Insulin signals control the competence of the *Drosophila* female germline stem cell niche to respond to Notch ligands

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**Abstract**

Adult stem cells reside in specialized microenvironments, or niches, that are essential for their function in vivo. Stem cells are physically attached to the niche, which provides secreted factors that promote their self-renewal and proliferation. Despite intense research on the role of the niche in regulating stem cell function, much less is known about how the niche itself is controlled. We previously showed that insulin signals directly stimulate germline stem cell (GSC) division and indirectly promote GSC maintenance via the niche in *Drosophila*. Insulin-like peptides are required for maintenance of cap cells (a major component of the niche) via modulation of Notch signaling, and they also control attachment of GSCs to cap cells and E-cadherin levels at the cap cell–GSC junction. Here, we further dissect the molecular and cellular mechanisms underlying these processes. We show that insulin and Notch ligands directly stimulate cap cells to maintain their numbers and indirectly promote GSC maintenance. We also report that insulin signaling, via phosphoinositide 3-kinase and FOXO, intrinsically controls the competence of cap cells to respond to Notch ligands and thereby be maintained. Contrary to a previous report, we also find that Notch ligands originated in GSCs are not required either for Notch activation in the GSC niche, or for cap cell or GSC maintenance. Instead, the niche itself produces ligands that activate Notch signaling within cap cells, promoting stability of the GSC niche. Finally, insulin signals control cap cell–GSC attachment independently of their role in Notch signaling. These results are potentially relevant to many systems in which Notch signaling modulates stem cells and demonstrate that complex interactions between local and systemic signals are required for proper stem cell niche function.

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**Introduction**

The microenvironment (niche) where stem cells reside provides physical contact and local signals to retain and modulate stem cells. Systemic factors that vary with physiological changes also influence stem cells either directly or by altering the niche (Drummond-Barbosa, 2008). It is largely unknown, however, how systemic factors interact with local signals to maintain the niche.

The *Drosophila* female germline stem cell (GSC) niche, located in the anterior germarium of each ovariole, is well described. The GSC niche is composed of cap cells, terminal filament cells, and escort cells (Kirilly and Xie, 2007). Cap cells are major cellular components of the niche, as they are directly attached to GSCs through E-cadherin (Song et al., 2002) (Fig. 1A). GSCs self-renew and produce cystoblasts that divide to form 16-cell cysts (Kirilly and Xie, 2007). One cell becomes the oocyte, the others become nurse cells, and follicle cells surround the cyst to generate a developing egg chamber (Spradling, 1993).

GSC number correlates with cap cell number (Hsu and Drummond-Barbosa, 2009; Xie and Spradling, 2000), which in turn is regulated by Notch signaling (Song et al., 2007; Ward et al., 2006). The Notch receptor and its ligands are transmembrane proteins, thus requiring cell–cell contact for signaling. *Drosophila* has one Notch receptor (encoded by *N*) and two ligands, Delta and Serrate (encoded by *Dl* and *Ser*, respectively), and full ligand activity requires the E3 ubiquitin ligase Neuralized (encoded by *neur*) in signal-producing cells (Fiuza and Arias, 2007). Ligand stimulation induces proteolytic cleavage of Notch and translocation of its intracellular domain into the nucleus, where it regulates gene expression (Fiuza and Arias, 2007). Notch inactivation leads to cap cell and GSC loss (Song et al., 2007), whereas overexpression of Delta in the germline or of activated Notch in somatic cells of the germarium has the opposite effect (Song et al., 2007; Ward et al., 2006). A report that GSCs mutant for *neur*, *Dl*, and *Dl Ser* are lost from the niche led to the model that Notch ligands produced in GSCs signal to cap cells to maintain their own niche (Ward et al., 2006). It remained experimentally untested, however, whether Notch ligands produced...
in GSCs control cap cell number, or whether Notch activation is cell autonomously (i.e., intrinsically) required for cap cell maintenance.

Insulin signaling ties diet to function of the GSC niche, at least in part via modulation of Notch signaling (Hsu and Drummond-Barbosa, 2009). The evolutionarily conserved insulin/insulin-like growth factor (IGF) pathway controls processes linked to nutrient sensing (Goberdhan and Wilson, 2003; Hafen, 2004). Insulin-like peptides activate the Drosophila insulin receptor (encoded by InR), leading to phosphorylation of the insulin receptor substrate (encoded by chico). Subsequent phosphoinositide 3-kinase (PI3K) stimulation leads to cytoplasmic retention of the transcriptional factor FOXO, thus preventing target gene activation (Oldham and Hafen, 2003). We previously showed that systemic insulin-like peptides promote both the maintenance of cap cells, via positive regulation of Notch signaling, and cap cell–GSC attachment, likely via E-cadherin (Hsu and Drummond-Barbosa, 2009). It remained unclear, however, how insulin and Notch signaling interact to control niche size, or if Notch modulates E-cadherin.

Here, we show that the insulin pathway and Notch signaling are both intrinsically required to maintain cap cell numbers. Notch ligands produced in GSCs are not required to activate Notch or to maintain cap cells or GSCs. Instead, ligands are produced within the niche itself to stimulate Notch signaling in cap cells. Further, our results demonstrate that insulin-like peptides, acting via PI3K and FOXO, directly control the competence of cap cells to respond to Notch ligands. Finally, Notch does not control cap cell–GSC attachment, indicating that this is a Notch-independent role of insulin signaling. These results connect systemic factors to the competence of niche cells to receive local signals and highlight the complexity of systemic effects on the function of niches and their stem cells.

