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STEM CELLS AND REGENERATION

RESEARCH ARTICLE

Diet regulates membrane extension and survival of niche escort cells for germline homeostasis via insulin signaling

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ABSTRACT

Diet is an important regulator of stem cell homeostasis; however, the underlying mechanisms of this regulation are not fully known. Here, we report that insulin signaling mediates dietary maintenance of *Drosophila* ovarian germline stem cells (GSCs) by promoting the extension of niche escort cell (EC) membranes to wrap around GSCs. This wrapping may facilitate the delivery of bone morphogenetic protein stemness factors from ECs in the niche to GSCs. In addition to the effects on GSCs, insulin signaling-mediated regulation of EC number and protrusions controls the division and growth of GSC progeny. The effects of insulin signaling on EC membrane extension are, at least in part, driven by enhanced translation of Failed axon connections (Fax) via Ribosomal protein S6 kinase. Fax is a membrane protein that may participate in Abelson tyrosine kinase-regulated cytoskeletal dynamics and is known to be involved in axon bundle formation. Therefore, we conclude that dietary cues stimulate insulin signaling in the niche to regulate EC cellular structure, probably via Fax-dependent cytoskeleton remodeling. This mechanism enhances intercellular contact and facilitates homeostatic interactions between somatic and germline cells in response to diet.

KEY WORDS: Insulin/IGF, PI3K, S6K, Fax, Failed axon connections, Escort cell, GSC, Niche

INTRODUCTION

Germline stem cells (GSCs) and other germ cells require proper interactions with surrounding somatic cells for maintenance, growth and differentiation (Ables et al., 2012; Laws and Drummond-Barbosa, 2016). Like other stem cells, GSCs reside in a specialized microenvironment, called a niche, which supplies diffusible factors and physical contacts to control stem cell identity (Jones and Wagers, 2008). Both stem cells and niche cells may be regulated by environmental cues that affect systemic factors to influence stem cell physiology. For example, diet is known to control GSC growth and differentiation (Ables et al., 2012), but whether and how diet affects soma-germline interactions, including GSC-niche interactions, remains unclear.

The *Drosophila* ovary is an excellent model for studying the effects of diet on stem cells, because GSCs quickly respond to diet (Drummond-Barbosa and Spradling, 2001) and both GSCs and niche cells are well characterized (Fig. 1A). An ovary is composed of 15-20 egg-producing functional units, called ovarioles (Spradling, 1993). In each ovariole, the anterior-most structure is the germarium, which is subdivided into regions 1, 2a, 2b and 3. Region 1 contains two or three GSCs that directly contact cap cells, which comprise the majority of the GSC niche and provide both physical (Song and Xie, 2002) and Dpp/BMP signals to maintain GSC identity (Xie and Spradling, 1998). Each GSC contains a fusome, an organelle that is juxtaposed to the GSC-cap cell interface (Xie and Spradling, 2000). Asymmetric GSC division produces a cystoblast, which undergoes four rounds of incomplete division in region 2a to form a 16-cell cyst (Kirilly and Xie, 2007). In region 2b, the 16-cell cyst acquires a layer of follicle cells, and then the entire structure buds off from the germarium to become an egg chamber (region 3) that, eventually, develops into an egg (Spradling, 1993). Importantly, escort cells (ECs) populate the lateral parts of regions 1 and 2a, occupying the spaces between cap cells and follicle cells with their cell bodies and long, germ cell-wrapping membranous protrusions (Kirilly et al., 2011). The anterior-most ECs, which are in direct contact with GSCs, are considered to be niche components and contribute to GSC maintenance (Chen et al., 2011). Meanwhile, each germ cell cyst is wrapped and encapsulated by multiple posterior ECs (Sahai-Hernandez and Nystul, 2013), which ensure the differentiation of GSC progeny (Eliazer et al., 2014; Kirilly et al., 2011).

The insulin/insulin-like growth factor pathway is highly conserved and controls processes linked to nutrient sensing (Goberdhan and Wilson, 2003; Hafen, 2004). In *Drosophila*, the binding of insulin-like peptides to the insulin receptor (encoded by *dInR*) causes the phosphorylation of the insulin receptor substrate homolog (encoded by *chico*), which activates phosphoinositide 3-kinase (PI3K) and other downstream components of the insulin signaling pathway. Further downstream effects include the cytoplasmic retention of Foxo, a transcription factor that negatively regulates insulin signaling (Oldham and Hafen, 2003), and activation of Ribosomal protein S6 kinase (S6K) to promote protein synthesis (Nagarajan and Grewal, 2014). It has previously been reported that insulin signaling through Foxo mediates dietary support of GSCs by maintaining niche cap cells (Hsu and Drummond-Barbosa, 2009, 2011); however, the effects of diet and/or insulin signaling on niche ECs have not been reported.

Here, we show that diet reversibly controls germ cell-EC interactions via insulin signaling. After being fed a protein-poor diet, flies exhibit fewer ECs and incomplete wrapping of germ cell

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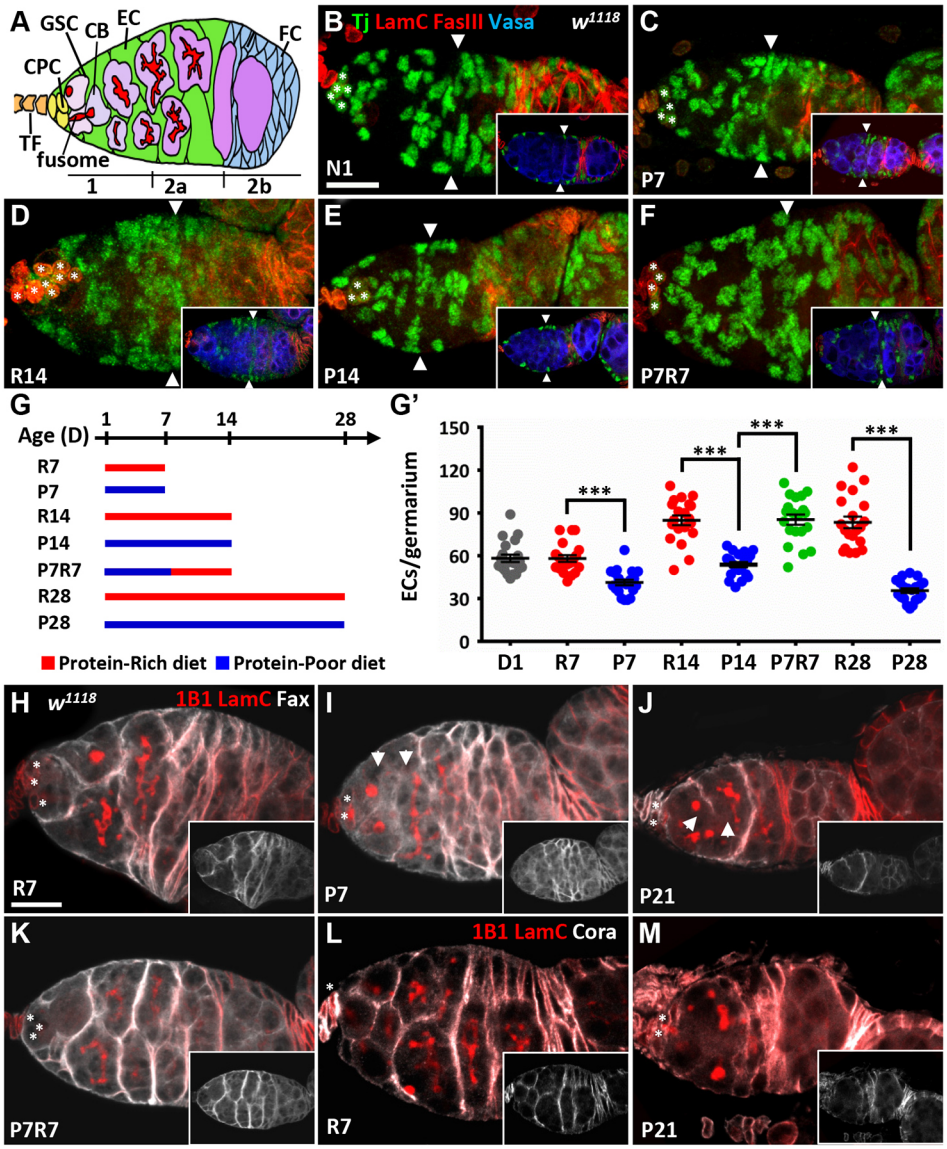

