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FOXO/Fringe is necessary for maintenance of the germline stem cell niche in response to insulin insufficiency

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ABSTRACT

The stem cell niche houses and regulates stem cells by providing both physical contact and local factors that regulate stem cell identity. The stem cell niche also plays a role in integrating niche-local and systemic signals, thereby ensuring that the balance of stem cells meets the needs of the organism. However, it is not clear how these signals are merged within the niche. Nutrient-sensing insulin/FOXO signaling has been previously shown to directly control Notch activation in the *Drosophila* female germline stem cell (GSC) niche, which maintains the niche and GSC identity. Here, we demonstrate that FOXO directly activates transcription of *fringe*, a gene encoding a glycosyltransferase that modulates Notch glycosylation. Fringe facilitates Notch inactivation in the GSC niche when insulin signaling is low. We also show that the Notch ligand predominantly involved is GSC niche-derived Delta. These results reveal that FOXO-mediated regulation of *fringe* links the insulin and Notch signaling pathways in the GSC niche in response to nutrition, and emphasize that stem cells are regulated by complex interactions between niche-local and systemic signals.

Introduction

Tissue homeostasis is maintained through the tight regulation of stem cells at multiple levels. First, intrinsic factors regulate stem cell identity and activity. Second, the stem cell niche regulates stem cells by providing physical contact and local signals. Third, stem cells are regulated by environmental and physiological fluctuations via the effect of systemic factors on either the stem cell or its niche (Drummond-Barbosa, 2008). However, how such regulatory factors are coordinated remains poorly understood.

The availability of powerful genetic approaches for the fruit fly *Drosophila melanogaster* make it ideally suited for studying the effect of different environmental conditions on cellular responses. In addition, the *Drosophila* ovary carries well-characterized GSCs and GSC niches (Fig. 1A), making it an excellent model in which to study the interaction between stem cells and their niche (Kirilly and Xie, 2007). A single *Drosophila* ovary is composed of 16 to 20 egg-producing ovarioles (Spradling, 1993). At the anterior of the ovariole is the germarium, which contains the GSC niche; this structure is composed of terminal filament cells, cap cells, and anterior escort cells (Chen et al., 2011; Kirilly and Xie, 2007). GSCs directly contact with cap cells through E-cadherin-mediated cell–cell adhesion (Song and Xie, 2002), and the GSC fusome, an organelle with a membranous-like structure, is juxtaposed to the interface between cap cell and GSC (Xie and Spradling, 2000). GSC division gives rise to a cystoblast, which subsequently undergoes four rounds of incomplete division to form a 16-cell cyst, in which the cells are interconnected with branched fusomes (Spradling, 1993). One cell develops into the oocyte, while the others become nurse cells. A layer of follicle cells proceeds to surround the 16-cell cyst, and the entire structure buds off from the germarium to become an egg chamber. The egg chamber then passes through 14 developmental stages to form a mature egg.

Cap cell and GSC maintenance requires the Notch signaling pathway (Song et al., 2007; Ward et al., 2006), which is highly conserved between species (Fiuza and Arias, 2007). The Notch receptor and its ligands are single-pass transmembrane proteins, and therefore Notch activation requires cell contact (Fiuza and Arias, 2007). *Drosophila* has one Notch receptor (encoded by *N*) and two Notch ligands, called Delta and Serrate (encoded by *Dl* and *Ser*). Notch is synthesized as a proform, which undergoes the following post-translational modifications: cleavage, *O*-fucose glycosylation, *N*-acetylglucosamine glycosylation (a process mediated by fringe

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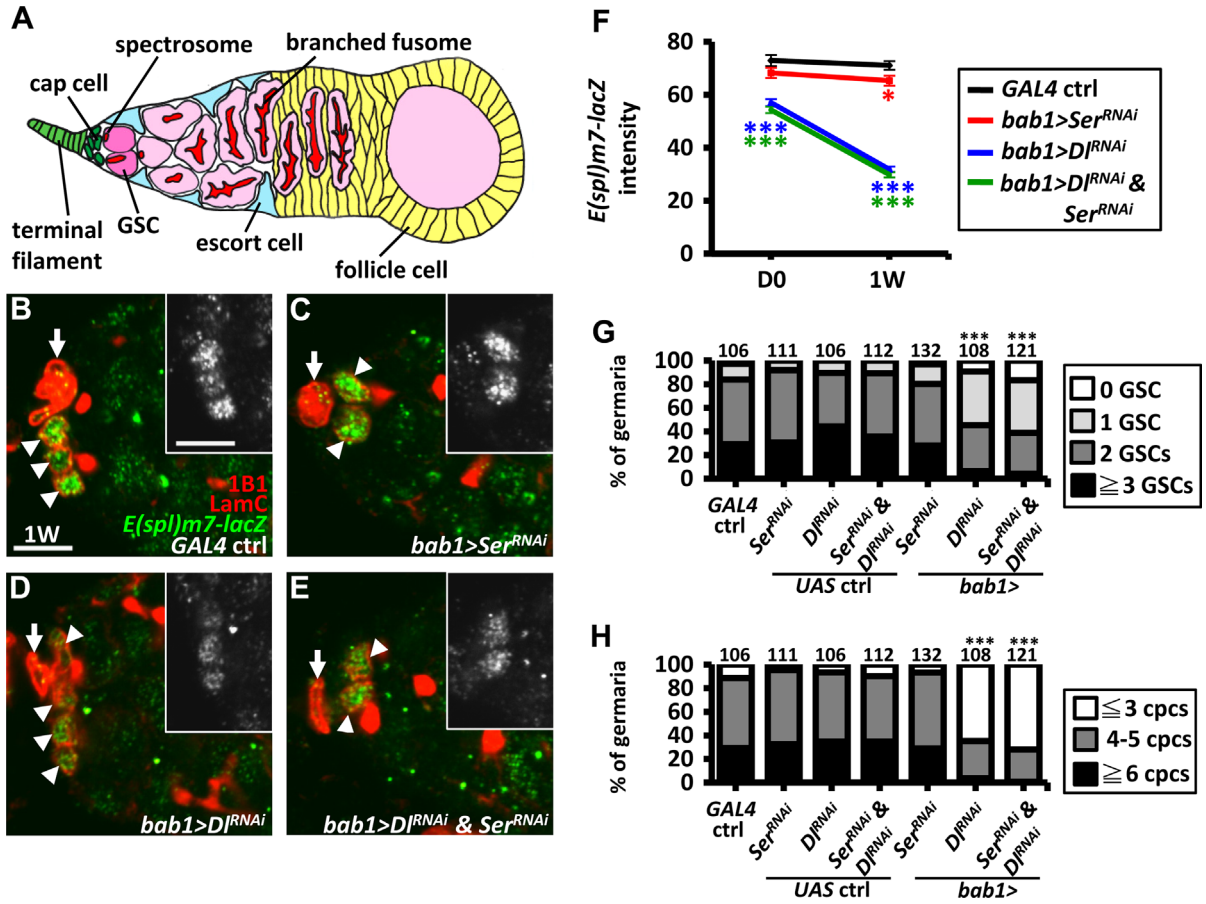

Fig. 1. Niche-derived Delta controls Notch activation in cap cells, and the numbers of GSCs and cap cells. (A) *Drosophila* germarium. Terminal filament, cap cells, and anterior escort cells form the GSC niche. Each GSC contains a spectrosome (fusome). A single GSC division generates a cystoblast; this develops into a germline cyst, which contains a branched fusome. The cyst is subsequently surrounded by somatic follicle cells. ((B)–(E)) One-week (1W)-old *GAL4* control (ctrl) (B), *bab1* > Ser^{RNAi} (C), *bab1* > Dl^{RNAi} (D), and *bab1* > Ser^{RNAi}& Dl^{RNAi} (E) germaria labeled with 1B1 (red, fusomes), LamC (red, terminal filament and cap cell nuclear envelopes), and β-gal (green, *E(spl)m7-lacZ* Notch reporter). Inserts are β-gal signals shown as grayscale images. Arrows indicate terminal filament cells and arrow heads indicate cap cells. Scale bar, 10 μm. (F) Average *E(spl)m7-lacZ* intensity in cap cells of newly eclosed and 1W-old *GAL4* control, *bab1* > Ser^{RNAi}, *bab1* > Dl^{RNAi}, or *bab1* > Ser^{RNAi} & Dl^{RNAi} females. *, $P < 0.05$; ***, $P < 0.001$. Error bars, mean ± S.E.M. ((G) and (H)) Number of GSCs (G) and cap cells (H) in 1W-old *GAL4* ctrl, *UAS* ctrls, *bab1* > Ser^{RNAi}, *bab1* > Dl^{RNAi}, or *bab1* > Ser^{RNAi} & Dl^{RNAi} females. The number of analyzed germaria is shown above each bar. cpc, cap cell. ***, $P < 0.001$. *GAL4* ctrls are flies with the genotype *bab1-GAL4*/+ in (B), and *GAL80*^{ts}/+; *bab1-GAL4*/+ in (E) and (F); *UAS* ctrls are flies with the genotypes *UAS-*Ser^{RNAi}/+, *UAS-*Dl^{RNAi}/+, or *UAS-*Ser^{RNAi}/+; *UAS-*Dl^{RNAi}/+.