Materials and methods

Drosophila strains and culture

Drosophila stocks were maintained at 22–25 °C on standard medium. yw is a wild-type control. Null InR339, DiRevF10, SerRX82, neur1, neur11, chico1, foxo21, and foxo25 alleles; hypomorphic N55e11 and InR16 alleles; and the temperature-sensitive Nts2 allele have been described (Hsu et al., 2008; Shellenberger and Mohler, 1975; Wang et al., 2007; Ward et al., 2006). Di-lacZ, Ser-lacZ, E(spl)m7-lacZ, c587-Gal4, bab1-Gal4, UAS-Dp110, and UAS-Dp110CAAX have been described (Bachmann and Knust, 1998; Grossniklaus et al., 1989; Bolivar et al., 2006; Hsu and
Drummond-Barbosa, 2009; Leevers et al., 1996). InR1/F1/InR339 females expressing UAS-Dp110 were raised at 18 °C (to reduce transgene expression during development) and shifted to 25 °C after eclosion. Other genetic elements are described in Flybase (http://flybase.bio.indiana.edu).

Genetic mosaic analyses

Genetic mosaics were generated by flipase (FLP)/FLP recognition target (FRT)-mediated mitotic recombination (Xu and Rubin, 1993). To generate GSC clones in adults, 2-day-old females were heat shocked for 1 h at 37 °C twice a day for 3 days to induce hs–FLP (LaFever and Drummond-Barbosa, 2005). To generate GSC clones during development, third-instar larvae or early pupae were heat shocked for 1 h at 37 °C for 2 days (Ward et al., 2006). For cap cell and terminal filament cell clones, UAS-FLP driven by bab1-Gal4 was used instead, and females raised at 25 °C were transferred to yeasted fresh food daily until dissection.

Homozygous cells were identified by the absence of β-gal or GFP. GSCs were identifiable by the anterior position of their fusome (recognized by 1B1 labeling), which is juxtaposed to cap cells (terminal filament and cap cell nuclear envelopes recognized by LamC labeling) (Hsu and Drummond-Barbosa, 2009). GSC and cap cell maintenance was determined by comparing the fraction of germinaria carrying at least one β-gal/GFP-negative GSC or cap cell, respectively, between different time points as described (Xie and Spradling, 1998).

Immunostaining and fluorescence microscopy

Ovaries were dissected, fixed, and immunostained as described (Hsu et al., 2008). For terminal filament analyses, dissected ovaries were teased apart only after fixation and immunostaining. An additional wash in 0.5% Triton X-100 for 30 min was included before incubation with anti-E-cadherin antibodies. The following primary antibodies were used: mouse 1B1 (Developmental Studies Hybridoma Bank, DSHB, 1:10), mouse anti-Lamin (Lam) C (DSHB, 1:100), mouse anti-Delta (DSHB, 1:100), rat anti-Serrate (a gift from P. Irvine, Rutgers University, 1:1,000), mouse anti-Notch (DSHB, 1:100), rat anti-β-catenin (DSHB, 1:3), rabbit anti-Vasa (a gift from P. Lasko, McGill University, 1:1,000), mouse anti-β-gal (Sigma, 1:500), rabbit anti-β-gal (Cappel, 1:1,000), and rabbit anti-GFP (Torrey Pines, 1:2,000). Alexa Fluor 488- or 568-conjugated goat anti-mouse and -rabbit secondary antibodies (Molecular Probes, 1:400) were used. The following primary antibodies were used: mouse 1B1 (Developmental Studies Hybridoma Bank, DSHB, 1:100), mouse anti-Dp110 (DSHB, 1:3), rabbit anti-Dp110 (a gift from R. Pletcher, Stanford University, 1:1,000), mouse anti-Dlβ-catenin (DSHB, 1:100), goat anti-Dl-β-catenin (Cell Signaling, 1:1,000), mouse anti-β-gal (Sigma, 1:500), rabbit anti-β-gal (Cappel, 1:1,000), and rabbit anti-GFP (Torrey Pines, 1:2,000). AlexaFluor 488- or 568-conjugated goat anti-mouse and -rabbit secondary antibodies (Molecular Probes, 1:400) were used. All samples were stained in 0.5 μg/ml DAPI (Sigma), mounted in Vectorshield (Vector Labs), and analyzed using Zeiss LSM 510 or LSM 700 confocal microscopes.

For quantification of E(spl)m7-lacZ expression, the average β-gal fluorescence intensity was measured in arbitrary units in confocal sections at the largest cap cell nuclear diameter using AxioVision (Zeiss). For E-cadherin quantification, five to six optical sections were taken along 3–4 μm of the Z-axis of the E-cadherin-rich interface between cap cell and GSC. The average intensity of E-cadherin signal for the region of contact between a GSC and cap cell was measured using AxioVision.

Results

Notch ligands produced by GSCs are not required for their self-renewal

Notch signaling controls the number of cap cells (a major niche component), and cap cell number largely determines how many GSCs are maintained in the niche (Song et al., 2007; Ward et al., 2006). We previously showed that insulin-like peptides promote Notch signaling in terminal filament and cap cells within the niche (Hsu and Drummond-Barbosa, 2009). It remained unclear, however, how insulin signaling controls the ability of cells to communicate via Notch. As a first step, we asked where Notch ligands are required for proper GSC niche function. Notch signaling requires cell–cell contact (Fiuza and Arias, 2007), and three cell types directly contact cap cells, namely GSCs, terminal filament, and escort cells; however, none of them contacts every cap cell (Fig. 1A). Paradoxically, Notch signaling can be detected in all cap cells using the Notch transcriptional reporters E(spl)m7-lacZ and E(spl)m7-lacZ (Hsu and Drummond-Barbosa, 2009; Song et al., 2007) (see Fig. S1A in Supplementary material), suggesting that every cap cell directly contacts a Notch-ligand-producing cell. Nonetheless, a recent study showed that DI and Ser are required in GSCs for their maintenance, presumably via cap cells (Ward et al., 2006).

