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Report

Stem Cells Signal to the Niche through the Notch Pathway in the *Drosophila* Ovary

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Summary

Stem cells are maintained and retain their capacity to continue dividing because of the influence of a niche. Although niches are important to maintain "stemness" in a wide variety of tissues, control of these niches is poorly understood. The *Drosophila* germline stem cells (GSCs) reside in a somatic cell niche [1, 2]. We show that Notch activation can induce the expression of niche-cell markers even in an adult fly; overexpression of *Delta* in the germline, or activated *Notch* in the somatic cells, results in extra niche cells, up to 10-fold over the normal number. In turn, these ectopic niche cells induce ectopic GSCs. Conversely, when GCSs do not produce functional Notch ligands, Delta and Serrate, the TGF-β pathway is not activated in the GSCs, and they differentiate and subsequently leave the niche. Importantly, clonal analysis reveals that the receiving end of the Notch pathway is required in the somatic cells. These data show that a feedback loop exists between the stem cells and niche cells. Demonstration that stem cells can contribute to niche function has far-reaching consequences for stem cell therapies and may provide insight into how cancer can spread throughout an organism via populations of cancer stem cells.

Results and Discussion

Stem cells retain the ability to self-renew and produce differentiating cells throughout the lifetime of an animal. Adult stem cells retain these "stemness" characteristics because of signals generated by a special environment, called a niche, that supports stem cell development [1, 3]. However, studies on the control of this microenvironment are just emerging [4]. A signal from stem cells has been proposed as a mechanism that maintains the niche, but the pathway remains elusive [3, 5].

Here, we use the *Drosophila* germline stem cell (GSC) niche as a model system and show that GSCs signal to the niche through the Notch signaling pathway. *Drosophila* oogenesis depends on the presence of self-renewing GSCs in the adult ovary [1, 2]. Adult GSCs are also found in *Drosophila* and mammalian testes and might exist in a mammalian ovary as well [6–10]. The *Drosophila* ovary consists of approximately 16 ovarioles, each with a structure called the germarium located at the anterior tip. Each germarium contains two to three GSCs occupying a niche of three to six cap cells (CpC) and possibly five to seven escort stem cells (ESC) (Figure 1 and Table 1; see also Figure S3 and Movie S1) [1, 11]. We now show that the Notch ligands Delta and Serrate signal from the GSCs to activate the Notch receptor in the overlaying somatic cells and thereby contribute to the niche function.

The Notch pathway plays an important role in many stem cell niches, including the hematopoietic system, gut, mammary gland, and muscles [12–18]. However, it is not clear in all cases which cells send the ligand and which cells receive the signaling activity. The *Drosophila* Notch ligands, Delta and Serrate, require an ubiquitin-dependent internalization process for full activation. This process is regulated by *neuralized (neur)*, an ubiquitin ligase that acts in the signal sending cell [19–21]. Upon activation, the ligand interacts with the Notch receptor in the neighboring cell and activates the receptor by rendering it accessible to protease cleavage steps. Subsequent to Notch receptor cleavage, a cytoplasmic portion of Notch is released from the membrane and transported to the nucleus. In the nucleus, Notch forms a complex with the transcription factor Suppressor of Hairless, and together, they regulate transcription of target genes [22].

To date, work has focused on understanding signaling from niche cells to stem cells; however, microarray data [5, 23] suggest that stem cells may also express signaling molecules. This finding raises the possibility that two-way communication exists between stem cells and niche cells. To investigate this, we analyzed the role of the Notch signaling pathway in GSC and cap-cell populations. We initiated these studies by engineering a *Delta* overexpression construct that allows germline expression, expressing it early in germline cells (*pUASpDelta nanos-Gal4*), and analyzing the effect in the niche and GSCs.

