Drosophila glypicans regulate the germline stem cell niche

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tem cells are maintained in vivo by short-range signaling systems in specialized microenvironments called niches, but the molecular mechanisms controlling the physical space of the stem cell niche are poorly understood. In this study, we report that heparan sulfate (HS) proteoglycans (HSPGs) are essential regulators of the germline stem cell (GSC) niches in the Drosophila melanogaster gonads. GSCs were lost in both male and female gonads of mutants deficient for HS biosynthesis. dally, a Drosophila glycan, is expressed in the female GSC niche cells and is responsible for maintaining the GSC niche. Ectopic expression of dally in the ovary expanded the niche area, showing that dally is required for restriction of the GSC niche space. Interestingly, the other glypican, dally-like, plays a major role in regulating male GSC niche maintenance. We propose that HSPGs define the physical space of the niche by serving as trans coreceptors, mediating short-range signaling by secreted factors.

Introduction

Understanding how cells receive positional information during tissue assembly and maintenance is a central problem in cell and developmental biology. Spatially controlled extracellular signals can convey this positional information in distinct ways. For example, in a morphogen system, cells in a tissue receive the same signal but in different amounts; this modulation of signal dosage in turn specifies distinct cell fates. In the stem cell niche, a signal is delivered only to cells within a specialized microenvironment, giving them the characteristics of stemness. The mechanism that spatially restricts this signaling and thus defines the physical space of the stem cell niche remains to be elucidated.

The Drosophila melanogaster gonadal niches provide excellent models to investigate how stem cell niches are regulated in vivo. In the Drosophila ovary, germline stem cells (GSCs) are located at the anterior edge of the gerarium, directly contacting somatic niche cells called cap cells. The cap cells produce decapentaplegic (Dpp), which regulates GSC maintenance by repressing a target gene, bag of marbles (bam), in GSCs (Xie and Spradling, 1998; Chen and McKearin, 2003; Song et al., 2004). After a GSC divides asymmetrically, Dpp represses bam expression in the daughter cell contacting the cap cells, which will remain a GSC (Deng and Lin, 1997; Xie et al., 2005). In the other daughter cell, which has lost contact with the cap cells, Dpp signaling is not activated, and bam directs differentiation into a cystoblast.

The GSC niche is formed at earlier developmental stages (Zhu and Xie, 2003; Asaoka and Lin, 2004). GSCs are derived from primordial germ cells (PGCs) in the embryonic gonads, which proliferate during larval and pupal stages. At the anterior edge of the early pupal ovary, the cap cells differentiate and secrete Dpp, which represses bam expression in the anterior PGCs (Zhu and Xie, 2003). Dpp thus prevents differentiation of the PGCs in close proximity to the cap cells, allowing them to become GSCs. Eventually, only PGCs directly contacting the cap cells will be GSCs in the adult ovary. Although Dpp is a secreted molecule, in this context, it mediates short-range or contact-dependent signaling in both the pupal and adult stages. The mechanism that spatially limits Dpp signaling and therefore the size of the niche is unknown.

Abbreviations used in this paper: Bam, bag of marbles; BMP, bone morphogenetic protein; Dip, Dally-like; Dpp, decapentaplegic; E-Cad, epithelial cadherin; FasIII, fasciclin III; Gbb, Glass-bottom boat; GSC, germline stem cell; HS, heparan sulfate; HSPG, HS proteoglycan; Hts, Hu-li tai shao; JAK/STAT, Janus kinase/signal transducers and activators of transcription; PGC, primordial germ cell; sft, sulfateless; tb, toulvelu; UAS, upstream activation sequence; Upd, Unpaired; Vas, Vasa.
signaling in trans (Kramer and Yost, 2002; Jakobsson et al., 2006), although the general biological significance of HSPGs as trans coreceptors needs to be determined. In this study, we investigate the role of HSPGs in the Drosophila GSC niches. We propose a model in which the differential activities of HS-dependent factors in long- and short-range signaling can be achieved, at least partly, by the differential (canonical and trans coreceptor) activities of HSPGs.