To investigate the source of Notch ligands, we first sought to confirm the requirement for DI and Ser in GSCs for their maintenance. We created genetic mosaic females carrying homozygous mutant GSCs (recognized by the absence of β-galactosidase, β-gal) via mitotic recombination in adults, and measured GSC maintenance (Fig. 1B–F; see Table S1 in Supplementary material). For wild-type control mosaic germaria, most β-gal-negative GSCs observed at 1 week after clone induction were retained at 2 weeks. N3Ser11 and InR339 GSCs were maintained normally, consistent with the indirect requirement for InR and N in GSC maintenance (Hsu and Drummond-Barbosa, 2009; Ward et al., 2006), and with the markedly low levels of Notch reporter in GSCs (Fig. 2; see Fig. S1A in Supplementary material). Surprisingly, there was no significant decrease in the maintenance of DlRevF10 or DlRevF10 SerRX82 GSCs, in contrast to the earlier report of increased loss of DlRevF10 and DlRevF10 SerRX82 GSCs induced in early pupal stages (Ward et al., 2006).

To determine if the discrepancy in results was due to specific stages at which clones were generated, we assayed for GSC maintenance using clones induced in early pupae (Fig. 1F; see Table S1 in Supplementary material). Again, our results indicated normal maintenance of DlRevF10 and DlRevF10 SerRX82 GSCs. We confirmed the presence of DlRevF10 and DlRevF10 SerRX82 mutations because we observed fused egg chambers in DlRevF10 and DlRevF10 SerRX82 mosaic ovaries (see Fig. S2 in Supplementary material) (Lopez-Schier and St Johnston, 2001). Thus, our results unequivocally indicate that Delta and Serrate ligands produced by GSCs are not required for GSC maintenance.

We also generated GSCs homozygous mutant for neur, which mediates ligand internalization in signal-sending cells (Fiuza and
Interestingly, neur1 and neur11 GSCs exhibited severely decreased maintenance (Fig. 1F; see Table S1 in Supplementary material), as previously reported (Ward et al., 2006). neur mutant cysts were very large and had excessively branched fusomes (Fig. 1E), which in some cases retained close juxtaposition to cap cells (see Fig. S3 in Supplementary material). Combined with the normal maintenance of DI and Ser mutant GSCs, these results show that neur is required for GSC maintenance and cyst development independently of Notch signaling.

Notch ligands produced by GSCs are not required for cap cell maintenance or Notch activation in the niche

To carefully examine what cell types in the niche express Notch ligands, we analyzed cap cells in germaria from control, DlRevF10, or DlRevF10 SerRX82 mosaics in which all GSCs were either β-gal-positive (i.e., wild-type control) or negative (i.e., control, homozygous DlRevF10, or DlRevF10 SerRX82, respectively). In control, DlRevF10, and DlRevF10 SerRX82 mosaics, numbers of cap cells were unaffected by the β-gal status of GSCs (Fig. 1G). These results show that Notch ligands from GSCs do not control niche size (i.e., cap cell number).

It is conceivable that Notch ligands from GSCs partially contribute to Notch activation in cap cells, even if this contribution is not essential for cap cell maintenance or function. To test this possibility, we used the E(spl)m7-lacZ reporter to monitor Notch signaling directly in cap cells of germaria from DlRevF10 SerRX82 mosaics carrying all GFP-positive or -negative mutant GSCs (Fig. 2). As a control, we analyzed InR19 mosaic germaria and found that E(spl)m7-lacZ intensity in cap cells did not depend on the GSC genotype, consistent with the non-cell-autonomous role of InR in GSC maintenance (Hsu and Drummond-Barbosa, 2009). Similarly, the intensity of E(spl)m7-lacZ in cap cells was indistinguishable between germaria with wild-type GSCs and those with DlRevF10 SerRX82 GSCs, indicating that Notch activation in cap cells does not depend on Notch ligands from GSCs. We thus infer that Notch ligands produced within the niche itself (i.e., terminal filament and/or cap cells themselves) induce Notch activation in cap cells.

Insulin signaling does not control transcription of Notch ligands in the niche

Delta expression has been reported in terminal filament cells in late third-instar larvae and in adults (Song et al., 2007), whereas the Serrate expression pattern in the GSC niche has remained unknown. To carefully examine what cell types in the niche express Notch ligands, we first attempted to use available antibodies against Delta and Serrate (Papayannopoulos et al., 1998; Song et al., 2007). Unfortunately, the signals detected by these antibodies were not specific in the germarium (see Fig. S4 in Supplementary material), precluding our analysis of protein expression. We instead examined transcription of Notch ligands using Di-lacZ, a DI enhancer trap line, and Ser-lacZ, a transgenic line carrying lacZ downstream of the Ser promoter region (Bachmann and Knust, 1998; Grossniklaus et al., 1989) (Fig. 3). We found that, in addition to the strong terminal filament expression, Di-lacZ was also detected in a subset of cap cells from 1-week-old females (Fig. 3A). Ser-lacZ was strongly expressed in some terminal filament cells, but not in cap cells (Fig. 3C). We did not detect Di-lacZ or Ser-lacZ expression in escort cells.