To analyze the niche and its activity, we used four markers for the somatic cap cells. Here, we will refer to the cap cells as a major component of the niche. However, other somatic cells, such as ESCs, may also contribute to the niche [11]. The transcription factor Engrailed (EN), adhesion molecules DE-Cadherin (CAD) and β-catenin/armadillo (ARM), and nuclear Lamin C are coexpressed in wild-type cap cells [24–26] (Figures 1A, 1B, 1D, and 1E and Movie S1). On average, 4.4 EN/CAD- and 4.5 Lamin C-expressing cells are observed per wild-type germarium (Table 1). The niche supports the function of GSCs that are identified by accumulation of pMAD in the nucleus and an anteriorly oriented spectrosome containing Adducin in the cytoplasm (Figures 1A and 1C). The markers for these two cell types underscore the interconnectedness of the cap cells and GSCs. The GSCs receive a TGF-β signal from the niche and this

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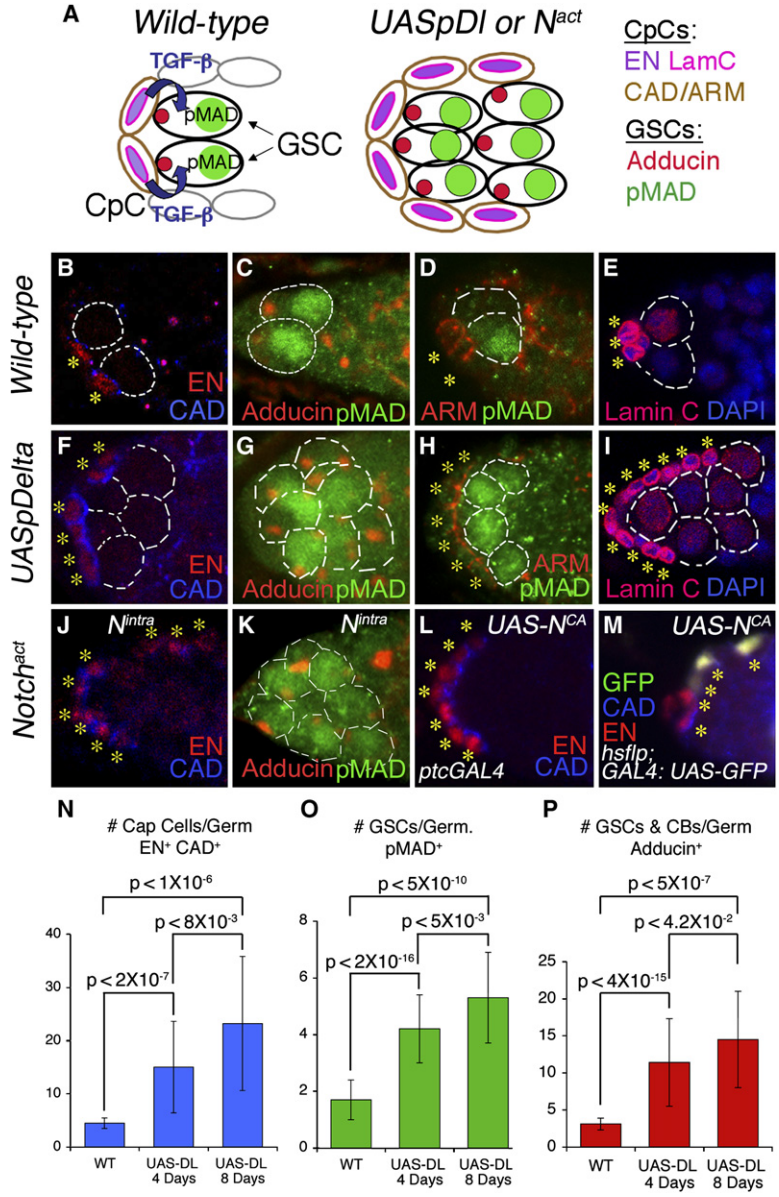

Figure 1. Activation of Notch Signaling Produces Extra Cap Cells and GSCs

(A) Diagrams of wild-type and *UASp-Delta*, or constitutively active Notch, germaria showing the expansion of cap cell and GSC markers when the Notch pathway is activated.

(B–E) Wild-type germaria, GSCs outlined by white dashes; yellow asterisks mark the cap cells. (B) Cap cells express EN and CAD. (C) Confocal projection showing GSCs with Adducin-containing spectrosomes in the anterior of the cell and nuclear pMAD. (D) ARM and (E) Lamin C also accumulate in cap cells. Note that Lamin C also accumulates in the GSCs.

