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

Sheng-An Yang a,1, Wen-Der Wang b, Ciao-Ting Chen b, Chen-Yuan Tseng a,c, Yi-Ning Chen d, Hwei-Jan Hsu a,*

a Institute of Cellular and Organismic Biology, Academia Sinica, Taipei 11529, Taiwan
b Department of Biogastronomic Science, Chiayi University, Chiayi City 60004, Taiwan
c Graduate Institute of Life Sciences, National Defense Medical Center, Taipei 11490, Taiwan
d Institute of Molecular Biology, Academia Sinica, Taipei 11529, Taiwan

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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.

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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 gerarium, 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 DI and Ser). Notch is synthesized as a proform, which undergoes the following post-translational modifications: cleavage, O-fucose glycosylation, N-acetylgalactosamine glycosylation (a process mediated by fringe...
(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 functions (Goberdhan and Wilson, 2003; Hafen, 2004). In Drosophila, insulin-like peptides activate the insulin receptor (encoded by dmem), 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: dinrt359, DppRt1, Ser752, fng13, dinrt19, fng859, fng-lacZ854 and fng-lacZ2350+ (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-Nfull, UAS-Nm1, UAS-Nm2, UAS-fng, UAS-Nfull, UAS-Nm1, UAS-Nm2, UAS-fng (Bloomington #8553), UAS-myoxo1, and UAS-dfoxo-A3.
Luciferase reporter assay

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(Nhel site) AGAGGAAAGGGGAGATCTG-3′ and 5′-GGGGGG(HindIII site) AACGGTTAGGAGCTACCC-3′. The Nhel 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-fng luciferase reporter gene in pGL4.15 (Promega). A transgene was generated using a tub-GAL80F transgene under the control of the tubulin promoter. At 4 h after transfection, CuSO4 was added to a 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.

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 fssome (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 Esp [s]m7-lacZ, fng[Br5] or fng[s]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 Esp[s]m7-lacZ and fng[s]1) or in escort cells (for fng[Br5]). 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′-TCGGCTGTTACAGATCTACTTCAAC-3′; igf1ra MO 2, 5′-TGAATTCACGGAAAGACAGAGATTATAC-3′; igf1rb MO 1, 5′-TGACTCTATGGACACATAC-3′; igf1rb MO 2, 5′-AGAATTCTGAAGTTCGAGAC-3′.

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

(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 Di (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-GAL4/UASpsh1 strain (Fig. S1). Flies expressing UAS constructs also carried a tub-GAL80F transgene (except where otherwise indicated) and were raised at 18 °C to inhibit the expression of transgenes during development (GAL80F suppresses the binding of GAL4 onto the UAS element); newly eclosed flies were then switched to 29 °C to allow transgene expression (as GAL80F 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(Nhel site) AGAGGAAAGGGGAGATCTG-3′ and 5′-GGGGGG(HindIII site) AACGGTTAGGAGCTACCC-3′. The Nhel 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(Nhel site) AAGAGAAGGGGAGATCTG-3′ and 5′-GGGGGG(HindIII site) AAGCCTAAGGACCACTACCC-3′, 5′-GTTTT-TGGTAGGACGATTTTGC-3′ and 5′-GGCAAAATCTGCTCTAAACAAAAC-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 pMVT5-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 × 10^5 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 pMVT5-HisA) using Cellfectin II reagent (Invitrogen). At 4 h after transfection, CuSO4 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.