Results and discussion

**dally regulates maintenance of the female GSC niche**

As a first step to study the role of HSPGs in the female GSC niche, we determined expression patterns of two glypican genes, dally and dally-like (dlp), in the adult ovary using enhancer-trap lines. We detected highly specific dally enhancer-trap expression in the anterior-most part of germarium (Fig. 1). These dally-positive cells had nuclei with a flattened shape, directly contacted GSCs, and expressed lamin C, which is a marker for the cap cells (Fig. 1, A–A′; Xie and Spradling, 2000). Based on
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expression (Fig. S2, A–B). Remarkably, Dpp protein levels in somatic cells were normal even in an empty germarium (Fig. S2, C–D). These observations strongly suggest that dally is required for Dpp signal transduction to properly maintain the GSC niche. dally is required for the establishment of the female GSC niche

We next asked whether dally is involved in GSC formation at earlier developmental stages. From the third instar larval to pupal stages, like in the adult ovary, dally enhancer-trap expression was detected in somatic niche cells (Fig. S3, B and B). At the early pupal stage, bam expression was repressed in the anterior PGCs of control animals (Fig. 2, A and D). In contrast, in dally mutant ovaries, the most anterior PGCs abutting the somatic cells expressed bam and had branched fusomes (Fig. 2, B, C, E, and F). Both phenotypes are characteristic of differentiating germ cells (Xie et al., 2005). This premature differentiation of PGCs in dally mutant ovaries suggests that Dally is involved in the formation of the niche, which maintains PGCs in an undifferentiated state.

HS biosynthesis is required for female GSC niche formation

To determine whether Dally’s role in GSC niche formation depends on HS modifications, we examined pupal ovaries in these characteristics, we concluded that the dally-expressing cells are the cap cells that support the GSC niche. Expression of dlp was not detectable in the cap cells (unpublished data).

To test whether dally participates in GSC maintenance, we examined dally mutant ovarioles. In control animals, each germarium contained two or three GSCs with a mean number of 2.6 (n = 75). dally mutant ovarioles had significantly fewer GSCs (mean = 1.8; n = 77; significance: P < 0.005). Strikingly, 14% of these ovarioles had a germarium with no germ cells (Fig. 1 C). This so-called “empty germarium” phenotype results from failure of GSC maintenance (Xie and Spradling, 1998; Ward et al., 2006). Ovarioles with an empty germarium are likely to be lost from the ovary over time; as a result, dally mutants have fewer ovarioles, and this phenotype becomes more severe with age (Fig. S1 A). Collectively, the dally mutant phenotypes suggest that Dally regulates GSC niche maintenance.

To determine whether dally controls Dpp signaling in the ovary, we used the reporter dad-lacZ (Tsuneizumi et al., 1997). In a control germarium, GSCs showed high levels of dad-lacZ expression (Fig. 1, D and D). In contrast, no signal was detected in 30% of the most anterior germ cells directly contacting the somatic cells in dally mutant germaria (Fig. 1, E and E). Staining for Dpp protein revealed that dally mutations reduced Dpp signaling without affecting the ligand expression (Fig. S2, A–B). Remarkably, Dpp protein levels in somatic cells were normal even in an empty germarium (Fig. S2, C–D). These observations strongly suggest that dally is required for Dpp signal transduction to properly maintain the GSC niche.

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These and other results support the model that Dally maintains the GSC niche by inhibiting Dpp signaling in the somatic cells that abut the GSCs. This model is consistent with previous studies that have shown that Dally regulates Dpp signaling in the ovary through its interaction with Dorsal (Dau et al., 1999; Tsuneizumi et al., 1997). In this model, Dally inhibits Dpp signaling by competing with dorsal for binding to the Dpp receptor, thereby preventing Dpp from activating the Dorsal signaling pathway (Keller et al., 1996; Tsuneizumi et al., 1997). This model is supported by the observation that expression of dally in the somatic cells that abut the germarium represses Dpp signaling in the adjacent germ cells (Fig. S2, C–D). These results are consistent with the idea that Dally regulates Dpp signaling by inhibiting Dorsal activation in the somatic cells that abut the GSC niche.
The severe phenotype of sfl mutants suggests that other HSPGs may play a partially redundant role in this process. We examined the GSC phenotype of dally dlp double mutants but observed no enhancement of the dally phenotype (Fig. S1 C). It is possible that other HSPGs such as syndecan share this function, but the strong phenotype of dally mutants shows that Dally is a key player in this process.