Fig. 1. Diet exerts reversible control over EC number and membrane extension. (A) In the *Drosophila* germarium, terminal filament (TF) cells, cap cells (CPCs) and escort cells (ECs) make up the germline stem cell (GSC) niche. Each GSC division produces a cystoblast (CB), which matures to become a 16-cell cyst. Mitotic germ cells reside in region 1, whereas the post-mitotic 16-cell cyst passes through region 2a and is enveloped by follicle cells (FCs) in region 2b. (B-F) 3D reconstructions of wild-type (w^{1118}) germaria of control 1-day-old (D1) flies (B), flies maintained on a protein-poor diet for 7 days (P7) (C), flies on a protein-rich diet for 14 days (R14) (D), flies on a protein-poor diet for 14 days (P14) (E) and flies on a protein-poor diet for 7 days that were switched to a protein-rich diet for an additional 7 days (P7R7) (F). Germaria were labeled for Tj (green: CPC, EC and FC nuclei), LamC (red: TF and CPC nuclear envelopes) and FasIII (red: FC membranes). Asterisks indicate CPCs. Insets show the largest longitudinal section of germaria with Tj, LamC, FasIII and Vasa (blue: germ cells) staining. Arrowheads indicate the 2a/2b boundary. Scale bar: 10 µm. (G) The feeding scheme: newly eclosed flies were maintained on standard medium for 1 day, and then cultured on either a protein-rich diet (standard medium plus a wet yeast paste) or a protein-poor diet (5% molasses) for the indicated times. (G′) Number of ECs in w^{1118} germaria from flies on different diets. ***P<0.001. Data are means±s.e.m. (H-M) Germaria from wild-type flies on a 7-day protein-rich diet (H,L), a 7-day protein-poor diet (I), a 7-day protein-poor diet switched to a 7-day protein-rich diet (J) and a 21-day protein-poor diet (K,M). Germaria were labeled for 1B1 (red: fusomes), LamC (red), Fax (H-K, gray: EC membranes) and Cora (L,M; gray: EC membranes). Asterisks indicate CPCs. Arrows point to the incomplete wrapping of germ cell cysts by ECs. Scale bar: 10 µm.

cysts. Similar phenomena can be observed in flies carrying insulin signaling-defective ECs. Interestingly, re-feeding or overexpression of insulin signaling components in starved flies restores these defects. We also report that insulin signaling in ECs maintains cell survival and stimulates membrane extension. The formation of EC protrusions is, at least in part, mediated by Failed axon connections (Fax), a membrane protein that was previously identified in a screen for diet-regulated proteins in the *Drosophila* ovary (Hsu and Drummond-Barbosa, 2017). In response to diet, insulin signaling via S6K enhances Fax expression, and knockdown of *fax* results in shortened EC protrusions and GSC loss, without changing the EC number. Our results are the first to show that insulin signaling modulates the appearance of cellular protrusions *in vivo* and that such modulation is required for GSC maintenance and soma-germline interactions.

RESULTS

Diet controls EC number and germ cell wrapping

ECs wrap germ cells with cellular extensions (Kirilly et al., 2011), creating a close physical association. In order to evaluate whether this interaction may be affected by diet, we first examined the number of ECs in flies cultured on either a protein-rich or a protein-poor diet (Fig. 1B-G′; Table S1). ECs were identified as cells that are positioned anterior to the 2a/2b boundary in the germarium (Fig. 1A) and stain positive for Traffic jam (Tj) (cap cells, ECs and follicle cell lineage) but negative for both LamC (cap cells) and FasIII (follicle cell lineage) (Lia et al., 2017). Newly eclosed (D1) and 7-day-old flies cultured on a protein-rich diet (R7) carried similar numbers of ECs (Fig. 1G′). However, 7-day-old flies fed with a protein-poor diet (P7) exhibited a 30% reduction in ECs compared with R7 flies (Fig. 1G′). Although 14- and 28-day-old flies on a protein-rich diet exhibited a 1.5-fold increase in ECs compared with newly eclosed or R7 flies, 14- and 28-day-old flies on a protein-poor diet showed a 37% or 58% reduction in EC number compared with control R14 or R28 flies (Fig. 1G′). These results clearly demonstrate that diet affects EC number. Interestingly, the number of ECs in newly eclosed flies that were fed first with a protein-poor diet for 7 days and then were switched to a protein-rich diet for 7 days was similar to that of R14 flies, indicating that the dietary effects on EC number are reversible. We also observed that diet affected the wrapping of cysts by ECs. In well-fed flies (Fig. 1H), each cyst was encased by ECs with long

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membrane extensions that were marked by Fax, a membrane protein expressed in ECs (Buszczak et al., 2007; Decotto and Spradling, 2005). However, flies kept on a protein-poor diet for 7 days showed incomplete separation between cysts (Fig. 1I). This phenomenon became more pronounced when flies were starved for 21 days (Fig. 1J), and could be completely restored when flies that had been starved for 7 days were switched to a protein-rich diet for 7 days (Fig. 1K). We confirmed the effect of diet on EC membrane extension using the cell junction marker Coracle (Cora), which is expressed in both cap cells and ECs (Fig. 1L,M) (Maimon et al., 2014). Together, our results show that diet affects EC number and membrane protrusions, thus modulating the interaction between ECs and germ cells.

Insulin signaling mediates dietary control of EC survival and germ cell wrapping

Insulin-PI3K signaling is a highly conserved mediator of dietary effects (Britton et al., 2002; Hietakangas and Cohen, 2009; Shim et al., 2013). To assess whether insulin-PI3K signaling modulates dietary effects on ECs, we used the *c587-GAL4* driver to overexpress the wild-type insulin receptor, $dInR^{WT}$, or a constitutively active form of the PI3K catalytic subunit, $dp110^{CAAX}$ (Leevers et al., 1996), in ECs. We then examined the EC number of these flies when kept on a protein-poor diet (Fig. 2A-D; Table S1). To address whether insulin signaling functions in adult ECs, we raised flies at 18°C and transferred newly eclosed flies to 29°C, enabling GAL4-driven expression of the transgene for 1 or 2 weeks. At eclosion (D0), *c587>gfp* and $c587>dInR^{WT}$ flies exhibited a comparable number of ECs, whereas $c587>dp110^{CAAX}$ flies exhibited a trend (P=0.08) towards a higher number of ECs (Fig. 2D). Consistent with our previous results, diet affected EC number in *c587>gfp* flies. However, flies on a protein-poor diet only showed reduced EC number after 14 days of starvation (Fig. 2D). The prolonged time to observe the effect may have been caused by temperature effects. Importantly, overexpression of $dInR^{WT}$ or $dp110^{CAAX}$ in ECs of starved flies partially prevented EC reduction (Fig. 2D) and restored incomplete EC-germ cell wrapping (Fig. 2F-H). Thus, insulin-PI3K signaling in ECs functionally responds to dietary protein content.

Next, we suppressed expression of *dInR* in adult ECs with an *RNAi* line driven by *c587-GAL4*. One week after knockdown, $c587>dInR^{RNAi}$ germaria were shorter and exhibited a significantly lower number of ECs compared with *GAL4* and *UAS* controls (Fig. 2E,I,J). Because ECs are slow-cycling cells, we used the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay to examine whether EC reductions in $c587>dInR^{RNAi}$ germaria were due to cell death. In 1-week-old *GAL4* controls (Fig. 2K), 26.3% of germaria (n=19) exhibited dying cells, whereas 35% of $c587>dInR^{RNAi}$ germaria (n=77) contained dying cells. Interestingly, the dying cells in control samples consisted of one or two dying somatic cells (ECs or pre-follicle cells) located near the 2a/2b boundary. However, $c587>dInR^{RNAi}$ germaria exhibited a larger number of dying cells and, in addition to those at the 2a/2b boundary, other dying ECs were marked by Fax and lacked a uniform location (Fig. 2L). Some of the dying cells in $c587>dInR^{RNAi}$ germaria did not express Fax, perhaps reflecting that Fax had been degraded or that they were germ cells (yellow arrow in Fig. 2L). Similar to starved flies, $c587>dInR^{RNAi}$ flies exhibited incomplete wrapping of germ cell cysts by ECs (Fig. 2L,N). These results suggest that insulin signaling controls EC maintenance and EC-germ cell interactions in a cell-autonomous manner.