(F–I) *UASp-Dl* overexpression in the germline with *nanosGAL4* produces extra cap cells expressing (F) EN/CAD, (H) ARM and (I) Lamin C. Note that generally only a subset of the cap cells are in a single focal plane. (G and H) The enlarged niche supports additional GSCs as monitored by nuclear pMAD accumulation and (G) round Adducin-containing spectrosomes. Note that G is a confocal projection.

(J) Overexpression of the Notch intracellular domain (*hsFLP;Act5C>y+>Notch^{intra}*; 4 days after heat shock) produces extra cap cells expressing Engrailed and DE-Cadherin.

(K) Confocal projection showing that based upon Adducin and pMAD accumulation, additional GSCs are produced when *Notch^{intra}* is expressed in the germarium.

(L) Somatic expression of *Notch^{CA}* is sufficient to induce an enlarged niche (*patched-GAL4;UAS-Notch^{CA};GAL80^{ts}*).

(M) Adult overexpression of *UAS-Notch^{CA}* (marked with GFP, *y hsp70-FLP;Act>CD2>GAL4,UAS-GFP*) can induce ectopic expression of cap-cell markers expressing EN/CAD in a cell-autonomous manner.

(N–P) Graphs showing that overexpression of *UASp-Delta* in the germline is sufficient to increase cells expressing cap-cell markers and GSCs. In (O)–(P), the mean and SD (error bars) of 18–59 germaria (see Table 1) are reported.

leads to phosphorylation and nuclear transport of the transcription factor MAD necessary for cystoblast differentiation [27, 28]. In the absence of TGF-β signaling, the GSCs divide less frequently, differentiate, and ultimately leave the niche [29]. Finally, the GSCs and cap cells maintain close contact with one another via the homophilic cell adhesion molecule Cadherin, which is required to anchor GSCs in the niche [25].

We find that *Delta* overexpression in the germline leads to an increase in somatic niche cells. By using a *nanos-GAL4* germline driver to overexpress *Delta*, a Notch ligand, we observe a dramatic increase in EN/CAD, Lamin C, and ARM-positive cap cells in the germaria of mutant ovaries compared to the control ovaries (Figures 1F, 1H, 1I, and 1N and Table 1; 10-fold increase, 8 days, $p < 1 \times 10^{-6}$). These data suggest that *Delta* overexpression in the germline is sufficient to induce cap-cell markers. Furthermore, a statistically significant increase was observed in the number of cells staining with these markers from the fourth day to the eighth day time-point in adulthood (Figure 1N and Table 1; 1.5-fold increase from the fourth day to the eighth day, $p < 8 \times 10^{-3}$). Thus, Delta from the germline is sufficient to induce cap-cell markers during adult life. Therefore, overexpressing a Notch-ligand in the germline is sufficient for transmission of a signal to the overlaying somatic cells and thus increases the number of cells expressing cap-cell markers.

Because numerous studies demonstrate that the niche cells signal to the underlying germline to promote GSC maintenance, we wished to determine whether the enlarged niche observed in ovaries overexpressing *Delta* could support additional GSCs. Normally, a niche supports two to three GSCs (Figures 1A and 1C and Table 1). However, when *Delta* is overexpressed in the germline, we observed an increase in the number of GSCs in the niche based upon Adducin-containing spectrosomes and pMAD expression (Figures 1G, 1H,

Table 1. Overexpression of Delta in the Germline Induces Ectopic Niche Cells and GSCs

	Cap Cells	Cap Cells	GSCs and Cbs	GSCs
Genotype	En$^+$ Cad$^+$	Lamin C$^+$	Adducin^{+a}	pMAD$^+$
Wild-type	4.4 ± 1.0 n = 23	4.5 ± 1.0 n = 21	3.1 ± 0.8 n = 37	1.7 ± 0.7 n = 55
NGT40/+ ;nanosGAL4/ UASpDl 4 days post-eclosion	15 ± 8.6 n = 28	37 ± 18 n = 18	11.4 ± 5.9 n = 56	4.2 ± 1.2 n = 36
NGT40/+ ;NanosGAL4/ UASpDl 8 days post-eclosion	23.2 ± 12.6 n = 20	45 ± 23[b] n = 24	14.5 ± 6.5 n = 18	5.3 ± 1.6 n = 20

n = Number of germaria analyzed.
[a] All round spectrosomes were counted, and therefore cystoblasts are included in this category.
[b] Example of a germarium with over 50 Lamin C$^+$ cells shown in Figure S1A.