We next evaluated Dpp signaling in mutant pupal ovaries. Corroborating the result in dally mutant adults (Fig. 1, D–E’), dad-lacZ reporter expression was markedly decreased in sfl mutant ovaries (Fig. S3, C and D). Thus, the loss of GSCs was caused by impaired Dpp signaling both in pupal and adult gonads. sfl mutations caused no detectable change in the expression of lamin C (Fig. S3, E and F), indicating that differentiation of the cap cells was not disrupted. Together, these results place
Ectopic expression of dally expands the GSC niche

To examine the effect of ectopic dally expression in GSC niche formation, we expressed upstream activation sequence (UAS–dally using c587-Gal4 and monitored bam expression. c587-Gal4 drives expression in most somatic cell populations, including inner germarial sheath precursors that directly contact most germ cells (Song et al., 2007). In control ovaries, bam-GFP was expressed in posterior PGCs at the early pupal stage (Fig. 3 A). In striking contrast, ectopic dally completely eliminated bam-GFP expression in c587>dally pupal gonads (Fig. 3 B), suggesting that germ cells failed to differentiate. Similarly, no bam-GFP expression was detected in c587>dally adult ovaries (Fig. 3 D). Instead, they have an increasing number of GSC-like cells with a spectrosome. When cultured at 30°C, the penetrance of these phenotypes was 100%: all c587>dally germlaria (n = 50) had >10 GSC-like cells and no bam-GFP signal, whereas no control germlarium (n = 35) showed these abnormalities. In addition to expanding the area of undifferentiated cells in both the pupal and adult stages, c587>dally abnormally activated Dpp signaling in the posterior part of the gerarium, as revealed by dad-lacZ staining (Fig. 3, F–G’). These results are consistent with the idea that in wild-type ovary, cap cell–specific dally expression spatially restricts Dpp signaling to define the physical space of the GSC niche.

HS biosynthesis is essential for male GSC niche formation

Our finding that Dally plays a role in the female GSC niche raised an important, interesting question: do HSPGs also regulate the male GSC niche? To address this question, we examined the male GSC phenotype in the HS-deficient mutants. In the anterior larval male gonad, the hub cells were surrounded proximally by GSCs (Fig. 4, A–A’; Xie et al., 2005). Cysts, which are large, differentiating germ cells that express Bam protein and bear branched fusomes, were located more distally. ttv mutant larval testes were substantially smaller than wild-type gonads largely because of a reduced number of germ cells. ttv mutation decreased the number of GSCs without affecting the number of hub cells (Fig. 4, B–D’; and Fig. S1 D). The hub cells directly contacted Bam-positive germ cells in the mutants, suggesting that ttv mutants fail to form or maintain a normal GSC niche and, consequently, that germ cells differentiate prematurely. These observations demonstrated that Drosophila HSPGs regulate the GSC niche in both sexes.

dlp regulates the male GSC niche

We next asked whether glypicans regulate the male GSC niche as in females. Both dally and dlp were specifically expressed in the hub cells (Fig. 4, E–F’); in particular, Dlp was detected at high levels. In dlp mutant gonads, the hub cells directly contacted cysts with branched fusomes (Fig. 4, G and G’), showing that GSCs are lost, as in the HS-deficient mutants. Based on the number of GSCs in a testis, the dlp phenotype was equivalent in severity to that of ttv mutants (Fig. S1 E). The number of GSCs was reduced only modestly in dally mutants, and the difference between dlp and dally dlp double mutants was not statistically significant. We concluded that Dlp plays a primary role in the male GSC niche and that Dally may have a minor redundant function.

The dlp mutant phenotype suggests that Dlp affects one or both of the pathways known to control the male GSC niche: JAK/STAT and/or Gbb signaling. Gbb is a member of the BMP family; because Dally serves as a coreceptor for another BMP, Dpp (Fujise et al., 2003; Akiyama et al., 2008), HSPGs may potentially regulate Gbb signaling. Upd, a ligand for the JAK/STAT pathway, is a heparin-binding protein (Harrison et al., 1998), so it is possible that Upd-JAK/STAT signaling is HS dependent in vivo. Further studies are needed to explore the molecular basis for male GSC control by HSPGs.

Mechanisms of glypican function in the GSC niche

Although it has been shown that secreted signaling molecules play fundamental roles as niche factors in many stem cell systems, the mechanisms by which these molecules can spatially control a restricted niche area are poorly understood. In this study, we showed that HSPGs are essential components of the GSC niches in the Drosophila gonads. We propose that HSPGs define the physical space of the niche by restricting the action range of growth factor signaling to the immediate vicinity of the niche cells.

