The glypican Dally is required in the niche for the maintenance of germline stem cells and short-range BMP signaling in the Drosophila ovary

Zheng Guo1,2 and Zhaohui Wang1,*

The Drosophila ovary is an excellent system with which to study germline stem cell (GSC) biology. Two or three female GSCs are maintained in a structure called a niche at the anterior tip of the ovary. The somatic niche cells surrounding the GSCs include terminal filament cells, cap cells and escort stem cells. Mounting evidence has demonstrated that BMP-like morphogens are the immediate upstream signals to promote GSC fate by preventing the expression of Bam, a key differentiation factor. In contrast to their morphogenic long-range action in imaginal epithelia, BMP molecules in the ovarian niche specify GSC fate at single-cell resolution. How this steep gradient of BMP response is achieved remains elusive. In this study, we found that the glypican Dally is essential for maintaining GSC identity. Dally is highly expressed in cap cells. Cell-specific Dally-RNAi, mutant clonal analysis and cell-specific rescue of the GSC-loss phenotype suggest that Dally acts in the cap cells adjacent to the GSCs. We confirmed that Dally facilitated BMP signaling in GSCs by examining its downstream targets in various dally mutants. Conversely, when we overexpressed Dally in somatic cells outside the niche, we increased the number of GSC-like cells apparently by expanding the pro-GSC microenvironment. Furthermore, in a genetic setting we revealed a BMP-sensitivity distinction between germline and somatic cells, namely that Dally is required for short-range BMP signaling in germline but not in somatic cells. We propose that Dally ensures high-level BMP signaling in the ovarian niche and thus female GSC determination.

KEY WORDS: Glypican, Germline stem cell, Niche, BMP, Short-range signaling

INTRODUCTION

Sustained gamete production during adulthood depends on germline stem cell (GSC) self-renewal, which is also restrained to its proper position to avoid the risk of tumorigenesis. The Drosophila ovary provides an excellent system to study GSC fate determination because its unique anatomical layout makes lineage tracing relatively easy in a complex cell context (for reviews, see Li and Xie, 2005; Spradling et al., 2005). Each ovary consists of 16-20 ovarioles that are basically parallel “assembly lines” of oocyte production. At the anterior tip of each ovariole (Fig. 1A), two to three GSCs are restricted in a space called a niche, the somatic components of which include terminal filament cells (TF), cap cells and escort stem cells (ESCs) (Decotto and Spradling, 2005; Xie and Spradling, 2000). To keep egg production going, each GSC divides to generate two daughter cells: one remains attached to the cap cells in the niche, thus retaining GSC fate; the other moves out of the niche and becomes a differentiated cystoblast, which becomes the start of the egg-production line. The early germ cells in the germarium are readily distinguished by the morphology of a germline-specific and spectrin-rich structure known as a fusome.

BMP morphogens promote GSC fate by preventing the expression of Bag of marbles (Bam), a key differentiation factor, so that Bam is repressed in GSCs but expressed in cystoblasts (Chen and McKearin, 2003a; Chen and McKearin, 2003b; McKearin and Spradling, 1990; Ohlstein and McKearin, 1997; Song et al., 2004). Transcriptional silencing of bam is established by a protein complex including BMP downstream effectors (Chen and McKearin, 2003a; Pyrowolakis et al., 2004; Song et al., 2004) and a nuclear envelope component Ote (Jiang et al., 2008). The direct upstream signals are apparently BMP morphogens, which have been proposed to be provided by the somatic niche cells, or more specifically by the cap cells (Kai and Spradling, 2003; Song et al., 2004; Xie and Spradling, 1998). The high BMP signaling is precisely restricted to GSCs in the niche and is turned down in the immediately adjacent cystoblast. Clearly, this represents a typical short-range BMP signaling. As Decapentaplegic (Dpp), one of the Drosophila BMP homologs, has been shown to act as a long-range morphogen in many developmental contexts (Entchev et al., 2000; Muller et al., 2003; Teleman and Cohen, 2000), how is the steep gradient of BMP response achieved in a span of only two cells? Casanueva et al. suggested that Dpp proteins are present throughout the anterior germarium, and Bam negatively feeds back to Dpp signaling, thus maintaining the differentiated state of germ cells in the germarium outside the niche (Casanueva and Ferguson, 2004). However, this does not explain how the differential expression of Bam is established in the first place. Nevertheless, even if BMP molecules are highly expressed in the niche, it is still difficult to understand how BMP molecules evoke an “on-or-off” outcome in terms of Bam expression and specifying distinct cell fate between the adjacent GSCs and cystoblasts.