(*fng*), and heterodimerization. Upon ligand binding, the Notch receptor translocates into the nucleus and regulates transcription of target genes.

The insulin/insulin-like growth factor (IGF) signaling pathway is also evolutionarily conserved, and mediates several biological processes, such as tissue growth, metabolic regulation, and ovarian function (Goberdhan and Wilson, 2003; Hafen, 2004). In *Drosophila*, insulin-like peptides activate the insulin receptor (encoded by *dinr*), which results in phosphorylation of the insulin receptor substrate homolog (encoded by *chico*) and subsequent activation of the insulin pathway. This in turn results in cytoplasmic retention of FOXO, a transcription factor which negatively regulates insulin signaling (Oldham and Hafen, 2003). It was previously reported that insulin/IGF signaling affects the response of niche cap cells to Notch ligands via FOXO, and that this process is independent of GSC-derived Notch ligands (Hsu and Drummond-Barbosa, 2011). However, the mechanisms underlying these processes remain unclear.

Here, we show that niche-derived Delta predominately activates Notch signaling in the GSC niche. We also show that FOXO suppresses Notch signaling by activating *fng* transcription in cap cells when insulin signaling is low. Over-expression of mouse FOXO1 has the same effect, indicating that mammalian FOXO may also regulate *fng* transcription. In addition, we also observed FOXO-*fng* regulation in ovarian polar cells. Moreover, mutation or over-expression of *fng* decreased Notch signaling in niche cap cells, suggesting that Notch activation is tightly regulated by its glycosylation. Finally, we demonstrate that FOXO activates *fng* transcription by binding to its promoter. In summary, our results uncover the molecular mechanism by which systemic and niche-local signals are integrated in the stem cell niche.

Materials and methods

Drosophila strains and culture

Drosophila stocks were maintained at 22–25 °C on standard media, unless otherwise indicated. The *yw* strain was used as a wild-type control. The following strains were described previously: $dinr^{339}$, Dl^{RevF10}, Ser^{RX82}, fng^{13}, $dinr^{E19}$, fng^{M69}, $fng\text{-}lacZ^{RF854}$ and $fng\text{-}lacZ^{35^{UZ-1}}$ (used to examine *fng* expression) (Grammont and Irvine, 2001; Irvine and Wieschaus, 1994), *bab1-GAL4* (mainly expressed in the GSC niche and follicle cells in adult ovaries), *dpp-GAL4UAS-phy1* (*dpp* > *phy1* enhances Notch loss-of-function phenotypes in the wing; a gift from Dr. H. Pi), *UAS-*N^{full}, *UAS-*$N^{\triangle ECN}$, *UAS-*N^{intra}, *UAS-fng*, *UAS-*N^{full}, *UAS-*$N^{\triangle ECN}$, *UAS-*N^{intra}, *UAS-fng* (Bloomington #8553), *UAS-mfoxo1*, and *UAS-dfoxo-A3*

(Baker and Schubiger, 1996; Frise et al., 1996; Hsu and Drummond-Barbosa, 2009, 2011; Kim et al., 1995; Kramer et al., 2003; Matsuno et al., 2002; Pi et al., 2011; Wessells et al., 2004; Grammont and Irvine, 2001; Irvine and Wieschaus, 1994). The *E(spl)m7-LacZ* line was used to monitor Notch signaling activity (Song et al., 2007). *UAS-RNAi* lines against *Dl* (v37288), *Ser* (v27174) and *fng* (v51799) were obtained from the Vienna *Drosophila* RNAi Center. The efficiency of each RNAi line was determined by examining their effect on the wing in a *dpp-GAL4UASphy1* strain (Fig. S1). Flies expressing *UAS* constructs also carried a *tub-GAL80^{ts}* transgene (except where otherwise indicated) and were raised at 18 °C to inhibit the expression of transgenes during development (GAL80 suppresses the binding of GAL4 onto the *UAS* element); newly eclosed flies were then switched to 29 °C to allow transgene expression (as GAL80ts is degraded) until dissection (McGuire et al., 2004). Other genetic elements are described in Flybase (http://flybase.bio.indiana.edu).

Generation of Drosophila fng promoter constructs

A fragment of the *Drosophila fng* promoter (positions −1040 to +207) containing a putative FOXO responsive element (FRE) (−50 to −42) was amplified from a BAC clone containing the *fng* gene (RP98-3J2, BACPAC Resources Center) by polymerase chain reaction (PCR) using the following primers: 5′-GGGGGG(*NheI site*) AGGAGGAACGGGAAGATACTG-3′ and 5′-GGGGGG(*HindIII site*) AACGGTTACGGACCACTACGC-3′. The NheI and HindIII sites were used to insert the 1054 bp *fng* promoter upstream of the firefly luciferase reporter gene in pGL4.15 (Promega). A *fng* promoter sequence bearing a mutated FRE was generated through PCR-based site directed mutagenesis of pGL4.5-*fng* with the following primers: 5′-GGGGGG(*NheI site*)AGGAGGAACGGGAAGATACTG-3′, 5′-GGGGGG(*HindIII site*)AACGGTTACGGACCACTACGC-3′, 5′GGTTT-TTGTTTAGAGGACGATTTTCGC-3′and 5′-GCGAAAATCGTCCTCTAAA-CAAAAACC-3′.

Luciferase reporter assay

Drosophila S2 cells were cultured with Schneider′s *Drosophila* media, containing 10% fetal bovine serum and 10% streptomycin. A gene encoding a constitutively active form of *Drosophila* FOXO (*dfoxoA3*) was cloned into the pMTV5-HisA vector (Invitrogen), under the control of the *Drosophila* metallothionein promoter (Puig et al., 2003). Actin5C-*Renilla* luciferase reporter (a gift from Dr. M.T. Su) was used as an internal control. A total of 5×10^6 S2 cells were transfected with 2.5 μg of reporter constructs (2.4 μg of *luciferase* and 0.1 μg of *Renilla*) and 0.5 μg of expression vector (pMTdFOXOA3 or pMTV5-HisA) using Cellfectin II reagent (Invitrogen). At 4 h after transfection, $CuSO_4$ was added to a final concentration of 500 μM to induce dFOXOa3 expression. After induction, 5×10^5 of the cells were cultured for 48 h in a 24-well plate, and luciferase activity was subsequently measured using a Dual-Glo luciferase assay kit (Promega). Firefly luciferase activity was normalized to *Renilla* luciferase activity. Data represent the mean ± s.d. of three independent experiments.

Chromatin immunoprecipitation (ChIP)

ChIP was performed using a Magna ChIP™ A/G kit (Millipore), in accordance with the manufacturer′s instructions. Approximately 2×10^7 S2 cells were transfected with 6 μg of pMTdFOXOA3, as described above. After transfection, cells were plated onto a 10 cm dish and cultured for 24 h, before being harvested and fixed with 1% formaldehyde. Nuclear extracts were isolated and sonicated on ice, to generate DNA fragments between 200 and 500 bp in length (pulse 8 s/pause 30 s, 25 cycles). Sonicated DNA was diluted 10 times and incubated at 4 °C overnight with either anti-V5 Agarose Affinity Gel (Sigma; 1:20) or anti-mouse IgG (1: 500) plus 20 μl of protein A/G beads. DNA was subsequently immunoprecipitated and purified for use in PCR. The following primers were used to amplify fragments of the *fng* (−81 to +205) or *4EBP* (−260 to −2) promoter: *fng*: -5′-TCACCGCTTACTGGTCTTTCTGGT-3′ and 5′ACGGACCACTACGCGCACA-TTGAA-3′, *4EBP*: 5′-CCCCTTATCATCTAGAACCTCCGA-3′ and 5′-GCGGT-ATTACGAAGTGTGGCTCTA-3′.

Immunostaining and fluorescence microscopy

Ovaries were dissected, fixed, and immunostained as previously described (Hsu et al., 2008). The following primary antibodies were used: mouse 1B1 (Developmental Studies Hybridoma Bank, DSHB, 1:10), mouse anti-Lamin (Lam) C (DSHB, 1:25), rabbit anti-Vasa (Santa Cruz, 1:1000), mouse anti-β-gal (Sigma, 1:1000), and rabbit anti-GFP (Torrey Pines, 1:2000). Alexa Fluor 488- or 568-conjugated goat anti-mouse and anti-rabbit secondary antibodies (Molecular Probes, 1:400) were used. Samples were stained with 0.5 μg/ml DAPI (Sigma), mounted with Vectashield (Vector Labs), and examined using Zeiss LSM 510 or Leica SP5 confocal microscopes.

For GSC and cap cell analyses, GSCs were identified by the anterior position of their fusome (recognized by 1B1 labeling), which is juxtaposed to cap cells, whose nuclear envelopes are ovoid and recognized by LamC labeling. All data were subjected to chi-square statistical analyses.