Both Dally and Dpp are critical for making the female GSC niche. Previous studies have shown that Dally acts as a (canonical) coreceptor for Dpp in the developing wing (Fujise et al., 2003; Belenkaya et al., 2004; Akiyama et al., 2008). However, in the GSC niche, unlike typical HSPG coreceptors, dally is expressed in the dpp-expressing cells (cap cells), not in the receiving cells, suggesting that Dally activates signaling in trans. By its nature, trans signaling mediates signaling only to neighboring cells, providing a potential mechanism for contact-dependent Dpp signaling during GSC niche formation. Because Dally coreceptors on the cap cell surface and Dpp receptors on the surface of germ cells meet only at the contacting membranes of these cells, efficient signal transduction and thus the active field of Dpp signaling are limited to this area (Fig. 5 A). This idea is strongly supported by the observation that ectopic expression of dally can expand the GSC niche (Figs. 3 and 5 A’). Thus, the trans coreceptor activity of Dally could account for spatially restricted Dpp signaling.

In addition to its trans coreceptor activity, Dally may independently limit the size of the niche by retaining Dpp protein on the surface of the cap cells. In this model, Dally would serve as a membrane-bound anchor for Dpp, restricting the distribution of Dpp protein (Fig. 5 B). Future studies visualizing Dpp protein distribution will be required to test this possibility. Furthermore, we cannot exclude the possibility that in addition to Dpp, Dally might affect signaling mediated by other HS-dependent factors. Although the signaling pathway controlled by Dlp is still unknown, Dlp is also expressed in the signal-sending cells (hub cells), suggesting that Dlp serves as a trans coreceptor in the male GSC niche.

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Figure 4. **Dlp is required for the GSC niche in the male gonad.** (A–D) HS is required to form the male GSC niche. Testes from *ttv* heterozygous (A and A’) and homozygous (B–D’) third instar larvae stained with anti-FasIII (hub cells; red), anti-Vas (germ cells; blue), and anti-Bam (differentiating germ cells; green). (A’) Schematic diagram of the image shown in A. Hub cells, GSCs, Bam-negative differentiating germ cells, and Bam-expressing germ cells are shown in orange, blue, light blue, and green, respectively. (E and E’) Specific expression of Dlp in the hub cells. Adult testis stained with anti-Dlp and anti–E-Cad (hub cell marker). (F and F’) Expression of *dally* in the hub cells. Adult testis stained with anti-FasIII (hub cell). (G and G’) The male GSC niche in a *dlp* mutant. *dlp* heterozygous (G) and homozygous (G’) larval testes stained with anti-Hts (green) and anti–E-Cad (red) antibodies. Germ cells were labeled with anti-Vas antibody (blue). In the *dlp* mutant, the hub cells directly contact cysts that have a branched fusome (yellow arrowhead). Anterior is to the left. Bars, 10 µm.
HSPGs as a general regulator of the stem cell niche

The Mammalian Reproductive Genetics Database (http://mrg.genetics.washington.edu/) shows that most mouse and rat glypicans genes are expressed in the testis with differential patterns. Recently, human glypican 3 has been established as a novel marker for testicular germ cell tumors (Zynger et al., 2006). These previous studies together with our observations suggest that glypicans have evolutionarily conserved roles in germline development and stem cell niches both in vertebrates and invertebrates.

Materials and methods

Fly strains

Detailed information for the fly strains used in this study is described in Flybase (http://flybase.bio.indiana.edu/) except where noted. The wild-type strain used was Oregon R. Other strains used were dally^+/+, a dally enhancer-trap line; dally^pm and dally^p327, loss-of-function alleles of dally; dlp^1 and dlp^p327, null alleles of dlp; stf^ma, a null allele of stf; and tv^214, a null allele of the c587-Gal4 (gift from T. Kai, National University of Singapore, Singapore, Republic of Singapore; Kai and Spradling, 2003) and UAS-dally were used to ectopically express dally. bam-GFP (gift from D. McKearin, University of Texas Southwestern Medical Center, Dallas, TX; Chen and McKearin, 2003) and dally-Gal4 were used to monitor expression of bam and dally, respectively.

