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Notch signaling controls germline stem cell niche formation in the *Drosophila* ovary

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Stem cells, which can self-renew and generate differentiated cells, have been shown to be controlled by surrounding microenvironments or niches in several adult tissues. However, it remains largely unknown what constitutes a functional niche and how niche formation is controlled. In the *Drosophila* ovary, germline stem cells (GSCs), which are adjacent to cap cells and two other cell types, have been shown to be maintained in the niche. In this study, we show that Notch signaling controls formation and maintenance of the GSC niche and that cap cells help determine the niche size in the *Drosophila* ovary. Expanded Notch activation causes the formation of more cap cells and bigger niches, which support more GSCs, whereas compromising Notch signaling during niche formation decreases the cap cell number and niche size and consequently the GSC number. Furthermore, the niches located away from their normal location can still sufficiently sustain GSC self-renewal by maintaining high local BMP signaling and repressing *bam* as in normal GSCs. Finally, loss of Notch function in adults results in rapid loss of the GSC niche, including cap cells and thus GSCs. Our results indicate that Notch signaling is important for formation and maintenance of the GSC niche, and that cap cells help determine niche size and function.

KEY WORDS: Notch, Stem cell, Germ line, *Drosophila*, Ovary, Niche

INTRODUCTION

Adult tissues undergo continuous cell turnover throughout an organism's lifetime. Stem cells, a group of undifferentiated cells residing in adult tissues, are responsible for generating differentiated cells for maintaining tissue homeostasis due to their unique self-renewal ability. The stem cells are controlled by their specialized local regulatory microenvironments, known as niches, that are formed by their neighboring stromal cells (Li and Xie, 2005; Spradling et al., 2001; Watt and Hogan, 2000). The signals from niche cells work with intrinsic factors to control stem cell self-renewal, proliferation and differentiation (Molofsky et al., 2004; Xi et al., 2005). Although the identification of stem cells remains challenging, due to their rarity and lack of unique molecular markers in mammalian systems, several niches are roughly defined based on their proximity to stem cells (Calvi et al., 2003; Nishimura et al., 2002; Tumbar et al., 2004; Zhang et al., 2003). However, little is known about how niche formation is genetically controlled, even though niche structure is defined. In this study, we show that *Notch* (*N*) signaling directly controls formation of the germline stem cell (GSC) niche in the *Drosophila* ovary.

The *Drosophila* ovary is one of the best-studied stem cell systems because of its easily identified stem cells and powerful genetic tools (Xie et al., 2005). There reside three types of stem cells: GSCs, somatic stem cells (SSCs) and newly identified escort stem cells (ESCs), which are responsible for generating differentiated germ cells, follicle cells and escort cells, respectively (Decotto and Spradling, 2005; Lin and Spradling, 1993; Margolis and Spradling, 1995; Wieschaus and Szabad, 1979). GSCs have been shown to be situated in the niche, which is composed of cap cells, and possibly terminal filament (TF) cells and ESCs (Cox et al., 1998; Decotto and Spradling, 2005; Kretzschmar et al., 1999; Xie and Spradling, 1998; Xie and Spradling, 2000) (Fig. 1A). Recent findings show that the number of cap cells is closely correlated with the GSC number in the normal ovary (Xie and Spradling, 2000) and that GSCs must be anchored to cap cells in order to be maintained as stem cells through DE-cadherin-mediated cell adhesion (Song et al., 2002). This supports the idea that cap cells are an important component of the GSC niche. *dpp*, *gbb*, *Yb* [*fs*(*1*)*Yb* – FlyBase], *piwi* and *hh*, known to be important for GSC maintenance, are expressed not only in cap cells but also in TFs and/or inner germarial sheath (IGS) cells (Cox et al., 1998; Cox et al., 2000; Kiger and Fuller, 2001; King and Lin, 1999; King et al., 2001; Song et al., 2004; Song et al., 2002; Xie and Spradling, 1998). These findings point to a crucial function of cap cells in the GSC niche, but it remains unclear how cap cell formation is genetically controlled.

N signaling plays an important role in regulating proliferation and differentiation of many different cell types (Artavanis-Tsakonas et al., 1999; Lai, 2004). In the *Drosophila* ovary, it was first shown to be required for maintaining follicle cells in their precursor stage and for specification of polar cells that mark the ends of the egg chamber (Grammont and Irvine, 2001; Larkin et al., 1996; Xu et al., 1992). During late oogenesis, *N* signaling is required for the switch from the mitotic cycle to the endocycle and differentiation of follicle cells by negatively regulating the *cut* gene (Shcherbata et al., 2004; Sun and Deng, 2005), and it is also required for patterning the anterior egg shell (Dobens et al., 2005). In this study, we have shown, for the first time to our knowledge, that *N* signaling is necessary and sufficient for controlling formation of the GSC niche.

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MATERIALS AND METHODS

Drosophila genetics

The following *Drosophila* stocks were used in this study: *c587-gal4* (Zhu and Xie, 2003); *UAS-Dl^{30B}*, *UAS-Dl^{36}* and *hs-gal4* (Bloomington *Drosophila* Stock Center); two *UAS-N^{int}* lines, *UAS-N^{B2A2}* and *UAS-N^{33c3}* (kindly provided by Dr Gary Struhl, Columbia University, New York City, NY);

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N^{264-39}, N^{54l9}, N^{ts1} and *hh-lacZ* (Bloomington *Drosophila* Stock Center); *UAS-Dl*^{DN} (Parks et al., 2000); *UAS-mam*^{H} and *UAS-mam*^{N} (Helms et al., 1999); *Dl-lacZ* and *E*(*spl*)*-CD2* (kindly provided by Dr Leonard Dobens, University of Missouri, Kansas City, MO).

Generation of the marked IGS cells overexpressing *dpp* and an activated *N* in the adult *Drosophila* ovary

IGS cells overexpressing *dpp* or N^{ICD} were generated using a technique that combines the FLP-FRT and UAS-GAL4 systems (Ito et al., 1997). *hs-flp; AyGal4* (*act>>y>>gal4*) *UAS-GFP/CyO* virgin females were crossed with either *UAS-dpp/TM3*, *UAS-N*^{B2A2}*/CyO* or *UAS-N*^{33C3}*/TM3* males, respectively. Clones were induced by two 1-hour heat shock treatments of 2-day-old females at 37°C separated by an interval of 5 hours. The heat-shock-treated females were cultured at room temperature for 1 week with daily supplied fresh food, and their ovaries were dissected out and processed for immunostaining with monoclonal anti-Hts (1B1) and rabbit anti-GFP antibodies as described previously (Xie and Spradling, 1998).

BrdU labeling of germline stem cells

The 2-day-old *hh-lacZ/+* control, *c587-gal4/+; UAS-Dl*^{30B}*/+;hh-lacZ /+*, *c587-gal4/+; UAS-Dl*^{36}*/hh-lacZ*, *c587-gal4/+;UAS-N*^{B2A2}*/+;hh-lacZ/+*, *c587-gal4/+; UAS-N*^{33c3}*/hh-lacZ* females were fed on wet yeast paste mixed with 20 mg/ml BrdU solution for 3 consecutive days with fresh BrdU yeast paste each day. The ovaries from these flies were processed for immunostaining with anti-BrdU, anti-Hts and anti-β-galactosidase (β-gal) antibodies according to our published procedures (Song et al., 2002). The rest of the flies were then transferred to fresh food with yeast flakes containing no BrdU every day for 3 consecutive weeks, and their ovaries were processed for immunostaining with the same antibodies.