ChIP 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′-TCACCCCTACTTGTTCGTTG-3′ and 5′-AGCAGACATACGCCACAGAACATGAAA-3′; 4EBP: 5′-CCCTTTATCCTAGAAACTTCCCGA-3′ and 5′-GGGTTATTACAACTGTGGGCT-3′.
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 (DI and/or Ser) in the GSC niche, and monitored Notch signaling using the E(sp)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 DI knock-down (57.0 ± 1.0 arbitrary units, n=54, P = 7.9 × 10^{-3}) and DI and Ser knock-down flies (54.3 ± 1.0 arbitrary units, n=53, P = 3.0 × 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 DI alone (31.9 ± 1.0 arbitrary units, n=62, P=1.2 × 10^{-29}) or both DI and Ser (29.8 ± 1.0 arbitrary units, n=61, P=4.1 × 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 GAL80Δts 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 DI knock-down and DI/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 over-expressed various Notch constructs in the GSC niche of dincE19/Δts.
dim^{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 bab1-GAL4 line controlled by GAL80ts to express (i) full length Notch (Nfull), (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\(^{\text{intra}}\)). Newly eclosed dinr\(^{E19}/\text{dinr}^{339}\) 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\(^{E19}/\text{dinr}^{339}\) mutants had decreased further, and this was unaffected by over-expression of Nfull. 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\(^{E19}/\text{dinr}^{339}\) (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\(^{35UZ}\)/C0 fringe reporter). Arrowheads indicate cap cells. Scale bar, 10 μm. (A′)–(C′) are β-gal signals. (D) Average fng\(^{35UZ}\) intensity in cap cells of heterozygous (Het.) ctrl, 1W-old dinr\(^{E19}/\text{dinr}^{339}\), and chico\(^{1}\) females. The number of cap cells analyzed is indicated above each bar. ***, P < 0.001. Error bars, mean ± S.E.M. Ctrl is flies with the genotypes dinr\(^{E19}/\text{fng}^{35UZ}\)/TM3 in (A), and dinr\(^{E19}/\text{fng}^{35UZ}\)/+ and chico\(^{1}\); fng\(^{35UZ}\)/+ in (D).
N^{ECN} or N^{Intra} significantly suppressed the loss of these cells in *dim^{E19}/dim^{339}** mutants (Fig. 2). Clearly, our results show that Notch downstream signaling remains functional in *dim^{E19}/dim^{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 to modulate Notch function, we examined published microarray data for genes induced by FOXO or by reduced insulin signaling (Oldham and Hafen, 2003; Puig et al., 2003). To identify targets of FOXO, we next investigated the mechanism by which FOXO affects Notch glycosylation in the GSC niche via FOXO-mediated regulation of *fng*.

![Fig. 4](image-url) - 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 *bab1 > dfoxo-A3* germline (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^{RF854}* in (D) and (E)). (A’), (B’), (D’) and (E’) are β-gal signals. Arrows indicate TF; asterisks indicate cap cells. Scale bar, 30 μm. ((C) and (F)) Average *fng^{35UZ-1}* (C) and *fng^{RF854}* (F) intensity in cap cells of 1W-old control and *bab1 > dfoxo-A3* females. The number of cap cells analyzed is shown above each bar. ***,* P < 0.001. Error bars, mean ± S.E.M. Ctrl is flies with the genotypes UAS-dfoxo-A3/++; *fng^{35UZ-1}*/+ in (A) and (C), and UAS-dfoxo-A3/++; *fng^{RF854}*/+ in (D) and (F).

**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, *dim^{E19}/dim^{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 *dim^{E19}/dim^{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 × 10^{-4} (Fig. 3A, B, and D). To confirm this result, we examined *fng^{35UZ-1}* expression in the insulin receptor substrate mutant *chico*<sup>1</sup>, in which insulin signaling is disrupted. Expression of *fng^{35UZ-1}* was also increased in the cap cells of one-week old *chico*<sup>1</sup> mutants as compared to the controls (107.7 ± 3.4 (n = 167) vs. 77.4 ± 2.6 (n = 163) arbitrary units, respectively, P = 6.9 × 10^{-22} (Fig. 3A, C, and D). The same result was also observed using another *fng* reporter line, *fng^{RF854}* (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}*
expression was detected in the polar cells of previtellogenic egg chambers in \textit{dim}^{19}/\textit{dim}^{339} mutants (Fig. S5), suggesting that insulin signaling may negatively regulate \textit{fng} expression in both polar and niche cells.

**FOXO nuclear activity promotes \textit{fng} transcription in the GSC niche**

We next investigated whether FOXO transcriptional activity underlies the increase in \textit{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 \textit{bab}1-GAL4 under the control of \textit{GAL80}'. We then examined expression of \textit{fng}^{3SUZ-1} within the niche (Fig. 4A–C). After culturing adult flies for one week at 29 °C, \textit{fng}^{3SUZ-1} expression was significantly increased in the cap cells of \textit{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 × 10^{-5}). A similar result was obtained using the \textit{fng}^{RPS64} reporter (Fig. 4D–F). These results indicate that FOXO nuclear activity promotes \textit{fng} expression in niche cap cells. As expected, cap cells were also reduced in flies over-expressing \textit{dfoxo-A3}, as compared to controls (Fig. S6). Our results indicate that dFOXO activates \textit{fng} transcription in the GSC niche, thereby resulting in a decrease of GSCs and cap cells. Over-expression of mouse \textit{foxo}1 also increased \textit{fng} expression in fly cap cells (Fig. S7), implying that mammalian FOXO1 may have a similar role.