Insulin signaling controls EC membrane extension for germ cell wrapping

ECs wrap around GSCs and their progeny with long cellular processes (Decotto and Spradling, 2005; Kirilly et al., 2011). To further explore how insulin signaling controls membrane extension in ECs, we generated *dInR*-knockdown (KD) EC clones (identified by the presence of GFP) in newly eclosed flies by applying a 30 min heat shock to activate *flip-out GAL4* (*actin promoter-FRT-CD2-FRT-GAL4*) (Fig. 3A). We then examined EC morphology in 1-week-old control germaria (Fig. 3B-B″) and found that the most-anterior EC, which directly contacts GSCs, had a short and simple membrane protrusion that wraps around the GSC (the longest protrusion: 3.95±1.7 µm, n=13). The membrane processes of individual ECs were increasingly longer and more elaborated as germ cell cysts became more developed, with the protrusions eventually encasing each germ cell cyst (Fig. 3C-E″). Thus, we confirmed the findings of a previous report showing that ECs at different locations within the germarium have distinct morphological characteristics, which correspond to the status of the germ cell cyst (Kirilly et al., 2011). Strikingly, *dInR*-KD ECs displayed blunted extension of membrane processes (Fig. 3F-I″), although such effects were milder in ECs that encased lens-shaped 16-cell cysts near the 2a/2b boundary (Fig. 3I-I″). We were unable to measure protrusion length in the most-anterior *dInR*-KD ECs, as the cells were triangular with no obvious protrusions (n=9) (Fig. 3F). These results suggest that insulin signaling autonomously promotes EC membrane extension to regulate EC-germ cell interaction in response to diet.

Insulin signaling-defective ECs cause GSC loss and delay in germ cell growth

To study the effect of insulin signaling on adult ECs, we examined GSC numbers in $c587>dInR^{RNAi}$ germaria of newly eclosed 1- and 2-week-old flies (Fig. 4A-C; Table S2). Notably, the GSC number in newly eclosed $c587>dInR^{RNAi}$ flies grown at 18°C was similar to that of day 1 *GAL80ts bab1-GAL4>*$dInR^{RNAi}$ and *GAL80ts bab1-GAL4/+* control flies grown at 18°C, suggesting that, if there is any RNAi expression during development, it does not affect GSCs. Two weeks after eclosion, most GSCs were retained in control germaria, whereas only 64% of GSCs remained in $c587>dInR^{RNAi}$ germaria. We obtained a similar result when *dInR* expression was suppressed by another EC driver, *ptc-GAL4* (Fig. 4C; Table S2). Knockdown of *dInR* in ECs did not affect niche cap cells (Fig. S1, Table S3), which are the major GSC niche constituents (Kirilly and Xie, 2007). Our results suggest that ECs directly receive insulin signals to maintain GSCs. To test whether insulin signaling in ECs influences germ cell division, we examined 5-ethynyl-2′-deoxyuridine (EdU) incorporation in control ovaries and those carrying *dInR*-KD ECs (Fig. 4D,E). In control germaria, 8.5±1.7% of GSCs (n=593) were EdU positive, whereas only 2.1±1.9% of GSCs (n=440, P<0.001) in $c587>dInR^{RNAi}$ germaria were positive (Fig. 4F). Control germaria (n=20) had 2.5±0.3 germ cell cysts that were positive for EdU labeling, in contrast to 1.1±0.3 positive germ cells in $c587>dInR^{RNAi}$ germaria (n=28, P<0.001; Fig. 4F′). Furthermore, in the control germaria (Fig. 4G), there were several germ cell cysts and a stage 1 egg chamber (arrow in Fig. 4G), which would bud off from the germarium and become a stage 2 egg chamber (arrowhead in Fig. 4G). In contrast, $c587>dInR^{RNAi}$ germaria were usually smaller with fewer germ cell cysts. These germaria also lacked a stage 1 egg chamber, but contained a stage 4 egg chamber (asterisks in Fig. 4G,H) that was identified by the five-blob phenotype of nurse-cell DNA (Jia et al., 2016). These results reflect slow division

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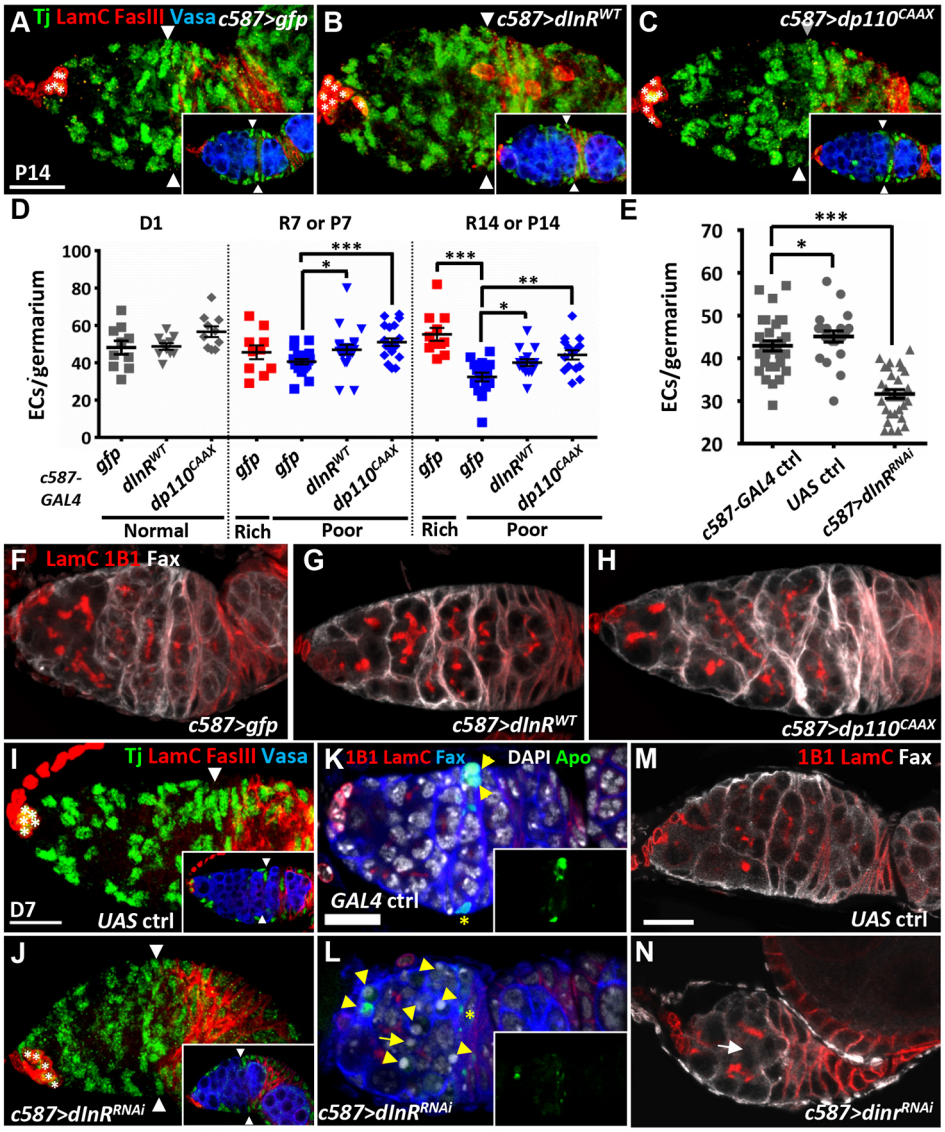

