ROLE OF HEPARAN SULFATE PROTEOGLYCANS IN DROSOPHILA FOLLICLE STEM CELL MAINTENANCE AND NICHE COMPETITION

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DEDICATION

This thesis is dedicated to my mentor and advisor Hiroshi Nakato. This work would not have been possible without his support and training. I am very grateful for his mentorship and the opportunity to learn from him.

ABSTRACT

Adult stem cells reside in specialized microenvironments, niches, which provide signals for the stem cells to maintain their undifferentiated and self-renewing state. To maintain stem cell quality, stem cells are sometimes replaced by progenitor cells through niche competition. However, the cellular and molecular basis for stem cell competition for niche occupancy are largely unknown. Here, we used the epithelial follicle stem cells (FSCs) system in the *Drosophila* ovary to study how FSC maintenance and niche competitive behaviors are regulated by heparan sulfate proteoglycans (HSPGs) and their post-translational modification. We found that a class of HSPGs, glypicans, regulates FSC maintenance and FSC competitiveness for niche occupancy. Furthermore, Notum, a secreted hydrolase known to cleave glypicans from the cell surface, is also a regulator of FSC niche competitive behavior. Our work highlights the significance of glypcans in adult stem cell systems and will further propel the study of stem cell maintenance and stem cell competition for niche occupancy.

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LIST OF ABBREVIATIONS

BMP: bone morphogenetic protein

Dally: division abnormally delayed

Dlp: dally-like protein

dphs: days post heat shock

FGF: fibroblast growth factor

FSC: follicle stem cell

GFP: green fluorescent protein

GPI: glycophosphatidylinositol

Hh: hedgehog

HS: heparan sulfate

HSPG: heparan sulfate proteoglycan

JAK/STAT: janus kinase/signal transducer and activator of transcription

MARCM: mosaic analysis with a repressible cell marker

Sdc: syndecan

Sfl: sulfateless

Trol: terribly reduced optic lobes

Upd: unpaired

Wg: wingless

INTRODUCTION

ADULT STEM CELLS AND STEM CELL NICHE

Adult stem cells divide to self-renew and to produce differentiated progeny that replace damaged or lost cells in a tissue. Adult stem cells reside in specialized microenvironments called “niches” that are critical for maintaining adult stem cells in an undifferentiated and self-renewing state. The composition and architecture of stem cell niches vary in different tissues (Losick and Spradling, 2011). However, the niches generally consist of these common components: niche cells that provide self-renewal signals and extracellular components such as basement membrane. Stem cells that maintain physical contact with the niche will maintain their stem cell identity, while stem cells that lose contact with the niche will differentiate.

Although stem cell activity is maintained into old age, individual stem cells often are not. Adult stem cells have limited lifespans and are replaced regularly in order to ensure the niche is always inhabited with functional stem cells. A possible mechanism for stem cell replacement is competition for niche occupancy between stem cells and their replacement-competent daughters (Jin et al., 2008). Competition for stem cell niche occupancy could result in two major consequences: 1) the niche is always inhabited with healthy stem cells for tissue homeostasis and regeneration, and 2) the stem cell could acquire spontaneous mutations that enhance stem cell competitiveness for niche occupancy, resulting in a phenomenon that resembles early steps of cancer formation: the spontaneous, competitive mutations enable the mutant stem cell and its mutant progenitors to outcompete neighboring stem cells from their niches and aggressively expand over the tissue (Nystul and Spradling, 2007). Additionally, competition for stem cell niche occupancy between cancer cells and genuine stem cells

has been observed to promote metastasis where cancer cells target a stem cell niche and displace genuine stem cells out of the niche, thus driving the differentiation of genuine stem cells and reducing stem cell number (Shiozawa et al., 2011). Previous studies have shown that a subset of tumor cells that are capable of self-renewal, cancer stem cells, requires that the stem cell niche maintains self-renewal and proliferative capability (Vermeulen et al., 2010; Yauch et al., 2008; O'Brien et al., 2010; Malanchi et al., 2012). Therefore, disruption of the stem cell niche occupancy competition behavior of cancer cells may cause the cessation of tumor formation and propagation. It is now critical to identify the mutations and signaling pathways responsible for regulating stem cell competition for niche occupancy and to understand their molecular mechanisms for the advancement of cancer biology and future development of cancer therapies.

To study the interaction between adult stem cells and stem cell niches *in vivo*, we turn to the highly tractable model, *Drosophila melanogaster*.

DROSOPHILA OVARIAN FOLLICLE STEM CELLS

Genetic studies of *Drosophila* stem cells, with sophisticated cell lineage tracing techniques, have helped define basic principles of stem cell biology. *Drosophila* ovarian epithelial follicle stem cells (FSCs) have served as a model for studying stem cell behavior in an epithelial tissue for more than a decade (Sahai-Hernandez et al., 2012). The FSCs are the source of the epithelia that surround each developing follicle in the Drosophila ovary.

The *Drosophila* ovary consists of 14 to 20 discrete substructures called ovarioles. During adulthood, follicles (or egg chambers) are produced continually from a specialized structure at the anterior tip of the ovariole named the germarium. The germline stem cell (GSC) niche and the FSC niche are both located in the germarium.

Production of a new follicle begins at the GSC niches, which are at the anterior tip of the germarium. After division from the GSCs, the nascent germ cells undergo four incomplete mitoses to become interconnected 16-cell germline cysts. The nascent germ cells and germline cysts are enclosed by stromal cells called escort cells. Germline cysts migrate posteriorly as they mature. When the cysts reach the FSCs at the border of regions 2a and 2b of the germarium (Fig. 1), the germ cells in the 16-cell cyst exit mitosis and one of these cells become the oocyte. The oocyte enters meiosis and the remaining 15 germ cells will become polyploid nurse cells that support oocyte development. After meiosis initiation, the germline cysts widen and line up in a single file. They also replace their escort cell covering with undifferentiated "prefollicle" cells. The germline cyst and prefollicle cells continue to move posteriorly as the cyst matures and the prefollicle cells divide and differentiate gradually.

There are two FSCs in each germarium between germarial regions 2a and 2b and each FSC resides in its own niche (Fig. 1). The FSCs are identified using a combination of criteria: the FSCs are always one of the most anterior labeled cells in a mature FSC clone (Margolis and Spradling, 1995); they are consistently found on each side of the germarium, contacting the basement membrane at the border of regions 2a and 2b; FSCs are triangular shaped, with a broad basal surface and a lateral surface that tapers toward the apical side of the cell (Nystul and Spradling, 2007). In addition, FSCs usually have low levels of fasciclin III (fasIII) compared to prefollicle cells in region 2b (Spradling et al., 1997).

The FSCs divide to produce prefollicle cells which will differentiate into three cell types: epithelial cells surrounding each egg chamber, stalk cells that connect the developing egg chambers, and specialized polar cells. Each FSC produces

approximately half of the follicle cells in the ovariole, thus both FSCs are equally active (Nystul and Spradling, 2007). FSC daughter cells move away from the FSC niche either posteriorly into region 2b or laterally toward the opposite FSC niche (Nystul and Spradling, 2007).