Immunohistochemistry

Immunostaining of the *Drosophila* ovaries was performed according to previously published procedures (Song et al., 2002). Primary antibodies used in this study are as follows: rabbit anti-β-galactosidase antibody (1:200, Cappel), rabbit anti-GFP antibody (1:200, Molecular Probes), mouse monoclonal anti-CD2 antibody (1:100, Serotec), mouse monoclonal anti-Hts antibody, 1B1 (1:4, DSHB), a mouse monoclonal anti-Dl antibody, c594.9B (1:3, DSHB), two mouse monoclonal anti-N antibodies, F461.3B and c458.2H (1:3, DSHB), rabbit anti-Vasa antibody (1:1000, a gift from Dr Paul Lasko, McGill University, Montreal, Canada), rat anti-DE-cadherin (1:4, DSHB) and sheep anti-BrdU antibody (1:100, Capralogies). The secondary antibodies used in this study are the Alexa 568-, Alexa 468- and Alexa 596-conjugated goat or donkey anti-mouse, rabbit, rat or sheep antibodies (1:200, Molecular Probes). All the images were taken using a Leica TCS SP2 confocal microscope.

RESULTS

N signaling is sufficient to induce cap cell formation

As *N* signaling plays an important role in regulating specification of many different cell types (Artavanis-Tsakonas et al., 1999; Lai, 2004), we sought to test whether *N* signaling regulates the formation of cap cells in the *Drosophila* ovary by manipulating the signaling pathway in somatic cells of developing female gonads using the GAL4-UAS system (Brand and Perrimon, 1993). Ectopic expression of a truncated *N* intracellular domain (N^{int}) or its ligand *Delta* (*Dl*) can activate *N* signaling in ectopic locations (Struhl et al., 1993), and the *c587-gal4* driver can drive a UAS-GFP specifically in most, but not all, of the somatic cells of developing female gonads (Zhu and Xie, 2003) (Fig. 1B,B′). A *hedgehog* (*hh*)*-lacZ* line (the bacterial *lacZ* gene inserted in the *hh* gene) is highly expressed in five to seven cap cells and eight to ten TF cells (Forbes et al., 1996a) (Fig. 1C). GSCs are identified by their direct association with cap cells and the presence of an anteriorly anchored spherical spectrosome (Fig. 1C). Their immediate daughters, cystoblasts, also contain a spherical spectrosome but are positioned away from the cap cells, while other further differentiated progeny, germ cell cysts, can be identified by the presence of a branched fusome (Fig. 1C). The spectrosome and the branched fusome are identified by their expression of Hu-li tai-shao (Hts) (de Cuevas et al., 1997). By contrast to the five to seven cap cells in a normal germarium (Fig. 1C), we observed more *lacZ*-positive cap cells at the tip of either *Dl*- or N^{int}-overexpressing germaria using two independent transgenic *UAS-Dl* or *UAS-N*^{int} lines (Fig. 1D,D′). Note that overexpression of *Dl* and N^{int} gave similar phenotypes, although overexpression of the latter generated a stronger phenotype than that of the former. In the germaria with increased cap cells, spectrosome-containing single germ cells, which were later shown to behave like GSCs, also increased at the germarial tip (Fig. 1D,D′). This result shows that *N* signaling is sufficient to induce cap cell formation and supports the idea that cap cells are a key niche component for controlling GSC self-renewal.

In addition to increased cap cells at the germarial tip, we frequently observed one or more patches of strongly *lacZ*-positive somatic cells away from the germarial tip when *Dl* or N^{int} was overexpressed in the developing gonads (Fig. 1E-F′). These *lacZ*-positive somatic cells appeared to be functional cap cells at ectopic locations, as spectrosome-containing single germ cells (later shown to be GSCs) were closely associated with them (Fig. 1E-F′). The ectopic GSCs associated with the ectopic cap cells also anchored their spectrosome on the side that contacts cap cells, as observed in a normal GSC context. Some of these ectopic cap cells were surrounded by IGS cells (Fig. 1E,E′) or somatic follicle cells (Fig. 1F,F′), and it appeared that both types of ectopic cap cells could sufficiently maintain GSCs. In rare cases of N^{int} overexpression, *lacZ*-positive cap cells completely occupied the anterior half of the germaria instead of IGS cells, and consequently, GSCs were everywhere in the anterior half of the germarium (Fig. 1G,G′). Germ cells moving away from the cap cells could still differentiate, as indicated by the presence of the branched fusomes. These observations further indicate that signals from the GSC niche directly repress differentiation of GSCs close to cap cells, allowing germ cells moving away from the cap cells to differentiate, because they are beyond the influence of short-range signals from the cap cells. The ectopic *lacZ*-positive cells and their associated GSCs could persist for at least 5 weeks (the longest time we had tested), suggesting that these expanded or ectopic cap cells are stable and sustain GSCs like normal cap cells. Together, these results demonstrate that *N* signaling is sufficient to induce cap cell formation. Furthermore, our observation that ectopic cap cells without TF cells or IGS cells are able to sustain GSC self-renewal indicates that cap cells are a key component to establish the niche for sustaining GSC self-renewal.

To further verify that the ectopic *hh-lacZ*-positive cap cells exhibit known properties of normal cap cells, we examined the expression of other markers for cap cells. A *wingless* (*wg*) enhancer trap line, *wg-lacZ*, is known to be expressed in some but not all cap cells (Forbes et al., 1996b) (see Fig. S1A in the supplementary material). In agreement with our hypothesis, some of these ectopic cap cells expressed *wg-lacZ* whether they were surrounded by IGS cells or somatic follicle cells (see Fig. S1B,C in the supplementary material). Nuclear lamin C is expressed highly in nuclear membranes of TF cells and cap cells (Xie and Spradling, 2000) (see Fig. S1D in the supplementary material), and the cap cells in normal or ectopic locations highly express this marker (see Fig. S1E in the supplementary material). Cap cells also express and accumulate DE-cadherin at their junction with GSCs in keeping GSCs in the niche