To test if insulin-like peptides regulate transcription of Notch ligands, we examined Di-lacZ and Ser-lacZ expression in InR19/InR339 females, which have reduced Notch signaling in the niche (Hsu and Drummond-Barbosa, 2009). Expression of Di-lacZ and Ser-lacZ was similar in control and InR19/InR339 females (Fig. 3). We obtained equivalent results in chic0 females (see Fig. S5 in Supplementary material). These results indicate that insulin signaling does not affect transcription of Di-lacZ and Ser-lacZ in the GSC niche.

Delta from basal terminal filament cells controls the formation of cap cells during development

In addition to contacting GSCs, cap cells are in direct contact with each other and with basal terminal filament cells (Fig. 1A). Given that Notch ligands are expressed in terminal filament and cap cells, and DI and Ser are not required in GSCs, we tested if Notch ligands from basal terminal filament cells contribute to Notch activation in cap cells. We first examined cap cell numbers in control and DlRevF10 mosaic germaria. It is not possible to generate mutant terminal filament and cap cell clones in adults because these cells are post-mitotic at that stage. Instead, clones were induced in early larval through pupal stages through recombination mediated by bric-a-brac (bab1) Gal4 driving UAS-FLP (bab1–FLP) in developing niche cells (Bolivar et al., 2006). Clones were recognized by the absence of β-gal. To analyze the requirement for DI in basal terminal filament cells, we grouped mosaic germaria into three categories: germaria with no β-gal-negative terminal filament cells, germaria with β-gal-negative non-basal terminal filament cells, and germaria with β-gal-negative basal terminal filament cells (Fig. 4A–C). In control mosaic germaria, cap cell numbers were similar among the three groups, and there was also no significant difference in cap cell numbers at 1 day or 1 week after eclosion. In contrast, DlRevF10 mosaic germaria with basal terminal filament cell clones had significantly fewer cap cell numbers relative to germaria with either non-basal or no β-gal-negative terminal filament cells 1 day after eclosion (Fig. 4D). A similar trend was observed at 1 week, although differences do not reach statistical significance, presumably due to smaller sample size. These results suggest that

Fig. 3. InR does not control the expression levels of Di-lacZ and Ser-lacZ in the GSC niche. (A–D) One-week-old germaria labeled with β-gal (green, Di-lacZ in A and B, and Ser-lacZ in C and D), 1B1 (red, fusomes), and LamC (red, cap cell and terminal filament nuclear envelopes). TF, terminal filament. Arrowheads indicate β-gal-positive cap cells, and arrows indicate β-gal-negative cap cells. Asterisks indicate out-of-focus terminal filament cells. Scale bar, 10 μm.
Delta from basal terminal filament cells helps modulate the number of cap cells that are specified during development of the niche.

It is possible that Delta produced by basal terminal filament cells also controls cap cell maintenance. Cap cell numbers in germaria containing DlRevF10 basal terminal filament cells, however, were comparable between 1 day and 1 week (Fig. 4D). Unfortunately, we were unable to analyze terminal filament clones at later times due to the difficulty in preserving older female terminal filament morphology through the immunostaining procedure. Serrate produced in basal terminal filament cells might also compensate for the absence of Delta during adult stages; however, we could not test this possibility because DlRevF10 or InR339 mosaic containing “No”, “Non-basal”, or “Basal” β-gal-negative TF cells 1 day or 1 week after eclosion. The number of analyzed germaria is shown above each bar. Error bars, mean ± SEM. *P < 0.05; **P < 0.01.

Unlike Dl, InR is not required in basal terminal filament cells for cap cell formation because we found that InR339 mosaic germaria containing either basal, non-basal, or no terminal filament cell clones had similar cap cell numbers at 1 day or 1 week (Fig. 4D). These results agree with the normal levels of Dl-lacZ under low insulin signaling and further suggest that InR is not required for transcriptional or post-transcriptional steps of Dl expression. Additionally, given that 1-week-old InR339/InR339 females show a significant decrease in cap cell numbers compared to their newly eclosed counterparts (Hsu and Drummond-Barbosa, 2009), unlike the case for InR339 terminal filament clones, InR is likely not required in the terminal filament to control cap cell maintenance.

InR and N are cell autonomously required for cap cell maintenance

Our results showing that InR is not required in GSCs or terminal filament cells suggested the possibility that InR might be required in cap cells themselves for their maintenance. In apparent contradiction, we had previously used heat-shock-induced FLP-mediated recombination to generate InR mutant cap cell clones during late third-instar larvae or early pupae and did not observe a reduction in average cap cell clone size between 1 and 2 weeks after eclosion (Hsu and Drummond-Barbosa, 2009). Nevertheless, the majority of cap cell clones contained just one or two cap cells, raising a potential caveat to our comparison of clone size averages over time. Specifically, if one-cap-cell clones disappear (and are thus not included in the calculation of average clone size) and two-cap-cell clones are converted to one-cell clones due to accelerated loss of InR mutant cap cells, the average clone size might remain deceptively similar over time.