Fig. 2. Diet controls ECs via insulin signaling. (A-C) Germaria of 1-day-old (D1) *c587>gfp* (A), *c587>dInR^{WT}* (B) and *c587>dp110^{CAAX}* (C) flies on a protein-poor diet for 14 days (P14). White arrowheads indicate the 2a/2b boundary. Germaria were labeled for Tj (green: CPC, EC and FC nuclei), LamC (red: TF and CPC nuclear envelopes) and FasIII (red: FC membranes). (D) Number of ECs in germaria from 1-day-old *c587>gfp*, *c587>dInR^{WT}* and *c587>dp110^{CAAX}* flies that were fed for 7 days or 14 days with protein-rich or protein-poor diets. (E) Number of ECs in 7-day-old control and *c587>dInR^{RNAi}* germaria. **P*<0.05, ***P*<0.01, ****P*<0.001. Data are means±s.e.m. (F-H) Germaria of 1-day-old *c587>gfp* (F), *c587>dInR^{WT}* (G) and *c587>dp110^{CAAX}* (H) flies on a protein-poor diet for 14 days. Germaria were labeled for 1B1 (red: fusomes), LamC (red: TF and CPC nuclear envelopes) and Fax (gray: EC membranes). (I,J) 7-day-old *UAS* control (I) and *c587>dInR^{RNAi}* (J) germaria with Tj (green), LamC (red) and FasIII (red) staining. (K,L) 7-day-old *GAL4* control (K) and *c587>dInR^{RNAi}* (L) germaria labeled for 1B1 (red), LamC (red) and Fax (blue: EC membranes), and with DAPI (gray: DNA) and ApoTag (green: apoptotic cells). Yellow arrowheads indicate dead cells. Yellow asterisks indicate non-specific signals. Yellow arrows indicate apoptotic cells with Fax expression. (M,N) 7-day-old *UAS* control (M) and *c587>dInR^{RNAi}* (N) germaria with 1B1 (red), LamC (red) and Fax (gray) staining. Germ cell cysts are incompletely wrapped by ECs. Insets in I and J show the largest longitudinal section of germaria with Tj, LamC, FasIII and Vasa (blue: germ cells) staining. Insets in K and L show the ApoTag channel alone; white arrows indicate incomplete separation of cysts and white asterisks indicate cap cells. Scale bars: 10 µm (bar in M applies to F-H,M,N; bar in I applies to I,J; bar in K applies to K,L). The genotype of the *UAS* control in E, I and M is *UAS-dInr^{RNAi}*/+; the genotype of the *GAL4* control in E and K is *c587-GAL4*/+.

and growth of germ cells when they are associated with insulin signaling-defective ECs.

Fax expression in ECs is controlled by insulin signaling through S6K

Failed axon connections (encoded by *fax*) is a highly conserved membrane protein (Fig. S2) that functions in axon development during embryogenesis (Hill et al., 1995; Liebl et al., 2000), but its role in the ovary is unknown. Fax-GFP (YC0036) carries a GFP artificial exon in the first intron of the *fax* gene, creating an in-frame GFP-Fax fusion (Fig. S2) (Buszczak et al., 2007). Interestingly, Fax-GFP is expressed in ECs (Buszczak et al., 2007; Decotto and Spradling, 2005), and its expression is affected by diet within 24 h (Hsu and Drummond-Barbosa, 2017).

Because little information is available about the *fax* gene, we first performed an *in silico* analysis. From the NCBI database, we found that the *fax* gene is located on the left arm of the third chromosome and contains seven exons and six introns, spanning around 9 kb (from nucleotide 16,395,896 to 16,404,939) of the genome (Fig. S2A). Information obtained from FlyBase showed that the

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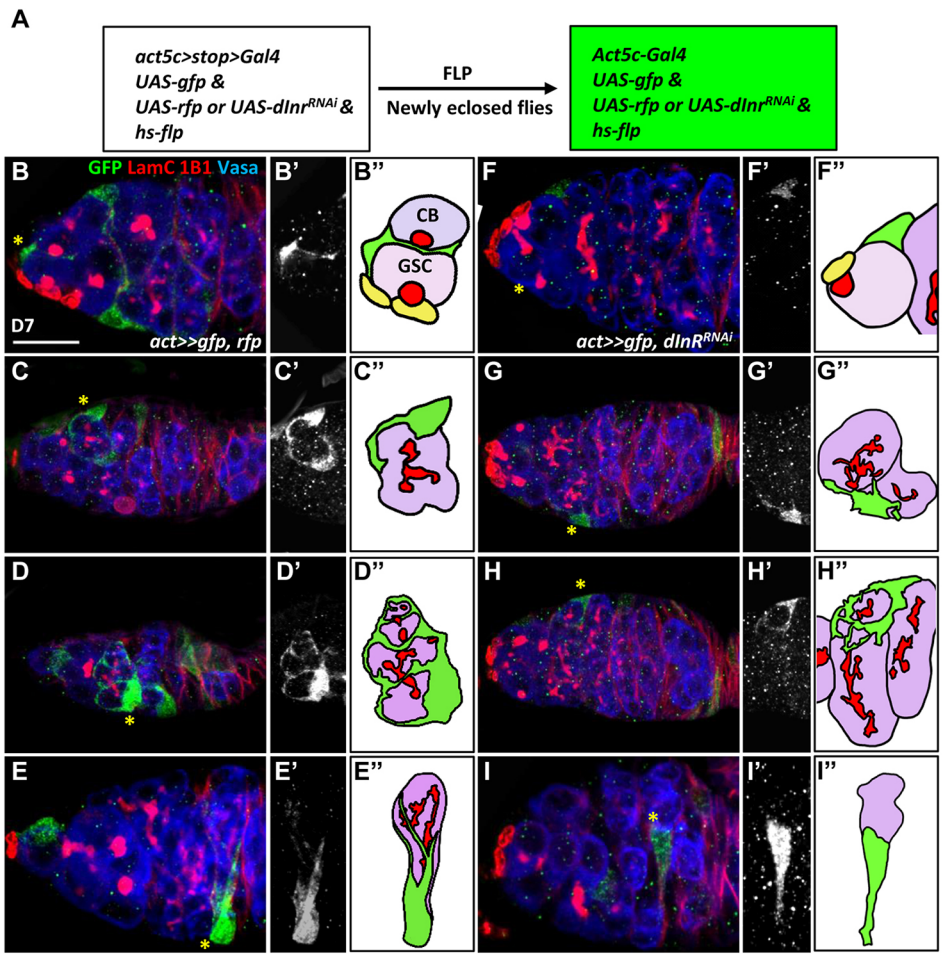

Fig. 3. Insulin signaling in ECs controls EC membrane extension. (A) Schematic diagram for the generation of GFP^{+}RFP^{+} or GFP^{+}*dInR*-KD cells. In females carrying an *actin* promoter-driven *FRT*-flanked flip-in *GAL4* construct (*act5C>STOP>GAL4*), GAL4 is not expressed, preventing expression of *UAS* transgenes. GAL4 expression is turned on by removing the stop cassette through Flipase-mediated recombination, which, in turn, activates expression of *UAS* transgenes. (B-I) 3D reconstructions of 7-day-old (D7) control mock (B-E) and *dInR*-KD mosaic germaria (F-I) with LamC (red: CPC nuclear envelopes), 1B1 (red: fusomes), Vasa (blue: germ cells) and GFP (green: flip-out clones) staining to show membrane extension of ECs marked by asterisks. (B′-I′) Membrane extensions of ECs are marked by asterisks in B-I with GFP channel alone shown in gray. (B″-I″) Schematic diagrams of asterisk-marked ECs in B-I and their association with cap cells (yellow), GSCs, cystoblasts (CB) and germ cell cysts. Red indicates fusomes. Green in B′-I′ indicates asterisk-marked ECs in B-I; purple in C′-I′ indicates germ cell cysts. Scale bar: 10 µm.

fax gene produces six transcript variants, A to F, from three different transcriptional start sites (Fig. S2A,B). *fax* A/C, *fax* B/D and *fax* E/F have transcriptional start sites located on exons 1, 2 and 3, respectively; each of them is then spliced and fused with exon 4, followed by exons 5, 6 and 7. Compared with *fax* C, D and E, *fax* A, B and F transcript variants include an additional nine bases in exon 5, which encodes three amino acids: Trp, Phe and Gln (Fig. 2B′). Fax GST-N and GST-C domains share ∼64% and 55% similarity with Fax orthologs in vertebrate FAXC (including human, mouse, rat and zebrafish) (Fig. S2C). Moreover, *Drosophila* Fax lacks a transmembrane domain that is present in vertebrate FAXC proteins (red boxes in Fig. S2C). The significance of this missing domain is unknown, as no functional analysis has been reported. To examine the evolutionary relationships of the *Drosophila* Fax genes with those from other species, we built a phylogenetic tree using mRNA sequence alignments of nine selected Fax sequences (Fig. S2D). The *Drosophila fax* gene was grouped with its ortholog in *Caenorhabditis elegans*, but was separated from other species. These results indicate that Fax is present and conserved from worms and flies to mice and humans.