Like other adult stem cells, FSCs are occasionally lost and replaced by a daughter of the remaining stem cell (Nystul and Spradling, 2007). To measure the rate of FSC replacement, Nystul and colleagues generated mitotic clones at low frequency in adult ovaries so that most of the germaria have either zero or one FSC labeled; most germaria are heterogeneous with one labeled FSC and one unlabeled FSC. Over time, interniche FSC replacement occurs, causing a decrease in the frequency of heterogeneous germaria. Using this method, the half-life of wild-type FSCs is observed to be approximately 12 days (Nystul and Spradling, 2007). Additionally, this lineage tracing method allowed Nystul and colleagues to observe that in wild-type ovaries, laterally cross-migrating FSC daughter cells (cmcs) regularly come into contact with the FSC on the opposite side of the germarium. These cmcs appear to be capable of displacing a FSC from its niche, suggesting that they compete for niche occupancy with the resident stem cell.

Previous genetic mosaic studies have provided evidence and insight into the mechanism of FSC maintenance by identifying mutations that alter the half-life of the mutant stem cell. Several signaling pathways, summarized below, are found to be essential in regulating normal FSC maintenance and competitive behavior.

Hedgehog (Hh) signaling

The Hh ligand is produced and secreted by GSC niche cells, cap cells, and escort cells. Hh signaling is required for FSC maintenance, as indicated by the

observation that mutant FSCs for *smoothened*, a positive regulator of Hh signaling, have a reduced lifespan compared to wild-type (Zhang and Kalderon, 2001; Vied and Kalderon, 2009). Hh signaling also affect FSC competitive behavior as it was observed that mutant FSCs lacking *patched* induced higher competitiveness (Vied and Kalderon, 2009; Zhang and Kalderon, 2001). Additionally, Hh regulates proliferation of prefollicle cells to promote development toward the stalk and polar lineages (Forbes et al., 1996). However, a recent study observed that Hh signaling is not specific for the FSC niche. Instead, it is a general signal that regulates proliferation and differentiation in FSCs and prefollicle cells (Sahai-Hernandez and Nystul, 2013).

Wingless (Wg) Signaling

Wingless ligands are produced and secreted from escort cells to specifically act on FSCs (Sahai-Hernandez and Nystul, 2013). Wg signaling regulates both FSC maintenance and proliferation of FSC lineage (Song and Xie, 2003). FSCs lacking *disheveled* or *armadillo*, positive regulators of Wg signaling, produce fewer follicle cells, indicating that proliferation is affected in FSCs, prefollicle cells, or both. These mutant FSCs are also lost from the niche more rapidly compared to wild-type FSCs. Overproliferation of the FSC lineage was observed when negative regulator of Wg signaling, either *axin* or *shaggy*, was removed. However, mutant FSCs with constitutive active Wg signaling are prematurely lost. A possible explanation is that a precise intermediate level of Wg signaling is required for normal FSC maintenance.

Bone Morphogenetic Protein (BMP) signaling

BMP signaling pathway is essential for FSC maintenance, but not proliferation or differentiation of the FSC lineage (Kirilly et al., 2005). Mutant FSCs for BMP pathway receptor *punt*, *thickveins*, or *saxophone* are rapidly lost from the niche compared to wild-

type control FSCs. Conversely, overexpression of constitutively active *thickveins* in FSCs exhibited a prolonged lifespan. In addition, Kirilly et al. found that overexpression of constitutively active *thickveins* in FSCs rescued the rapid loss of *disheveled* mutant FSCs to wild-type levels, but not that of *smoothened* mutant FSCs. This suggests that BMP signaling may act in conjunction with Wg signaling, but in parallel to Hh signaling.

JAK/STAT Signaling

JAK/STAT signaling is required for FSC maintenance and regulation of competitive behavior (Vied et al., 2012). FSCs lacking JAK (*Hopscotch*), or STAT, (*DStat92E*), are prematurely lost from the niche. In contrast, FSCs with overexpression of Hop have a prolonged lifespan, and hyper-competitiveness for niche occupancy. Like the BMP signaling pathway, JAK/STAT signaling may be acting in conjunction with other signaling pathways, Hh, BMP, and Wg pathways (Vied et al., 2012). Hyperactivity of the JAK/STAT pathway compensated for the loss of activity in Hh, Wg, or BMP pathway, suggesting that JAK/STAT signaling may be collaborating with multiple pathways to influence FSCs (Vied et al., 2012).

Studies have been performed to identify which signaling pathway(s) is(are) necessary and sufficient for FSC maintenance. In one study, Hh and JAK/STAT pathways were shown to be the key regulators for FSC maintenance since their signaling levels need to be within a precise range for normal FSC behavior (Vied et al., 2012). However, a more recent study showed evidence that the Wg signaling pathway is the key and specific pathway for FSC maintenance, while Hh is a general signal for all cell types in the germarium (Sahai-Hernandez and Nystul, 2013). The signaling ligands known to regulate FSC maintenance emanate from different distant sources, so one would expect a shallow gradient across the FSC niche (Zhang and Kalderon, 2001;

Hayashi et al., 2012). However, there is evidence that the primary signal for FSC maintenance is produced and released from escort cells, which are immediately adjacent to FSCs and are physically contacting FSCs (Sahai-Hernandez and Nystul, 2013). The model presented by Sahai-Hernandez and Nystul suggests that the FSC niche has a similar architecture to the GSC niche where the niche factor is produced and released by niche cells directly in contact with the GSCs (Hayashi et al., 2009). Nonetheless, FSC behavior in response to these signals is dosage-dependent, so reception of signal ligands at FSCs is tightly regulated (Vied et al., 2012). One common theme among these signaling pathways is that the ligands for these signaling pathways are known to associate with heparan sulfate proteoglycans (HSPGs).

HEPARAN SULFATE PROTEOGLYCANS (HSPGS) AND THEIR POST-TRANSLATIONAL MODIFICATIONS

HSPGs are a class of carbohydrate-modified proteins that play an essential role in proper distribution and activity of extracellular signaling molecules. They can be found on the cell surface and in the extracellular matrix (ECM). HSPGs consist of a core protein with long, unbranched HS chains of repeating disaccharide units covalently attached at serine residues. There are three evolutionarily conserved classes of HSPGs: glypicans, syndecans, and perlecans. Glypicans and syndecans are cell surface HSPGs and are linked to the plasma membrane by a glycosylphosphatidylinositol (GPI) anchor or a transmembrane domain, respectively. Perlecans are secreted HSPGs that are found in the ECM. The *Drosophila* genome encodes two glypicans, division abnormally delayed (dally), and dally-like protein (dlp), a single syndecan (sdc), and a single perlecan, terribly reduced optic lobes (trol). *dally*

and *dally-like protein* (*dlp*), have been shown to control Hh, Upd, Wg, and BMP signaling (Kirkpatrick and Selleck, 2007; Yan and Lin, 2009, Williams et al., 2010; Kim et al., 2011; Hayashi et al., 2012).

There are two known types of HSPG post-translational modifications: 1) modification of HS chains and 2) core protein modification, i.e. glypican shedding. HS chains undergo modification following polymerization. These modifications include 1) *N*-deacetylation and *N*-sulfation of GlcNAc units catalyzed by HS *N*-deacetylase/*N*-sulfotransferase (NDST, *sulfateless* (*sfl*) in *Drosophila*), 2) *C5*-epimerization of GlcA residues catalyzed by HS *C5*-eipmerase (Hsepi), and 3) *O*-sulfation at different positions catalyzed by HS *O*-sulfotransferases (*Hs2st* and *Hs6st*). Genetic studies showed that mutations in HS biosynthetic/modification enzymes result in defects in various signaling pathways in Drosophila and mice (Gorsi and Stringer, 2007; Ori et al., 2008). After HSPGs are transported to the cell surface, secreted 6-*O*-endosulfatases (Sulfs) catalyze the removal of 6-*O*-sulfate group within the HS chains in the extracellular space. The 6-*O* sulfate group is the only known HS structural modification that occurs after HSPGs are presented on the cell surface. This allows the cell to modulate the activity of different HS-dependent signaling pathways. Functional studies of vertebrate *Sulfs* and *Drosophila Sulf1* have shown that they regulate Wnt, FGF, BMP and Hh signaling (Ai et al., 2003; Viviano et al., 2004; Kleinschmit et al., 2010; Wojcinski et al., 2011). These findings suggest that 6-*O* sulfate groups are a key component of the binding site on HS for most protein ligands.