Division abnormally delayed (Dally) is a glypican member of heparan sulphate proteoglycan (HSPG) and all glypicans possess a glycosylphosphatidylinositol (GPI) moiety, a hydrophobic modification anchoring the core protein to the cell surface (Nakato et al., 1995; Selleck, 2000; Tsuda et al., 1999). Dally has been demonstrated to regulate the Dpp gradient in the imaginal epithelia (Akiyama et al., 2008; Belenkaya et al., 2004; Crickmore and Mann, 2007; Fujise et al., 2003; Jackson et al., 1997), and physical interaction between Dally and Dpp was detected (Akiyama et al., 2008). Misexpression of the cell-surface-anchored, but not the...
secreted form, of Dally enhances Dpp signaling, and the secreted Dally has rather a weak dominant-negative effect (Takeo et al., 2005). Immobilized Dally seems to have the ability to concentrate/stabilize Dpp or increase the cellular response to Dpp (Akiyama et al., 2008).

Here we present genetic evidence to support the argument that Dally is responsible for establishing the steep gradient of BMP response from GSC to cytoplasm. By altering the pattern of Dally expression, we were able to manipulate the number and position of the GSC-like cells. We also demonstrate that Dally defines a distinction between germline and somatic cells in their sensitivity to BMP signals.

MATERIALS AND METHODS
Fly genetics
P[PZ]dally06464, bab1-GAL4 (BL#6803), en-GAL4, ptc-GAL4, nos-Gal4 on III, UAS-GFP and balancers were obtained from the Bloomington Stock Center; c587GAL4 from Ting Xie (Kawase et al., 2004); FRT2A dally29 from Xinhua Lin (Belenkaya et al., 2004); dally385, dally406, UAS-TM-dally, UAS-Sec-dally from Hiroshi Nakato (Nakato et al., 1995; Takeo et al., 2005); hs-bam, FRT2A histone-GFP, FRT2A, ham-GFP, UASp-Tkv9 (constitutively active Tkv), and bam92 from Dahua Chen (Chen and McKearin, 2003a; Chen and McKearin, 2003b; Jiang et al., 2008); UAS-dally06464 (Jackson et al., 1997), UAS-dallyRNAi (Vienna, Austria) and UAS-dlpRNAi (Vienna, Austria) from Michael A. Crickmore (Crickmore and Mann, 2007); Dad-lacZ from Rongwen Xi (Tsuneizumi et al., 1997; Zhao et al., 2008); esg-lacZ from Shigeo Hayashki. UASp-Dally was constructed by PCR cloning Dally cDNA from UASp-Dally and inserting the cDNA into UASp vector. None of the dally alleles used in this study is a strict null or amorph. All stocks and crosses except the overexpression experiments were cultured at 25°C. Overexpression of Dally and dallyRNAi were set up at 29°C.

Generation of female germline clones
FLP-FRT-mediated mitotic recombination was used to generate dally80 mutant GSC clones. To generate GSC clones and avoid TF and Cap cells clones in adult ovaries, 3-day-old females (w hs-flp; FRT2A histone-GFP/FRT2A dally29) were heated at 37°C for 1 hour twice a day for 5 consecutive days. w hs-flp; FRT2A hist GFP/FRT2A flies were treated in parallel as controls. Ovaries were dissected at day 2 and day 10 post-heat induction. GSC clones were identified by the lack of GFP and the presence of the anterior-positioned dot fusome (spectrosome) in the germline cells (Yang et al., 2007).

RESULTS
X-gal staining of β-gal activity
Adult ovaries and testes were fixed with 0.5% glutaraldehyde in PBS for 2 minutes, and then subjected to the standard X-gal color reaction for 12 hours at 37°C.

BrdU labeling
Ovaries were dissected in PBS, incubated for 1 hour in PBS containing 100 μg/ml BrdU (Sigma) at 25°C, then fixed. The rest of the procedure was described previously (Li et al., 2007).

Cell death assays
For TUNEL assay, after fixation in 4% formaldehyde/PBS, samples were incubated in the mixture of Enzyme and Label solutions (Roche Kit, 1 684 795) at room temperature for at least 3 hours.

For Acridine Orange staining, samples were incubated in 1.6 μM Acridine Orange/PBS for 20 minutes at room temperature.

For Caspase detection, rabbit anti-Active Caspase-3 (CM1, from BD Pharmingen) was used at 1:5000.