1O, and 1P and Table 1). Significantly, the extra GSCs appear to be in a typical niche because most of them are in close contact with the cap cells (Figures 1F–1I and Figures S1C and S1D). However, we do not know whether the extra stem cells are associated with escort stem cells. On average, there is a 4.7-fold increase in GSCs and cystoblasts based upon Adducin accumulation in round spectrosomes (Figure 1P and Table 1; 8 days, $p < 5 \times 10^{-7}$). Similarly, there is a 3.1-fold increase in extra GSCs identified by nuclear accumulation of pMAD (Figure 1O and Table 1; 8 days, $p < 5 \times 10^{-10}$). Furthermore, similar to the cap cells, there is a significant increase in GSCs during adulthood (Figures 1G, 1H, 1O, and 1P and Table 1; 1.3-fold increase for pMAD and Adducin from the fourth day to the eighth day, $p < 5 \times 10^{-3}$ and $p < 4 \times 10^{-2}$, respectively).

To test whether other components of the Notch pathway are involved in the same process, we overexpressed activated forms of Notch. Overexpression of the intracellular domain of *Notch* (*Notch^{intra}*) in the adult ovary also leads to an increased number of EN/CAD-positive cap cells (Figure 1J), and a corresponding increase in GSCs (Figure 1K), suggesting that Delta acts through the canonical Notch pathway in this context. To test whether activation of Notch in somatic cells is sufficient to induce niche cells, we expressed *UAS-Notch^{CA}* with *patched-GAL4,GAL80^{ts}*. This scenario induced extra EN/CAD-positive cells (Figure 1L), suggesting that expression of *Notch^{CA}* in somatic cells is sufficient to induce extra niche cells. To test whether Notch acts cell-autonomously to induce niche cells, we created flies with *hsFLP;UAS-Notch^{CA};Act>CD2>GAL4, UAS-GFP* to drive ectopic *Notch^{CA}* in GFP-marked cells. In all analyzed germaria, the GFP-marked cells in region 1 of the germarium stained with EN/CAD, suggesting that activated Notch is sufficient to induce cap-cell markers in a cell-autonomous manner (Figure 1M).

The gain-of-function data show that overexpression of components in the Notch pathway is sufficient for increase of the number of cap cells, presumably producing a larger niche, and this can support a greater number of GSCs. To test whether the Notch pathway is required for this process, and if so, in which cell type, we analyzed GSCs lacking Notch-pathway components. First, to test whether the sending side of the Notch pathway is required in the GSCs for niche function and GSC maintenance, we analyzed these cell types when the GSCs were mutant for *Delta* and *Serrate* or defective in their activation (*Dl^{RevF10};Dl^{RevF10} Ser^{RX82};neur^{KX9}* and *neur^{9B9}* clones). To test whether the receiving side of the pathway is required in GSCs for niche and GSC fate, we analyzed GSCs lacking *Notch^{55e11}* or *Su(H)^{del47}*.

Importantly, the data show that components in the sending side of the Notch signaling pathway are required in the GSCs, whereas the receiving end of the Notch pathway is not required in the GSCs but is required in the somatic niche cells. GSC clones of *neuralized*, *Delta*, and *Delta Serrate* are not maintained in the niche (Figures 2A–2C and 2I). When both GSCs are mutant, they will differentiate and leave the niche; this results in a significantly reduced germarium (Figures 2A and 2B) compared to normal. When one GSC is mutant and the other is wild-type, the mutant GSC will differentiate and leave the niche (Figure 2C). These phenotypes were observed in *neuralized*, *Delta*, and *Delta Serrate* mutant germlines. During a 6-day period, 84% of the control stem cell clones remained in their niche, whereas only 58% of the *Delta$^-$*, 44% of the *Delta$^-$,Serrate$^-$* and 23% of *neuralized$^-$* GSC clones remained in their niches (Figure 2I and Table S1). Consistent with a requirement for *Delta* function in GSCs, *Delta* mRNA is enriched in GSCs isolated from ovaries [23]. In addition, *neuralized* GSC clones induced in adult females are also lost from the niche (15.4% per day lost in adult-induced clones versus 12.8% per day lost in larval-induced clones; Table S1), indicating that *neur* is required in GSCs for GSC maintenance during adulthood. Conversely, components required for reception of the Notch signal are not required in GSCs for maintenance in the niche (Figures 2D, 2F, and 2I and Table S1). GSC mutants for *Notch* or *Su(H)* are maintained in the niche similarly to wild-type control clones (3.1%–3.3% per day lost for *Notch* or *Su(H)* GSC compared to 2.7% per day lost for wild-type GSC; Table S1). In addition, GSCs defective for ligand presentation also divide more slowly (Figure S2).