Glypicans can be cleaved at their GPI-linkage and shed from the cell surface. Previous studies found that a secreted hydrolase, Notum, releases Dally and Dlp from the cell surface to modulate Hh and Wg signaling activities as a negative feedback

(Giraldez, 2002; Kreuger, 2004; Han, 2005; Ayers, 2010). Notably, it is known that Notum can convert glypican activity from a negative to a positive factor (Kreuger, 2004; Kirkpatrick, 2004). Therefore, Notum-mediated glypican shedding is another key event regulating signaling activities. Interestingly, it has been recently reported that Notum is specifically expressed in the FSCs (Sahai-Hernandez, 2013).

PURPOSE OF STUDY

Since signaling pathways that regulate FSC maintenance and competitive behavior identified thus far are HS-dependent, we postulate that HSPGs and their post-translational modifications have a role in regulating FSC competitive behavior. The objective of this study is to define the functions and mechanisms of HSPGs and their post-translational modifications in stem cell maintenance and competition for niche occupancy. Adult stem cells are central to both normal homeostasis and the progression of disease such as cancer. Thus, understanding how they are regulated in their native, *in vivo* contexts is important to provide a foundation for advancement of stem cell and cancer biology, as well as future development of cancer treatments and regenerative medicine-based therapies.

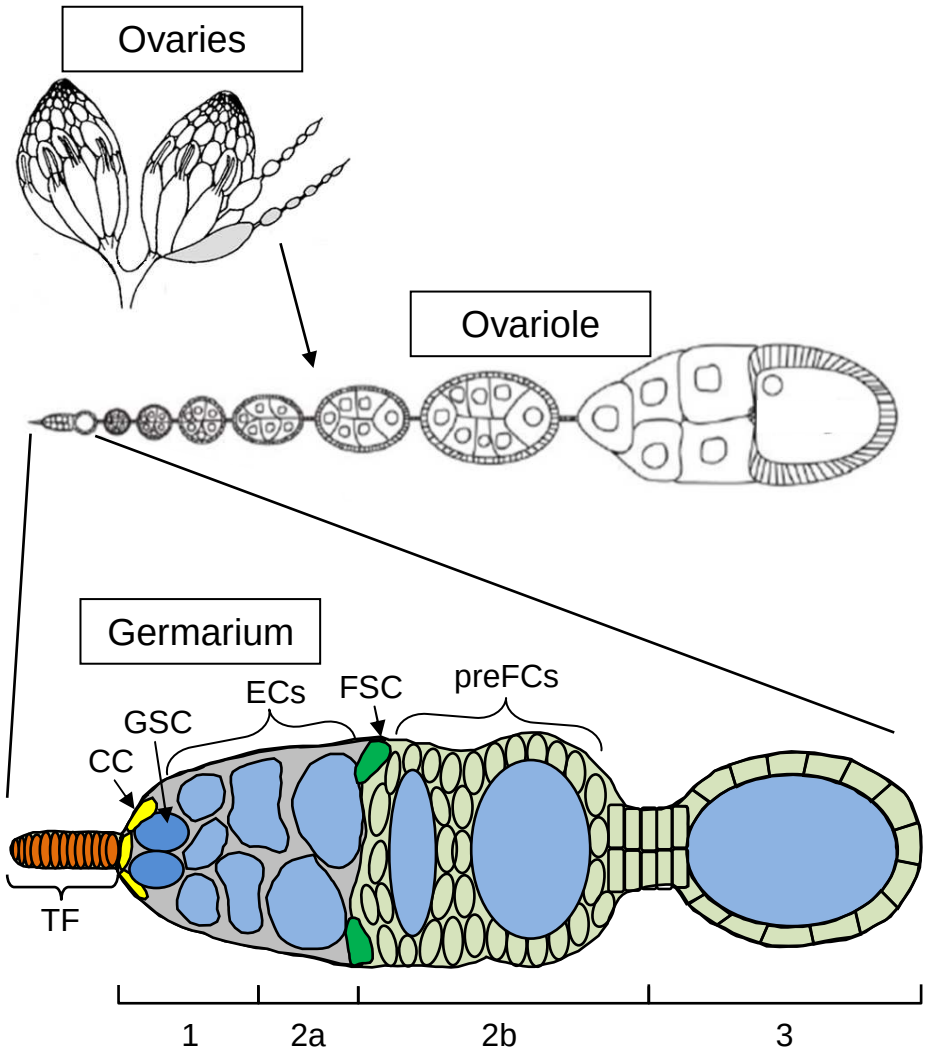

Figure 1: The *Drosophila* ovary. The *Drosophila* ovary is composed of multiple units of follicle production called ovarioles. At the anterior tip of each ovariole, a structure called the germarium houses the two populations of stem cells, germline stem cells (GSCs, dark blue) and follicle stem cells (FSCs, dark green). In addition, there are multiple cell types in the germarium: terminal filament cells (TF, orange), cap cells (CCs, yellow, niche cells for the GSCs), escort cell (ECs, gray, surround germ cell cysts which are shown in light blue), prefollicle cells (preFCs, light green), and follicle cells (light green). The germarium is divided in regions 1, 2a, 2b and 3, which are defined by the developmental stages of the germline cysts. There are two FSCs in each germarium and they reside in separate niches, one on each side of the germarium at the border of regions 2a and 2b.

MATERIALS AND METHODS

Fly strains

Flies were raised at 25℃. Enhancer trap lines used for *dally* and *dlp* expression detection are *dally*^{P1} and *y*^{1} *w*^{67c23}*; P{w*$^{+mC}$*=GSV6}GS9658* (DGRC, Kyoto, Japan), respectively. Protein trap lines used to detect expression of Trol and Sdc: *trol*^{ZCL1700} (DGRC, Kyoto, Japan), *sdc*^{CC00871} (Bloomington Drosophila Stock Center, Bloomington, IN, USA). Notum expression: *yw;Notum-lacZ* (gift from Dr. Todd Nystul, UCSF, San Francisco, CA, USA). MARCM clone induction: *y w hs-FLP tub-GAL4 UAS-GFP[nls];; FRT2A tub-GAL80* (gift from Dr. Huaqi Jiang, University of Texas Southwestern Medical Center, Dallas, TX, USA), *w;FRT2A, sfl*^{9B4} *FRT2A, dally*^{80}*dlp*^{A187} *FRT2A, dally*^{gem} *FRT2A, dlp*^{A187} *FRT2A. UAS-Notum RNAi (*VDRC #103775, Vienna, Austria).