For quantification of *E(spl)m7-lacZ*, *fng^{RF584}* or *fng^{35UZ-1}* expression in cap cells, the average β-gal fluorescence intensity in confocal sections at the largest cap cell nuclear or cellular diameter was measured using Image J software. To avoid variation in immunostaining between samples, we only analyzed germaria with comparable expression levels of β-gal signals in polar cells (for *E(spl)m7-lacZ* and *fng^{35UZ-1}*) or in escort cells (for *fng^{RF584}*). Data were analyzed by Student′s *t*-test.

Zebrafish culture, morpholino injection, and in situ hybridization

Zebrafish were raised and maintained under standard laboratory conditions (Westerfield, 1993). Embryos were staged and fixed as previously described (Kimmel et al., 1995). The sequences of the antisense morpholino oligonucleotides (MO) (Gene Tools) used to knock down *igf1ra* and *igf1rb* were as follows (Schlueter et al., 2006):

igf1ra MO 1, 5′-TCGCTGTTCCAGATCTCATTCCTAA-3′; *igf1ra* MO 2, 5′-TGAAATTGCAGAAAAACGCGAGGCT-3′; *igf1rb* MO 1, 5′-TGTTTG-CTAGACCTCATTCCTGTAC-3′; *igf1rb* MO 2, 5′AGAAATTAGGGAGAGA-CACCTCAAC-3′. One- or two-cell stage embryos obtained by natural mating were injected with 2.5 ng of each of the two *igf1ra* MOs (5 ng MO/embryo) and 4 ng of each of the two *igf1rb* MOs (8 ng MO/embryo) (Schlueter et al., 2006).

Results

Niche-derived Delta predominately activates Notch in the GSC niche

Notch signaling, which requires cell–cell contact, is required for the maintenance of cap cells (a major component of the niche), which in turn facilitate the retention of GSCs (Hsu and Drummond-Barbosa, 2009; Song et al., 2007). Insulin signals directly control the ability of cap cells to respond to Notch ligands (Hsu and Drummond-Barbosa, 2011); however, the source of Notch ligands in the niche is unknown. Given that Notch signaling is active in every cap cell (Hsu and Drummond-Barbosa, 2011), the relevant Notch ligands must be produced by cells in direct

contact with cap cells. These cells include GSCs and cells within the niche itself, including basal terminal filament cells, anterior escort cells, and cap cells (Fig. 1A). Earlier work demonstrated that Notch ligands produced from GSCs are not required for Notch activation in cap cells (Hsu and Drummond-Barbosa, 2011), suggesting that the required Notch ligands may be produced by the niche itself.

To test this hypothesis, we used the *bab1-GAL4* driver to knock down Notch ligands (*Dl* and/or *Ser*) in the GSC niche, and monitored Notch signaling using the *E(spl)m7-lacZ* reporter (Fig. 1B–F). To specifically address the requirement for Notch ligands in adult cap cells, we raised flies at 18 °C, and transferred newly eclosed flies to 29 °C to enable GAL4-driven expression of *RNAi* for one week. At eclosion (D0), Notch signaling activity in cap cells was similar between control and *Ser* knock-down flies (72.9 ± 1.6 ($n=64$) vs. 68.3 ± 1.9 ($n=61$) arbitrary units, respectively, $P=0.1$; Fig. 1F), but activity in the cap cells of *Dl* knock-down (57.0 ± 1.0 arbitrary units, $n=54$, $P=7.9 \times 10^{-9}$) and *Dl* and *Ser* knock-down flies (54.3 ± 1.0 arbitrary units, $n=53$, $P=3.0 \times 10^{-11}$) was only ~70% of the control. This result indicates that GAL4 retains minor activity at 18 °C, and Delta activates Notch in the developing niche, in agreement with previous reports (Hsu and Drummond-Barbosa, 2011; Song et al., 2007). One week after eclosion, Notch signaling activity in *Ser* knock-down cap cells was slightly decreased as compared to control (65.3 ± 1.9 ($n=76$) vs. 71.1 ± 1.6 arbitrary units ($n=74$), respectively, $P=0.02$; Fig. 1B, C and F). Knock down of *Dl* alone (31.9 ± 1.0 arbitrary units, $n=62$, $P=1.2 \times 10^{-29}$) or both *Dl* and *Ser* (29.8 ± 1.0 arbitrary units, $n=61$, $P=4.1 \times 10^{-30}$) resulted in a dramatic decrease of Notch signaling activity in cap cells (Fig. 1D–F). This indicates that Delta produced from the niche itself predominantly activates Notch signaling in cap cells.

GSCs are maintained by Delta–Notch-mediated control of cap cell number

We proceeded to examine the number of GSCs and cap cells in knockdown flies raised at 18 °C. These flies carried *GAL80^{ts}* to prevent leaky expression of GAL4 (Fig. 1G–H, Fig. S2, and Tables S1 and S2). At eclosion, all backgrounds exhibited comparable numbers of GSCs and cap cells (Fig. S2). One week after the switch to 29 °C, the numbers of GSCs and cap cells in control and *Ser* knock-down flies were largely unchanged, while they were decreased by comparable amounts in *Dl* knock-down and *Dl*/*Ser* double-knock-down flies (Fig. 1G–H). These results confirm that niche-derived Delta, but not Serrate, regulates GSC identity via cap cell maintenance.

Insulin signaling controls the numbers of GSCs and cap cells by regulating Notch cellular processing or trafficking in cap cells

Insulin/FOXO signaling controls the cellular responses of niche cap cells to Notch ligands, but the mechanism is currently unclear (Hsu and Drummond-Barbosa, 2011). We hypothesized that insulin signaling may affect Notch activation at the levels of Notch synthesis, cytoplasmic processing, membrane trafficking, or cleavage upon ligand binding. To identify the relevant step, we overexpressed various Notch constructs in the GSC niche of *dinr^{E19}*/

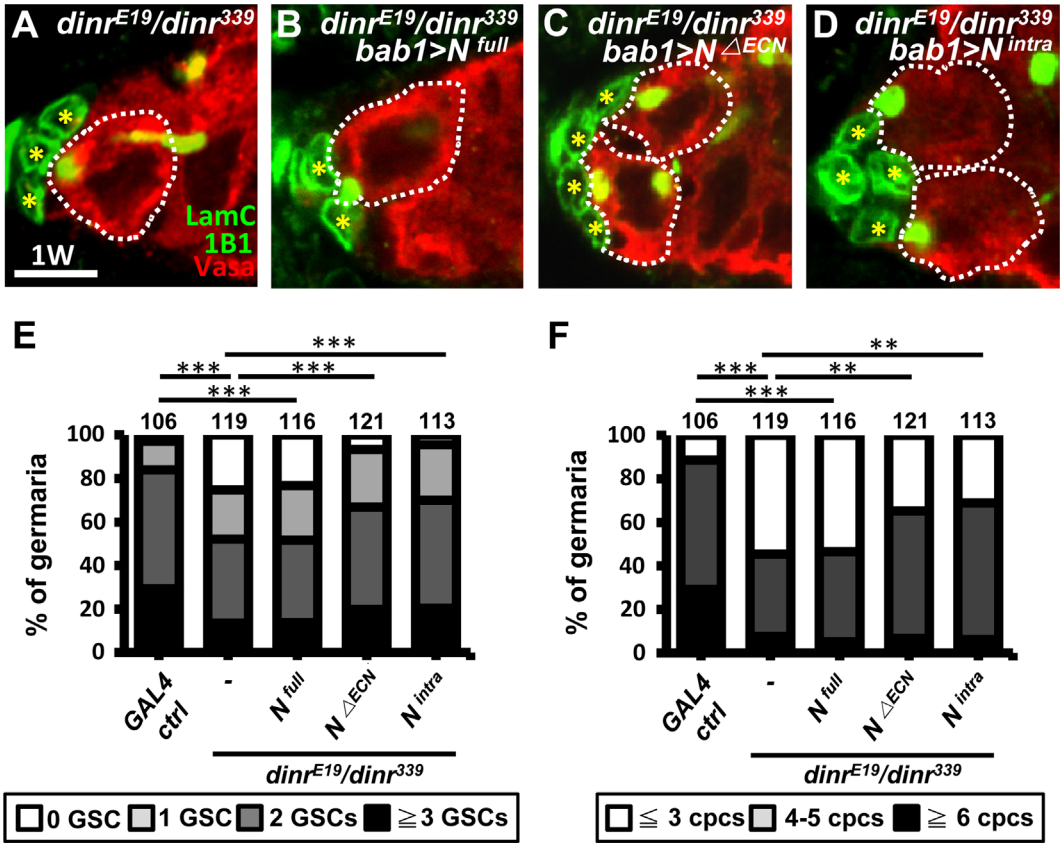

Fig. 2. Insulin signaling controls GSC and cap cell numbers by regulating Notch processing or trafficking. ((A)–(D)) One-week (1W)-old *dinr^{E19}/dinr^{339}* (A), *dinr^{E19}/dinr^{339}* with *bab1* > full length Notch (N^{full}) (B), membrane-bound Notch (*N$^{\triangle ECN}$*) (C), or Notch intracellular domain (*N^{intra}*) (D) germaria labeled with 1B1 (green, fusomes), LamC (green, terminal filament and cap cell nuclear envelopes), and Vasa (red, germ cells). Asterisks indicate cap cells and dashed circles mark GSCs. Scale bar, 10 μm. ((E) and (F)) Numbers of GSCs (E) and cap cells (F) in 1W-old *GAL4* control (ctrl), *dinr^{E19}/dinr^{339}*, and *dinr^{E19}/dinr^{339}* with *bab1* > *N^{full}*, *N$^{\triangle ECN}$* or *N^{intra}* females. The number of germaria analyzed is shown above each bar. Data for *GAL4* ctrl (*GAL80^{ts}*/+; *bab1-GAL4*/+) are the same data as shown in Fig. 1 (E) and (F). cpc, cap cell. **, $P < 0.01$; ***, $P < 0.001$.