Immunohistochemistry and microscopy
All samples were dissected in PBS, fixed and stained as described previously (Li et al., 2007). Primary antibodies were used at the following dilutions: mouse anti-c-tensor at 1:50 (a gift from Rongwen Xi) (Zhao et al., 2008); mouse anti-Hs-p (1B1, developed by Howard D. Lipshitz, DSHB); rabbit anti-GFP (gifts from Dahua Chen) (Jiang et al., 2008); mouse anti-FasIII (developed by P. A. Beachy, DSHB) at 1:50; mouse anti-β-spectrin at 1:50 (a gift from Rongwen Xi) (Zhao et al., 2008); guinea pig anti-pMAD at 1:4000 (E. Lauffer and T. Jessell, Columbia University, New York, NY) (Crickmore and Mann, 2007); rabbit anti-GFP at 1:5000 (Invitrogen); mouse anti-β-gal (JE7, developed by T. L. Mason, DSHB) at 1:200; and rabbit anti-β-gal at 1:10000 (Cappel). Alexa-Fluor-conjugated secondary antibodies were used at 1:4000 (Molecular Probes, Invitrogen). Fluorescent images were collected using a Zeiss ApoTome microimaging system. 63X Oil Plan-Apochromat lens was used to visualize the details in gerarium.

daily is required in the somatic niche cells for the ovarian GSC maintenance
We discovered the glypican Dally as one of the candidates that may be involved in enhancing the BMP response in the female GSC niche when we examined the expression pattern of an enhancer trap lacZ in the daily gene (P[PZ]dally06464) (Fig. 1B). In gerarium, Dally-β-gal is expressed mainly in the cap cells, to which the GSCs are attached in the niche (Fig. 1C). Unlike Dally, another Drosophila glypican, Dally-like (Dlp), is ubiquitous in the gerarium, as shown in the immunostaining of the protein distribution (see Fig. S1A in the supplementary material).
To determine if *dally* is involved in GSC fate regulation, we counted the GSC numbers in the ovaries of various *dally* mutant alleles. In wild-type ovaries at adult day 10, two to three GSCs were normally present in the niche and germaria containing none or one GSC were rarely found (Table 1). In the homozygous or trans-heterozygous mutants of *dally*, GSC loss was obvious after 10 days into adulthood, and was consistent in all mutants, although at different severity (Fig. 2; Table 1). As early as day 3 after eclosion, complete germ cell loss due to lack of GSC renewal was observed in the strong *mutant* alleles (Fig. 2F,H; Table 1).

The loss of GSC was unlikely to be a result of cell death, because advanced egg chambers were present in the same ovarioles displaying GSC loss (Fig. 2B). Using assays to evaluate DNA fragmentation or caspase activation for the signs of cell death, no difference between the wild type and *dally* mutant was found (Fig. 2L,J). In both wild type and mutant when germ cells were still present in the gerarium, programmed cell death was detected in a few sporadic somatic cells in the germarial region in which the egg chamber starts to form but not in the cap cells or germline (Fig. 2L,J; see also Fig. S2 in the supplementary material), similar to that reported previously (Decotto and Spradling, 2005). Additionally, *dally* mutant clones in GSCs remained in the niche 10 days after clone induction (see Fig. S3 in the supplementary material), indicating that Dally is not autonomously required in the germline for the viability of GSC.

As Dally is highly expressed in the cap cells and required for GSC maintenance, we wonder if Dally is essential for the viability and/or identity of the cap cells. We therefore compared the cap cells in wild type and *dally* mutant by Lamin C staining, which labels the TF and cap cells of the niche. However, we did not detect a loss of cap cells in *dally* mutant even when GSCs were completely lost (Fig. 2G,H). The average cap cell number per ovariole was 5.5 (n=46) in the wild type, and 5.6 (n=41) in the *mutant*.

To check whether Dally acts specifically in the somatic niche cells, we tried to rescue *dally* mutant phenotype by expressing Dally in these cells. We took advantage of two Gal4 lines, which drive targeted expression in a slightly different range in the niche (see Fig. S4 in the supplementary material). *bab1*Gal4 is strong in both TF and cap cells (Fig. 2K; see also Fig. S4 in the supplementary material) (Bolivar et al., 2006), and its induction of Dally significantly restored the GSCs in *mutant* (Fig. 2E; Table 1). Different from *bab1*Gal4, *en*Gal4 is highly expressed in TF cells but was barely detected in the cap cells (Fig. S4 in the supplementary material) (and personal communications with Dahua Chen and A. Gonzalez-Reyes), and its ability to rescue GSC loss in the *mutant* is less than that of *bab1*Gal4 (Table 1). Nevertheless, the cell-specific rescue of *dally* mutant phenotype implicates that Dally acts in the somatic niche to maintain GSC fate.