Immunohistochemistry and microscopy

Ovaries were dissected as previously described (Fujise et al., 2001; Hayashi et al., 2009; Hayashi et al., 2012). Antibodies used were: mouse anti-Fas3 [1:200, Developmental Studies Hybridoma Bank (DSHB), chick and mouse anti-β-galactosidase (1:200, abcam and DSHB, respectively), rabbit anti-GFP (1:1000, Invitrogen), rabbit and rat anti-Vasa (1:1000 and 1:50, respectively, gifts from A. Nakamura, RIKEN Center for Developmental Biology, Kobe, Japan, and DSHB). Secondary antibodies conjugated with Alexa-488, Alexa-546, or Alexa-633 were used in 1:500 dilutions (Molecular Probes). Stained ovaries were mounted in Vectashield (Vector Laboratories) and imaged by confocal microscopy (Nikon C1).

Clone induction

Adult female flies that were 2-4 days old after eclosion were heat shocked twice for 1 hour (about 8 hours apart) at 37℃ and then were ke pt at 25℃ for 7-21 days before dissection.

Replacement assay calculation

A = decrease in germaria containing 1 marked FSC clone between 7 dphs and 21 dphs.

B = increase in germaria containing 0 marked FSC clone between 7 dphs and 21 dphs.

C = increase in germaria containing 2 marked FSC clone between 7 dphs and 21 dphs.

In our observation, A = B+C. The relative proportions were calculated by the following:

For increase in germaria containing 0 marked FSC clone: B/A

For increase in germaria containing 2 marked FSC clone: C/A

RESULTS

Glypicans, Dally and Dlp, are expressed in posterior escort cells, FSCs, and follicle cells

To determine whether HSPGs regulate FSC maintenance and competitive behavior, we examined whether HSPGs are expressed in the germarium. Using enhancer trap lines, we found that glypicans, *dally* and *dlp,* have the same expression pattern in the germarium: *dally* and *dlp* are expressed in escort cells in germarial region 2a, FSCs, and all follicle cells in the germarium (Fig. 2A-B). We also detected expression of Sdc and Trol in the germarium using protein trap lines. Sdc is expressed on the cell surface of germ cells as well as in the basement membrane (Fig. 2C-C'). Trol is expressed in the basement membrane and the muscle sheath encapsulating the ovariole (Fig. 2D-D').

HS biosynthesis and glypicans are required for FSC maintenance and niche competitive behavior regulation

Since glypicans, *dally* and *dlp*, are expressed in the FSCs, we tested whether they function in FSC maintenance and niche competition. FSC maintenance and competition behavior can be measured by examining the persistence of marked FSC lineages of defined genotype that are generated by heat-shock-induced FRT mediated mitotic recombination in young adults using Mosaic Analysis with a Repressible Cell Marker (MARCM, Lee and Luo, 2001). FSC lineages are marked by GFP and ovaries are examined at 7, 14 and 21 days post heat shock (dphs). FSC daughters proliferate, differentiate and exit the ovariole within 5 days at 25℃ (Margolis and Spradling, 1995). Hence, all marked clones examined 7 or more days after heat shock must derive from recombination events induced in FSCs ("FSC clones"). Any reduction or increase in the

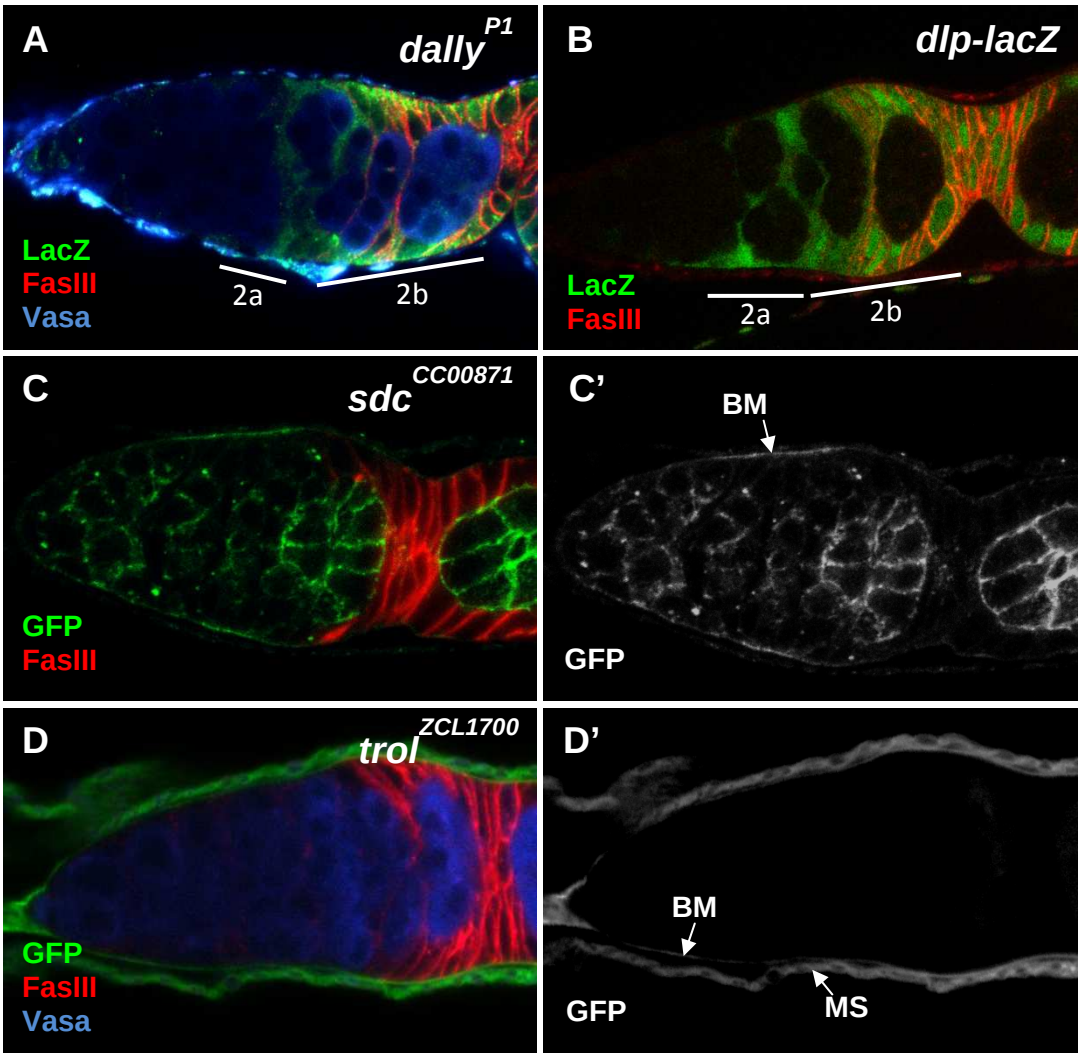

Figure 2: **Expression patterns of HSPGs in the germarium.** (A and B): glypicans are expressed in a similar pattern in the germarium as showed by *dally* and *dlp* enhancer trap lines. *dally* and *dlp* are expressed in posterior escort cells in region 2a, FSCs, and follicle cells in the germarium. (C and C'): Sdc is expressed on the cell surface of germ cells and the basement membrane (BM). (D and D'): Trol is expressed in the basement membrane (BM) and the muscle sheath (MS) surrounding the ovariole.

frequency of marked mutant FSC clones relative to marked control clones at 7 days or thereafter reflect a selective loss or gain of FSC clones, respectively, that could be attributed to their mutant genotypes. Marked, or GFP-positive, FSC clones can be induced with heat shock conditions where the majority of the germaria containing GFP-positive clones have only one marked FSC in the germarium, which allows the examination of competitive behavior (Vied et al., 2012). In wild-type control ovaries, a slow decrease in marked FSC clone frequency is observed due to normal slow turnover (Zhang and Kalderon, 2001; Song and Xie, 2003; Kirilly et al., 2005; Vied et al., 2009 & 2012, Fig. 4A). If a mutation in FSC causes fast turnover or loss of FSC maintenance (less competitive phenotype), the frequency of mutant, GFP-labeled FSC clones will decrease (Fig. 3A). In contrast, if a mutation in FSC causes hyper-competition, the frequency of mutant, GFP-labeled FSC clones will remain at the initial frequency of GFP-positive clones (Fig. 3A). As an extreme case, the GFP-positive progenitors occupy the entire epithelial sheet ("all marked" phenotype). Since only one FSC is being induced, "all marked" ovariole would demonstrate that the induced (GFP-marked) FSC has invaded the other FSC niche and replaced the unmarked FSC to produce an "all marked" ovariole.