To directly address the requirement for insulin signaling in cap cells, we generated InR339 cap cells using bab1→FLP, reported to efficiently

Fig. 4. Dl, but not InR, is required in basal terminal filament cells for the establishment of the correct number of cap cells. (A–C) One-day-old control mosaic germaria labeled with β-gal (green, non-recombined cells), 1B1 (red, fusomes), and LamC (red, cap and terminal filament nuclear envelopes). Asterisks indicate β-gal-negative terminal filament (TF) cells. Arrow indicates a β-gal-negative cap cell in (C). Scale bar, 10 μm. (A′–C′) Schematic presentations of the types of mosaics shown above: germarium with no β-gal-negative TF cells (A′, “No”), germarium with exclusively non-basal β-gal-negative TF cells (B′, “Non-basal”), and germarium with basal β-gal-negative TF cell (C′, “Basal”). (D) Average cap cell number in DlRevF10 or InR339 mosaic containing “No”, “Non-basal”, or “Basal” β-gal-negative TF cells 1 day or 1 week after eclosion. The number of analyzed germaria is shown above each bar. Error bars, mean ± SEM. *P < 0.05; **P < 0.01.
induce cap cell clones (Bolivar et al., 2006) (Fig. 5; see Table S2 in Supplementary material). Because the average cap cell clone size was still only 1–2 cells, we assayed for loss of InR339 cap cells by measuring the percentage of germaria carrying cap cell clones over time. For control mosaics, 61% of germaria carried at least one β-gal-negative wild-type cap cell at 1 day after eclosion, and there was a small decline at 1 (52%) and 2 (56%) weeks after eclosion. By contrast, only 42% of germaria (n = 94) contained β-gal-negative InR339 cap cells 1 day after eclosion. These results agree with the reduced cap cell number of newly eclosed InR339 females (Hsu and Drummond-Barbosa, 2009) and further suggest that insulin signaling is cell autonomously involved in cap cell formation. In addition, the decrease in the percentage of germaria with InR339 cap cells at 1 (30%) and 2 (22%) weeks was significantly more severe than for control mosaics (Fig. 5A–C; see Table S2 in Supplementary material). These results indicate that insulin-like signals directly stimulate cap cells to promote their maintenance.

Overexpression of the intracellular domain of Notch in somatic cells of the gerarium induces ectopic formation of cap cells (Song et al., 2007; Ward et al., 2006), suggesting a cell autonomous requirement for N in cap cell formation. Temperature-sensitive Nts1 adult females exhibit a reduction in cap cell number at the restrictive temperature, showing that N is also required for cap cell maintenance (Song et al., 2007). We also observed reduced cap cell and GSC numbers in N55e11 females (see Fig. S6 in Supplementary material). To test if N controls cap cell maintenance intrinsically, we generated N55e11 cap cells and measured the frequency of germaria carrying mutant cap cells over time (Fig. 5D; see Table S2 in Supplementary material). As for InR339 cap cell clones, there was a reduced incidence of germaria with N55e11 cap cells at 1 day after eclosion and a marked decline at 1 and 2 weeks relative to control mosaics (Fig. 5D). Germaria from DlRevF10 mosaics showed no increase in mutant cap cell loss relative to controls (Fig. 5C), consistent with the expected non-cell autonomous role of Notch ligands. These results show that N, similar to InR, is intrinsically required in cap cells both for their formation and maintenance.

InR controls the competence of cap cells to respond to Notch ligands likely upstream of the nuclear translocation of the intracellular domain of Notch

Because both InR and N are required intrinsically within cap cells for their formation and maintenance, we hypothesized that activation of insulin signaling within cap cells may directly regulate their competence to respond to Notch ligands. To test this hypothesis, we generated bab1-FLP-induced InR339 cap cells and monitored Notch activation within each cap cell using the E(spl)m7-lacZ transcriptional Notch

Fig. 5. InR and N are cell autonomously required for cap cell maintenance. (A and B) One-day-old mosaic germaria labeled with β-gal (green, wild-type cells), 1B1 (red, fusomes), and LamC (red, cap and terminal filament cell nuclear envelopes). GSCs are outlined. Asterisks indicate β-gal-negative cap cells. Scale bar, 10 μm. (C) Percentage of germaria carrying control, InR339 or DlRevF10 β-gal-negative cap cell clones 1, 7, or 14 days after eclosion. (D) Percentage of germaria carrying control or N55e11 GFP-negative cap cell clones 1, 7, or 14 days after eclosion. *P < 0.05; **P < 0.01; ***P < 0.001.
reporter (Fig. 6). Relative to neighboring GFP-positive wild-type cap cells, E(spl)m7-lacZ expression in GFP-negative InR339 cap cells was significantly reduced in 1-week-old mosaic females (Fig. 6A, D), indicating that InR controls Notch activation in cap cells cell autonomously.

Expression of the intracellular domain of Notch within the niche of InR19/InR339 females results in rescue of cap cell and GSC maintenance (Hsu and Drummond-Barbosa, 2009), suggesting that insulin signaling affects Notch activation upstream of the nuclear translocation of its intracellular domain. To test if insulin signaling affects Notch expression, we used available antibodies against Notch for immunofluorescence. In control females, Notch signals were detected in follicle cells of developing egg chambers as described (Jordan et al., 2006), and levels were unaltered in InR19/InR339 females (see Fig. S7A, B in Supplementary material). We could not compare the expression of Notch in control versus InR19/InR339 germaria, however, due to the very low signal-to-noise ratio (see Fig. S7C, D in Supplementary material). It remains therefore conceivable that insulin signaling affects Notch pathway activation in the niche at the level of either Notch synthesis, processing and trafficking to cell membrane, or cleavage of its intracellular domain upon ligand binding.