To confirm the action of Dally in the niche, we also employed cell-specific RNAi to reduce Dally levels in somatic niche cells. *bab1*Gal4-driven Dally RNAi led to a complete GSC loss on day 10 in ~30% of the germline scored (Table 1, n=122). On the contrary, Dlp RNAi in the same cells did not have any effect on GSC number in the niche (see Fig. S1A in the supplementary material and more than 100 ovarioles scored). Thus, data obtained from various *dally* mutations, cell-specific RNAi, germline mutant clones, and cell-specific rescue of *dally* mutations indicate that glypican Dally is required for GSC maintenance and probably it exerts this function in somatic niche cells.

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### Table 1. Scoring of GSC-like cells in *dally* mutants and Dally-RNAi flies

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Dissection time after eclosion</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 3</td>
</tr>
<tr>
<td></td>
<td>2-3 GSCs/niche</td>
</tr>
<tr>
<td><em>w^{1118}</em></td>
<td>126 (100%)</td>
</tr>
<tr>
<td><em>daily</em>los</td>
<td>126 (77%)</td>
</tr>
<tr>
<td><em>daily</em>emm</td>
<td>47 (16%)</td>
</tr>
<tr>
<td><em>daily</em>ko</td>
<td>14 (31%)</td>
</tr>
<tr>
<td><em>daily</em>ko/daily*ko</td>
<td>52 (84%)</td>
</tr>
<tr>
<td><em>daily</em>omm/daily*omm, UAS-daily</td>
<td>51 (20%)</td>
</tr>
<tr>
<td><em>en-GAL4; daily</em>omm/daily*emm, UAS-daily</td>
<td>107 (56%)</td>
</tr>
<tr>
<td><em>bab1-GAL4, daily</em>omm/daily*emm, UAS-daily</td>
<td>248 (80%)</td>
</tr>
<tr>
<td><em>CSB7-GAL4; daily</em>omm/daily*emm, UAS-daily</td>
<td>251 (81%)</td>
</tr>
<tr>
<td><em>daily</em>emm, Dad<em>1883</em></td>
<td>66 (66%)</td>
</tr>
<tr>
<td>*bab1-GAL4&gt;UAS-dailyRNAi</td>
<td>85 (94%)</td>
</tr>
<tr>
<td><em>bab1-GAL4&gt;UAS-sec-daily</em></td>
<td>ND</td>
</tr>
</tbody>
</table>

*Denotes otherwise stated, adult day 10 samples were used.
*About half of these ovarioles contained more than three GSCs.
*Approximately 80% of these ovarioles contained more than three GSCs.
ND, not determined.

GSCs were identified by the morphology of fusome staining (drops or Hts). Numbers in the table are the counts of germarium and their percentage of the total (n) germaria examined at the same stage of adulthood. Flies of RNAi experiments were raised at 29°C.
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transcription was de-repressed in the GSCs or germ cells occupying ovaries, pMad staining was barely above background and pathway effectors (Jiang et al., 2008). GSC is directly repressed by the protein complex containing BMP-Bam is required for germ-cell differentiation, and its transcription in BMP signaling and is phosphorylated upon ligand-receptor binding. (phosphorylated Mad) or Bam. Mad is the intracellular effector of 2005). The contrast in BMP response between GSC and cystoblast ovary, the niche-restricted response of Dpp but not Hh or Wg plays (Hedgehog) and Wg (Wingless) in the imaginal epithelia (for reviews, see Lin, 2004; Nybakken and Perrimon, 2002). In the Dally has been demonstrated to regulate the gradient of Dpp, Hh (Hedgehog) and Wg (Wingless) in the imaginal epithelia (for reviews, see Lin, 2004; Nybakken and Perrimon, 2002). In the GSC determination, we generated a double mutant of dally and bmp provides evidence for their functional interaction in oogenesis. Further, restoration of GSCs in dally mutant by expressing the activated Tkv (a BMP receptor) in the early germline cells also supports the idea that Dally regulates BMP response in the germarial niche (Fig. 3D). Bam-independent GSC differentiation has been reported previously (Chen and McKearin, 2005; Szakmary et al., 2005; Xi et al., 2005). Mutations in pumilio and pelota lead to GSC loss without affecting Bam expression, and pelota also modulates BMP signaling. To exclude the possibility that Dally is involved in the Bam-independent pathway in addition to the BMP-Bam pathway, we generated dally bam double mutant. dally bam double mutant produced GSC-like over-proliferation in the germinarium, a genotype indistinguishable from that in the bam single mutant (Fig. 3E). Thus, we genetically illustrate that Dally acts in the Bam-dependent pathway. Again, GSC-like cell accumulation in dally bam double mutant ovaries also supports the argument against the possibility that dally mutations cause GSC death. No difference in TUNEL and Caspase3 signals was detectable between bam and dally bam mutants (see Fig. S2E-H in the supplementary material).