To examine the role of HSPGs in FSC maintenance and competitive behavior, we first generated FSC mutant clones for *sulfateless (sfl),* analyzed the FSC clone frequency and compared with that of wild-type control FSC clones. *sfl* encodes the only *Drosophila* homolog of heparan sulfate *N*-deacetylase/*N*-sulfotransferase (NDST). Since *N*-sulfation of glucosamine residues is essential for subsequent modifications of HS chains, *sfl* null mutations will disrupt most, if not all, activities of HS chains and in turn impair all known HS-dependent pathways such as BMP, Hh and Wnt signaling

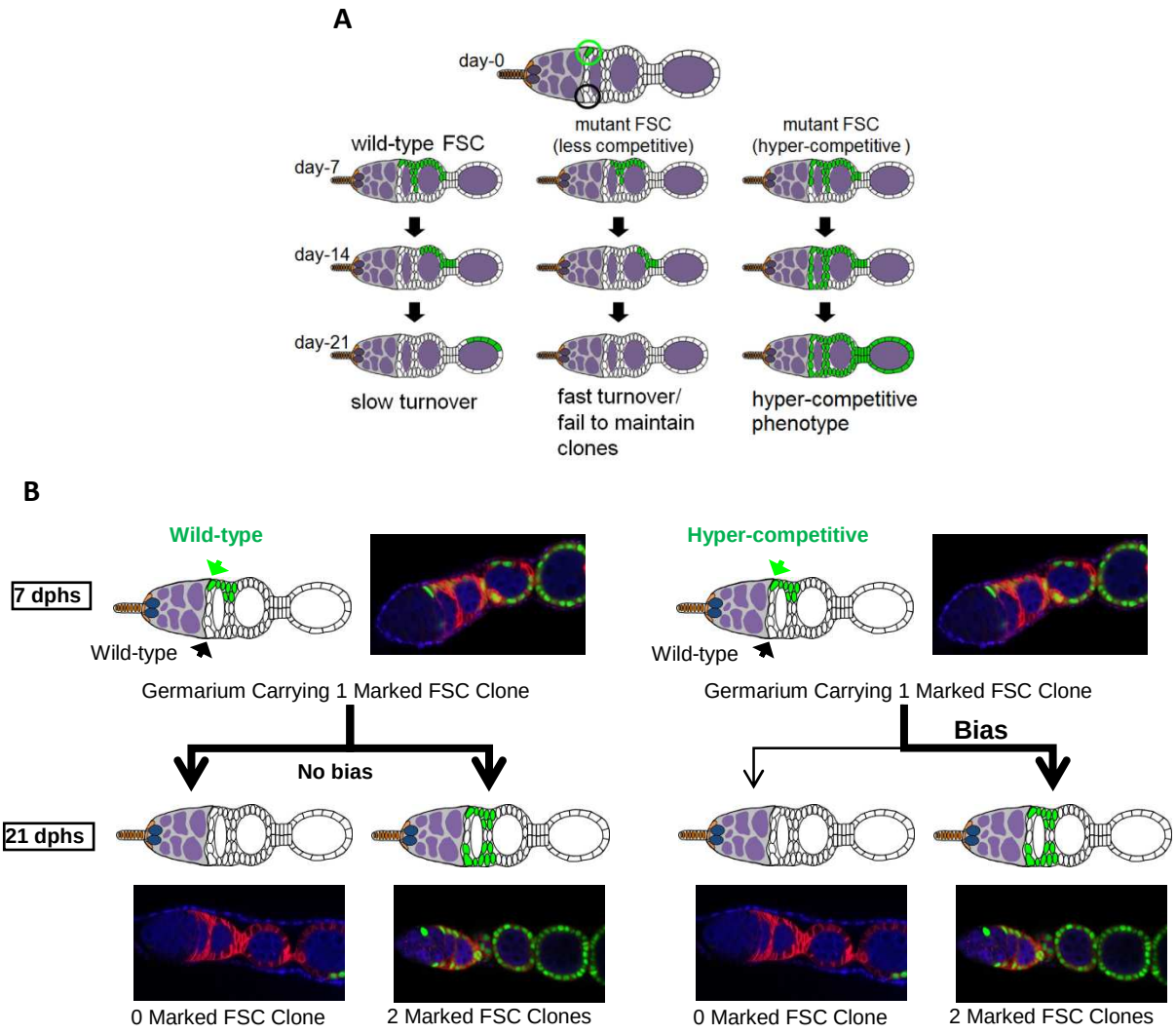

Figure 3: Experimental strategies using MARCM system. (A): FSC maintenance assay. The presence of GFP(+) FSC clones are measured and compared between control and mutants over time. (B): FSC replacement assay. The number of GFP(+) FSC clones are counted in this assay. The increase in zero or 2 marked FSC clones between 7 and 21 dphs are being analyzed to determine if the marked cells have niche competitive advantage or disadvantage compared to control.

pathways (Lin et al., 1999; Lin and Perrimon, 1999). As expected, a slow decrease in GFP-positive FSC clone frequency is observed for wild-type control ovaries (Fig. 4A). Our data showed that *sfl* null mutation caused a significant decrease in the frequency of mutant FSCs over time compared to control, indicating that HS is essential in FSC maintenance (Fig. 4A). This result also suggests that signaling ligands interact with HS chains of HSPGs in order to regulate FSC behavior. Since *sfl* mutants lack *N*-, 6-*O*-, and 2-*O*- sulfate groups in the disaccharide units, HS sulfation state could also play a role in regulating FSC maintenance.

Drosophila glypicans, *dally* and *dlp*, are also regulators of FSC maintenance. The frequency of *dally* mutant FSC clones decreased more rapidly than control from 7 dphs to 21 dphs, thus *dally* is required for FSC maintenance (Fig. 4A). Interestingly, the frequency of *dlp* mutant FSCs did not change significantly over time (Fig. 4A). This phenotype suggests that *dlp* mutant FSCs and possibly FSC daughter cells are more likely to remain in the niche. In many instances, we found that the marked *dlp* mutant cells occupy the entire follicular epithelium, exhibiting the “all-marked” phenotype. This raises the possibility that *dlp* mutants are more competitive for the stem cell niche. We observed that *dally-dlp* double mutant FSC clones also remained at approximately the same frequency over time, indicating that *dally* and *dlp* could be acting in an epistatic relationship (Fig. 4A).