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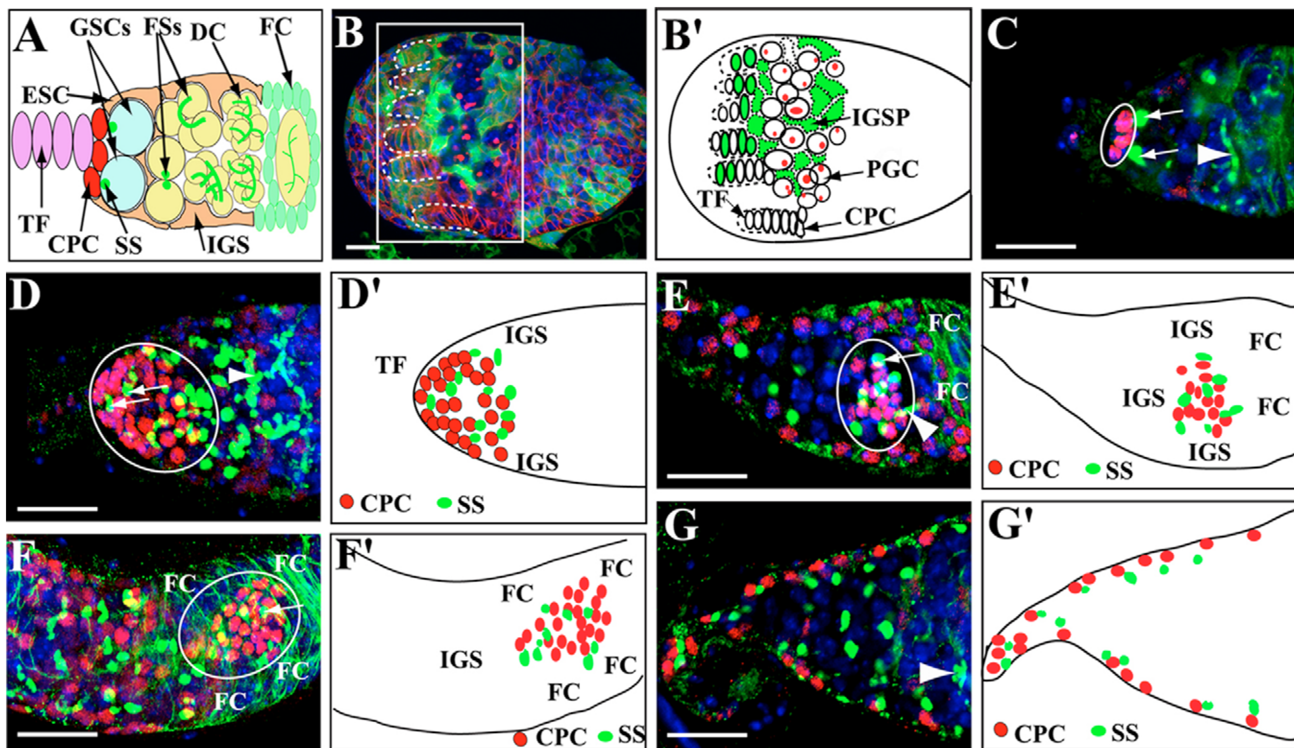

Fig. 1. Forced expression of an activated *N* can sufficiently expand niche sizes and generate ectopic niches. (**A**) Schematic of a cross-section of the anterior part of the germarium. (**B**,**B′**) A confocal section of a *c587-gal4; UAS-GFP* female gonad at the larval-pupal transition stage labeled for Hts (red), GFP (green) and DNA (blue), showing that the *c587-gal4* driver expresses GFP in most, but not all, somatic cells of the gonad including TFs and IGS precursors. (B′) Schematic of the region highlighted by a rectangle in B. Panels C,E,G represent one confocal section of the anterior portion of the germaria labeled for β-gal (red) and Hts (green), while D,F represent overlayed images. (D′-F′) Schematic presentations of the areas highlighted by ovals in D-F, respectively; (G′) schematic presentation of G. (**C**) A *hh-lacZ*/+ germarial tip showing cap cells (oval) and two GSCs indicated by spectrosomes (arrows). β-gal-positive TFs are not shown on this confocal section. Arrowhead indicates a branched fusome. (**D**,**D′**) A *c587-gal4*/+; *UAS-N^{int}*/+; *hh-lacZ*/+ germarial tip showing over 40 *lacZ*-positive cap cells at the normal location close to the TF (oval), 15 GSCs evidenced by the presence of spectrosomes (two indicated by arrows) and differentiated germ cell cysts evidenced by the presence of branched fusomes (one indicated by an arrowhead). (**E**,**E′**) A *c587-gal4*/+; *UAS-N^{int}*/+; *hh-lacZ*/+ germarial tip showing that a group of 12 *lacZ*-positive cap cells (oval) located away from the tip support six GSCs (one spectrosome denoted by an arrow and one elongated spectrosome by an arrowhead). (**F**,**F′**) A *c587-gal4*/+; *UAS-N^{int}*/+; *hh-lacZ*/+ germarial tip showing that a group of 24 *lacZ*-positive cap cells (oval) surrounded by follicle cells (FC) support eight GSCs (one spectrosome indicated by an arrow). (**G**,**G′**) A *c587-gal4*/+; *UAS-N^{int}*/+; *hh-lacZ*/+ germarial tip showing that *lacZ*-positive cap cells covering the surface of the anterior half of the germarium support the contacting GSCs indicated by spectrosomes, and a differentiated germ cell cyst evidenced by the presence of a branched fusome (arrowhead). Scale bars: 10 μm. CPC, cap cell; DC, developing cyst; ESC, escort stem cell; FC, follicle cell; FS, fusome; GSC, germline stem cell; IGS, inner germarial sheath cell; IGSP, IGS precursor; SS, spectrosome; TF, terminal filament cell; PGC, primordial germ cell.

(Song et al., 2002). Indeed, DE-cadherin proteins significantly accumulated between ectopic cap cells and their associated GSCs (see Fig. S1F in the supplementary material), which might also function to anchor GSCs. Therefore, our molecular evidence strongly indicates that these ectopic cap cells behave like normal cap cells.

Expanded and ectopic niches can maintain germline stem cells

To investigate whether the spectrosome-containing single germ cells associated with expanded cap cells resemble normal GSCs, we examined the expression of *bam-GFP* and *Dad-lacZ*, which were used to monitor *bam* and *Dad* transcription (Chen and McKearin, 2003; Kai and Spradling, 2003; Song et al., 2004). As in the wild-type niche (Fig. 2A), the spectrosome-containing single germ cells associated with those ectopic cap cells did not express *bam-GFP*, which resembles the property of normal GSCs (Fig. 2B,C). Interestingly, germ cells lying one cell away from ectopic GSCs were often germline cysts, as they contained branched fusomes, indicating that the progeny of the ectopic GSCs probably undergo normal differentiation (Fig. 2B,C). However, too many cap cells (more than seven) at the normal location (Fig. 2D) or at an ectopic site (Fig. 2E) often caused the accumulation of spectrosome-containing single germ cells located two or more cell diameters away, and *bam* expression was also repressed in those single cells, indicating that these extra single cells also resemble GSCs. Normally, *bam* is only repressed in GSCs due to the short-range BMP signal, which may be caused by a limited amount of DPP protein produced by five to seven cap cells in a wild-type germarium. As we have previously demonstrated that *dpp* overexpression in the germarium causes the accumulation of single cells that are negative for *bam* expression (Song et al., 2004; Xie and Spradling, 1998), these observations further suggest that cap cells are the major source of BMP, and more cap cells can produce more BMP, which could help it diffuse farther than the normal distance.