InR controls Notch activation in cap cells and cap cell number via PI3K and FOXO

The PI3K branch of the insulin pathway stimulates growth and proliferation downstream of chico during Drosophila development and

Fig. 6. Insulin signaling regulates the expression of E(spl)m7-lacZ through PI3K and FOXO. (A–C) One-week-old InR339 (A), InR339 with bab1-Gal4-induced Dp110CAAX (B), and foxo21 InR339 (C) mosaic germaria labeled with GFP (green, wild-type cells), β-gal (fuschia, E(spl)m7-lacZ), 1B1 (blue, fusomes), and LamC (blue, cap and terminal filament cell nuclear envelopes). Asterisks indicate GFP-negative InR339 cap cells. Arrows indicate GFP-positive wild-type cap cells. Scale bar, 10 μm. (D) Average intensity of E(spl)m7-lacZ reporter in wild-type (GFP+) and mutant (GFP−) cap cells of each mosaic genotype. The number of analyzed cap cells is shown above each bar. Error bars, mean ± SEM. (E) Percentage of germaria carrying β-gal-negative cap cells 1, 7, or 14 days after eclosion. Data for FRT82B control and InR339 are the same data shown in Fig. 5D. *P<0.05; **P<0.01; ***P<0.001.
oogenesis (Hsu et al., 2008; Oldham and Hafen, 2003). To test if PI3K also mediates the responsiveness of cap cells to Notch ligands, we generated GFP-negative InR<sup>239</sup> cap cells in mosaic females expressing a constitutively active form of the PI3K catalytic subunit, Dp110<sup>CAAX</sup> (Leevers et al., 1996), driven by bab1-Gal4 in somatic cells of the gerarium (bab1–Dp110<sup>CAAX</sup>). Strikingly, the levels of the Notch reporter E(spl)m7-lacZ in InR<sup>239</sup> cap cells were significantly restored by Dp110<sup>CAAX</sup> (Fig. 6A, B, D), indicating that PI3K promotes Notch signaling downstream of InR.

The restoration of Notch signaling by activated PI3K in InR<sup>239</sup> cap cells predicts that PI3K should also rescue low cap cell numbers of InR<sup>239</sup> mutants. To test this prediction using an independent strategy, we overexpressed wild-type Dp110 in somatic cells of the gerarium of InR<sup>239</sup> females using the c587-Gal4 driver instead and examined cap cell numbers (see Fig. S8 in Supplementary material). In 1-week-old InR<sup>239</sup> females, the average number of cap cells per gerarium was 3.5 ± 1.1, and each gerarium carried 1.8 ± 0.7 GSCs (n = 99 geraria). Overexpression of Dp110 in InR<sup>239</sup> females significantly increased those averages to 5.4 ± 1.4 cap cells and 2.1 ± 0.8 GSCs per gerarium (n = 86 geraria; P < 0.001) (see Fig. S8 in Supplementary material for distributions). Taken together, these results indicate that insulin-like peptides signal through PI3K to control the competence of niche cells to respond to Notch ligands and thereby modulate cap cell numbers and GSC maintenance.

Insulin-like peptides directly stimulate GSCs to progress through the cell cycle via PI3K inhibition of the transcriptional factor FOXO (Hsu et al., 2008). We therefore tested if insulin/PI3K signaling controls Notch activation in cap cells through negative regulation of FOXO. We previously reported that, in addition to maintaining cap cell numbers, insulin signaling also controls the levels of E-cadherin at the GSC–cap cell junction, and the attachment of GSCs to cap cells (Hsu and Drummond-Barbosa, 2009). Insulin and Notch signaling are intrinsically required for cap cell maintenance (this study; see Fig. 5), and insulin signaling is also required within cap cells to promote their attachment to GSCs (Hsu and Drummond-Barbosa, 2009). To test if Notch is required for proper attachment of cap cells to GSCs, we measured the cap cell–GSC interaction index for N<sup>Scel</sup><sup>11</sup> and InR<sup>239</sup> cap cells in mosaic females over time (Fig. 7A, B). We calculated the cap cell–GSC interaction index as the percentage of [l-gal–] or GFP-negative cap cells juxtaposed to a GSC.
fusome divided by the average number of GSCs per mosaic gerarium (see Table S3 in Supplementary material). In agreement with our previous study (Hsu and Drummond-Barbosa, 2009), InR293 cap cells showed a reduced cap cell–GSC interaction index over time. In addition, we found that foxo21 InR293 cap cells interacted with GSCs similarly to control cap cells (Fig. 7A), indicating that insulin signaling controls cap cell–GSC attachment through FOXO. In contrast, the cap cell–GSC interaction indices of NtS21 cap cells at 1 day, 1 week, and 2 weeks were comparable to those of control cap cells (Fig. 7A, B; see Table S3 in Supplementary material). These results indicate that insulin signaling regulates cap cell–GSC attachment through a separate mechanism that is independent of Notch signaling. Further, these results suggest that Notch signaling and cap cell–GSC attachment are likely modulated by distinct FOXO targets.