**FOXO mediates up-regulation of \textit{fng} in the GSC niche when insulin signaling is low**

To further examine if endogenous FOXO mediates the increase in \textit{fng} expression when insulin signaling is low, we disrupted FOXO function in \textit{dim}^{19}/\textit{dim}^{339} mutants and examined \textit{fng} expression in cap cells using \textit{fng}^{3SUZ-1} (Fig. 5). As we previously observed, \textit{fng}^{3SUZ-1} expression in cap cells was higher in \textit{dim}^{19}/\textit{dim}^{339} mutants than in \textit{dim}^{19}/\textit{TMS} controls (85.1 ± 4.6 (n = 99) vs. 40.1 ± 2.6 (n = 85) arbitrary units, respectively, P = 1.5 × 10^{-14}). This increase in \textit{fng}^{3SUZ-1} expression was suppressed in \textit{foxo}25\textit{dim}^{19}/\textit{foxo21dim}^{339} mutants, in which FOXO function is disrupted (34 ± 2.2 arbitrary units, n = 91). Similar results were obtained using \textit{fng}^{RPS64} as a reporter (Fig. S8). Therefore, inactivation of insulin signaling leads to increased FOXO nuclear activity, which enhances \textit{fng} transcription in the GSC niche.

**Over-expression of \textit{fng} disrupts Notch activation in the GSC niche, thereby decreasing GSCs and cap cells**

Over-expression of \textit{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 × 10^{-18}) (Fig. 6A–C). The numbers of GSCs and cap cells were similar in newly eclosed control and \textit{fng}-over-expressing flies raised at 18 °C, but significantly decreased in \textit{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 \textit{fng} expression.

**FOXO stimulates \textit{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 \textit{fng} expression in the adult GSC niche of \textit{dim}^{19}/\textit{dim}^{339} mutants, which have elevated FOXO nuclear activity. We then examined Notch signaling using the \textit{E(spl)m7-lacZ} transgene (Fig. 7A–D). One week after the switch to 29 °C, expression of \textit{lacZ} was significantly reduced in \textit{dim}^{19}/\textit{dim}^{339} mutants (Fig. 7A) while \textit{fng} expression was not significantly altered relative to controls (Fig. 7B). One week after the switch to 29 °C, 

\[ \text{expression} = (\text{control} - \text{mutant}) / \text{control} \]

**Fig. 5.** dFOXO mediates up-regulation of \textit{fng} expression in cap cells of \textit{dim} mutants. (A)–(C) One-week (1W)-old control (Ctrl) (A), \textit{dim}^{19}/\textit{dim}^{339} (B) and \textit{foxo25/dim}^{19} \textit{foxo21/dim}^{339} germaria (C) labeled with \textit{1B1} (red, fusomes), \textit{LamC} (red, terminal filament (TF) and cap cell nuclear envelopes), and \textit{β}-gal (green, \textit{fng}^{3SUZ-1} fringe reporter). (A')–(C') are \textit{β}-gal signals. Arrows indicate cap cells. Scale bar, 10 μm. (D) Average \textit{fng}^{3SUZ-1} intensity in cap cells of 1W-old sibling ctrl, \textit{dim}^{19}/\textit{dim}^{339}, and \textit{foxo25/dim}^{19} \textit{foxo21/dim}^{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 \textit{dim}^{19}/\textit{fng}^{3SUZ-1}/\textit{TMS} 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 dinrE4339 cap cells (48.9 ± 1.9 arbitrary units, n = 96). Notch activity in the cap cells of fng knock-down dinrE4339 mutants was 71.5% that of controls. This partial rescue may be due to other factors involved in this regulatory process; uneven expression of fngRNAi (Fig. S1). Furthermore, we found that knockdown of fng in dinrE4339 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 chico1 or dinrE4339/dinr339 upon removing one functional copy of fng (Tables S1 and S2). This indicates that fng is upregulated 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. 5B). We identified a putative FOXO responsive element (FRE, TT(G/A)TGTAT) 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 *euca-
yotic initiation factor 4E-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-IF4 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. 5C). 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-
Recent studies have 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-acetylgalactosamine 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. 59). These results indicate...
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 \(\beta\)-D-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 *mfoxo1* 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 *igf1a* or *igf1b* results in a dramatic decrease in the expression of the Notch downstream target *her4* 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 (Fuiuza 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 DI-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.

Acknowledgements


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.

References