dinr^{339} insulin receptor mutants, which exhibit reduced Notch signaling and decreased numbers of GSCs and cap cells (Hsu and Drummond-Barbosa, 2009). We used a *bab*1-*GAL*4 line controlled by *GAL*80^{ts} to express (i) full length Notch (N^{full}), (ii) a constitutively active membrane-bound Notch that lacks the extracellular domain and requires γ-secretase to release its intracellular domain (N$^{\triangle ECN}$), or (iii) the Notch intracellular domain (N^{intra}). Newly eclosed *dinr*^{E19}/*dinr*^{339} mutants raised at 18 °C contained fewer GSCs and cap cells than the respective controls, and the numbers of these cells were unaffected by Notch over-expression in this background (Fig. S3, Tables S1 and S2); these findings are in agreement with an earlier report implicating insulin signaling in niche formation (Hsu and Drummond-Barbosa, 2009). One week after the switch to 29 °C, the numbers of GSCs and cap cells in *dinr*^{E19}/*dinr*^{339} mutants had decreased further, and this was unaffected by over-expression of N^{full}. In contrast, over-expression of

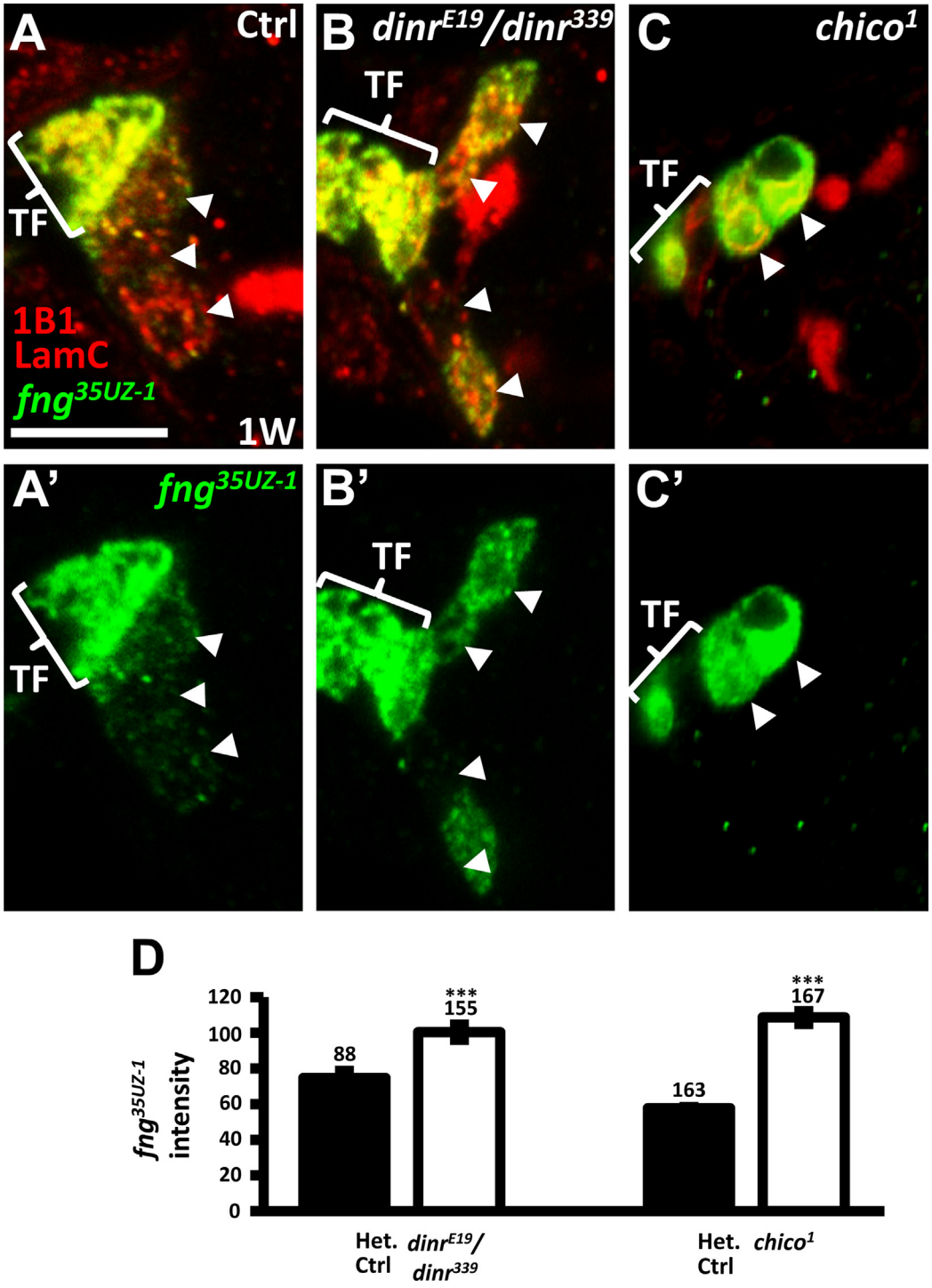

Fig. 3. Expression of *fng* in cap cells is increased by mutations in insulin signaling. ((A)–(C)) One-week (1W)-old control (Ctrl) (A), *dinr*^{E19}/*dinr*^{339} (B), and *chico*^{1} (C) germaria labeled with 1B1 (red, fusomes), LamC (red, terminal filament (TF) and cap cell nuclear envelopes), and β-gal (green, *fng*$^{3UZ-1}$ fringe reporter). Arrow heads indicate cap cells. Scale bar, 10 μm. (A′)–(C′) are β-gal signals. (D) Average *fng*$^{35UZ-1}$ intensity in cap cells of heterozygous (Het.) ctrl, 1W-old *dinr*^{E19}/*dinr*^{339}, and *chico*^{1} females. The number of cap cells analyzed is indicated above each bar. ***, $P < 0.001$. Error bars, mean ± S.E.M. Ctrls are flies with the genotypes *dinr*^{E19}*fng*$^{35UZ-1}$/*TM*3 in (A), and *dinr*^{E19}*fng*$^{35UZ-1}$/+ and *chico*^{1}/+; *fng*$^{35UZ-1}$/+ in (D).

$N^{\triangle ECN}$ or N^{intra} significantly suppressed the loss of these cells in $dinr^{E19}/dinr^{339}$ mutants (Fig. 2). Clearly, our results show that Notch downstream signaling remains functional in $dinr^{E19}/dinr^{339}$ mutant cap cells, suggesting that insulin/FOXO signaling may affect Notch processing or trafficking.

Insulin and FOXO regulate expression of fringe, a modulator of Notch glycosylation

We next investigated the mechanism by which FOXO affects Notch cellular processing or trafficking in the GSC niche. When insulin signaling is low, FOXO translocates into the nucleus and activates genes required for the response to decreased nutrient availability (Oldham and Hafen, 2003; Puig et al., 2003). To identify targets of FOXO that modulate Notch function, we examined published microarray data for genes induced by FOXO or by reduced insulin signaling (Gershman et al., 2007; Juhasz et al., 2007). We discovered that expression of *fng* was up-regulated by high FOXO nuclear activity (or nutrient depletion), but decreased by low FOXO nuclear activity, indicating that its expression is linked to nutritional input. The *fng* gene encodes a glycosyltransferase, fringe, which adds *N*-acetylglucosamine to an *O*-linked fucose on the Notch EGF domain (Fiuza and Arias, 2007). This finding raises the possibility that insulin signaling may affect Notch glycosylation in the GSC niche via FOXO-mediated regulation of *fng*.