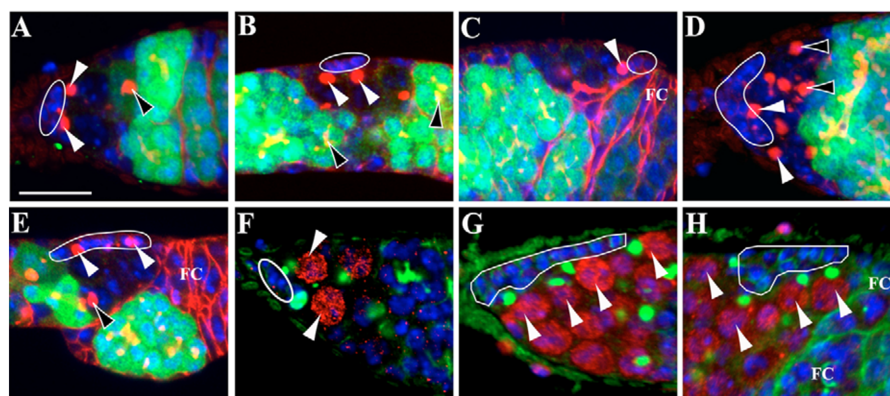

Fig. 2. Ectopic GSCs associated with ectopic niches behave like normal GSCs. The panels A-C and E-H represent one confocal section, whereas D represents an overlayed image of multiple confocal sections. The germaria in A-E are labeled for GFP (green), Hts (red) and DNA (blue), while the ones in F-H are labeled for β-gal (red), Hts (green) and DNA (blue). (**A**) A wild-type germarial tip showing that two GSCs (white arrowheads) in contact with cap cells (oval) do not express *bam-GFP* (black arrowhead). (**B**) Part of a *c587-gal4*/+; *UAS-N^{int}*/+; *bam-GFP*/+ germarium showing that two spectrosome-containing GSCs (white arrowheads) that are associated with ectopic cap cells (oval) fail to express *bam-GFP*. Black arrowheads indicate branched fusomes in differentiated cysts. (**C**) A middle portion of a *c587-gal4*/+; *UAS-N^{int}*/+; *bam-GFP*/+ germarium showing that ectopic cap cells (oval) support one *bam-GFP*-negative spectrosome-containing GSC (arrowhead). (**D**) The tip of a *c587-gal4*/+; *UAS-N^{int}*/+; *bam-GFP*/+ germarium showing that the expanded cap cells (indicated by unbroken outline) repress *bam* expression not only in GSCs (white arrowheads) but also in the spectrosome-containing single germ cells lying more than one cell diameter away (black arrowheads). (**E**) *c587-gal4*/+; *UAS-N^{int}* /+; *bam-GFP*/+ germarium showing that the ectopic cap cells (indicated by unbroken outline) repress *bam* expression not only in GSCs (white arrowheads) but also in the spectrosome-containing single germ cells lying more than one cell diameter away (black arrowhead). (**F**) The tip of a *Dad-lacZ*/+ germarium showing that two GSCs (arrowheads) are in contact with cap cells (oval) and express high levels of *Dad-lacZ*. (**G**) The tip of a *c587-gal4*/+; *UAS-N^{int}*/+; *Dad-lacZ*/+ germarium showing that an increased number of cap cells (indicated by unbroken outline) induced by the expression of an activated *N* support an increased number of GSCs (arrowheads) and cystoblasts that are positive for *Dad-lacZ*. (**H**) A middle portion of a *c587-gal4*/+; *UAS-N^{int}*/+; *Dad-lacZ*/+germarium showing that a group of ectopic cap cells (indicated by unbroken outline) close to follicle cells also support ectopic GSCs (arrowheads) that are also positive for *Dad-lacZ*. Scale bar: in A, 10 μm for all images.

A short-range BMP signal from cap cells specifically activates its signaling cascade in GSCs to activate expression of *Dad* (Casanueva and Ferguson, 2004; Kai and Spradling, 2003; Song et al., 2004) (Fig. 2F). As expected, all the GSCs that directly contact the expanded cap cells at the germarial tip highly expressed *Dad-lacZ*, indicating that the expanded cap cells have the capacity to produce a BMP signal in a similar manner to normal cap cells (Fig. 2G). Interestingly, many spectrosome-containing single germ cells not directly associated with the expanded cap cells (more than ten cap cells) also expressed *Dad-lacZ*, further supporting the idea that the increased number of cap cells leads to production of more diffusible BMP (Fig. 2G). When the ectopic cap cells are surrounded by IGS cells or near follicle cells, their associated GSCs also highly expressed *Dad-lacZ* as in normal GSCs (Fig. 2H), confirming the idea that ectopic cap cells can also emit the BMP signal like normal cap cells. These results suggest that cap cells are the source of active BMP and that the spectrosome-containing single germ cells associated with expanded or ectopic cap cells resemble GSCs.

Ectopic GSCs are capable of dividing and self-renewing

A GSC generates two daughters that remain connected to each other via a contractile ring, through which an elongated spectrosome passes (de Cuevas and Spradling, 1998). In our earlier experiments, we observed that many cases of ectopic GSCs carried an elongated spectrosome, indicating that those ectopic GSCs are probably capable of dividing and generating differentiated germ cells. To further confirm that these extra spectrosome-containing germ cells behave like GSCs, we labeled them with BrdU, a nucleotide analog, for 3 days and chased for 3 weeks. As a control, 96.4% of the germaria (*n*=57) of the *hh-lacZ*/+ heterozygotes contained one or more BrdU-labeled GSCs after 3 days of BrdU feeding, while 84.5% of the total GSCs (*n*=136) were labeled by BrdU. Consequently, most of the control germaria contained two or three labeled GSCs (Fig. 3A). After the females were fed for 3 more weeks on normal food without BrdU, the GSCs in the control germaria (*n*=35) completely lost their BrdU label (Fig. 3B), indicating that the BrdU label is completely diluted out as the labeled GSCs continuously divide for 3 weeks.

Similarly, 98.8% of the germaria (*n*=85) (developed from the female gonads overexpressing *Dl* or *N^{int}*) carried one or more BrdU-labeled GSCs close to the cap cells at the normal location or at ectopic cap cells (Fig. 3C,D). Among them, 94.1% of the expanded niches (more than seven cap cells carrying four or more GSCs; *n*=51) at the normal location (close to TFs) carried one or more BrdU-labeled GSCs (Fig. 3C,C′), while 96.0% of the ectopic niches (*n*=25) carried one or more BrdU-labeled GSCs (Fig. 3D), indicating that extra GSCs in the expanded niches as well as in the ectopic niches are mitotically active like normal GSCs. After the 3 week chase, 85.9% of the germaria (*n*=120) did not carry any BrdU-labeled GSCs at the normal location or at ectopic sites (Fig. 3E), whereas 14.1% of them carried one or more BrdU-labeled GSCs (Fig. 3F), also indicating that the expanded or ectopic GSCs remain active for over 3 weeks. It also appeared that those GSCs did not divide as frequently as normal GSCs. Perhaps this is due to their excessive number at each niche. These results strongly suggest that the GSCs at the expanded niche or at the ectopic niche can continuously divide and generate differentiated germ cells like normal GSCs.

Notch signaling induces formation of ectopic cap cells only during the late third-instar larval and early pupal stages

Cap cells normally form during the larval-pupal transition and in the early pupal stage (Zhu and Xie, 2003). To further determine when ectopic cap cells form, we used an *hs-gal4* transgene (the *gal4* gene under the control of the heat shock protein 70 promoter) to drive the expression of *Dl* during gonadal development by heat shock treatments. When *Dl* expression was induced during the second instar larval stage or after the mid-pupal stage, about 98% of the germaria carried five to seven cap cells in the normal location, just like in the wild type (*n*=603), whereas about 2% of the germaria had

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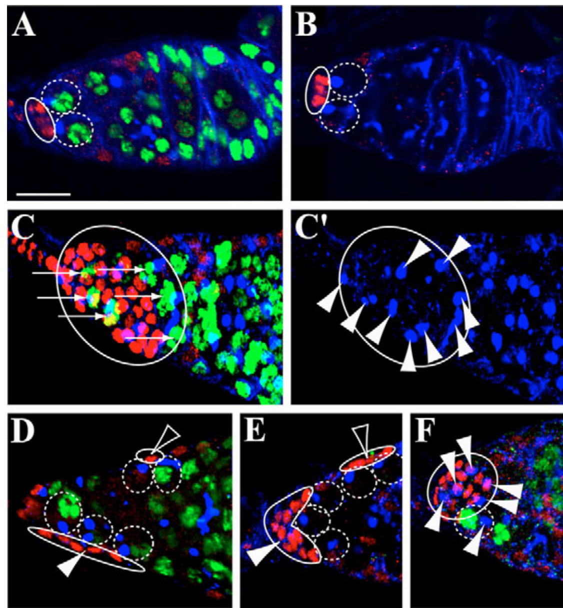