Although the *fax* gene is predicted to produce six mRNA isoforms, we wanted to know which of these was most highly expressed in ovarian ECs. We performed qRT-PCR on ovarian tissue, using primer pairs located on the first, second and third exon to distinguish between *fax* A/C, B/D and E/F. The results showed that *fax* A/C transcripts were 22-fold higher than those of *fax* B/D and *fax* E/F transcripts, which were below the limit of detection (Fig. S2E). This result suggests that *fax* A and/or C are predominately expressed in the ovary. These two variants encode Fax A (418 amino acids) and Fax C (415 amino acids), respectively. To validate the expression of Fax-GFP in the YC0036 line, we extracted lysates from wild-type *faxCRISPR* (a genetic null mutant; see details in the Materials and Methods), *faxM7* (a 92-amino acid deletion mutant in the GST-C domain) (Hill et al., 1995) and YC0036 ovaries, and performed western blots using antibodies against Fax (Fig. S2A,F). Fax was present in wild-type (∼63 kDa) and was larger than its predicted size (47 kDa), whereas Fax protein in the *faxCRISP* mutants was not detected. Truncated Fax was weakly present in *faxM7* mutants, which is in agreement with a previous report (Hill et al., 1995) and demonstrates the specificity of the anti-Fax antibody. In YC0036 flies, Fax was detected at a greater size compared with wild-type Fax, as it was fused with GFP (Fig. S2F). Because the GFP exon was inserted into the first intron of the *fax* gene, we conclude that the GFP-containing fusion protein expressed in YC0036 flies represents Fax A/C, which is predominantly expressed in the ovary.

Because we suspected that Fax mediates the observed insulin signaling effects, we first confirmed that diet decreases Fax-GFP expression in ECs (Hsu and Drummond-Barbosa, 2017). Fax-GFP signal was strong in ECs of flies that were fed with a protein-rich diet for 6 days (R6) (Fig. 5A), but in starved flies (R2P4: protein-rich diet for 2 days, then a protein-poor diet for 4 days) it was reduced to 37% of control levels (Fig. 5B,D). Interestingly, starved flies that were later fed with a protein-rich diet (R2P2R2: protein-rich diet for 2 days, followed by a protein-poor diet for 2 days and a protein-rich diet for 2 days) exhibited levels of Fax-GFP that were similar to those of R6 flies (Fig. 5A,C,D). These results indicate that a

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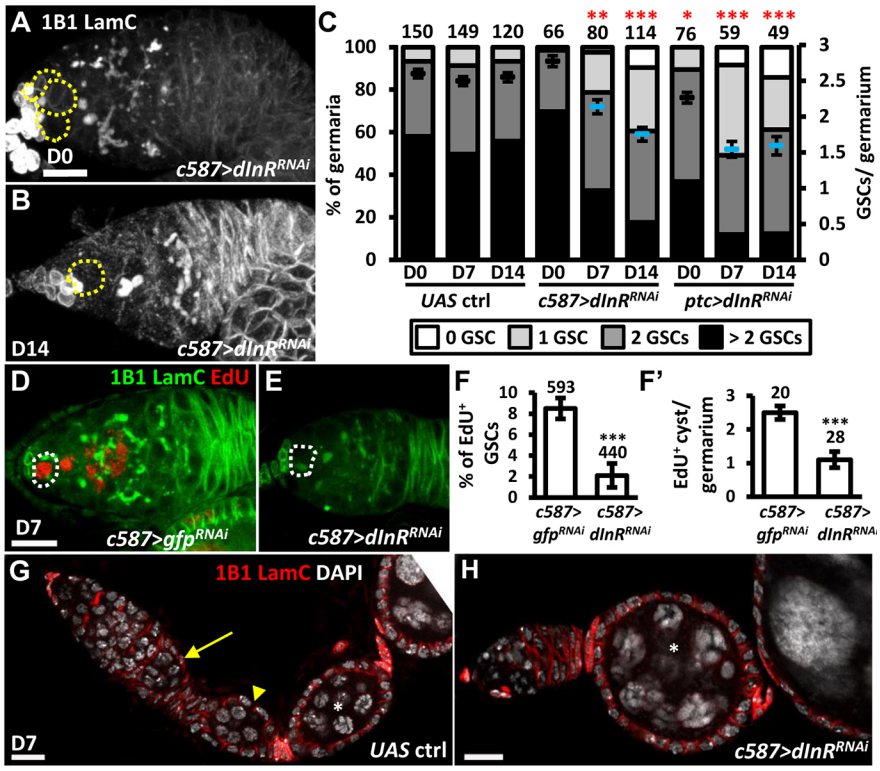

Fig. 4. Knockdown of *dInR* in ECs results in loss of GSCs, decreased proliferation and delayed growth of germ cell cysts. (A,B) Newly eclosed (D0) (A) and D14 *c587>dInR*^{RNAi} (B) germaria with 1B1 (gray: fusomes) and LamC (gray: TF and CPC nuclear envelopes) staining. Dashed circles outline GSCs. (C) Number of GSCs in newly eclosed (D0), 7-day-old (D7) and 14-day-old *UAS* control (ctrl), *c587>dInR*^{RNAi} and *ptc>dInR*^{RNAi} germaria. A blue line for the mean value indicates that the number of GSCs is significantly lower than in the D0 within the same genotype. Asterisks indicate significant differences between the knockdown groups and *UAS* controls. (D,E) 7-day-old *c587>gfp*^{RNAi} and *c587>dInR*^{RNAi} germaria with 1B1 (green), LamC (green) and EdU (red: proliferation marker) staining. Dashed circles outline GSCs. (F,F′) Average number of EdU-positive (EdU^{+}) GSCs (F) or germ cell cysts (F′) in 7-day-old *c587>gfp*^{RNAi} and *c587>dInR*^{RNAi} germaria. (G,H) 7-day-old *UAS* control (G) and *c587>dInR*^{RNAi} (H) germaria with 1B1 (red), LamC (red) and DAPI (gray, DNA) staining. The arrow points to a stage 1 egg chamber; the arrowhead indicates a stage 2 egg chamber; asterisks indicate stage 4 egg chambers, which are identified by the five-blob structure of nurse-cell DNA. The number of germaria analyzed is shown above each bar. *P<0.05, **P<0.01, ***P<0.001. Data are means±s.e.m. Scale bars: 10 µm. The genotype of the control in A and G is *UAS-dInR*^{RNAi}/+.

protein-rich diet maintains high levels of Fax protein in ECs, and Fax-GFP level responds to diet in as few as 2 days. We further examined *fax* A/C mRNA levels by pRT-PCR in ovaries of flies on different diets. Our results showed that diets do not affect *fax* A/C mRNA expression levels in the ovary (Fig. 5E), indicating that dietary effects on Fax protein are at a post-transcriptional level.

We then investigated whether insulin signaling modulates the diet-induced alterations in Fax abundance. Fax-GFP was expressed in transheterozygous Insulin receptor mutant (*dInR*^{E19}/*dInR*^{339}) and Insulin receptor substrate mutant (*chico*^{1}) flies. We examined GFP signals in ECs after maintaining the flies on the R6 diet. Strikingly, Fax-GFP was almost completely absent in the ECs of *dInR*^{E19}/*dInR*^{339} mutants (Fig. 5F-H), and was found at only 18% of control levels in *chico*^{1} mutants (Fig. 5F,I). We obtained similar results in ECs of *ptc>dInR*^{RNAi} flies using anti-Fax antibody (Fig. 5J-L). To verify that the overall reduction of Fax expression in ECs of *dInR*, *chico* mutant or *ptc>dInR*^{RNAi} flies was not simply due to the loss of posterior ECs, where Fax was strongly expressed (Fig. 5H,I,K), we used *flip-out GAL4* to generate *dInR*-KD EC clones (identified by the presence of GFP) in the adults. Because multiple EC membranes overlap to wrap a germ cell cyst, and membrane protrusions of anterior ECs are simpler than those of posterior ECs, we limited our analysis of Fax expression to germaria with *dInR*-KD ECs that were in direct contact with GSCs. In 1-week-old control germaria (Fig. 5M), GFP-positive ECs exhibited similar levels of Fax expression to contralateral GFP-negative ECs (arrow in Fig. 5M). Consistent with our previous results, *dInR*-KD (GFP-positive) ECs that were in contact with GSCs displayed blunted membrane extension and reduced Fax expression compared with GFP-negative control ECs (arrow in Fig. 5N). These results indicate that insulin signaling within ECs controls Fax expression.