Because the receiving side of the Notch pathway was not required in GSCs, we tested whether the transcriptional read-out of Notch activity was required in the niche by inducing somatic clones mutant for *Su(H),* the transcription factor that forms a complex with Notch in the nucleus. Somatic niche cap cells cease dividing early in development, rendering them difficult to analyze by conventional clonal analysis. However, early clonal induction did result in *Su(H)* clones of the cap cells, ESC, and escort cells (Figures 2E and 2G, data not shown; n = 7, Table S2). In these mutant germaria, region 1 was reduced (contained fewer cysts) compared to wild-type, and only one GSC was observed (Figures 2E and 2G). Furthermore, the GSC spectrosomes were abnormal (smaller or fragmented) in these germaria (Figures 2E and 2G). Thus, the reduced region 1 in the *Su(H)* cap-cell mutant germaria presumably reflects a combination of GSC loss and reduced GSC division similar to the phenotypes observed when the signal-sending side of the Notch pathway is defective in GSCs. These phenotypes are most likely due to *Su(H)* activity in cap cells and ESCs because *Su(H)* is not

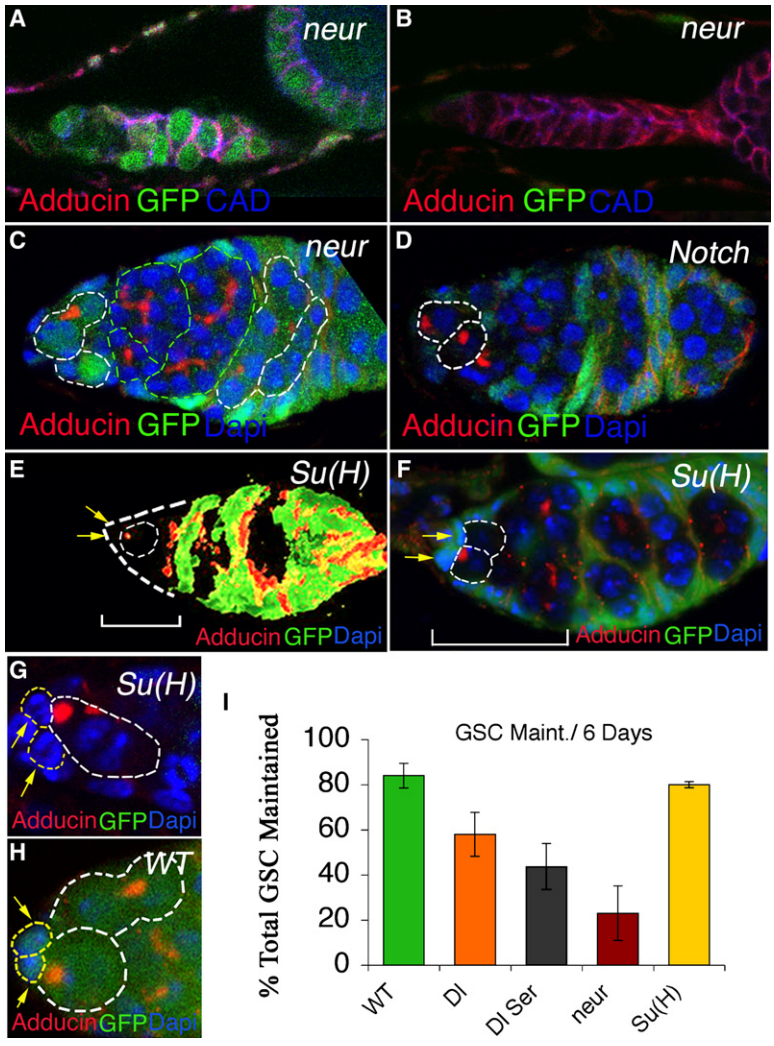

Figure 2. GSCs Require the Sending but Not the Receiving Side of the Notch Pathway

(A, B, and I) In the absence of Neur activity, GSCs are lost from the niche, ultimately resulting in an empty germarium phenotype. This phenotype reflects GSC loss from the niche (C, I) and a reduction in cell division (Figure S2A).