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**Daily is essential for BMP signaling in germline stem cells**

Dally has been demonstrated to regulate the gradient of Dpp, Hh (Hedgehog) and Wg (Wingless) in the imaginal epithelia (for reviews, see Lin, 2004; Nybakken and Perrimon, 2002). In the ovary, the niche-restricted response of Dpp but not Hh or Wg plays a major role in maintaining GSC fate (King et al., 2001; Li and Xie, 2005). The contrast in BMP response between GSC and cystoblast can be revealed by the BMP-downstream targets such as pMad (phosphorylated Mad) or Bam. Mad is the intracellular effector of BMP signaling and is phosphorylated upon ligand-receptor binding. Bam is required for germ-cell differentiation, and its transcription in GSC is directly repressed by the protein complex containing BMP-pathway effectors (Jiang et al., 2008).

To clarify whether the GSC loss in dally mutants is due to the compromised BMP signaling, we examined the status of pMad and Bam in germinarium. In GSCs, the presence of pMad and the absence of Bam reflect the activation of BMP signaling. These two events are normally associated with the GSC fate in the niche (Fig. 3A,B) (dally+/–; bamGFP, a bam promoter fused with GFP). In dally mutant ovaries, pMad staining was barely above background and bam transcription was de-repressed in the GSCs or germ cells occupying the niche (Fig. 3A,B) (dally+/–), indicating the downregulation of BMP signaling in these cells.

To obtain genetic evidence confirming the association of Dally and BMP signaling in GSC determination, we generated a double mutant of dally and Dad (Daughters against dpp). Dad is an antagonist of Dpp signaling and its expression is turned on by Dpp (Tsuneyizumi et al., 1997). We found that the Dad mutation partially rescued the GSC loss phenotype of dally mutant (Fig. 3C; Table 1; dally Dad double mutant). Additionally, every double mutant female laid eggs, whereas the homozygous female of this particular dally allele (dallygenom) rarely did. The genetic interaction between dally and Dad provides evidence for their functional interaction in oogenesis. Further, restoration of GSCs in dally mutant by expressing the activated Tkv (a BMP receptor) in the early germline cells also supports the idea that Dally regulates BMP response in the germarial niche (Fig. 3D).

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**Fig. 2. dally is required in the somatic niche cells for ovarian GSC maintenance.** Vas, a germline-specific marker; Spectrin, a fusome component used to distinguish GSC and the differentiated germ cells. (A) An ovariole (dally+/–) normally contains numerous Vas-positive germ line cells in the gerarium (yellow dotted line) and egg chambers of nearly consecutive stages. (B) Ovarioles of dally homozygous mutant (dally–/–, image of dallygenom allele) within 3 days after eclosion exhibit the typical GSC loss phenotype: a tiny gerarium missing germ cells (yellow dotted line, note the absence of Vas staining) at the anterior end, as well as developmentally advanced egg chambers present posteriorly with large stage gaps. (C) When viewed at higher magnifications, in dally–/– ovaries normally two to three GSCs are visible in the niche and each GSC contains a single αSpectrin dot positioned anteriorly (arrowheads). Germ cells mitotically dividing before reaching the 16-cell stage are interconnected by branched αSpectrin in the gerarium (dally+/–, Vas-positive cell clusters). (D) In the dally homozygous mutant (dally–/–, image of dallygenom allele), only two germ cells are left and the posterior one is apparently moving away from the niche (note the αSpectrin staining between the two green germ cells). Ovaries were dissected from 3-day-old flies. (E) A gerarium of genotype bab1-Gal4>dally,dallygenom 10 days after eclosion. bab1-Gal4 is active specifically in cap cells and TF cells (see bab1>GFP and cap cell staining in Fig. S4 in the supplementary material). (F) An example of dallygenom with more severe GSC loss within 3 days after eclosion; lack of Vas-staining indicates that no germline cell was present in the gerarium. (G,H) Lamin C (LamC) labels the TF and cap cells of the germarial niche. The number of cap cells remained similar between the control (G) and dallygenom (H). (I,J) The TUNEL assay detected programmed cell death, which was not detected in cap cells or germline cells in either control (I) or dallygenom (J) germlaria. (K): bab1-Gal4,UAS- GFP. (L) Daily Knockdown by bab1-Gal4-controlled RNAI non-autonomously led to the empty-gerarium phenotype, i.e. no Vas-positive cells were observed in the gerarium. The effect of RNAI is variable and one of the most severe cases is shown here.
Dally enhances BMP response in female GSC niche

Overexpression of membrane-bound Dally posterior to the niche induces GSC-like expansion and activation of BMP signaling