Insulin signaling suppresses fng expression in the GSC niche

To test our hypothesis, we examined *fng* expression levels in the GSC niche of an insulin receptor mutant, $dinr^{E19}/dinr^{339}$, using a *fringe* reporter line, fng^{35UZ-1} (35 kb of the *ubx* promoter region is inserted into the 5′ end of *fng* transcription unit) (Irvine and Wieschaus, 1994) (Fig. 3). Expression of fng^{35UZ-1} was observed in terminal filament cells, cap cells, and polar cells, as previously reported (Grammont and Irvine, 2001); however, the expression level of fng^{35UZ-1} varied from cap cell to cap cell, even within the same germarium (Figs. 3B and 5A). Nevertheless, average fng^{35UZ-1} expression in cap cells was enhanced in one-week old $dinr^{E19}/dinr^{339}$ mutants as compared to control (100.4 ± 4.8 ($n=155$) vs. 74.6 ± 4.6 ($n=88$) arbitrary units, respectively, $P=1.2 \times 10^{-4}$) (Fig. 3A, B, and D). To confirm this result, we examined fng^{35UZ-1} expression in the insulin receptor substrate mutant $chico^1$, in which insulin signaling is disrupted. Expression of fng^{35UZ-1} was also increased in the cap cells of one-week old $chico^1$ mutants as compared to the controls (108.6 ± 4.1 ($n=167$) vs. 57.7 ± 2.6 ($n=163$) arbitrary units, respectively, $P=6.9 \times 10^{-22}$) (Fig. 3A, C, and D). The same result was also observed using another *fng* reporter line, fng^{RF584} (an enhancer trap line) (Fig. S4). These results indicate that *fng* transcription is enhanced in niche cap cells when insulin signaling is low. In addition, fng^{35UZ-1} was not expressed in the follicle cells of vitellogenic cells or in the previtellogenic egg chamber of controls; however, fng^{35UZ-1}

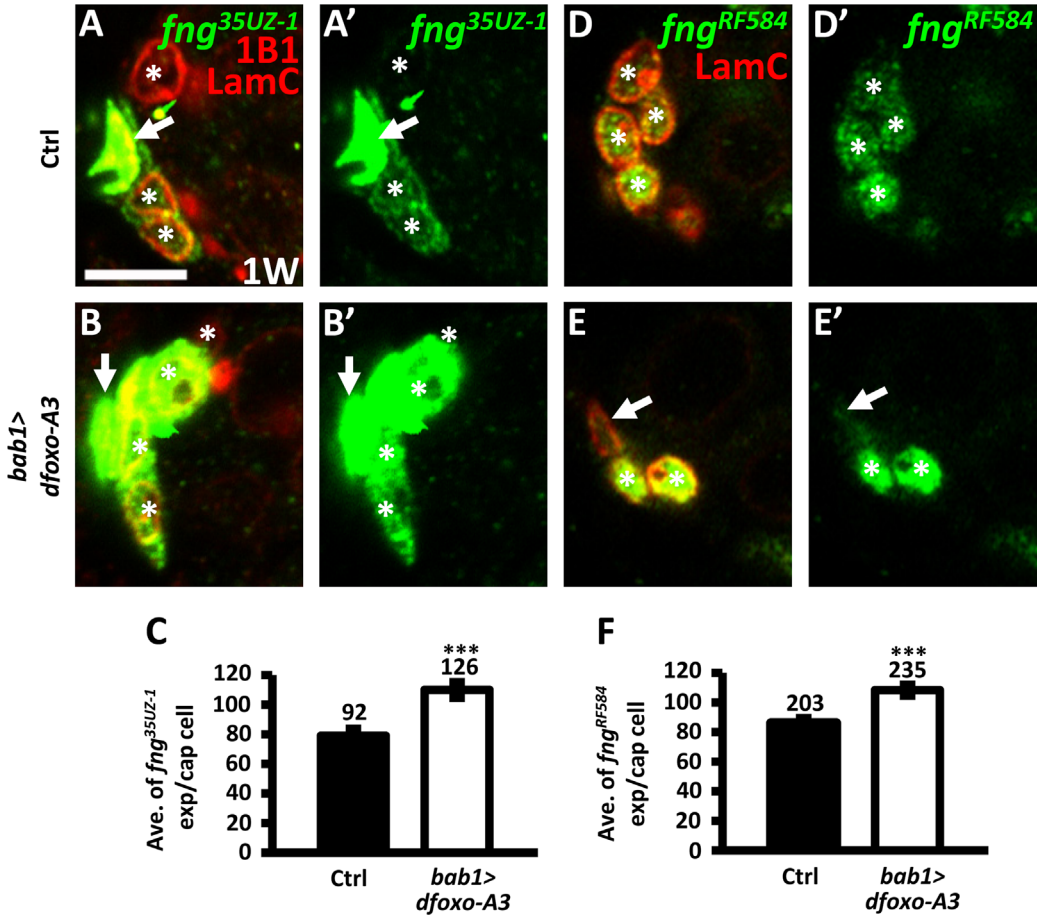

Fig. 4. Over-expression of a constitutively active form of dFOXO enhances *fng* expression in cap cells. ((A), (B), (D) and (E)) one-week (1W)-old control (Ctrl) ((A) and (D)) and *bab*1 > *dfoxo-A*3 germaria ((B) and (E)) labeled with 1B1 (red, fusomes), LamC (red, terminal filament (TF) and cap cell nuclear envelopes), and β-gal (green, fringe reporters: fng^{35UZ-1} in (A) and (B), fng^{RF584} in (D) and (E)). (A′), (B′), (D′) and (E′) are β-gal signals. Arrows indicate TF; asterisks indicate cap cells. Scale bar, 10 μm. ((C) and (F)) Average fng^{35UZ-1} (C) and fng^{RF584} (F) intensity in cap cells of 1W-old ctrl and *bab*1 > *dfoxo-A*3 females. The number of cap cells analyzed is shown above each bar. ***, $P<0.001$. Error bars, mean ± S.E.M. Ctrls are flies with the genotypes *UAS-dfoxo-A3*/+; fng^{35UZ-1}/+ in (A) and (C), and *UAS-dfoxo-A3* /+; fng^{RF584}/+ in (D) and (F).

expression was detected in the polar cells of previtellogenic egg chambers in $dinr^{E19}/dinr^{339}$ mutants (Fig. S5), suggesting that insulin signaling may negatively regulate *fng* expression in both polar and niche cells.

FOXO nuclear activity promotes fng transcription in the GSC niche

We next investigated whether FOXO transcriptional activity underlies the increase in *fng* expression in the GSC niche when insulin signaling is low. To this end, we over-expressed a constitutively-active form of *Drosophila* FOXO (dFOXO-A3, which is restricted to the nucleus due to mutations of three putative Akt phosphorylation sites) in the adult GSC niche using *bab*1-*GAL*4 under the control of $GAL80^{ts}$. We then examined expression of fng^{35UZ-1} within the niche (Fig. 4A–C). After culturing adult flies for one week at 29 °C, fng^{35UZ-1} expression was significantly increased in the cap cells of *dfoxo-A3*-over-expressing flies as compared to controls (110.8 ± 5.2 ($n=126$) vs. 79.9 ± 4.5 ($n=92$) arbitrary units, respectively, $P=1.1 \times 10^{-5}$). A similar result was obtained using the fng^{RF584} reporter (Fig. 4D–F). These results indicate that FOXO nuclear activity promotes *fng* expression in niche cap cells. As expected, cap cells were also reduced in flies over-expressing *dfoxo-A3*, as compared to controls (Fig. S6). Our results indicate that dFOXO activates *fng* transcription in the GSC niche, thereby resulting in a decrease of GSCs and cap cells. Over-expression of mouse *foxo*1 also increased *fng* expression in fly cap cells (Fig. S7), implying that mammalian FOXO1 may have a similar role.

FOXO mediates up-regulation of fng in the GSC niche when insulin signaling is low

To further examine if endogenous FOXO mediates the increase in *fng* expression when insulin signaling is low, we disrupted FOXO function in $dinr^{E19}/dinr^{339}$ mutants and examined *fng* expression in cap cells using fng^{35UZ-1} (Fig. 5). As we previously observed, fng^{35UZ-1} expression in cap cells was higher in $dinr^{E19}/dinr^{339}$ mutants than in $dinr^{E19}/TM3$ controls (85.1 ± 4.6 ($n=99$) vs. 40.1 ± 2.6 ($n=85$) arbitrary units, respectively, $P=1.5 \times 10^{-14}$). This increase in fng^{35UZ-1} expression was suppressed in $foxo^{25}$-$dinr^{E19}/foxo^{21}dinr^{339}$ mutants, in which FOXO function is disrupted (34 ± 2.2 arbitrary units, $n=91$). Similar results were obtained using fng^{RF584} as a reporter (Fig. S8). Therefore, inactivation of insulin signaling leads to increased FOXO nuclear activity, which enhances *fng* transcription in the GSC niche.

Over-expression of fng disrupts Notch activation in the GSC niche, thereby decreasing GSCs and cap cells

Over-expression of *fng* in the GSC niche for one week after eclosion resulted in reduced Notch signaling activity in cap cells, as compared to the sibling control (91.2 ± 2.9 ($n=112$) vs. 130.8 ± 2.4 ($n=72$) arbitrary units, respectively, $P=2.6 \times 10^{-18}$) (Fig. 6A–C). The numbers of GSCs and cap cells were similar in newly eclosed control and *fng*-over-expressing flies raised at 18 °C, but significantly decreased in *fng*-over-expressing flies one week after the switch to 29 °C (Fig. 6D and E). These results demonstrate that fringe negatively regulates Notch activation, thereby regulating cap cell maintenance and GSC identity. In addition, these findings support our hypothesis that when insulin signaling is inactive, FOXO suppresses Notch function in the GSC niche by increasing *fng* expression.