(B) Shows an empty germarium when both the germline and follicle cells are mutant.

(C) Shows a germarium 7 days after adult heat shock. Wild-type GSCs and cysts are outlined in white; *neur*^{kx9} mutant cysts are outlined in green. Typically, it takes 7 days for the progeny of a GSC to exit the germarium. The presence of *neur* mutant cysts in region 1 with two wild-type GSCs in the niche indicates that the *neur* mutant GSC has left the niche.

(D, F, and I) On the other hand, *Notch* and *Su(H)* are not required in the GSCs. (E and G) *Su(H)* is required in the somatic cells for proper GSC function.

(E) When cap cells lack *Su(H)*, region 1 of the germarium (indicated by brackets) is reduced compared to a normal germarium (compare with Figure 2F). Region 1 of this germarium contains one GSC and two, four-cell cysts, suggesting that *Su(H)* is required in the somatic cells for GSC maintenance and proper division. In addition, the spectrosome is smaller than normal. Note that this image was obtained with deconvolution software.

(F) GSCs lacking *Su(H)* divided normally.

(G) Abnormally dividing GSC is associated with *Su(H)* mutant cap cells.

(H) Wild-type GSCs and cap cells.

(I) Graph showing that *neur*, *Dl*, and *Dl Ser* GSC clones are not maintained in the niche during a 6-day period, whereas *Su(H)* clones are maintained (Table S1). GSCs are outlined in white dashes; cap cells are marked with arrows and outlined in yellow dashes. In (I), the mean and SD (error bars) for two or three experiments (see Table S1) are reported.

required in the GSCs (Figure 2F and Table S2). However, at least half of the escort stem cells can be mutant for *Su(H)* without affecting GSC maintenance (n = 18, Figure S3C and Table S2). Therefore *Su(H)* is required either in cap cells or both the cap cells and the ESCs. Later defects are observed in cysts associated with *Su(H)* mutant escort cells, indicating that the Notch pathway functions in this cell population (Figure S3C). Altogether, the data demonstrate that neither *Notch* nor *Su(H)* are required in the germline for GSC maintenance, whereas *Su(H)* is required in the somatic niche cells for proper GSC function.

Previous work demonstrates that TGF-β signaling from the niche is essential for the maintenance and division of GSCs [29]. These phenotypes are similar to those observed in *neuralized*, *Delta,* and *Delta Serrate* mutant GSC clones or *Su(H)* follicle cell clones. To determine if *neuralized* GSCs are lost because of a lack of proper TGF-β signaling from niche cells, we used nuclear pMAD accumulation to monitor TGF-β activity in *neuralized* mutant GSCs (Figures 3A–3C). *neuralized* mutant GSCs stained less frequently for pMAD than controls, suggesting that *neuralized* mutant GSCs are associated with defective TGF-β signaling from the niche (*neur*^{kx9} 18% [n = 27], wild-type 94% [n = 73], Figures 3A–3C). *Delta Serrate* GSC clones also stain less frequently with pMAD (Figure 3C, 66% [n=39]). In contrast, *Su(H)* GSC clones exhibit a normal frequency of pMAD staining, consistent with their normal maintenance and division kinetics (Figure 3C, 97% [n = 34]). These data are consistent with Notch activity being required for TGF-β signaling, either directly or indirectly.

To further test whether the ectopic Notch pathway acts through the TGF-β pathway in GSC induction, we compared the number of GSCs present in germaria overexpressing Delta with, and without, reduced TGF-β signaling. The number of Delta-induced GSCs was reduced when the TGF-β pathway was downregulated by overexpression of *Dad*, an inhibitor of TGF-β signaling (Figure 3D), suggesting that the Notch pathway requires a functional TGF-β pathway in this system.