To see whether Dally expression in somatic cells outside the niche is sufficient to induce BMP response and more GSC-like cells, we overexpressed Dally in the somatic cells posterior to the niche (Fig. 4, C587GFP; see also Fig. S4 in the supplementary material) and observed substantial accumulation of GSC-like cells in the germarium (Fig. 4, C587>dally; Table 2, C587>dally). Such phenotype is similar to that of bam mutant. This was confirmed by the targeted expression in the somatic cells under the control of a different Gal4 (see Fig. S4 in the supplementary material; Table 2, ptcGAL4>UAS-daily). Notably, it takes time for the GSC-like cells to accumulate and become evident (Table 2) (C587GAL4>UAS-daily and ptcGAL4>UAS-daily, day 5 versus day 15). These GSC-like cells hardly differentiated, leaving big stage-gaps between the germlarium and the adjacent egg chamber (Fig. 4D). In TF cells, Dally overexpression did not show any effect on GSC number or position (see Fig. S4 in the supplementary material; Fig. 4A, en>dally+GFP; Table 2, enGAL4>UAS-dally). Furthermore, Dally expression in germine cells also induced more GSC-like cells in the germarium (Fig. 4H; Table 2, nos-GAL4VP16>UASp-dally). Thus, overexpression of Dally outside niche causes GSC-like expansion.

To get a clue of whether GSC-like expansion induced by Dally expression in somatic germarium is simply due to an increase in GSC production at the anterior tip, or due to more cell divisions elsewhere in the escort cell-surrounded space, the mitotic activity of the GSC-like cells was evaluated by BrdU labeling (Fig. 4B). Wild-type germaria often contain BrdU-positive germ cells in a cluster of four or eight cells in the middle of the germarium, reflecting synchronized division of cystocytes (Fig. 4B, wild type). Interestingly, we observed single cells or two-cell clusters actively dividing along the germarial periphery (Fig. 4B) (C587>dally). It seems that cell proliferation posterior to the niche at least partially accounts for the GSC-like cell expansion induced by Dally from escort cells.

As Bam promotes differentiation and is repressed in the GSCs, we asked whether this protein is silenced in the GSC-like cells induced by Dally expansion. Bam proteins were present in a few cystocytes of almost every control germarium (Fig. 4C) (C587>GFP). Not surprisingly, Bam was drastically reduced in the germarium actively expressing Dally in the escort cells, and even in the germaria in which GSC-like cells had not completely replaced the differentiated germ cells (Fig. 4C,E) (C587>dally+GFP). Consistently, using bam-promoter-driven GFP as a reporter, C587Gal4 overexpression of Dally repressed the bamGFP expression in all GSC-like cells (Fig. 4F).
To determine whether BMP signaling is indeed elevated in the GSC-like cells induced by ectopic Dally expression, we examined the activation of Dad, another downstream effector of BMP signaling. In the wild-type controls, Dad expression revealed by Dad-β-gal was strong in the GSCs next to the cap cells and weaker in cystoblasts (Fig. 4G) (C587>GFP). As we predicted, expanded Dally expression was sufficient to enhance the Dad-β-gal signal in most of the GSC-like cells (Fig. 4G).

Full-length Dally is anchored on the cell surface through the GPI addition at its C-terminus, and Dally’s ability to regulate Dpp gradient depends on its membrane-anchor (Takeo et al., 2005). Nakado and colleagues had constructed the secreted form of Dally by deleting the GPI-linked C-terminus (sec-Dally), and then showed that this expression of Dally induced the accumulation of GSC-like cells in the gerarium (supposedly GPI-modified). In addition to the targeted expression of the wild-type Dally in the niche thus ensuring the GSC fate, we tried to express different forms of Dally at different positions in the germarium. In the process of studying the collaborative effect of Dally and BMP signaling in germline but not in somatic cells, we discovered a difference in BMP response, Dad-lacZ reporter (Fig. 5). As a downstream readout of BMP response, Dad-lacZ serves as a lacZ reporter in these flies. Dad-β-gal expression was dramatically upregulated in the GSC-like cells encapsulated by the C587>dally escort cells. Note how inflated the space of C587>dally+GFP is relative to the control on the right, owing to the overproliferation of GSC-like cells. C587Gal4 is expressed in somatic cells, specifically active in the early germline cells including GSCs (left panel). Daily expressed by nosGal4 induced an increase of GSC-like cells in the gerarium (right panel, 3 days after eclosion; the yellow arrowhead points to one of the GSC-like cells). Scale bars: 10 μm in D; 10 μm in A for all other panels.