To investigate the roles of insulin signaling effectors, we overexpressed dominant-negative S6K (S6K^{DN}) or constitutively active Foxo (FoxoA3) in ECs. Our results showed that *c587>S6K*^{DN}, but not *c587>foxoA3*, germaria exhibited decreased Fax expression and defective EC-germline wrapping (Fig. 5O-Q), with a coincident reduction in GSC number (Fig. S3, Table S2). Interestingly, the EC number in *dInR*^{E19}/*dInR*^{339} flies was rescued by removing Foxo (*dInR*^{E19}*foxo*^{21}/*dInR*^{339}*foxo*^{25}) (Fig. S4). Taken together, our results indicate that insulin/PI3K signaling promotes protein translation of Fax in ECs via S6K, and probably maintains EC survival by suppressing Foxo.

Fax functions in ECs to control GSC maintenance

We next asked whether Fax in ECs regulates GSCs and GSC progeny. We first knocked down Fax-GFP in adult ECs of the *YC0036* line with a *UAS-gfp*^{RNAi} line driven by *c587-GAL4*. In the *UAS* control, Fax-GFP was highly expressed in ECs (Fig. 6A). Conversely, Fax-GFP expression was abolished in ECs of *c587>gfp*^{RNAi} flies on the YC0036 background. In these flies, GFP was still detectable in cap cells and early germ cells (Fig. 6B,B′), demonstrating the efficiency of the *gfp*^{RNAi} line. In *UAS* or *GAL4* controls, GSCs were consistently retained in the niche for 2 weeks after eclosion, whereas we observed a significant loss of GSCs in *fax-gfp*-KD germaria at both time-points examined (Fig. 6E; Table S2). We also suppressed *fax* expression in adult ECs using *c587-GAL4* to drive a *UAS-fax*^{RNAi} line. Fax protein was significantly reduced in ECs of *c587>fax*^{RNAi} germaria (Fig. 6C,D,D′) and in ovarian tissue from global *fax*-KD flies (*actin>fax*^{RNAi}) compared with controls (Fig. S5A). Two weeks after eclosion, we also observed 30% loss of GSCs in *c587>fax*^{RNAi} germaria and no GSC loss in *UAS* controls (Fig. 6E; Table S2). A similar result was obtained using an independent *UAS-fax*^{RNAi} line (Fig. S5B-D, Table S2). In addition, cap cell number was not affected when *fax-gfp* or *fax* was knocked down in ECs (Fig. S6, Table S3). Further, disruption of *fax* expression during development did not affect GSC or cap cell numbers, and wrapping of germ cell cysts by ECs was only mildly disrupted (Fig. S7; Tables S2, S3).

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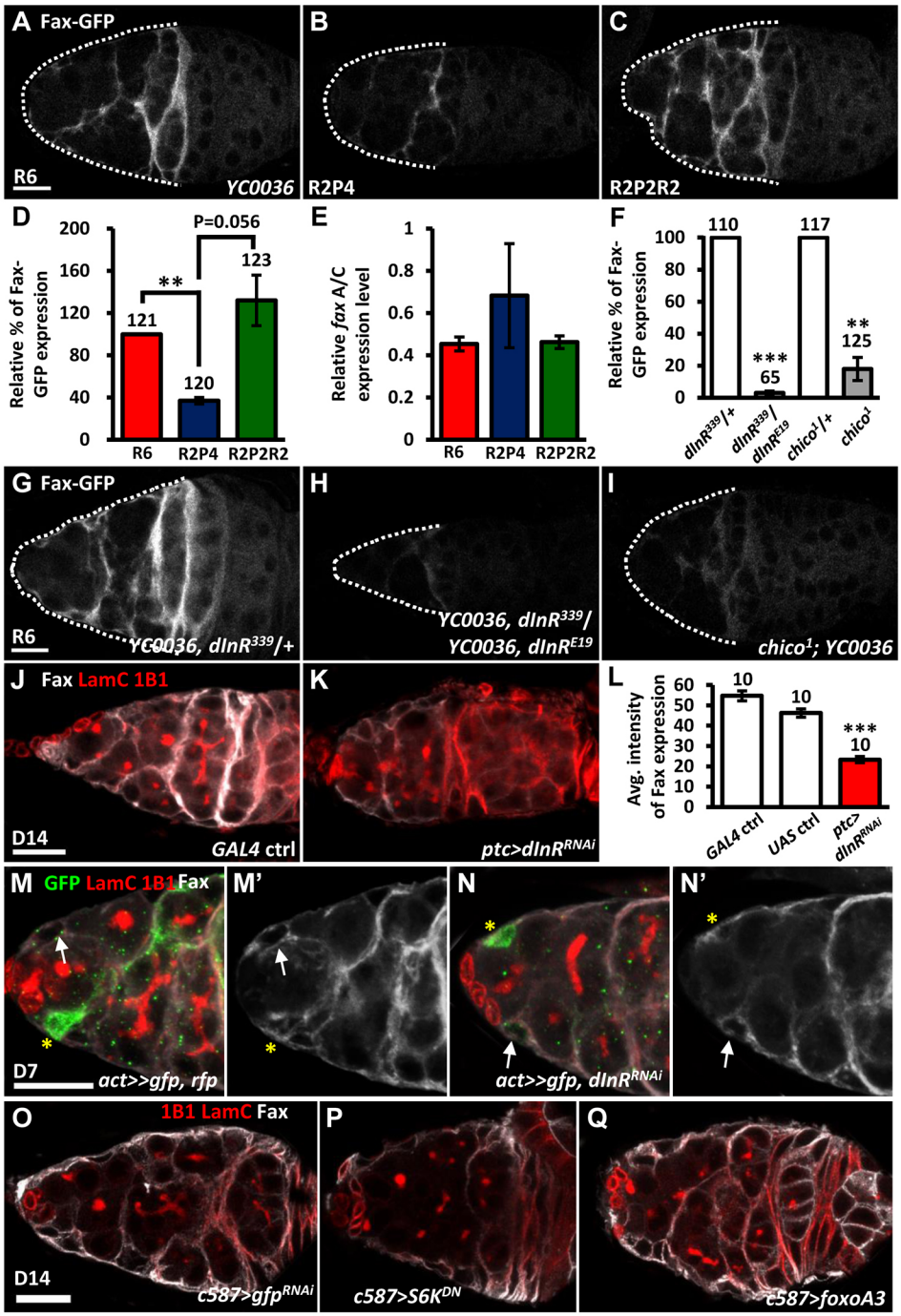

Fig. 5. Insulin signaling controls Fax expression in ECs in response to diet. (A-C) The expression of Fax-GFP in ECs of 8-day-old *YC0036* flies that were maintained on a protein-rich diet for 6 days (R6) (A), a protein-rich diet for 2 days followed by a protein-poor diet for 4 days (R2P4) (B), or a protein-rich diet for 2 days, a protein-poor diet for 2 days and a protein-rich diet for 2 days (R2P2R2) (C). (D) Expression of Fax-GFP in ECs of 8-day-old *YC0036* flies on R2P4 and R2P2R2 diets relative to flies on the R6 diet. (E) qRT-PCR quantification of *fax* A/C transcript levels in the ovaries of 8-day-old *YC0036* flies cultured under the three different diet conditions. The expression of *fax* A/C was normalized to *RPL19* (internal control). (F) Expression of Fax-GFP in ECs of 8-day-old $dlnR^{339}$/$dlnR^{E19}$ and $chico^{1}$ mutants relative to heterozygous controls. (G-I) The expression of Fax-GFP in ECs of 8-day-old $dlnR^{339}$ *YC0036*/+, $dinR^{339}$ *YC0036*/$dlnR^{E19}$ *YC0036* and $chico^{1}$; *YC0036* flies fed with an R6 diet. Dashed lines mark the anterior edge of the germaria. (J,K) 14-day-old control (ctrl) and *ptc>*$dlnR^{RNAi}$ germaria with Fax (gray), 1B1 (red: fusomes) and LamC (red: TF and CPC nuclear envelopes) staining. (L) Average intensity of Fax expression in control and *ptc>*$dlnR^{RNAi}$ germaria. (M-N′) 7-day-old mock control (M,M′) and *dlnR*-KD mosaic (N,N′) germaria with GFP (green: flip-out clones indicated by asterisks), Fax (gray), 1B1 (red) and LamC (red) staining. Arrows indicate wild-type ECs located opposite GFP-marked ECs in the germarium. (O-Q) 14-day-old *c587>*gfp^{RNAi} (O), *c587>*$S6K^{DN}$ (P) and *c587>foxo3A* (Q) germaria with Fax (gray), 1B1 (red) and LamC (red) staining. Scale bars: 10 µm. The number of germaria analyzed is shown above each bar. Data are means±s.e.m. $^{**}P<0.01$, $^{***}P<0.001$. The genotype of the *GAL4* control in J and L is *ptc-GAL4*/+; the genotype of the *UAS* control in L is *UAS-*$dinr^{RNAi}$/+.