Immunostaining

Antibody staining was performed according to standard procedures (Fujise et al., 2001). The following antibodies were used: mouse anti-Dllp (1:50; Developmental Studies Hybridoma Bank), mouse anti-fasciclin III (FasIII; 1:50; Developmental Studies Hybridoma Bank), rabbit anti-β-galactosidase (1:500; Cappel), mouse anti-Hu-li tai shao (Hts; 1:5; Developmental Studies Hybridoma Bank), rat anti-Bam (1:300, gift from D. McKearin), rat anti-epithelial cadherin (E-Cad; 1:10; Developmental Studies Hybridoma Bank; Song et al., 2002; Tazuke et al., 2002; Le Bras and Van Doren, 2006), mouse anti–lamin C (1:10, Developmental Studies Hybridoma Bank), mouse anti-Dpp (1:5; R&D Systems), rabbit anti-GFP (1:500; Invitrogen), and rat anti-Vasa (Vas; 1:50; Developmental Studies Hybridoma Bank). For anti-Dpp staining, we used Can Get Signal Solution B (TOYOBO) as antibody dilution solution. Stained ovaries and testes were mounted in Vectashield (Vector Laboratories) and imaged with a confocal microscope (TCS SP5; Leica).

Phenotypic analysis of mutant gonads

To analyze the differentiated status of germ cells, testes and somatic nuclei were stained with anti-Hts antibody. The GSCs were identified as spherical-shaped Vas-positive germ cells located at the distal-most part of a developing ovariole. Further differentiated germ cells are characterized by a branched fusome.

To examine the number of GSCs, ovaries were dissected from adult female flies at 2 d after eclosion and stained with anti-Vas and anti-Hts antibodies. The GSCs were identified as spherical-shaped Vas-positive cells that were in direct contact with the cap cells and had a spectrosome. To assess the empty germarium phenotype, the number of germaria with no detectable GSCs was counted. To analyze the age dependency of the daily phenotype, ovaries were dissected from adult females at the indicated day after eclosion, and the number of ovarioles bearing at least one developing egg chamber was counted.

To examine expression of bam, the differentiation marker of germ cells, in mutant PGCs, ovaries with the indicated genotypes were dissected at early pupal stage and stained with anti-Vas and anti-Hts antibodies. Bam expression was examined by either bam-GFP (a GFP reporter gene for bam transcription) or anti-Bam antibody. The most anterior PGCs were defined as Vas-positive cells located at the distal-most part of a developing ovariole in direct contact with the somatic niche cells. The number of ovarioles in which at least one developing egg chamber was counted.

To evaluate the effect of daily mutations on Dpp signaling in the GSC niche, ovaries were dissected from adult females bearing a dad-lacZ transgene and stained with anti-β-galactosidase, anti-Vas, and anti-Hts antibodies. The number of GSCs with detectable dad-lacZ expression was counted. To assess the effect of ectopic expression of daily on the GSC niche, c587-Gal4/UAS-dally; bam-GFP animals were cultured at 30°C. Adult ovaries were dissected and stained with anti-Vas and anti-Hts antibodies. The number of germlia in which no bam-GFP signal was detected in
Vas-positive cells (germaria without bam-GFP) was counted. Also, Vas-positive, round-shaped cells with a spectroscopic were referred to as GSC-like cells, and the number of germaria with >10 GSC-like cells was counted.

To count the number of GSCs and hub cells in mutant males, testes were dissected from late third instar larvae and stained with anti-Vas, anti-Hts, and anti–E-Cad antibodies. The hub cells were identified as E-Cad–positive cells at the anterior edge of the testes. GSCs were identified as spherical-and anti–E-Cad antibodies. The hub cells were identified as E-Cad–positive were dissected from late third instar larvae and stained with anti-Vas, anti-Hts, and 63x NA 1.20 lenses (Leica) and with acquisition software [Application Suite Advanced Fluorescence; Leica]. Acquired images were processed with Photoshop CS3 (Adobe).

Microscope image acquisition
Secondary antibodies were used conjugated with Alexa Fluor 488, 546, and 633 fluorochromes (Invitrogen). Stained samples were mounted with Vectashield and observed at room temperature. Images were acquired using a TCS SPS confocal microscope with Plan-Apochromat 20x NA 1.00 and 63x NA 1.20 lenses (Leica) and with acquisition software [Application Suite Advanced Fluorescence; Leica]. Acquired images were processed with Photoshop CS3 (Adobe).

Online supplemental material
Fig. S1 shows a quantitative summary of the GSC phenotype observed in mutants for dally, sfl, and 463ka-Z, and staining for dally-GFP, bam-GFP, and dally-lacZ.

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