**Dally is required for the short-range BMP signaling in germline but not in somatic cells**

In the process of studying the collaborative effect of Dally and BMP signaling on GSC-fate determination, we discovered a difference in BMP sensitivity between germline and somatic cells in the daily mutant carrying a Dad-lacZ reporter (Fig. 5). As a downstream readout of BMP response, Dad-β-gal signal is normally detected in the GSCs and to a lesser degree in cystoblasts (Fig. 4G; Fig. 5A), and could also be induced in the more differentiated germ cells in the niche after Bam overexpression (Fig. 5C). In daily mutants, when GSCs were completely lost in the gerarium, the somatic escort cells became in contact with cap cells and expressed Dad-β-gal, indicative of BMP response (Fig. 5B). In another sample, however, the remaining GSC was unable to show such response in the absence of Dally, whereas the somatic cells around this GSC displayed obvious Dad-β-gal expression in the niche (Fig. 5D).
Dally enhances BMP response in female GSC niche

Table 2. Targeted overexpression of Dally and its effect on GSC number

<table>
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<tr>
<th>Genotype</th>
<th>Dissection time after eclosion</th>
<th>&lt;7 spherical fusomes</th>
<th>≥7 spherical fusomes</th>
<th>&lt;7 spherical fusomes</th>
<th>≥7 spherical fusomes</th>
</tr>
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<tr>
<td></td>
<td>Day 5</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>n=194</td>
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</tr>
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<td>141 (73%)</td>
<td>53 (27%)</td>
<td></td>
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<tr>
<td>CS87GA4&gt;UAS-TM-dally</td>
<td></td>
<td>104 (88%)</td>
<td>14 (12%)</td>
<td>46 (75%)</td>
<td>15 (25%)</td>
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<td>54 (57%)</td>
<td>40 (43%)</td>
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Using pMad to reveal BMP pathway activation, we also observed BMP response in the somatic cells of dally mutant germaria (Fig. 5E,F). The most likely interpretation of this observation is that Dally is required for short-range BMP response in germline but not in somatic cells.

DISCUSSION
To understand how a steep gradient of BMP response is established and thus determines cell fate at single-cell resolution in the GSC niche of Drosophila ovary, we have taken genetic approaches to examine the role of the glypican Dally in the process. Based on our current data, we propose a model of how the expression pattern of Dally shapes the BMP-signaling range and consequently determines distinct cell fates in the ovarian niche (Fig. 6).

Female GSC fate requires high BMP signaling, which is provided in the ovarian niche. Dally is highly expressed in the cap cells that contact the GSCs. Cap-cell-localized and membrane-bound Dally either stabilizes/concentrates BMP molecules, or enhances BMP sensitivity to ensure that only the germ cells in contact with cap cells become GSC (Fig. 6, wild type). Removal of Dally, BMP concentration at the niche or BMP sensitivity in the germ cells adjacent to cap cells dissipates, and GSCs cannot be maintained and subsequently differentiate (Fig. 6, dally−/−). Conversely, when Dally is ectopically overexpressed in the escort cells posterior to the niche, BMP signaling or sensitivity increases in all germ cells encapsulated by these escort cells, and GSC-like cells accumulate in the germarium (Fig. 6, C87> dally).

Consistent with this model, the secreted form of Dally expressed from cap cells caused GSC loss, possibly by competing with endogenous membrane-anchored Dally for binding with BMP molecules or by interfering with BMP signaling (see Fig. S5 in the supplementary material; Table 1). Because secreted Dally expressed from somatic cells in addition to cap cells did not cause GSC expansion as the membrane-anchored Dally did (Table 2), it further supports the idea that Dally’s function in the GSC niche depends on the cap-cell-specific expression and membrane anchoring.

When somatic cells displace the differentiating germ cells in the niche and become close to or in contact with cap cells where the BMP morphogen is localized (Kai and Spradling, 2003), these somatic cells are able to respond to BMP when Dally is lacking (Fig. 5; Fig. 6, purple escort cell in dally−/−). Whether a cellular BMP response is Dally dependent or not distinguishes the germline and somatic cells.

The molecular aspects of how Dally modulates short-range BMP response
We have noticed that it took 15 days for Dally overexpression in somatic cells to make all germ cells GSC-like in the germarium (Table 1), although C587Gal4 was active since stage larval 3 at the latest. One possible explanation is that Dpp is limited and Dally stabilizes Dpp. This possibility is supported by a recent report demonstrating that Dally and Dpp physically interact with each other in the cultured S2 cells and that Dally stabilizes Dpp on the cell surface in the wing imaginal epithelia (Akiyama et al., 2008). Consistent with their theory of cell-surface-associated stabilization, the secreted Dally, although retaining the ability to bind Dpp, did not have the activity that the full-length Dally possesses in terms of enhancing GSC proliferation (Table 2, C87GA14>UAS-sec-dally). It suggests that Dally can only stabilize Dpp at the cell surface. Additionally, secreted Dally expressed in the same cells in which the endogenous Dally is produced had a weak dominant-negative effect (Table 1, bab1GAL4>UAS-sec-dally). By contrast, the secreted Dally expressed elsewhere did not have any detectable effect on GSC, suggesting that it did not compete with the endogenous Dally expressed from the cap cells. These results imply that the anterior tip of the germarium contains the main source of BMP molecules, which the secreted Dally from cap cells has a better chance to catch than that from elsewhere.