Interestingly, in *c587>*fax^{RNAi} flies, we did not observe the same significantly decreased cell division in GSCs and GSC progeny (Fig. 6F-H,H′) or growth delay of germ cells (data not shown) that was observed in germaria with *dInR*-KD ECs.

Fax regulates EC membrane extension but not EC number

We further asked whether Fax acts downstream of insulin signaling to control EC number and/or membrane extension. Unlike the experiments where we disrupted insulin signaling in ECs (see Fig. 2), we did not observe small germaria or decreased ECs in *c587>*fax^{RNAi} germaria of newly eclosed, 1- and 2-week-old flies (Fig. 7A-C). Thus, Fax is not required for EC maintenance. However, germaria of 1-week-old *c587>*fax^{RNAi} flies did contain incompletely wrapped cysts (Fig. 7D,E). Therefore, we generated *fax*-KD EC clones (identified by the presence of GFP) and used *flip-out GAL4* to examine whether Fax controls membrane extension in newly eclosed flies. In 1-week-old control mosaic germaria (Fig. 7F,F′,F″), the most-anterior ECs consistently displayed short, simple membrane protrusions that wrapped around GSCs, whereas the membrane processes of posterior ECs were extended to encase germ cell cysts (Fig. 7G,G′,G″). In *fax*-KD flies, ECs in contact with GSCs had severely blunted membrane processes (Fig. 7H,H′,H″). This blunting occurred to a similar degree as in *dInR*-KD ECs. Although membrane processes of posterior *fax*-KD ECs were also affected (Fig. 7I,I′,I″), the defect was less severe than that observed in *dInR*-KD flies. These results

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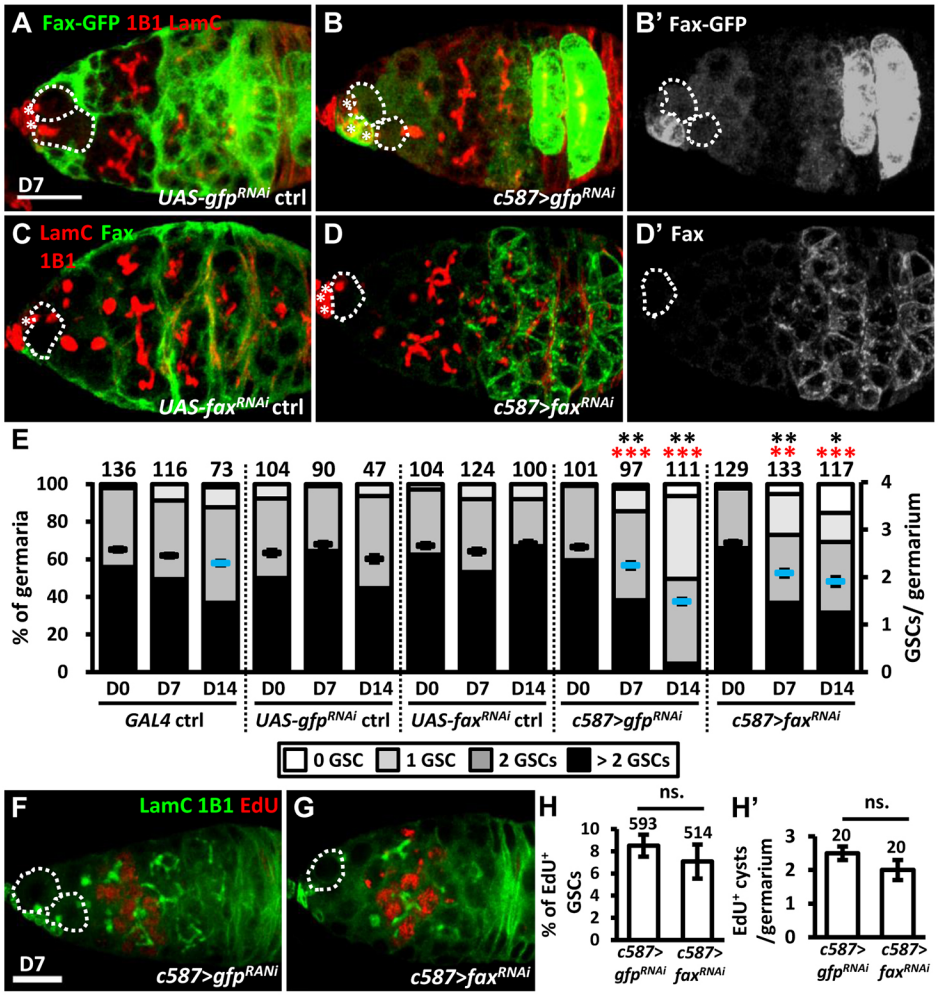

Fig. 6. Knockdown of *fax* in ECs results in GSC loss. (A-D′) 3D reconstruction of 7-day-old (D7) *UAS* ctrl (A,C), *c587>gfp*^{RNAi}, *YC0036* (B,B′) and *c587>fax*^{RNAi} (D,D′) germaria with GFP (green in A,B), Fax (green in C,D), LamC (red: TF and CPC nuclear envelopes) and 1B1 (red: fusomes) staining. Dashed lines mark GSCs. Asterisks indicate CPCs. (E) GSC numbers in newly eclosed (D0), 7- and 14-day-old *GAL4* ctrl, *UAS-gfp*^{RNAi} ctrl, *UAS-fax*^{RNAi} ctrl *c587>gfp*^{RNAi} and *c587>fax*^{RNAi} germaria at different ages. The blue bar indicates that the number of GSCs is significantly lower than D0 within the same genotype. Black and red asterisks show that the number of GSCs in the knockdown group is significantly lower than that in *GAL4* and *UAS* controls, respectively. (F,G) 7-day-old *c587>gfp*^{RNAi} (F) and *c587>fax*^{RNAi} (G) germaria with 1B1 (green), LamC (green) and EdU (red: proliferation marker) staining. (H,H′) Average number of EdU-positive (EdU^{+}) GSCs (H) and germ cell cysts (H′) in 7-day-old *c587>gfp*^{RNAi} and *c587>fax*^{RNAi} germaria. Scale bars: 10 µm. The number of germaria analyzed is shown above each bar. Data are means±s.e.m. **P*<0.05, ***P*<0.01, ****P*<0.001. The genotype of *UAS-gfp*^{RNAi} in A and E is *UAS-gfp*^{RNAi}*/+; YCOO36/+*; the genotype of *UAS-fax*^{RNAi} in C and E is *UAS-fax*^{RNAi}*/+*; the genotype of the *GAL4* control in E is *c587-GAL4/+*.

suggest that EC membrane extension is directly regulated by Fax and acts downstream of insulin signaling.

Insulin signaling and Fax in ECs promote Dpp signaling in GSCs

To investigate how insulin signaling in ECs controls GSC maintenance, we examined the expression of phospho-Mad (p-Mad), which is an effector of the BMP ortholog, Dpp. GSCs are maintained by Dpp that is provided from niche cap cells and ECs (Decotto and Spradling, 2005). Expression of p-Mad was significantly reduced in the nuclei of GSCs in 1-week-old germaria carrying *dInR*-KD or *fax*-KD ECs driven by *c587-GAL4* (Fig. 8A-D). To understand how insulin signaling in ECs affects Dpp signaling in GSCs, we first examined *dpp* transcript levels in control, *dInR*^{RNAi} and *c587>fax*^{RNAi} germaria using quantitative real-time PCR (qRT-PCR). Surprisingly, *dpp* mRNA was increased when *dInR* or *fax* was knocked down in ECs, although the increase did not reach statistical significance for *c587>fax*^{RNAi} flies (Fig. 8E). We then examined Dpp protein levels with a monoclonal antibody that recognizes a domain within mature Dpp (Asp457-Arg588) (Martinez et al., 2009). Ovarian tissue from flies carrying *dpp*-KD ECs (*c587>dpp*^{RNAi}) was used as a negative control. *Drosophila* Dpp is initially produced as an inactive 588-amino acid nonfunctional pro-protein, and the mature peptide is released after cleavage (Akiyama and Gibson, 2015; Künnapuu et al., 2009). In control ovaries, we observed two major bands on the blot; one was the pro-protein at ∼75 kDa, and the other was ∼45 kDa (Fig. 8F). Only the 45 kDa band was reduced in *c587>dpp*^{RNAi} lysate (Fig. 8F,F′), suggesting this band represents mature Dpp. These results indicate that neither insulin signaling nor membrane extension in ECs impairs Dpp production. Given that insulin signaling in ECs did not affect the number of cap cells (Fig. S1) and that cap cells are the major source of Dpp, we propose that the physical contact between ECs and GSCs may facilitate the receipt of Dpp by GSCs. This idea is strongly supported by a previous report showing that ECs produce seemingly low amounts of Dpp to maintain GSCs and promote differentiation of cystoblasts (Liu et al., 2015).

