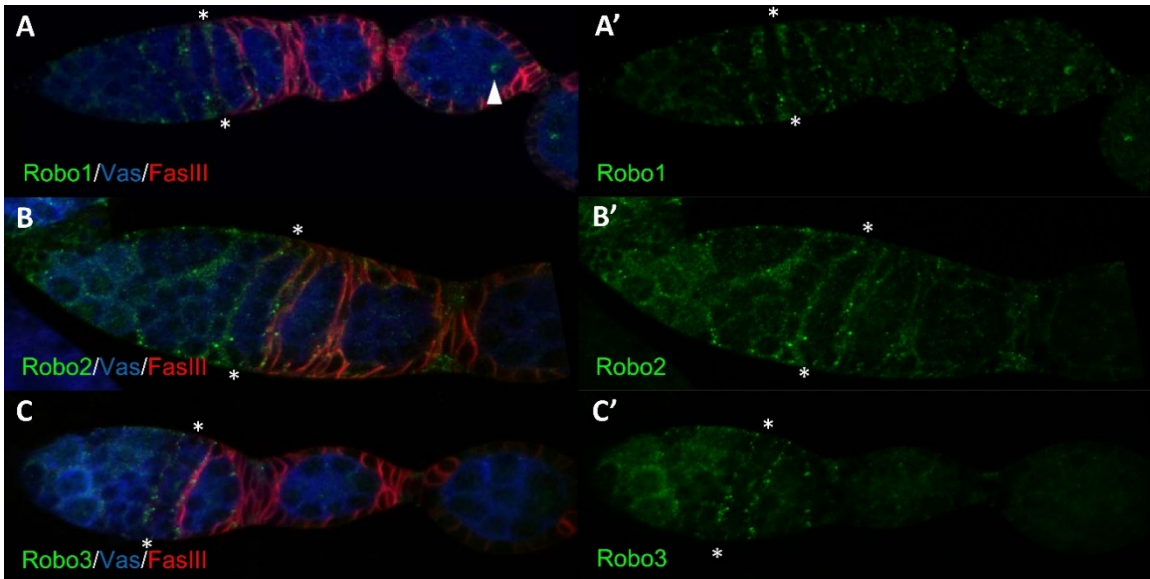


Figure S1. Robo receptor expression pattern in the germarium. (A-A') Expression pattern of an HA-tagged Robo1 receptor. Robo1 is expressed in the germarium and overlaps with the FSC regions. It is also expressed in the oocyte of the later egg chambers (arrowhead). (B-B') Expression pattern of an HA-tagged Robo2 receptor. Robo2 is expressed in the germarium and overlaps with the FSC regions. (C-C') Expression pattern of an HA-tagged Robo3 receptor. Robo3 is expressed in the germarium and overlaps with the FSC regions, but there is a higher expression in the anterior region than in the posterior region of the germarium.

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