Fig. 3. GSCs in the expanded or ectopic niche are mitotically active and are able to generate differentiated germ cells. The germaria in A-F are labeled for BrdU (green), β-gal (red) and Hts (blue). A,B,D,E represent a confocal section, whereas C,F are overlayed confocal images. (**A**) A *hh-lacZ*/+ germarium showing that two GSCs (broken lines) close to the cap cells (oval) are BrdU-positive after 3 days of BrdU feeding. (**B**) A *hh-lacZ*/+ germarium showing that two GSCs (broken lines) close to the cap cells (oval) have lost their BrdU label 3 weeks after BrdU feeding. (**C**,**C′**) A germarial tip showing that an expanded cap cell cluster (oval) supports nine GSCs (arrowheads), six of which are BrdU-positive (arrows). (**D**) The tip of a *c587-gal4/+; UAS-N^{int}/+; hh-lacZ/+* germarium after 3 days of BrdU feeding, showing that a cap cell cluster (oval and white arrowhead) near TF support three GSCs (broken lines), two of which are BrdU-positive, and an ectopic cap cell cluster (oval and black arrowhead) supporting two GSCs (broken lines), one of which is BrdU-positive. (**E**) The tip of a *c587-gal4/+; UAS-N^{int}/+; hh-lacZ/+* germarium showing that GSCs in an expanded cap cell cluster (unbroken outline indicated by a white arrowhead) and an ectopic cap cell cluster (oval and black arrowhead) are BrdU-negative 3 weeks after BrdU feeding. (**F**) The tip of a *c587-gal4/+; UAS-N^{int}/+; hh-lacZ/+* germarium showing that one (broken line) of six GSCs (arrowheads) in an expanded cap cell cluster (oval) remains BrdU-positive 3 weeks after BrdU feeding. Scale bar: 10 μm.

ectopic cap cells positioned away from TFs, as detected in the heat-shocked *hsgal4; hh-lacZ* control germaria (*n*=606). Also, overexpression of *Dl* in adult ovaries did not increase cap cell numbers and induce ectopic cap cells (*n*=212). Interestingly, when *Dl* was ectopically expressed during the third instar and early pupal stages, the number of cap cells at the tip of some germaria was increased (Fig. 4A,A′), and about 10% of these germaria carried ectopic cap cells (*n*=234) (Fig. 4B,B′), indicating that elevated *N* signaling can induce cap cell formation in the normal location as well as in the ectopic sites. In some of the germaria (*n*=27), cap cells were formed away from TFs and still maintained GSCs, but no GSCs were associated with TFs (Fig. 4B,B′), indicating that TFs alone are not sufficient to sustain GSC self-renewal. These results suggest that the IGS precursors in the developing gonad are competent to form cap cells in response to ectopic *N* signaling from the late third-instar larval stage to the early pupal stage. Note that these ectopic cap cells no longer expressed *Dl* or had the activated *N* signaling in the adult ovary but were stable and able to maintain GSCs, suggesting that ectopic activated *N* signaling by *c587-gal4* is not directly involved in controlling GSC self-renewal.

To rule out the possibility that the remaining activated *N* in the ectopic niches may directly control expression of *dpp* and thereby maintain ectopic GSCs, we used the previously established ‘flip-out’ cassette to express *dpp* and the activated *N* in IGS cells of the adult *Drosophila* ovary, which were labeled by GFP expression (Ito et al., 1997). Interestingly, when *dpp* was induced to be expressed in several of the IGS cells of adult *Drosophila* ovaries, many spectrosome-containing single germ cells accumulated, and those GFP-positive *dpp*-expressing IGS cells still maintained their normal morphology, suggesting that *dpp*-expressing IGS cells may sufficiently create ectopic GSC niches without being transformed into cap cells (see Fig. S2A in the supplementary material). By contrast, overexpression of the activated *N* in the GFP-positive IGS cells did not affect the differentiation status of the underlying germ cells, indicating that activation of *N* signaling in adult IGS cells alone does not sufficiently create ectopic GSC niches, unlike *dpp* overexpression (see Fig. S2B,C in the supplementary material). In light of the evidence we have presented so far, we conclude that ectopic expression of the activated *N* during niche formation leads to formation of ectopic niches and GSCs, but the continuous expression of the activated *N* in adult IGS cells might not directly contribute to the maintenance of ectopic GSCs.

Dl on newly formed TF cells activates *N* signaling in their adjacent somatic cells to induce cap cell formation

To gain further insight into how *N* signaling is involved in controlling cap cell formation, we examined *N* and *Dl* expression in the developing gonads from the late third instar larval stage to the early pupal stage. A *Dl-lacZ* line (the *lacZ* gene inserted in the *Dl* locus to recapitulate its expression) and an anti-Dl antibody were used to determine *Dl* expression in the developing female gonads (Grossniklaus et al., 1989). *Dl* was primarily expressed in newly formed TFs and other somatic cells anterior to the primordial germ cell zone (PGCZ) at high levels but not in primordial germ cells (PGCs) during the late third-instar larval stage or the larval-pupal transitional stage (Fig. 4C-D′). Interestingly, N protein was expressed at high levels in TFs and cap cells as well as in the somatic cells that are mingled with PGCs at lower levels, but not in PGCs (Fig. 4E,E′). *N* signaling is known to regulate expression of *E*(*spl*) complex genes (Bailey and Posakony, 1995); an *E*(*spl*)*mβ*-CD2 transgene has been used to monitor *N* signaling activity in several tissue types (Cooper and Bray, 1999; de Celis and Bray, 1997). Consistent with the idea that TFs and cap cells are capable of activating *N* signaling due to presence of both Dl and N, *E*(*spl*)*mβ*-CD2 was expressed almost exclusively in TFs and cap cells but not in the somatic cells that are mixed with PGCs (Fig. 4F,F′). These expression results suggest that developing TFs and cap cells are active in *N* signaling, which may be dependent on *Dl* in TFs.

Our earlier observation that the somatic cells that mingle with PGCs express *N* raises the interesting possibility that activated *N* signaling transforms those somatic cells normally destined to form IGS cells and/or follicle cells into cap cells. To further investigate this possibility, we used the *E*(*spl*)*mβ*-CD2 as a marker to study whether ectopic expression of *Dl* or *N^{int}* could induce expression of *E*(*spl*)*mβ*-CD2 in the somatic cells that are mingled with PGCs. When *N^{int}* or *Dl* was expressed by *c587-gal4* in most of the somatic cells in the gonad, clusters of