FOXO stimulates fng expression in response to insulin insufficiency

Insulin/FOXO signaling inhibits Notch activation in the GSC niche (Hsu and Drummond-Barbosa, 2011). To determine whether fringe acts downstream of FOXO to suppress Notch activation when insulin signaling is low, we suppressed *fng* expression in the adult GSC niche of $dinr^{E19}/dinr^{339}$ mutants, which have elevated FOXO nuclear activity. We then examined Notch signaling using the *E(spl)m7-lacZ* transgene (Fig. 7A–D). One week after the switch

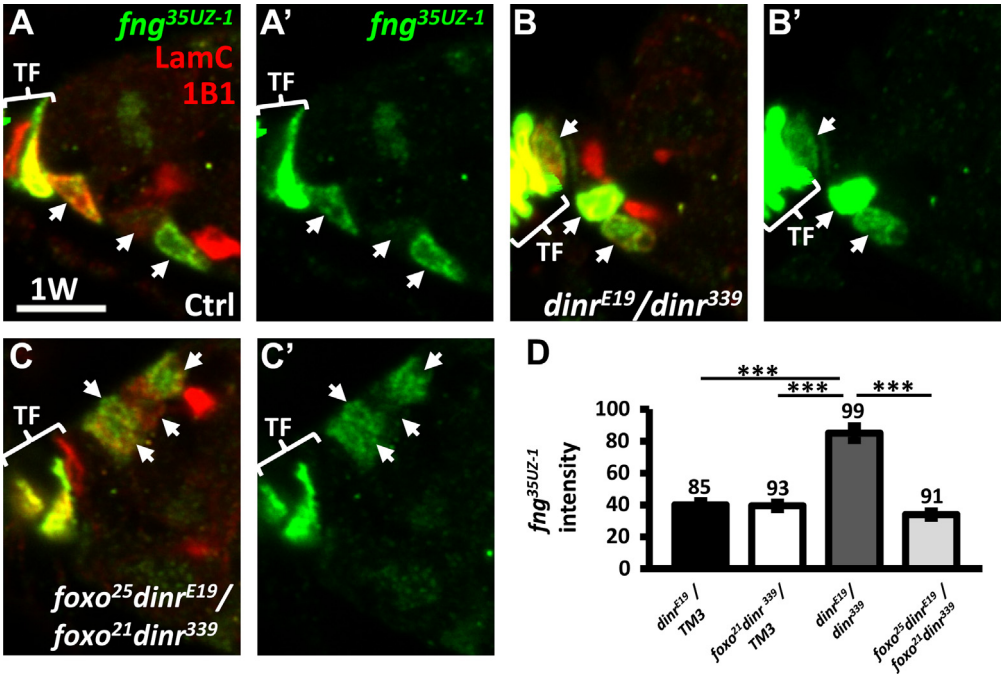

Fig. 5. dFOXO mediates up-regulation of *fng* expression in cap cells of *dinr* mutants. ((A)–(C)) One-week (1W)-old control (Ctrl) (A), $dinr^{E19}/dinr^{339}$ (B) and $foxo^{25}dinr^{E19}/foxo^{21}dinr^{339}$ germaria (C) labeled with 1B1 (red, fusomes), LamC (red, terminal filament (TF) and cap cell nuclear envelopes), and β-gal (green, fng^{35UZ-1} fringe reporter). (A′)–(C′) are β-gal signals. Arrows indicate cap cells. Scale bar, 10 μm. (D) Average fng^{35UZ-1} intensity in cap cells of 1W-old sibling ctrl, $dinr^{E19}/dinr^{339}$, and $foxo^{25}dinr^{E19}/foxo^{21}dinr^{339}$ females. The number of cap cells analyzed is shown above each bar. ***, $P<0.001$. Error bars, mean ± S.E.M. Ctrl indicates flies with the genotype $dinr^{E19}fng^{35UZ-1}/TM3$ in (A).

to 29 °C at eclosion, Notch activity was detected in every cap cell in the controls (sibling control #1: 83.2 ± 2.6 arbitrary units, $n=70$; sibling control #2: 91.7 ± 4.5 arbitrary units, $n=97$), and was reduced by 42–46% in $dinr^{E19}/dinr^{339}$ cap cells (48.9 ± 1.9 arbitrary units, $n=96$). Notch activity in the cap cells of *fng* knock-down $dinr^{E19}/dinr^{339}$ mutants was 71.5% that of controls. This partial rescue may be due to other factors involved in this regulatory process, uneven expression of *bab*1-*GAL4* in cap cells (data not shown), or low efficiency of fng^{RNAi} (Fig. S1). Furthermore, we found that knockdown of *fng* in $dinr^{E19}/dinr^{339}$ mutants significantly suppressed GSC and cap cell loss one week after eclosion (Fig. 7E and F, and Tables S1 and S2). Similar results were also observed in $chico^{1}$ or $dinr^{E19}/dinr^{339}$ upon removing one functional copy of *fng* (Tables S1 and S2). This indicates that *fng* is up-regulated in *dinr* mutants to suppress Notch signaling in the GSC niche, in turn affecting cap cell maintenance, and consequently GSC identity.

FOXO directly binds to the fng promoter

We subsequently investigated whether dFOXO regulates transcription of *fng* through binding to its promoter (Fig. 8). We identified a putative FOXO responsive element (FRE, TT(G/A) TTTAC) 42–50 bp upstream of the transcriptional start site of the *fng* gene (Fig. 8A). This FRE is similar to those in the promoters of the human *Glucose*-6-*Phosphatase*, and *Drosophila inr* and *eukaryotic initiation factor* 4*E-binding protein* (4EBP) genes, which were previously shown to bind FOXO4 and dFOXO, respectively (Puig et al., 2003; Schmoll et al., 2000). We used chromatin immunoprecipitation (ChIP) to determine if dFOXO-A3 (tagged with V5) binds to the FRE of the *fng* promoter in S2 cells (Fig. 8B). Anti-IgG was used as a negative control. We used PCR to determine dFOXO-A3 occupancy at three FRE tandem repeats within the *4EBP* promoter (−260 to +2) (as a positive control) or at the FRE of the *fng* promoter (−81 to +205). We report that antibodies against V5 efficiently immunoprecipitated the FREs of the *4EBP* and *fng* promoters. We also examined the effects of dFOXO binding on *fng* transcription using a promoter activity assay (Fig. 8C). We generated *luciferase* reporter genes driven by 1.2 kb of the *fng* promoter containing wild-type (TTGTTTAC) or mutant FRE (TAGAGGAC), and transfected them into S2 cells, with or without *dfoxo-A3*. The addition of *dfoxo-A3* increased luciferase expression three-fold in cells transfected with the wild-type *fng* reporter. However, no such increase was observed in cells transfected with the mutant *fng* reporter. Our results indicate that FOXO activates *fng* transcription by directly interacting with the FRE.

Discussion

Insulin/IGF and Notch signaling play central roles in several developmental processes, cancer progression, and stem cell self-renewal (Bolós et al., 2007; Clayton et al., 2011; Drummond-