To further investigate whether *dlp* mutants are more competitive for the FSC niche, we assessed FSC replacement between wild-type control and mutant cells in the germarium. After heat shock treatment to induce GFP-positive FSC clones, we quantified the number of germaria with 0, 1, or 2 marked FSC clones at 7 dphs. We found that the majority of germaria at 7 dphs either contain 0 or 1 marked FSC clone. In

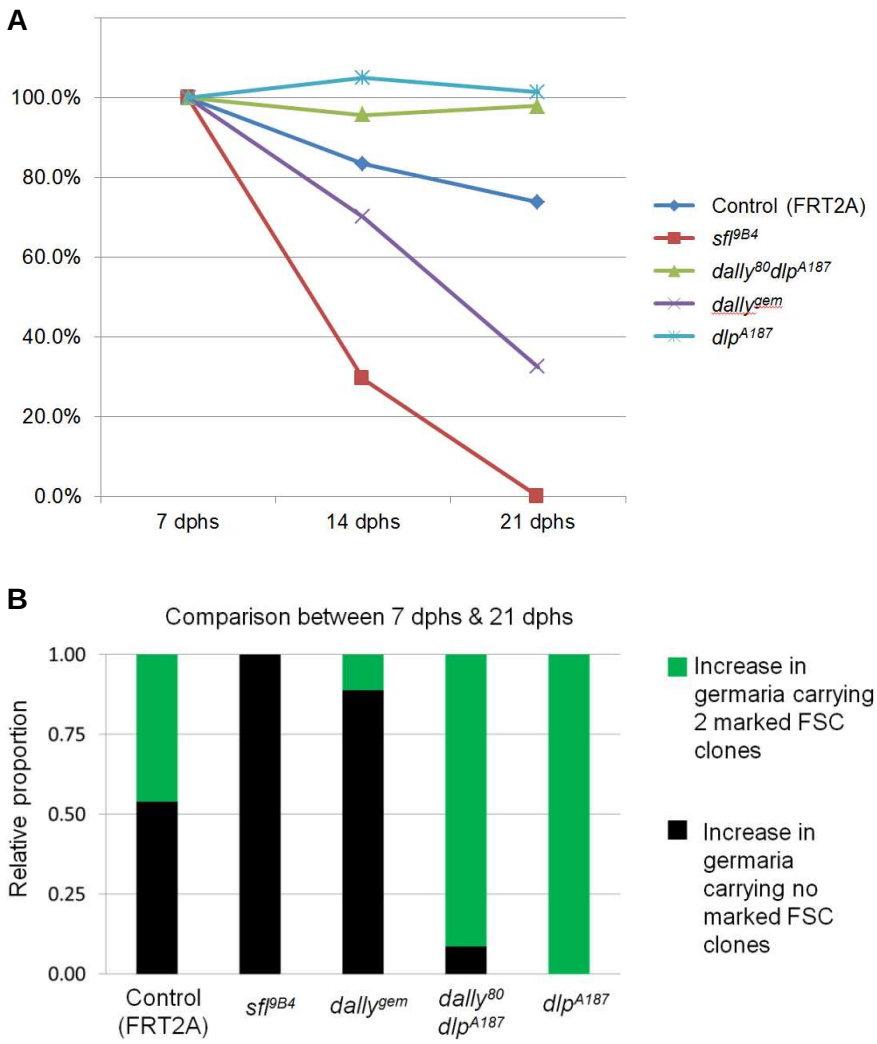

Figure 4: *sulfateless* and glypicans are required for regulating FSC maintenance and competitive behavior. (A): The percentage of germaria containing FSC clones for the control (dark blue), *sfl*^{9B4} (red), *dally*^{80}*dlp*^{A187} (green), *dally*^{gem} (purple), and *dlp*^{A187} (aqua) as a function of time after clone induction. The 7dphs values are normalized to 100% and the values for 14 dphs and 21 dphs are compared relative to the 7 dphs value. In the control, the percentage decreases over time due to natural stem cell turnover. A greater percentage of mutant stem cells are lost for *sfl*^{9B4} and *dally*^{gem}. The percentages of mutant FSC clones persist over time for *dally*^{80}*dlp*^{A187} and *dlp*^{A187}. (B): Results of the replacement assay as described in Fig. 3 and in the text. We compared between the increase in 0 or 2 marked FSC clones between 7 dphs and 21 dphs as proportions of the decrease in 1 marked FSC clone between 7 dphs and 21 dphs. Decrease in 1 marked FSC clone between 7 dphs and 21 dphs is normalized to 100%. Raw data for all genotypes are presented in Table 1.

a germarium with 1 marked FSC clone, there are two possible fates for the marked FSC: either the marked FSC is replaced by an unmarked FSC daughter resulting in germarium with no marked clone, or the marked FSC daughter replaces the unmarked FSC, producing 2 marked FSC clones (Fig. 3B). Therefore, when we examine the number of germaria with 0, 1, or 2 marked FSC clones at 21 dphs, we will see a decrease in the percentage of germaria with 1 marked FSC clone and increases in the percentages of germaria with 0 and 2 marked FSC clones compared to results obtained at 7 dphs (Fig. 3B). By comparing the increase of germaria containing 0 and 2 marked clones, we can determine if marked FSC daughter cell is more likely to replace unmarked FSC, or vice versa. The ratio of these two increases will reflect the competitiveness for niche occupancy, and how it is affected by mutation. In wild-type control, marked and unmarked cells do not have competitive advantage or disadvantage for niche occupancy. As a result, we observed that the increases of germaria with 0 or 2 marked FSC clones between 7 and 21 dphs for the control are comparable (54% and 46%, respectively), as we expected (Fig. 3B, 4B). In other words, there's no bias between increase in 0 or 2 marked FSC clones (Fig. 3B). For *dlp* and *dally-dlp* double mutants, we observed that the increase of germaria containing 2 marked FSCs are significantly higher than the increase in germaria containing 0 marked FSCs (Fig. 4B). Thus the marked mutant FSC daughter cells are replacing unmarked wild-type FSCs at a higher rate compared to the wild-type control. Therefore, our results suggest that *dlp* and *dally-dlp* double mutant FSC daughter cells are more fit to occupy the FSC niche compared to WT FSCs. As a confirmation, we also assayed the replacement of *sfl* and *dally* mutants. As expected, we observed a bias towards 0 marked FSCs for both *sfl*

and *dally* mutants, indicating that these mutant FSCs are less fit to be maintained in the niche (Fig. 4B).

Notum is a potential negative regulator of competitive behavior for FSC niche occupancy

Previously, it was found that Notum is expressed specifically in the FSCs (Sahai-Hernandez and Nystul, 2013), and we confirmed this expression pattern (Fig. 5). We are interested in learning about Notum's role in the FSCs because Notum is known to cleave glypicans at their GPI anchors to modulate Wg signaling activity (Giraldez, 2002; Kreuger et al., 2004; Han et al., 2005; Traister et al., 2008). In our next experiments, we sought to investigate if Notum has a role in regulating FSC maintenance and competitive behavior. Using MARCM again, we measured the persistence of marked mutant FSC clones with Notum knocked down using RNAi. In the Notum RNAi flies, we observed that the increase of germaria containing 2 marked FSCs is significantly higher than the increase in germaria containing 0 marked FSCs, compared to wild-type control (Fig. 5). Therefore, knockdown of Notum with RNAi may have enabled cells to better fit in the FSC niche and thus increased replacement of unmarked wild-type FSCs.