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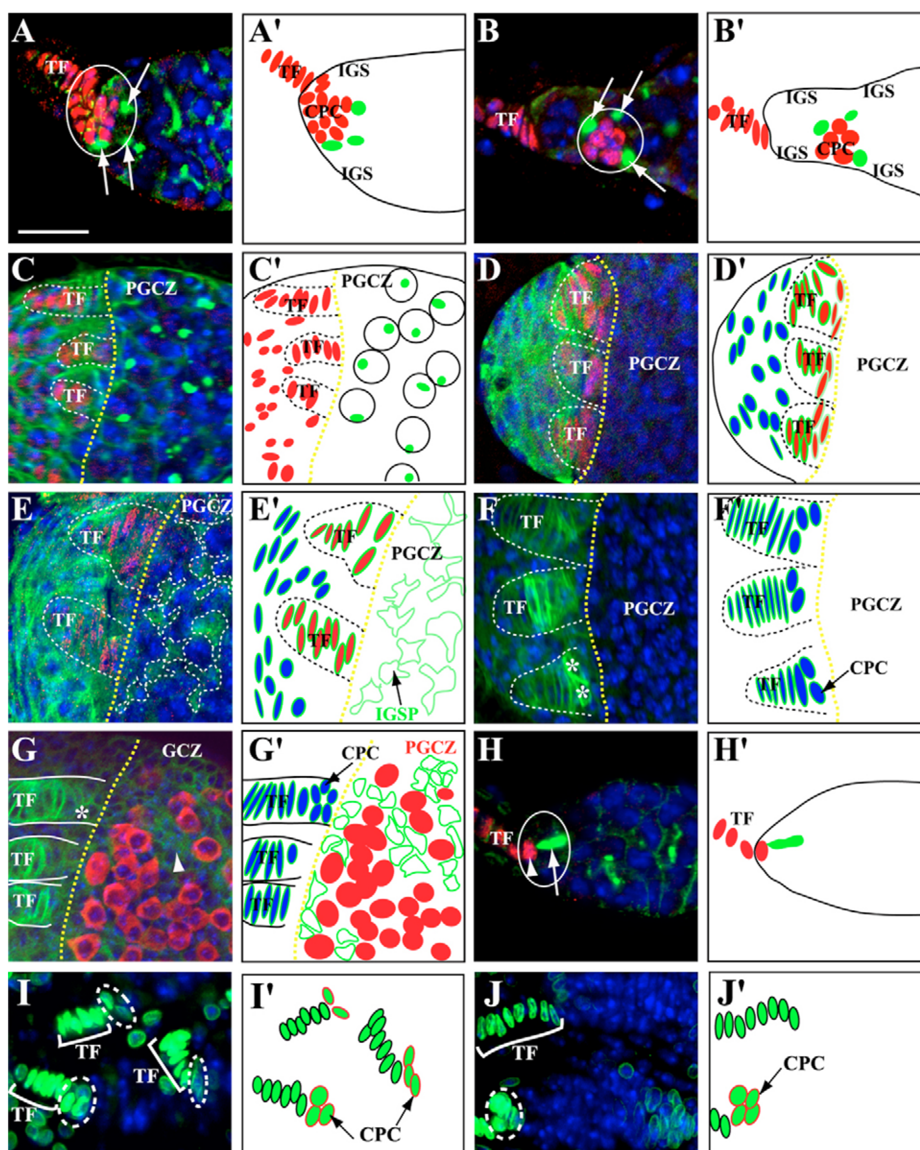

Fig. 4. *N* signaling controls niche formation in a developmental stage-dependent manner. The panels in A-J represent one confocal section of the adult germaria (A,B,H-J) or early female gonads (C-G) that are labeled for β-gal (red, A-E,H), Hts (green, C,H), N (green, E), Dl (green, D), CD2 (green, F,G), Vasa (red, G), Lamin C (green, I,J) and DNA (blue, A-J). (A′,B′,H′) Schematic presentations of the areas highlighted by ovals in A,B,H, respectively; (C′-G′) Schematic presentations of C-G, in which a yellow line indicates the borderline between TFs and the PGC zone. (**A**,**A′**) The tip of a *UAS-Dl*/+; *hs-gal4*/*hh-lacZ* germarium overactivating *N* signaling at the early pupal stage, showing three GSCs (arrows in A and green dots in A′) and the increased number of β-gal-positive cap cells (oval in A and CPC in A′). (**B**,**B′**) The tip of a *UAS-Dl*/+; *hs-gal4*/*hh-lacZ* germarium overexpressing activated *N* at the early pupal stage, showing three GSCs (arrows in B and green dots in B′) and a group of β-gal-positive ectopic cap cells (circle in B and CPC in B′). (**C**,**C′**) A part of a *Dl-lacZ*/+ female gonad at the larval-pupal transitional stage showing β-gal-positive TFs and β-gal-negative PGCs (circles with a green dot for spectrosome in C′). (**D**,**D′**) A part of a *hh-lacZ*/+ female gonad at the late third-instar larval stage showing that β-gal-positive TFs and surrounding anterior somatic cells (green ovals filled with blue in D′) express Dl protein. (**E**,**E′**) A part of a *hh-lacZ*/+ female gonad at the larval-pupal transitional stage showing that β-gal-positive TFs and IGS precursors (broken lines in PGC zone of E and green lines in PGC zone of E′) express N protein. (**F**,**F′**) A part of an *E(spl)mβ-CD2* female gonad at the larval-pupal transitional stage showing that TFs (white broken lines in F and black broken line in F′) and newly formed cap cells (asterisks in F and arrow in F′) express CD2. (**G**,**G′**) A part of an *E(spl)mβ-CD2* female gonad overexpressing the activated *N* at the larval-pupal transitional stage showing that some somatic cells (arrowhead in G and green lines in PGCZ of G′) express *E(spl)* in addition to TFs and newly formed cap cells (asterisk in G). (**H**,**H′**) A tip of a N^{264-39}/+; *hh-lacZ*/+ germarium showing two cap cells (arrowhead in H) and one GSC (its spectrosome indicated by an arrow in H). (**I-J′**) The 2-day-old ovo^{D1rS1} mutant germaria showing that many germaria have cap cells (broken lines) lying adjacent to TFs (brackets) (I, and the lower one in J), but some do not have cap cells (the upper one in J). Scale bar: 10 μm. IGSP, IGS precursor; PGCZ, PGC zone.

the somatic cells that were also closely associated with PGCs started to express *E(spl)mβ*-CD2 (Fig. 4G,G′). This suggests that ectopic *N* signaling in somatic cells, which is normally devoid of *N* signaling, induces them to assume cap cell fate.

Notch signaling is involved in controlling cap cell formation

To directly investigate whether *N* signaling is required for cap cell formation, we attempted to examine the number of cap cells in *N* and *Dl* temperature-sensitive mutants. Unfortunately, all the existing temperature-sensitive *Dl* and *N* mutant third-instar larvae or early pupae did not survive at a restrictive temperature (29°C) to adulthood. As *N* is known to be haploinsufficient in several developmental processes (Artavanis-Tsakonas et al., 1999), we examined the cap cell number in 2-day-old heterozygotes carrying strong or null *N* mutants, N^{5419} and N^{264-39}, using *hh-lacZ* to label TFs and cap cells. Because there is no or little cap cell and GSC turnover in 2-day old *Drosophila* females, the number of cap cells and GSCs should truly reflect the number of cap cells that form during the pupal stage. By contrast, with 2-day-old wild-type germaria with 5.5±1.3 cap cells and 2.5±0.6 GSCs (*n*=121), the 2-day-old N^{5419} heterozygous germaria carried 4.1±1.5 cap cells and 1.8±0.6 GSCs (*n*=131), while the N^{264-39} heterozygous germaria had 4.2±1.4 cap cells and 1.8±0.6 GSCs (*n*=97), indicating that decreased *N* signaling can significantly reduce cap cell number