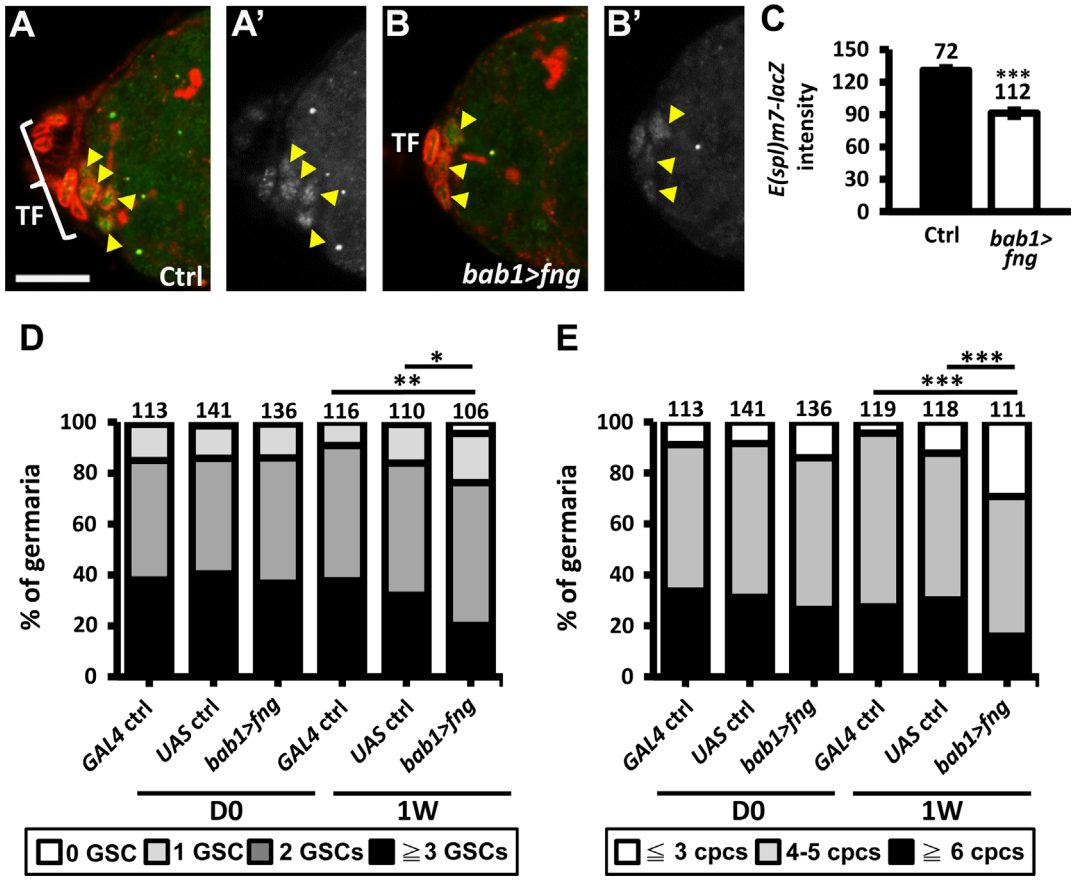

Fig. 6. Over-expression of fringe disrupts Notch activation and GSC and cap cell maintenance. ((A) and (B)) One-week (1W)-old control (Ctrl) (A) and *bab*1 > *fng* germaria (B) labeled with 1B1 (red, fusomes), LamC (red, terminal filament (TF) and cap cell nuclear envelopes), and β-gal (green, *E(spl)m7-lacZ* Notch reporter). (A′) and (B′) are β-gal signals shown as grayscale images. Arrow heads indicate cap cells. Scale bars are 10 μm. (C) Average *E(spl)m7-lacZ* intensity in cap cells of 1W-old sibling ctrl and *babi* > *fng* females. The number of cap cells analyzed is shown above each bar. Error bars, mean ± S.E.M. ((D) and (E)) Number of GSCs (D) and cap cells (E) in newly eclosed (D0) and 1W-old *GAL4* ctrl, *UAS* ctrl, and *bab*1 > *fng* females. The number of germaria analyzed is shown above each bar. cpc, cap cells. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$. Ctrl are flies with the genotypes *UAS-fng*/+; *E(spl)m7-lacZ* /+ in (A) and (C); *GAL4* ctrl are flies with the genotype $GAL80^{ts}$/+; *bab*1-*GAL4*/+; and *UAS* ctrl are flies with the genotype *UAS-fng*/+ in (D) and (E).

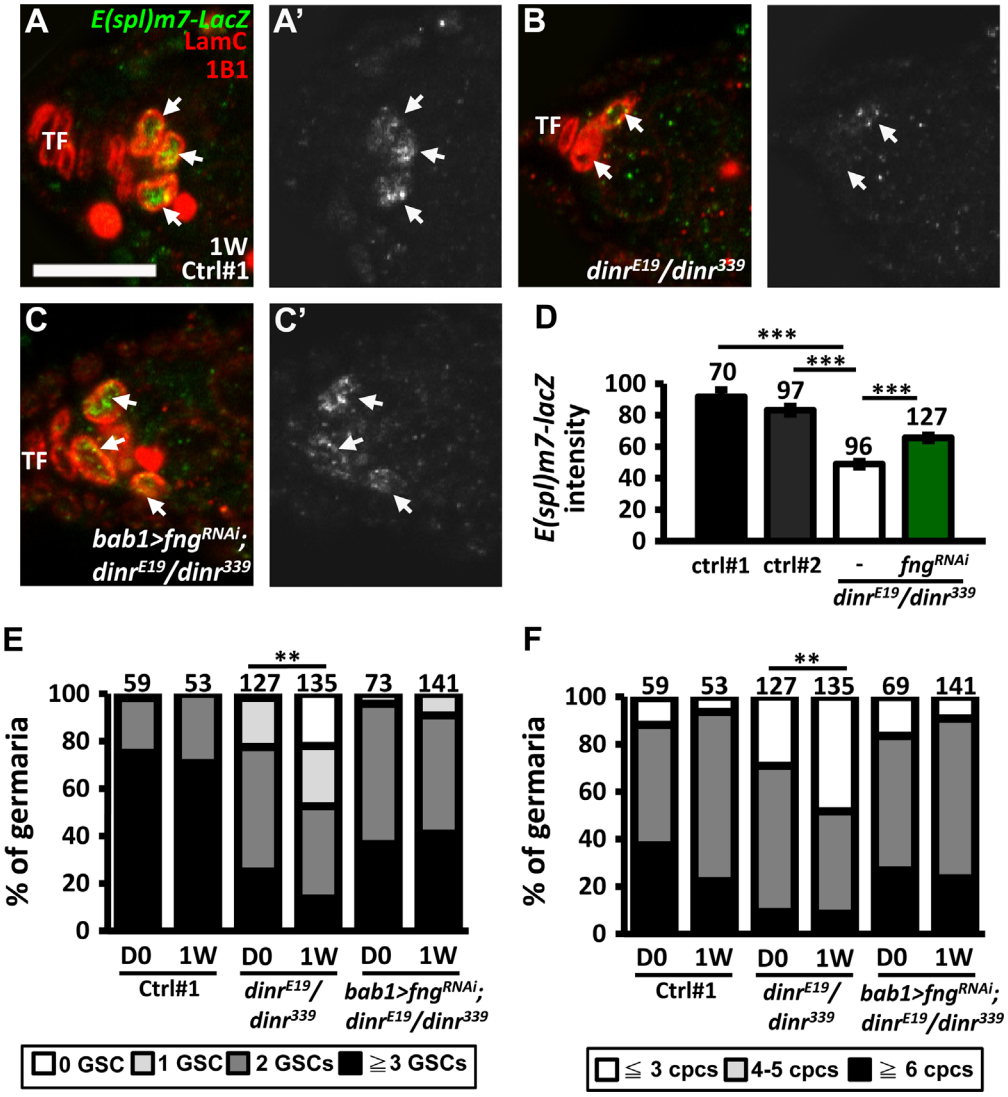

Fig. 7. Reduced *fng* expression in the GSC niche of *inr* mutants rescues Notch activity and GSC and cap cell numbers. ((A)–(C)) One-week (1W)-old control (Ctrl) (A), $dinr^{E19}$/$dinr^{339}$ (B) and *fng-knock down* $dinr^{E19}$/ $dinr^{339}$ (C) germaria labeled with 1B1 (red, fusomes), LamC (red, terminal filament (TF) and cap cell nuclear envelopes), and β-gal (green, *E(spl)m7-lacZ* Notch reporter). (A′)–(C′) are β-gal signals shown as grayscale images. Arrows indicate cap cells. Scale bar, 10 μm. (D) Average fng^{35UZ-1} intensity in cap cells of newly eclosed (D0) and 1W-old ctrl and $dinr^{E19}$/$dinr^{339}$ females with or without *bab1* > fng^{RNAi} expression. ***, $P < 0.001$. Error bars, mean ± S.E.M. ((E) and (F)) Number of GSCs (E) and cap cells (F) in newly eclosed (D0) and 1W-old ctrl and $dinr^{E19}$/$dinr^{339}$ females with or without *bab1* > fng^{RNAi} expression. The number of germaria analyzed is shown above each bar. cpc, cap cells. **, $P < 0.01$. Ctrl are flies with the genotype *E(spl)m7-lacZ*/+ in (A), (D), (E) and (F).

Barbosa, 2008; Morrison and Spradling, 2008). Recent studies have also shown that these two signaling pathways act directly on stem cell niches to regulate stem cells. For example, both IGF and Notch signaling maintain the hematopoietic stem cell niche to regulate hematopoiesis in mice (Mayack et al., 2010; Weber and Calvi, 2010). Therefore, an understanding of the mechanism by which systemic insulin signals are integrated with niche-local Notch signaling is central to stem cell biology. In *Drosophila*, Notch signaling controls niche cap cell number (Song et al., 2007; Ward et al., 2006), and we previously reported that insulin signaling controls Notch activation in niche cap cells (Hsu and Drummond-Barbosa, 2009, 2011). Here, we establish that niche-derived Delta stimulates Notch in the GSC niche, and we describe a novel regulatory mechanism in which a lack of nutrients causes FOXO to disrupt Notch activation in the GSC niche by directly up-regulating *fng* expression (Fig. 8D). These findings further our understanding of how organisms regulate stem cell behavior, via the modification of niche-local signaling by systemic factors in response to external environmental changes.