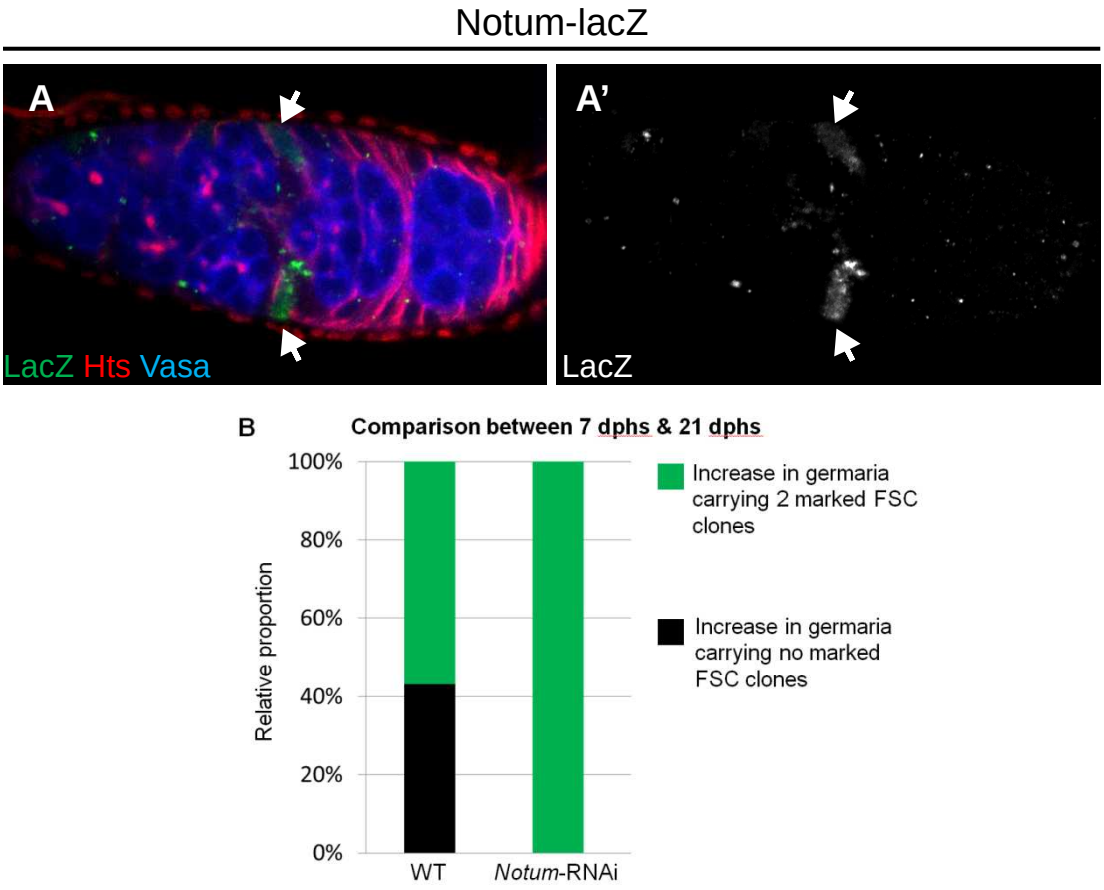

Figure 5: Notum is expressed in the FSCs and could be regulating FSC competitive behavior for niche occupancy. (A and A'): Notum-lacZ is specifically expressed in the FSCs (arrows). (B): Results of the replacement assay as described in Fig. 3 and in the text for Notum RNAi. This graph shows the comparison between the increase in 0 or 2 marked FSC clones between 7 dphs and 21 dphs as proportions of the decrease in 1 marked FSC clone between 7 dphs and 21 dphs. Decrease in 1 marked FSC clone between 7 dphs and 21 dphs is normalized to 100%. Raw data for all genotypes are presented in Table 1.

Table 1: Percentages of germaria containing 0, 1, or 2 marked FSC clones for all genotypes examined. "(1+2) Normalized" values at 14 dphs and 21 dphs are compared to that of 7 dphs.

	7 dphs					14 dphs					21 dphs				
	% of GFP (+) FSC Clones					% of GFP (+) FSC Clones					% of GFP (+) FSC Clones				
Genotype	**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	50.0%	39.8%	10.2%	50.0%	100.0%	58.3%	24.0%	17.6%	41.67%	83.33%	63.1%	15.5%	21.4%	36.9%	73.79%
***sfl*^{9B4}**	70.8%	28.3%	0.9%	29.2%	100.0%	91.4%	8.6%	0.0%	8.57%	29.35%	100.0%	0.0%	0.0%	0.0%	0.00%
***dally*^{9em}**	71.9%	24.8%	3.3%	28.1%	100.0%	80.3%	11.1%	8.5%	19.66%	69.96%	90.9%	3.4%	5.7%	9.1%	32.35%
***dlp*^{A187}**	59.5%	34.5%	6.0%	40.5%	100.0%	57.5%	18.0%	24.5%	42.50%	105.00%	58.9%	17.3%	23.8%	41.1%	101.47%
***dally*^{80}*dlp*^{A187}**	56.3%	34.8%	8.9%	43.8%	100.0%	58.1%	24.2%	17.7%	41.94%	95.85%	57.9%	15.1%	27.0%	42.1%	96.15%
Notum															
FRT2A	62.50%	31.25%	6.25%	37.5%	100.0%	N/A	N/A	N/A	N/A	N/A	70.00%	13.85%	16.15%	30.0%	80.00%
Notum RNAi	72.67%	24.22%	3.11%	27.3%	100.0%	N/A	N/A	N/A	N/A	N/A	70.00%	10.63%	19.38%	30.0%	109.77%

DISCUSSION

In this study, we have identified glypicans as regulators of FSC maintenance and niche competitive behavior. We found that the two glypicans in *Drosophila*, *dally* and *dlp*, have opposing phenotypes in the FSCs. Mutant FSCs of *dally* are lost from the FSC niche rapidly while *dlp* mutants are more likely to remain in the FSC niche as *dlp* mutant daughter cells are more likely to replace WT FSCs. Based on our double mutant results, it is likely that *dally* and *dlp* are in an epistatic relationship. More studies are warranted to further elucidate this relationship.

It will be of great interest and importance to identify the signaling pathways being regulated by *dally* and *dlp*. The two glypicans could each be controlling separate signaling pathways that can interact to regulate FSC maintenance. For example, Dally is the regulator for JAK/STAT signaling pathway and Dlp is the major regulator of Hh signaling (Kim et al., 2011; Hayashi et al., 2012). In addition, *dally* and *dlp* could be acting in an opposing fashion in the same signaling pathway. An example of *dally* and *dlp* having opposing roles in the same pathway was shown in the *Drosophila* imaginal wing disc. Dally is a positive regulator of the Wg signaling pathway while Dlp is a negative regulator. This is due to the presence of a secreted hydrolase, Notum, which is known to cleave glypicans at their GPI-linkage (Giraldez et al., 2002; Gerlitz et al., 2002; Kreuger et al., 2004; Han et al., 2005). In this study, our results showed that Notum RNAi FSC daughters exhibit hyper-competitive behavior for the FSC niche. The phenotype that we observed for Notum RNAi is the same as we observed for *dlp* mutants. Therefore, it is possible that Notum expressed in the FSCs modulate the function of Dlp. Our current model is depicted in Figure 6. Signaling proteins or niche factors are released from posterior escort cells (Sahai-Hernandez and Nystul, 2013),