E-cadherin is required for GSC retention in the niche (Song et al., 2002), and InR293 females have lower E-cadherin levels at the GSC–cap cell junction (Hsu and Drummond-Barbosa, 2009). To strengthen our finding that Notch does not control cap cell–GSC attachment, we examined E-cadherin levels at the cap cell–GSC junctions of temperature-sensitive NtS21 mutants, which display Notch signaling defects upon shifting from permissive (18 °C) to restrictive (29 °C) temperatures (Wang et al., 2007). After 1 week at 29 °C, approximately 80% of NtS21 ovarioles (n = 82) contained fused egg chambers, which were not observed at 18 °C, confirming that Notch signaling was disrupted. Although NtS21 females exhibited significantly reduced numbers of cap cells and GSCs after 12 days at 29 °C (see Fig. S6 in Supplementary material), E-cadherin expression levels in the cap cell and GSC junction remained similar (Fig. 7C, D). These results suggest that Notch does not modulate the attachment between cap cells and GSCs and that Notch signaling and cap cell–GSC attachment are independently controlled by the insulin/PI3K/FOXO pathway.

Discussion

The Notch pathway plays a central role in many stem cell systems (Morrison and Spradling, 2008), and how systemic signals impact Notch signaling in stem cell niches is a question of wide relevance to stem cell biology. Notch controls cap cell number in the Drosophila female GSC niche (Song et al., 2007; Ward et al., 2006), and our recent studies showed that insulin-like peptides control Notch signaling in the niche (Hsu and Drummond-Barbosa, 2009), although the underlying cellular mechanisms remained unclear. Here, we dissect the specific cellular requirements for Notch pathway components and the insulin receptor and reveal that insulin signaling controls cell–cell communication via Notch signaling within the niche.

To summarize, from this study in combination with our previous work, a fairly complex model emerges of how insulin-like peptides – systemic signals influenced by diet – impact the function of GSCs and their niche through multiple mechanisms (Fig. 8). In adult females under favorable nutritional conditions, insulin-like peptides signal directly to GSCs via PI3K to inhibit FOXO and thereby increase their division rates by promoting progression through G2 (Hsu et al., 2008; LaFever and Drummond-Barbosa, 2005). In parallel to this direct effect on GSC proliferation, insulin-like peptides also act directly on cap cells – a major cellular component of the GSC niche – to control two separate processes (this study). Stimulation of the insulin pathway, also via PI3K inhibition of FOXO, within cap cells intrinsically increase their responsiveness to the Notch ligand Delta (likely at a step upstream of nuclear translocation of the intracellular domain of Notch), which is likely produced by neighboring cap cells (see below). (A similar process likely occurs during niche formation in larval/pupal stages, although in this case, Delta produced in basal terminal filament cells clearly contributes to the specification of cap cells.) Notch signaling within cap cells leads to their maintenance and, indirectly, to GSC maintenance. Independently of its effect on Notch signaling, insulin/PI3K/FOXO pathway activation in cap cells intrinsically promotes stronger cap cell–GSC adhesion (presumably via E-cadherin; Hsu and Drummond-Barbosa, 2009), which also promotes GSC maintenance. Further, aging also appears to influence insulin signaling levels in Drosophila females (Hsu and Drummond-Barbosa, 2009), suggesting that physiological changes caused by diverse factors can impinge on this GSC regulatory network. Together, these studies underscore the importance of investigating how whole organismal physiology impacts stem cell function via effects on stem cells and on their niche, potentially via changes in local signaling.

Non-canonical Notch signaling within the GSC niche?

Notch signaling requires direct cell–cell contact because Notch ligands are membrane-bound proteins that induce Notch activation in neighboring cells (Fiuza and Arias, 2007). In addition to transactivating Notch in adjacent cells, the Notch ligand Delta also inhibits Notch in cis, thus creating a potent switch between high Delta expression/low Notch activity and high Notch activity/low Delta expression (Sprinzak et al., 2010). Differential Notch activation often underlies binary cell fate decisions. For example, during Drosophila sensory organ development, cells with high levels of Delta and low Notch activity become neurons, while those with elevated Notch activity and low Delta become epidermal cells (Bray, 1998).

In the Drosophila GSC niche, we detect Notch activity in all cap cells, and Dl-lacZ is expressed in all terminal filament cells. A subset of cap cells also expresses DI-lacZ, suggesting that some cap cells may express Delta and have high Notch activity simultaneously. The basal terminal filament cell, in which DI is required for cap cell formation, does not contact all cap cells directly, and we also found that DI and Ser are not required within GSCs for cap cell formation or maintenance. We therefore propose that cap cells may signal to each other via Delta.
to activate Notch signaling, and that, in cap cells, Delta might not consistently act in cis to inhibit Notch activation.

Our observation that a subset of cap cells can express Dl-lacZ and Notch activity simultaneously is consistent with recent findings. Human eosinophils express both Notch and its ligands, and autocrine Notch signaling controls their migration and survival (Radke et al., 2009). Similarly, Notch is co-expressed with its ligands in rat hepatocytes following partial hepatectomy (Kohler et al., 2004) and also in normal human breast cells (Stylianou et al., 2006), although it is unclear if autocrine signaling occurs. It is therefore conceivable that Delta expressed in cap cells may stimulate Notch signaling via both paracrine and autocrine manners.

Alternatively, Notch ligands might be secreted from terminal filament cells to stimulate Notch signaling in all cap cells and thereby promote their maintenance. In fact, a soluble form of Delta capable of stimulating Notch has been identified in Drosophila S2 cell cultures, and the ADAM disintegrin metalloprotease Kusbanian is required for the production of soluble Delta in culture. Further, Dl and kuzbanian genetically interact, raising the possibility that soluble forms of ligands might modulate Notch signaling in vivo (Qi et al., 1999).