Excessive sugar modification may disrupt Notch signaling

Fringe is a glycosyltransferase that transfers *N*-acetylglucosamine onto *O*-fucose at epidermal growth factor (EGF)-like domains of Notch (Bruckner et al., 2000; Moloney et al., 2000). This modification facilitates the binding of Notch to Delta, but suppresses the Notch–Serrate interaction (Bruckner et al., 2000; Panin et al., 1997). Conversely, Notch without this modification interacts with Serrate, but not Delta. Fringe-dependent Notch glycosylation is known to control the formation of the dorsal-ventral boundary in *Drosophila* eyes and wings (Cho and Choi, 1998; Dominguez and de Celis, 1998; Panin et al., 1997), and the establishment of the somite boundary in zebrafish and mouse (Barrantes et al., 1999; Prince et al., 2001), and is likely to control the differentiation of muscle fibers in *Drosophila* (Bernard et al., 2006).

In this study, we have demonstrated that niche-derived Delta activates Notch signaling in niche cap cells, and that fringe is required for this activation, as evidenced by low Notch signaling activity in the niche of *fng* mutants (Fig. S9). These results indicate

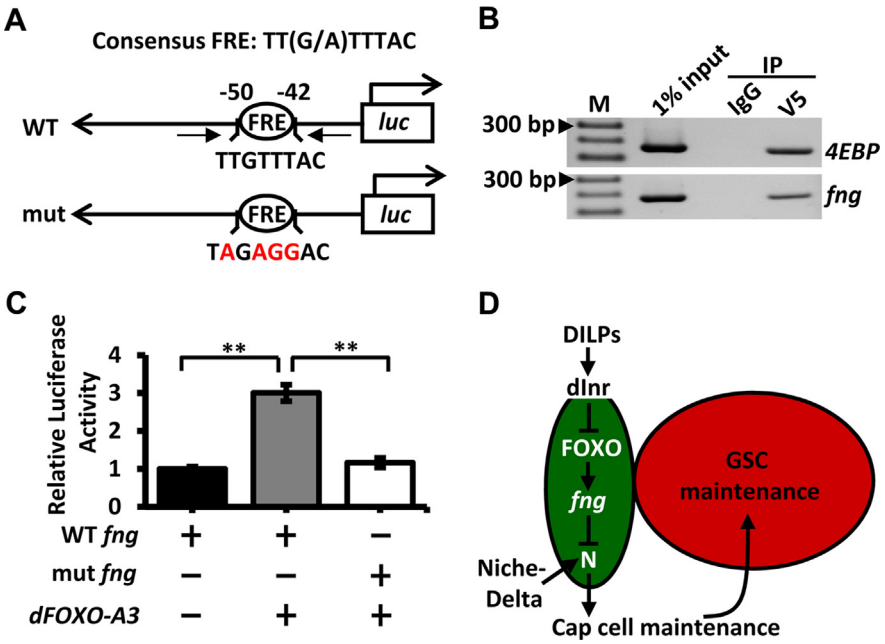

Fig. 8. FOXO binds to the FOXO-responsive element of the *fng* promoter and activates *fng* (A) Luciferase expression driven by the *fng* promoter carrying a wild-type (wt) or a mutated (mut) FOXO-responsive element (FRE). (B) ChIP analysis of FOXO binding. Soluble chromatin was prepared from S2 cells expressing dFOXO-A3 tagged with V5, and immunoprecipitated with antibodies against V5 or IgG. Co-precipitated DNA was analyzed by PCR using primers against positions −260 to +2 of the 4*EBP* gene and −81 to 250 of the *fng* gene. M, marker. (C) Luciferase reporter assay. S2 cells were transiently transfected with a wt or mut FRE *luciferase* reporter alone, or together with *dfoxo-A3*. The activity of the *fng/luciferase* reporter alone was set at 1. (D) Insulin and Notch signaling are integrated by FOXO-*fng* in the GSC niche. This serves to maintain cap cells, and thus GSCs. Niche-derived Delta stimulates Notch activity in cap cells.

that Delta interacts with fringe-modified Notch in niche cap cells. It is interesting to note that fringe over-expression does not enhance Notch signaling, but in fact decreases it. Fringe does not modify all of the *O*-fucose residues on Notch 1 EGF repeats in CHO cells (Shao et al., 2003), and we therefore speculate that increased fringe may result in excessive glycosylation, thereby disrupting Notch–Delta binding or Notch trafficking to the cell surface. Nevertheless, we cannot rule out the possibility that fringe may have additional effects on Notch activation independent of its glycosyltransferase activity.

FOXO controls cellular processes through Notch signaling

When nutrients are not available (i.e. insulin signaling is low), *Drosophila* females gradually lose GSCs due to loss of their niche cells (Hsu and Drummond-Barbosa, 2009); this presumably reflects a trade-off between reproduction and survival. This study identifies a novel mechanism by which diminished nutrient availability suppresses Notch signaling in the GSC niche via activation of insulin/FOXO signaling and fringe; this process results in cap cell loss, which in turn causes GSCs to be lost. In S2 cells, FOXO directly binds to the FRE of the *fng* promoter and transactivates *fng* expression. We therefore hypothesize that *fng* is a direct target of FOXO, and that FOXO-*fng* regulation bridges insulin/IGF and Notch signaling to control the cellular response to nutrient stress.

Other types of interaction between FOXO and Notch signaling have been reported. During muscle differentiation in myoblast culture, FOXO1 physically interacts with CSL (CBF1, Suppressor of Hairless, Lag-1) to activate Notch target genes; this suppresses serum withdrawal-induced myogenic differentiation, implying that FOXO and Notch cooperate to regulate muscle progenitor maintenance and differentiation (Kitamura et al., 2007). FOXO has also been reported to be a downstream target of Notch (Mandinova et al., 2008). Upon exposure to high energy UVB, Notch activity is highly induced in epidermal cells and primary keratinocytes. Notch decreases FOXO3 expression, thereby protecting the cell from apoptosis. These studies indicate that FOXO and Notch interact at multiple levels. We believe that the process of FOXO/Notch regulation observed in the GSC niche may also occur in other stem cell niches.

Insulin/FOXO/fringe/Notch regulation may be conserved between cell types and species

FOXO-mediated insulin/IGF signaling is evolutionarily conserved, and widely used by cells for nutrient sensing (Goberdhan and Wilson, 2003; Hafen, 2004). Notch signaling is also highly conserved, and involved in regulating developmental processes or stem cell function (Fiuza and Arias, 2007; Morrison and Spradling, 2008). This raises the possibility that the insulin/FOXO/*fringe*/Notch pathway reported here may be commonly used by different cell types or species. Indeed, *fng* expression is not only enhanced in the GSC niche, but also in polar cells of *insulin receptor* mutants (Fig. S5). In addition, over-expression of *mfoxo*1 in the GSC niche also results in elevated *fng* expression (Fig. S7). Two IGFs (IGF-1 and IGF-2) are present in zebrafish, but only IGF-1 receptors (IGF-1a and IGF-1b) have been cloned (Zou et al., 2009). In addition, there are three fringe genes in zebrafish, encoding lunatic fringe, radical fringe, and manic fringe (Qiu et al., 2004). Disruption of insulin/IGF signaling by injecting embryos with morpholinos against *igf*1*a* or *igf*1*b* results in a dramatic decrease in the expression of the Notch downstream target *her*4 gene during somitogenesis (Fig. S10), consistent with the hypothesis that IGF signaling controls Notch. Although it is not clear whether FOXO/fringe are involved in this process in zebrafish at present, putative FOXO-binding elements have been found in the *lunatic fringe* and *radical fringe* genes at the promoter regions −2562 to 2570 and −745 to 753, respectively. These results suggest that the interaction between the insulin/IGF and Notch signaling pathways, as mediated through the regulation of *fng* transcription by FOXO, may be a commonly employed strategy for the modulation of cellular behavior under nutrient stress.

Non-canonical Notch signaling regulates the GSC niche

Notch signaling typically requires direct contact between ligand- and receptor-producing cells, as both Notch ligands and receptors are transmembrane proteins (Fiuza and Arias, 2007). Notch ligands transactivate Notch in neighboring cells, but suppress it through *cis*-interactions (Sprinzak et al., 2010); thus cells with high levels of Notch activation may have low ligand expression levels, and vice versa. Such differential expression of Notch and its ligands are known to control many developmental processes, including cell fate decision and boundary formation.

Notch is activated in every cap cell in the GSC niche of female *Drosophila* (Hsu and Drummond-Barbosa, 2011), and Delta produced within the niche is required for this activation. Although *Dl-lacZ* is detected in only a subset of cap cells, Delta-producing cap cells may stimulate Notch signaling through an autocrine or paracrine manner. This possibility is supported by the observations that human eosinophils express both Notch and its ligands, and that autocrine Notch signaling controls their migration and survival (Radke et al., 2009). Notch and its ligands are also co-expressed in rat hepatocytes and in normal human breast cells (Kohler et al., 2004; Stylianou et al., 2006), suggesting that autocrine Notch signaling may also occur in these cells. Nevertheless, the identification of a soluble form of Delta capable of stimulating Notch in *Drosophila* S2 cells (Qi et al., 1999) means we cannot rule out the possibility that Notch ligands secreted from terminal filament cells may activate Notch in cap cells.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2013.07.018.

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