Dally-dependent short-range BMP signaling: the unique feature in germline cells
In the imaginal epithelia, glypican Dally and Dlp are essential for Dpp gradient formation but not for short-range Dpp signaling because one to two rows of cells in the glypican double mutant clone were able to respond to the nearby Dpp signals (Belenkaya et al., 2004). Similarly, in dally mutant ovary, in which the germarium was emptied due to GSC loss, we observed BMP response in the escort cells getting close to the cap cells, where the BMP source is supposed to be (Fig. 5B). However, the germ cell surrounded by the BMP-responsive somatic cells was refractory to BMP morphogen.

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<th>Genotype</th>
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<th>&lt;7 spherical fusomes</th>
<th>≥7 spherical fusomes</th>
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<td>n=74</td>
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Files were raised at 29°C. The number of spherical fusomes is equal to the number of GSC-like cells and cystoblasts. ‘?’ is used because generally 6 is the maximal number of spherical fusomes observed in wild type (three in GSCs and three in cystoblasts). Numbers in the table are the counts of germaria and their percentage of the total (n) examined at the same stage of adulthood.

sec, secreted; TM, transmembrane.

DEVELOPMENT
Is Dally involved in male GSC maintenance?

As in the testis the Dally-β-gal reporter is expressed in the hub cells (an equivalent of the female niche; see Fig. S6A in the supplementary material), we wondered whether dally is also involved in male GSC determination. We did observe a decrease in GSC number in the testis (see Fig. S6 in the supplementary material) (dally<sup>OE</sup>; and data not shown for dally<sup>P2</sup> and dally<sup>genm</sup>), but we could not exclude the possibility that such GSC loss was due to the malformation of dally mutant testis (see Fig. S6B in the supplementary material). Because the tubular structure of the testis and the external reproductive organ do not properly form without Dally (Nakato et al., 1995; Tsuda et al., 1999), this ‘no-exit’ cavity is crowded with continuously developing sperm cells. There is a strong possibility that the GSC loss around the hub was a result of the increased physical pressure inside the concealed testis. In addition, when Dally was overexpressed in the hub, cyst or early germ cells, no detectable effect was seen (our unpublished data). Due to the pleiotropic defects of dally mutants, Dally’s function in spermatogenesis remains to be clarified.

Controlling germline stem cell fate by manipulating glypican Dally

Dally belongs to the glypicans family of HSPG, part of the extracellular matrix involved in the signaling of many growth factors. We have provided evidence supporting the idea that Dally acts in trans to promote short-range BMP signaling and thus GSC fate in the Drosophila ovary. We also demonstrated that GSC fate can be manipulated by simply altering Dally expression patterns (Fig. 4; Table 2). Although targeting Dally to germline cells can increase the number of GSC-like cells in the germaria (Fig. 4H), we could not exclude the possibility that Dally acts in trans from neighboring germ cells instead of autonomously. Regardless of Dally acting autonomously or not, if Dally is localized on the GSC surface to enhance BMP signaling, it would be difficult to switch from high to low BMP response in the presence of ligands. Perhaps this is why Dally is abundant on the somatic niche cells but not on GSCs, which would shut down BMP signaling by simply leaving a Dally-rich environment.

Notably, being Dally dependent or not for short-range BMP signaling is different for germline and somatic cells. This phenomenon could be potentially valuable for in vitro manipulation of germ cells. Tremendous interest and efforts have been focused on the in vitro manipulation of cell fate, which often involves the
addition of growth factors. Applying specific HSPG in trans to modulate short-range cell response and to specify cell fate would be more amenable and probably impose fewer side effects than adding more growth factors.

Acknowledgements
We are grateful to Dahua Chen, Michael A. Crickmore, Shigeo Hayashi, E. Laufer, Xinhua Lin, Hiroshi Nakato, Rongwen Xi and Ting Xie for sharing files and reagents; Rui Zhao for technical help; and Dahua Chen and Rongwen Xi for critical reading of the manuscript. The Bloomington Stock Center and Developmental Studies Hybridoma Bank provided invaluable tools. This work is supported by National Basic Research Program of China (2007CB947503), National Science Foundation China (30771061), and the BailRen fund from Chinese Academy of Sciences.

Supplementary material
Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/136/21/3627/DC1

References