and they bind to Dlp or Dally, which are potentially functioning as co-receptors. As a response to high signaling level, the FSCs release Notum as a negative feedback. Notum then selectively cleaves Dlp, but not Dally, from the surface of FSC (selective cleavage of Dlp, but not Dally, has been observed previously (Kreuger et al., 2004)). The release of Dlp from the cell surface turns it into a negative regulator, thus maintaining an optimal signaling level. In *dlp* mutants, there is no Dlp for Notum to cleave, so this leads to an accumulation of signaling protein on the cell and higher signaling activity, which could explain the hyper-competitive behavior we observed. In response to Notum RNAi, Dlp is not released from the surface of FSCs and/or FSC daughters. This also causes accumulation of signaling proteins on the cell surface, again leading to high signaling activity and potentially leading to the observed hyper-competitive phenotype. Although our Notum results are intriguing, it should be noted that the same experiments should be repeated with null mutants to confirm this hyper-competitive phenotype we observed for RNAi knockdown. If confirmed, it will provide further insight into the mechanism of stem cell behavior regulation and also it will provide further evidence supporting the significance of glypican shedding.

Two other conserved classes of HSPGs, Sdc and Trol, are expressed in the germarium (Fig. 2C-D). We found that Sdc and Trol are both expressed in the basement membrane. FSCs, like other stem cells (e.g. Drosophila intestinal stem cells), have a distinct triangular shape with a broad basal surface and a lateral surface that tapers toward the apical side (Sahai-Hernandez and Nystul, 2010). A previous study showed that integrins anchor FSCs to the BM, enabling FSCs to maintain their characteristic morphology and position (O'Reilly et al., 2008). The authors also proposed that integrins

and their ligand, laminin A, affect FSC proliferation rates, suggesting a possible contribution of the BM to the FSC niche, possibly through Sdc and/or Trol as they have

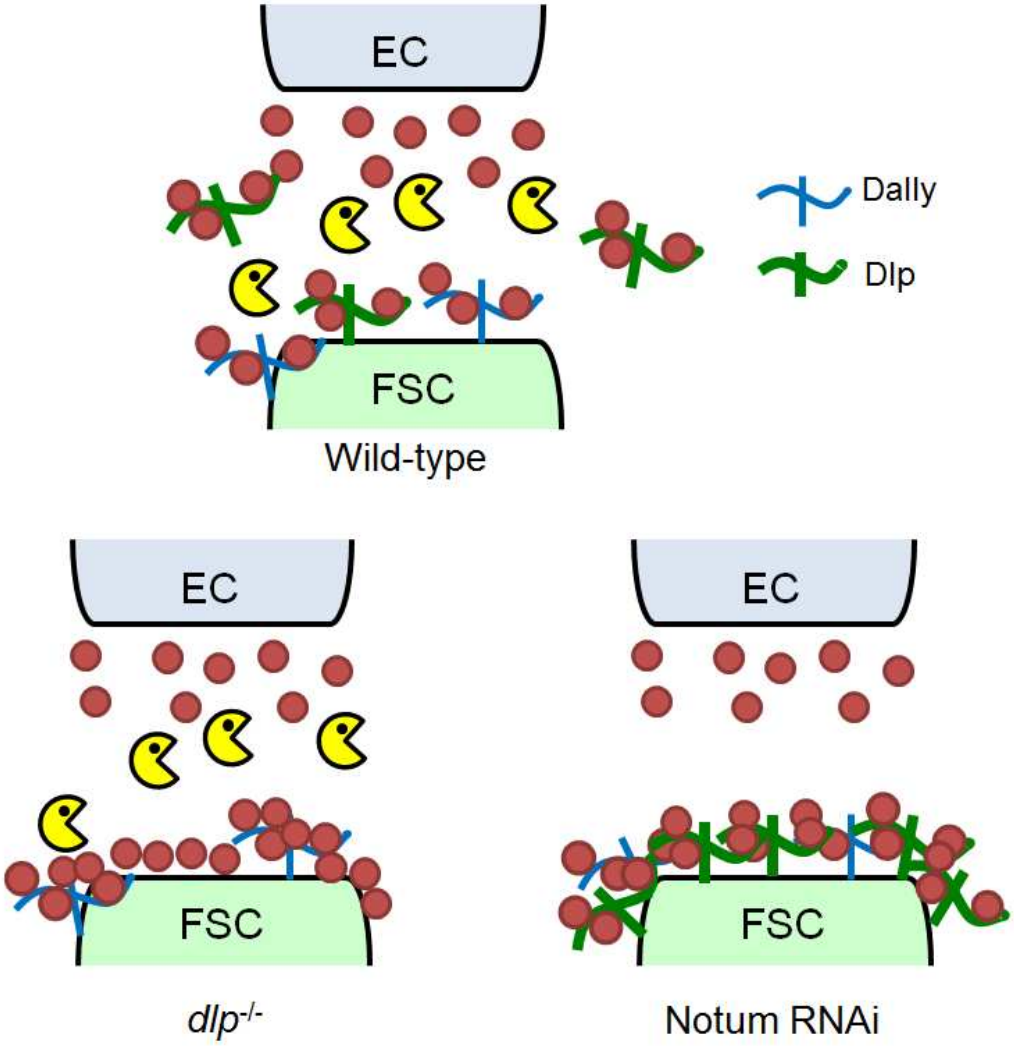

Figure 6: Current model of regulation of FSC behavior by glypicans and Notum. Signaling ligands (red), or niche factors, are secreted from escort cells (EC, blue cell). The FSC (green cell) receives the ligands via co-receptors Dally (blue) and Dlp (green). As signaling activity increase, Notum (yellow) is expressed and secreted by FSC as a negative feedback response. Dlp is cleaved by Notum from the cell surface, consequently maintaining signaling activity at optimal level. In both *dlp* mutants and Notum RNAi, signaling ligands are able to accumulate at the cell surface of FSC and FSC daughters, thus potentially causing the hyper-competitive phenotype.

previously been shown to interact with integrin and play a significant role in the ECM, respectively (Kirkpatrick and Selleck, 2007; Park et al., 2003).

HS chain modification (sulfation) could also be essential for FSC maintenance as indicated by our results in *sfl* mutants (Fig. 4). *sfl* mutants lack sulfation on the HS chains, including *N*-, 2-*O*-, and 6-*O*- sulfation on the disaccharide units, which are essential for regulating ligand binding. Functional studies of vertebrate and *Drosophila* sulfotransferases and sulfatases have shown that HS-sulfation regulates Wnt, FGF, BMP, and Hh signaling, thus it is likely that HS sulfation affects ligand binding on FSCs (Ai et al., 2003; Viviano et al., 2004; Kleinschmit et al., 2010; Wojcinski et al., 2011).

The *Drosophila* FSC niche offers an excellent system to study stem cell competition for niche occupancy and replacement, which is a major mechanism for stem cell quality control. Since abnormally competitive behaviors of stem cells resemble that of cancer cells, the FSC niche is also a powerful model for epithelial cancers in addition to a model for epithelial stem cell niche (Nystul and Spradling, 2007). In this study, we found that Dlp may be involved in suppressing competitive behavior for niche occupancy. It is important to continue to search for genes and mechanisms that regulate this type of stem cell behavior. Our knowledge gained in the *Drosophila* FSC niche will provide insights into the mechanisms by which an epithelial stem cell niche functions and by which cancer arises.

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