These data show that the GSCs require the sending end, but not the receiving end, of the Notch pathway, suggesting that Notch-receptor activity, induced by the Delta and Serrate ligands from the germline, is required in the niche for proper activity. More complex scenarios may occur, including relay signaling from the germline to the ESCs and then to the cap cells.

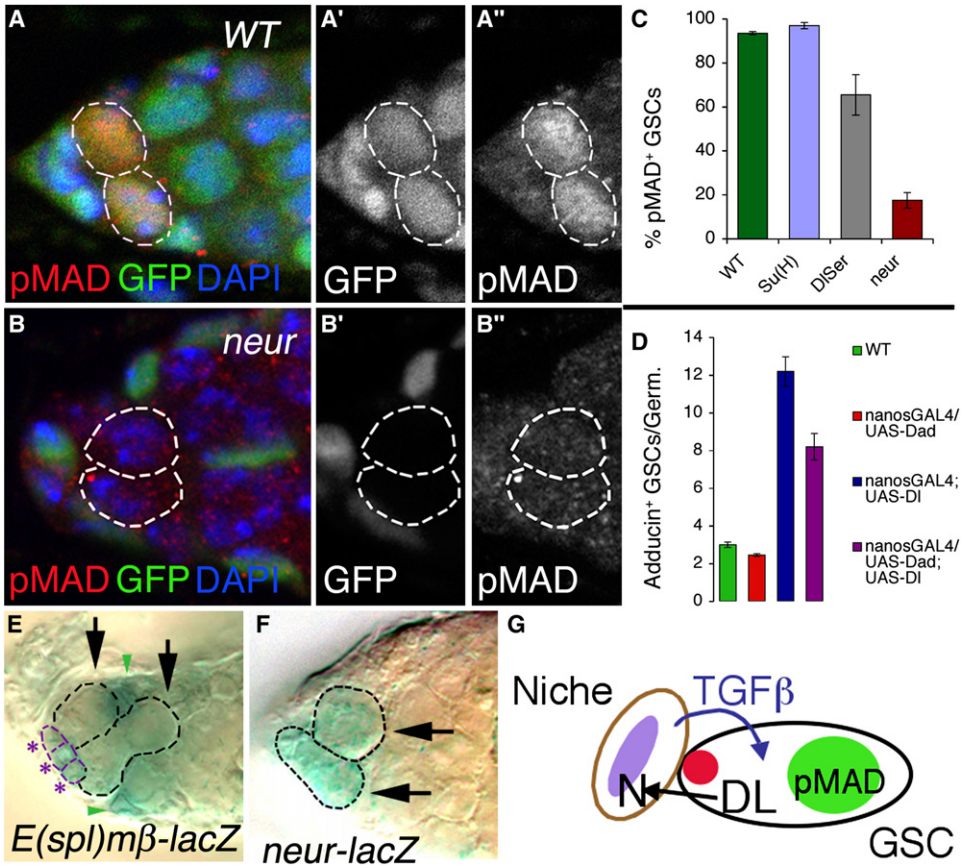

Figure 3. Notch and TGF-β Pathways Act Together for GSC Maintenance

(A) In response to TGF-β signaling from the cap cells, wild-type GSCs accumulate nuclear pMAD.

(B) *neuralized* GSC clones do not accumulate pMAD.

(C) Graph showing that *neuralized* and *Delta,Serrate* GSC clones 8 days after larval heat shock accumulate nuclear pMAD at a lower frequency than wild-type or *Su(H)* GSC clones.

(D) Graph showing that there are fewer GSCs induced by *Delta* overexpression with reduced TGF-β activity (*UAS-Dad/P{NGT40};UASp-Dl/[w+ nos-GAL4:VP16] A4-2 III*, blue, n = 78) compared to the sample with normal TGF-β activity (*P{NGT40}/+;UASp-Dl/[w+ nos-GAL4:VP16] A4-2 III/+*, purple, n = 101). Ectopic expression of Dad (*UAS-Dad/P{NGT40};[w+ nos-GAL4:VP16] A4-2 III/+*, red, n = 77) slightly reduces the number GSCs compared to the wild-type (green).