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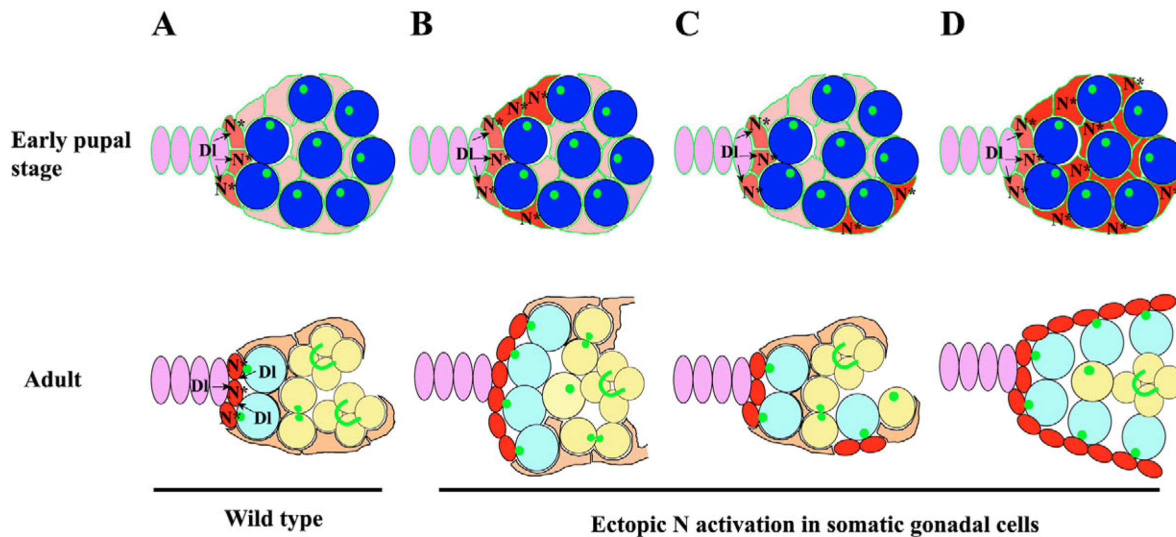

Fig. 5. Model explaining how *N* signaling controls GSC niche formation in the *Drosophila* ovary. (**A**) Newly formed TFs (purple) express Dl protein and activate *N* (*N**) signaling in neighboring somatic cells and induce them to form cap cells (red oval) supporting two GSCs (light blue circle), while the rest of the somatic cells that are not in contact with TFs form ESCs or IGS cells (brown). PGCs are depicted as dark blue round cells, while differentiated germ cells, including cystoblasts, are yellow round cells. *N* signaling remains active in cap cells of the adult ovary and is required for their maintenance. (**B**) When *N* signaling is expanded to the somatic cells that do not contact TFs but are adjacent to the somatic cells destined to become cap cells, these somatic cells will also become cap cells (and possibly ESCs) and thus increase the niche size in the normal location and the GSC number. (**C**) When *N* signaling is ectopically activated in the somatic cells a few cells distant from TFs, these somatic cells differentiate into cap cells (and possibly ESCs). Thus, ectopic niches that are surrounded by IGS cells or follicle cells are formed. (**D**) When *N* signaling is active in most, if not all, somatic cells of the gonad, the somatic cells that have active *N* signaling generate cap cells (and possibly ESCs), forming niche cells, which are able to sustain GSC self-renewal throughout the germaria.

(P<0.001) and consequently GSC number (P<0.001). Moreover, about 36% of the *N* heterozygous germaria had three or fewer cap cells, by contrast to the five to seven cap cells of the wild-type germaria (Fig. 4H,H′). To gain further evidence supporting the idea that *N* signaling is required for cap cell formation, we used the *c587-gal4* driver to overexpress dominant-negative mutants for *N* pathway components, such as *Dl* and *mastermind* (*mam*). The 2-day-old germaria developing from female gonads that overexpressed a dominant-negative *Dl* had an average of 3.3±1.6 cap cells and 1.6±0.6 GSCs (total 62 germaria examined), while the germaria developing from female gonads that overexpressed two dominant-negative forms of *mam*, mam^H and mam^N, carried an average of 3.2±1.2 cap cells and 1.6±0.6 GSCs (total 162 germaria examined) as well as 2.8±1.2 cap cells and 1.4±0.6 GSCs (total 128 germaria examined), respectively. As the dominant-negative forms of *Dl* and *mam* have been shown to specifically block *N* signaling in imaginal discs (Helms et al., 1999; Parks et al., 2000), our results further support the idea that *N* signaling is required for controlling cap cell formation.

Due to lack of the ability to remove *Dl* function specifically from TFs to test whether TF-expressing *Dl* controls cap formation, we investigated if cap cells could still form without germ cells. ovo^{D1rS1} mutant females contain no germ cells (Rodesch et al., 1995). Among the 2-day-old agametic ovo^{D1rS1} mutant germaria, many still contained one to five cap cells, although some completely lacked cap cells, which could have degenerated during the pupal period (Fig. 4I-J′). In the *osk* mutant agametic germaria, cap cells appear to form and persist (Margolis and Spradling, 1995). Together, these findings argue that TF-expressed Dl is responsible for cap cell formation in the agametic germarium, and possibly in the normal germarium as well.

Notch signaling is required for the maintenance of the niche and GSCs in the adult ovary

To investigate whether *N* signaling is also involved in regulation of niche and GSC functions in the adult ovary, we examined the expression of *Dl* using the *Dl-lacZ* line mentioned earlier. By contrast with the fact that *Dl* expression is restricted to the somatic cells anterior to the PGC zone in the third-instar larval stage, including newly formed TFs, *Dl* was also observed to be expressed at low levels in germ cells of adult germaria, including GSCs, in addition to its expression in TFs (see Fig. S3A in the supplementary material). *E*(*spl*)*m7*, a gene in the *E*(*spl*) complex, is a response gene of *N* signaling (Bray, 2006). *E*(*spl*)*m7-lacZ* was used to monitor *N* signaling activity in the germarium. Interestingly, *E*(*spl*)*m7-lacZ* was mainly detected in cap cells, possibly in GSCs at very low levels, suggesting that *N* signaling remains active in cap cells of the adult germarium (see Fig. S3B in the supplementary material). To further investigate whether or not *N* signaling is required for the maintenance or function of the GSC niche, we used a well-studied temperature-sensitive *N* allele (N^{ts1}) to determine the role of *N* signaling in the adult ovary. One previous study using this *N* allele showed that *N* signaling is required for proper differentiation of follicle cells and thus proper formation of egg chambers (Xu et al., 1992). As a control, the germaria from the wild-type females cultured at 29°C for 1 or 2 weeks had 2.4±0.5 (n=33) and 2.2±0.7 (n=39) GSCs, respectively, and germ cysts differentiated normally and egg chambers formed normally as well (see Fig. S3C,D in the supplementary material). By contrast, the germaria from the N^{ts1} mutant females cultured at 29°C for 1 or 2 weeks were generally small, and contained 1.1±1.0 (n=27) and 0.5±0.8 (n=36) GSCs, respectively. By contrast with 6.0±0.9 (1 week) and 5.8±1.0 (2 weeks) cap cells for the wild-type ovaries, the N^{ts1} mutant ovaries from the females cultured at 29°C for 1 or 2 weeks carried 4.6±1.3

and 2.7±1.4 cap cells, respectively. As complete loss of GSCs does not quickly eliminate cap cells (Xie and Spradling, 2000), our results suggest that GSC loss in the *N* mutant ovaries is probably due to loss of cap cells. Indeed, the germaria from the females cultured at 29°C for 1 week had more cap cells than the ones cultured for 2 weeks (see Fig. S3E-H in the supplementary material), and in some of the germaria, cap cells completely disappeared (see Fig. S3H in the supplementary material). In light of the evidence that *N* signaling activity in the adult ovary is restricted to cap cells, these results further suggest that *N* signaling is required for maintaining cap cells and thus GSCs.