**Notch-independent roles of Neuralized in the control of GSC maintenance and cyst development in Drosophila**

_neur_ encodes an E3 ubiquitin ligase that mediates the endocytosis of Notch ligands in signal-sending cells, thereby enhancing their signaling strength (Fiuza and Arias, 2007). Contrary to a previous report (Ward et al., 2006), we find no evidence that Notch ligands produced from GSCs are required for self-renewal. In contrast, _neur_ is intrinsically required for GSC maintenance. Similarly, in the Drosophila testis, _neur_, but not _Dl_ and Ser, is required for GSC maintenance (Terry et al., 2006), further indicating that Neuralized maintains GSCs via a Notch-independent pathway.

_neur_ mutant cysts exhibit large and highly branched fusomes, another Notch-independent phenotype. In principle, this aberrant fusome morphology might result from a defect in fusome growth and/or partitioning, or be secondary to an excessive number of cyst division rounds. Nevertheless, the close association of some of these abnormal fusomes with the cap cell interface (see Fig. S3 in Supplementary material) suggests that fusome defects might lead to GSC loss. Ubiquitination regulates many processes, including protein degradation and vesicular trafficking (Hicke and Dunn, 2003). It is therefore possible that Neuralized ubiquitinates specific substrates that regulate fusome-related vesicular trafficking during cyst division. Future studies should test whether _E_ ligase activity is indeed required for the role of _neur_ in early germline cysts, identify key ubiquitination targets, and elucidate the molecular mechanisms they regulate.

**FOXO has multiple roles in stem cell control**

Under low insulin signaling, the FOXO transcriptional factor is required for extended longevity, reduced rates of proliferation, and stress resistance, among other processes. FOXOs are conserved from yeast to humans, and they control many target genes, different subsets of which modulate distinct processes (Tothova and Gilliland, 2007). We previously showed that Drosophila FOXO negatively controls GSC division when insulin signaling is low (Hsu et al., 2008). We also showed that insulin signaling modulates niche-stem cell interactions and Notch signaling in the niche (to control cap cell number), and that insulin signaling declines as females become older, leading to stem cell loss (Hsu and Drummond-Barbosa, 2009). In this study, we find that FOXO is required to negatively regulate Notch signaling within cap cells under low insulin activity and that FOXO also modulates the physical interaction between cap cells and GSCs. The multiplicity of FOXO roles in stem cell regulation is further underscored by studies in other stem cell systems. For example, FOXOs regulate several processes, including cell cycle progression, oxidative stress, and apoptosis, in the hematopoietic stem cell compartment, thereby influencing stem cell number and activity (Tothova and Gilliland, 2007). It will be important to investigate how the specificity of FOXO is controlled and also whether or not FOXO regulates other stem cell niches, perhaps acting as a mediator of changes in niche size and/or activity during aging or cancer development.

**Insulin/IGF and Notch signaling are intertwined in both normal and cancerous cells**

This study suggests a potentially novel mechanism by which the Notch and insulin pathways interact. In the Drosophila female GSC niche, insulin signaling does not control ligand transcription, and it is not required for ligand function (i.e., _Dl_ is required in basal terminal filament cells during cap cell formation, but _InR_ is not). Instead, both _InR_ and _N_ are cell autonomously required for cap cell maintenance, and insulin receptor function (via repression of _FOXO_ is required for proper Notch signaling. Expression of the intracellular domain of Notch rescues the low cap cell and GSC numbers of _InR_ mutants (Hsu and Drummond-Barbosa, 2009), and ovarian Notch expression does not appear altered in _InR_ mutants. Therefore, we speculate that FOXO inhibits the ability of cap cells to respond to Notch ligands by regulating a target that negatively regulates the series of proteolytic events responsible for the release of the intracellular domain of Notch. We cannot, however, rule out the possibility that Notch and FOXO normally interact at the level of target gene regulation but that overexpression of the intracellular domain of Notch overrides the normal inhibition by FOXO.

Our findings contrast with other types of interactions between FOXO and Notch that have been reported. During muscle differentiation in myoblast cultures, FOXO promotes (instead of antagonizing) Notch activity via a physical interaction that leads to activation of Notch target genes (Kitamura et al., 2007). Positive interactions between Notch and PI3K signaling have also been reported. Specifically, activation of the PI3K pathway potentiates Notch-dependent responses in CHO cells, T-cells, and hippocampal neurons. The suggested mechanism, however, involves the inactivation of GSK3 by Akt phosphorylation upstream of FOXO (McKenzie et al., 2006), which is distinct from the involvement of FOXO in the insulin–Notch signaling interaction within the GSC niche. These examples illustrate the diversity of modes of interaction between Notch and insulin signaling. It is conceivable that the positive interaction that we describe between insulin and Notch signaling pathways in the GSC niche may occur in other stem cell niches.

Deregulated Notch signaling is associated with many types of cancers and, in some cases, it is thought that altered Notch signaling promotes cancer development by overstimulating the self-renewal of normal stem cells (Wang et al., 2009). Hyperactivation of insulin/IGF pathway is also linked to increased cancer risk and poor cancer prognosis (Pollak, 2008). The Notch and insulin/IGF pathways have been reported to interact in cancerous cells via yet another mechanism. Specifically, upregulation of the Notch ligand Jagged1 leads to PI3K activation in human papillomavirus-induced cancer lines (Veeraraghavalu et al., 2005). We speculate that additional types of interactions between Notch and insulin/IGF signaling, such as the positive regulation of Notch activity by the insulin/PI3K/FOXO pathway that occurs in the Drosophila GSC niche, may also contribute to cancer progression.

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Appendix A. Supplementary data

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References