(E) Consistent with Notch-receptor activity in the cap cells (purple asterisks), these cells express *E(spl)mb-lacZ*, a Notch-activity reporter line. In addition, the escort stem cells (green arrowheads) also express *E(spl)mβ-lacZ*.

(F) The GSCs express $neur^{A101}$*-lacZ*.

(G) Model showing that the Delta ligand in the germline signals to the Notch receptor in the cap cells, inducing the niche. In turn, the cap cells express TGF-β, which signals to the germline. In (C) and (D) mean and SD (error bars) of two experiments is reported.

Consistent with this, the cap cells and the escort stem cells express the Notch-activity marker *E(spl)mβ.5-lacZ* (Figure 3E). In this system, β-galactosidase expression indicates that the cell contains an active N^{intra}/Su(H)-complex capable of binding Su(H)-binding sites in the *E(Spl)* promoter [30]. On the other hand, the GSCs express $neur^{A101}$*-LacZ* (Figure 3F). Therefore, based upon the loss-of-function, gain-of-function, and expression data, GSC maintenance requires Delta and Serrate signaling from the GSCs and reception of the Notch signal in the somatic cells.

The data presented here suggest that when GSCs are defective for proper Delta and Serrate presentation, the niche cells do not receive a Notch signal, which is necessary for a fully functional niche. We propose that the ligands Delta and Serrate in the germline signal to the Notch receptor in the somatic cells. Activation of Notch in somatic cells leads to proper TGF-β signaling from the niche, and this signaling in turn induces GSC maintenance. These data show that a feedback loop between stem cells and their niche exists: Delta and Serrate from the stem cells and Su(H) in the cap cells (and possibly the ESCs) is required to maintain a functional niche, whereas the niche supports stem cell maintenance and division through TFG-β and Piwi pathways [31–33] (Figure 3G).

Notch signaling from GSCs to the niche is not essential for niche cell short-term survival because some of the niche markers are still observed up to 10–18 days after the GSCs leave the niche [34, 35]. We propose that either Notch signaling from the GSCs is required for long-term niche survival or in the short term for a fully functional niche. Previous work showed that somatic stem cells (SSC) (or their progeny) can enter the empty GSC niche and divide in response to niche signals, suggesting that the niche is functional in this case [35]. It is

possible and consistent with the present work that the SSCs, albeit the wrong stem cell group, can contribute a stem cell signal required for a functional niche.

Similar with the work shown here, plant stem cells also signal to their niche cells [36, 37]. In the shoot meristem, stem cells are maintained by intercellular communication between the apical stem cells and the underlying organizing center. The organizing center acts non-cell-autonomously to specify stem cell identity and is analogous to a niche. The stem cells express a secreted signaling molecule called CLAVATA3 (CLV3), which limits the niche by repressing the transcription factor WUSCHEL (WUS). WUS then acts to promote CLV3 expression in the stem cells, thereby establishing a negative regulatory feedback loop between the niche and stem cells [36, 37].

Previous work demonstrates that Notch controls stem cell differentiation in multiple stem cell systems [12–18]. Here, we show that the Notch pathway signals from GSCs to the niche cells, and in turn, the niche induces and maintains stem cell fate. It will be important to determine whether the Notch pathway affects stem cell differentiation by maintaining a functional niche in some other stem cell systems as well. Finally, stem cells share many similarities with cancer cells. They both are able to self-renew and proliferate for a long period of time. Cancer is thought to be a disease of stem cells [38, 39]. Furthermore, niche expansion is previously seen in colorectal tumors [40]. We now show that *Drosophila* adult stem cells are capable of signaling to the niche and that the Notch pathway is a key player in this process. Delta from the GSC signals to the neighboring somatic cells to maintain an active niche. These niche cells furthermore act as a functional niche to maintain the stem cells [31, 32]. It is tempting to speculate that the ability of stem cells to contribute to the niche function may help to explain how cancer stem cells can spread cancer throughout an organism.

Supplemental Data

Supplemental Data include additional Results and Discussion, Experimental Procedures, three figures, two tables, and one movie and can be found with this article online at http://www.current-biology.com/cgi/content/full/16/23/2352/DC1/.

Acknowledgments

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