DISCUSSION

Although it has recently been shown that several adult stem cell types are controlled by their neighboring stromal cells (Li and Xie, 2005), it remains unclear what constitutes a functional niche and how its formation is genetically controlled. In this study, we have revealed one novel molecular mechanism underlying GSC niche formation in the *Drosophila* ovary (Fig. 5). The newly formed TFs express *Dl*, which is probably responsible for activating *N* signaling in their neighboring somatic cells and for inducing niche formation. Ectopic *N* signaling is sufficient to induce niche formation in a developmental stage-dependent manner, and reduced *N* signaling results in reduction of cap cell and GSC numbers, demonstrating that *N* signaling is important for controlling GSC niche formation. As ectopic niche cells in different locations are stable and able to sustain GSC self-renewal, this leads us to conclude that the niche does not have to function in a fixed position. Finally, N signaling is also required for the survival of niche cells in adults (see Fig. S3 in the supplementary material). By analogy, *N* signaling may control niche formation in other systems, including mammals.

N signaling is required for formation of the GSC niche

At the onset of the larval-pupal transition, all of the 16 to 20 TF stacks have formed and initiate ovariole formation (Godt and Laski, 1995; King, 1970), while another group of somatic cells, cap cells, start to occupy a position between the TFs and the germ cells (Zhu and Xie, 2003). The PGCs in direct contact with cap cells are further anchored through E-cadherin and are further expanded through symmetric division and develop into permanent GSCs in the adult ovary (Song et al., 2002; Zhu and Xie, 2003). Actin-filament regulator, Cofilin/ADF, and ecdysone signaling, are required for TF formation (Chen et al., 2001; Hodin and Riddiford, 1998). However, no studies have been carried out to investigate the formation of cap cells, which are a key component of the GSC niche. In this study, we have investigated the role of *N* signaling in controlling cap cell formation.

N signaling controls cell fate determination in a variety of organisms (Artavanis-Tsakonas et al., 1999; Kadesch, 2004; Lai, 2004). In this study, we show that in late third-instar larval female gonads, Dl is expressed in newly formed TFs, while the N receptor is expressed in all the somatic cells, including TFs and cap cells. Consequently, *N* signaling is active in newly formed TFs and cap cells and its activation is sufficient to induce cap cell formation, suggesting that TF-expressed *Dl* activates *N* signaling to induce cap cell formation. To further support the idea that *N* signaling specifies cap cell fate, reduction of *N* signaling results in a reduced number of cap cells. Induction of cap cells by *N* signaling can only take place during the late third instar and early pupal stages, suggesting that active *N* signaling only promotes cap cell formation along with other factors provided at particular stages. Cap cells can still form without germ cells. This suggests that Dl is unlikely to be required in germ cells for cap cell formation. Therefore, we conclude that *N* signaling, activated probably by Dl from newly formed cap cells, specifies cap cell fate through direct induction. In this study, we also show that *N* signaling is required for maintaining the GSC niche in the adult ovary, as loss of *N* function results in rapid loss of cap cells and GSCs. Taken together, the results of this study demonstrate that *N* signaling is important for controlling niche formation as well as niche maintenance.

Expanded and ectopic niches are sufficient for controlling GSC self-renewal

Although niches have been defined for GSCs in the *Drosophila* ovary and testis, as well as in several tissue types of the mammalian systems, it remains unclear whether they still function properly for controlling stem cell self-renewal after their location and size are changed. In this study, we have provided two pieces of experimental evidence supporting the idea that expanded niches are functional for controlling GSC self-renewal. First, increased cap cells in the normal location express all known cap cell markers, such as *hh-lacZ*, *wg-lacZ*, Lamin C and E-cadherin, and behave like normal cap cells. Second, these expanded cap cells can support self-renewal of extra GSCs, which behave similarly to normal ones based on *Dad-lacZ* and *bam-GFP* expression, and their ability to self-renew and generate differentiated germ cells. Even when cap cells cover the anterior half of the germarium, the GSCs associated with the cap cells also appear to be capable of self-renewing and generating differentiated germ cells. Our findings show that GSC niche size can be expanded by adding more niche cells.

This study has also demonstrated that the GSC niche could function in ectopic locations. Ectopic cap cells, which are surrounded by IGS cells or follicle cells, also express known cap cell markers and sufficiently support functional GSCs, supporting the idea that TFs and IGS cells are not essential components of the GSC niche. This is consistent with early published studies showing that the numbers of cap cells and GSCs are closely correlated and that TFs and cap cells express the genes important for GSC self-renewal such as *piwi*, *Yb*, *hh* and *dpp/gbb* (Chen and McKearin, 2005; Cox et al., 2000; King et al., 2001; Song et al., 2004; Szakmary et al., 2005; Xie and Spradling, 1998). In light of the recent evidence that ESCs in direct contact with cap cells and GSCs are required for maintaining GSCs (Decotto and Spradling, 2005), it remains formally possible that some unidentified ESC cells associated with expanded or ectopic cap cells contribute to niche function. In any case, this study demonstrates that the size and location of the GSC niche can be genetically manipulated while it maintains its functions. Our ability to manipulate niche location and size will further increase our capacity to investigate how niche formation is controlled and how the niche controls stem cell function in general.

Limited amount of active BMP produced by five to seven cap cells may explain its short-range effect on GSC self-renewal

One of the major unsolved issues for the GSC niche in the *Drosophila* ovary is how BMPs function as a short-range signal to control GSC self-renewal and allow the GSC daughter adjacent to the GSC to differentiate at the same time. Several previous studies from us and others have shown that BMP signaling activity is primarily restricted to the GSC based on Mad phosphorylation and *Dad* expression, two indicators of BMP signaling (Casanueva and Ferguson, 2004; Kai and Spradling, 2003; Song et al., 2004). Our

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early work has also shown that *dpp* is primarily expressed in TF and cap cells, while *gbb* is expressed in TF and cap cells as well as in IGS cells (Song et al., 2004). In this study we show that BMP signaling activity can spread two or more cell diameters based on expression of *Dad-lacZ* and *bam-GFP* when more cap cells exist. Furthermore, when more cap cells accumulate in ectopic sites, the GSCs associated with the cap cells as well as the germ cells lying two or three cells away are capable of activating BMP signaling and repressing *bam* expression. One of the explanations for these observations is that cap cells are the source of BMP and more cap cells would produce more BMP to diffuse further away to repress differentiation of germ cells lying two or more cell diameters away. Another explanation is that the ratio of cap cells to ESCs or escort cells increases so that BMP inhibitors, such as Sog, produced by ESCs or escort cells, are diluted or deterred by more cap cells, and consequently more active BMP is available for reaching and activating cells lying more than two cell diameters away. In *Xenopus* gastrula-stage embryos, an effective BMP-4 activity gradient is established, not by diffusion of BMP-4 protein but by the long-range effects of two BMP-4 inhibitors, Noggin and Chordin (Jones and Smith, 1998). Finally, it is also possible that a combination of both mechanisms contributes to restriction of BMP signaling activity to one cell diameter in the GSC niche. Our observations from this study have suggested that a limited amount of active BMP produced by cap cells is probably responsible for its short-range effect on GSC self-renewal in the GSC niche.

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/134/6/1071/DC1

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