DISCUSSION

It is known that insulin signaling acts on both stem cells and niche cells to influence stem cell physiology in various tissues (Mah et al., 2014; Shim et al., 2013); however, the underlying mechanisms are incompletely described. In the *Drosophila* ovary, GSCs directly associate with niche cells, including cap cells and the anterior-most ECs (Kirilly and Xie, 2007). Cap cells anchor GSCs via E-cadherin-mediated cell-cell adhesion, but the role of ECs in maintaining GSCs is less clear. It has been shown that insulin signaling mediates dietary effects on GSCs and cap cells by directly controlling GSC division and maintenance (Hsu et al., 2008; LaFever and Drummond-Barbosa, 2005; Tseng et al., 2014). In cap cells, insulin signaling stimulates Notch and E-cadherin to maintain cell growth and survival, in addition to niche cap cell-GSC adhesion (Bonfini et al., 2015; Hsu and Drummond-Barbosa, 2009, 2011;

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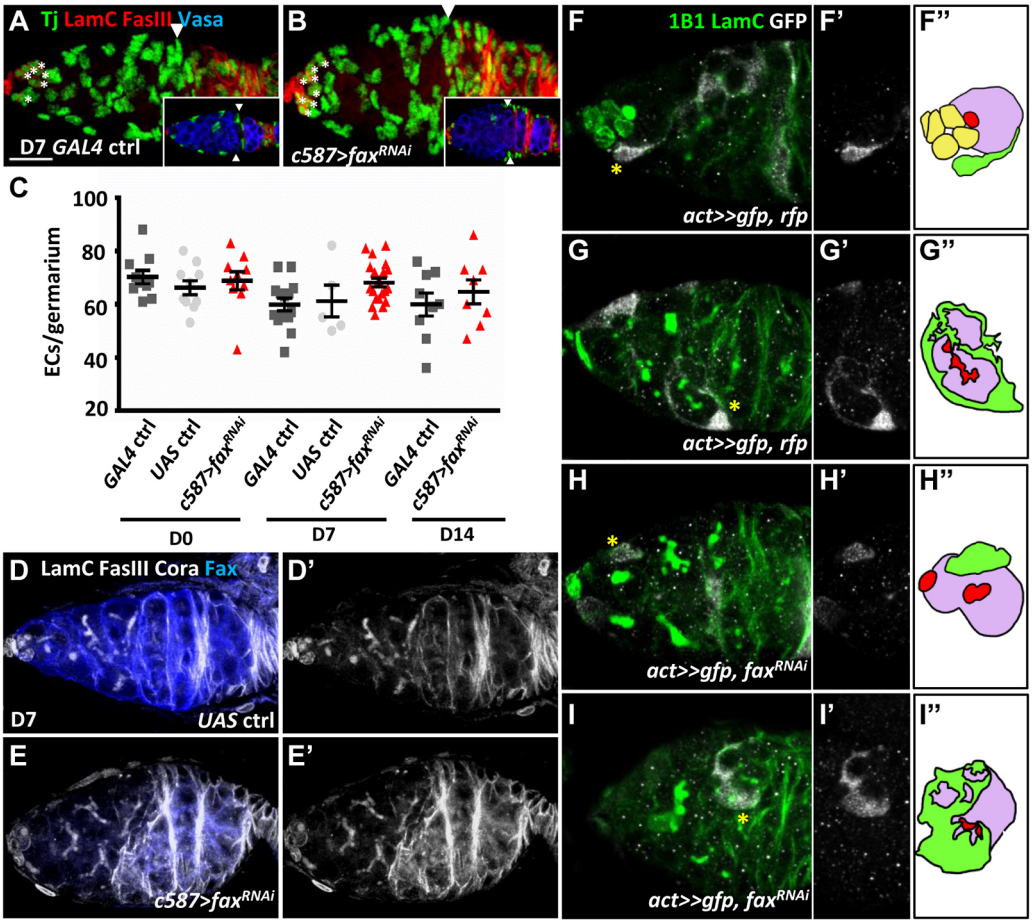

Fig. 7. Fax in ECs is required for EC membrane wrapping of germ cells. (A,B) 7-day-old (D7) control (ctrl) and *c587>fax*^{RNAi} germaria with Tj (green: CPC, EC and FC nuclei), LamC (red: TF and CPC nuclear envelopes) and FasIII (red: follicle cell membranes) staining. Insets are the largest longitudinal section of germaria with Tj, LamC, FasIII and Vasa (blue: germ cells) staining. Asterisks indicate cap cells. White arrowheads indicate the 2a/2b boundary. (C) Number of ECs in *GAL4* control, *UAS* control and *c587>fax*^{RNAi} germaria of newly eclosed, 7-day-old and 14-day-old flies. (D-E′) 7-day-old *UAS* control (D,D′) and *c587>fax*^{RNAi} (E,E′) germaria with LamC (red), 1B1 (red: fusomes), Cora (gray: ECs) and Fax (blue: ECs) staining. (F-I′′) 3D reconstruction images of 7-day-old mock (F-G′) and *faxKD* mosaic germaria (H,I) with LamC (green), 1B1 (green) and GFP (gray: flip-out clones) staining to show membrane extension of ECs marked by yellow asterisks. Scale bar: 10 µm. The genotype of the *GAL4* control in A and C is *c587-GAL4/+*; the genotype of the *UAS* control in C and D is *UAS-fax*^{RNAi}*/+*.

Yang et al., 2013). Here, we have shown that insulin signaling also modulates membrane extension in niche ECs, which allows the ECs to wrap and maintain GSCs, possibly by facilitating Dpp delivery (Fig. 8G). Insulin signaling also promotes membrane extension in posterior ECs that associate with GSC progeny to assist in their proliferation and growth. The control of EC membrane extension by insulin signaling occurs, at least in part, through S6K and Fax. In addition, insulin signaling maintains the proper number of ECs, which contributes to germ cell proliferation and growth, and also affects EC membrane protrusion (Kirilly et al., 2011). When nutritional inputs are limited (Fig. 8G′), insulin signaling is compromised in the GSC niche. Cap cells are not maintained and EC membrane extension is disrupted because of the decreased expression of Fax, leading to a loss of GSC wrapping and, eventually, to GSC loss. Decreased EC number, which is probably dependent on Foxo signaling, and blunted EC protrusions also cause a delay in germ cell proliferation and growth. The combined effects of starvation on GSCs and the GSC niche, as well as on GSC progeny, rapidly shut down the reproductive system to fit the needs of the organism. Our results suggest that insulin signaling modifies the cytoskeleton of niche cells to control stem cell behavior in response to dietary changes. Further, our study suggests that germ cells respond indirectly to insulin signaling in ECs via unknown factor(s). Overall, this work explains several molecular mechanisms underlying the long-known effects of diet on reproduction, the coordinated response of somatic and germline cells to diet, and the conserved functions of insulin signaling.

EC membrane extension controls GSC maintenance and cyst cell proliferation and growth

ECs are self-maintained, experience slow turnover and proliferation (Kirilly et al., 2011; Morris and Spradling, 2011), and extend membranous processes to wrap around GSCs and differentiated germ cells (Decotto and Spradling, 2005; Kirilly et al., 2011; Morris and Spradling, 2011). Previous studies have shown that ECs are the primary cellular constituents in the differentiation niche for GSC progeny. In their capacity as differentiation niche cells, multiple regulatory pathways ensure that Dpp signal is suppressed. First, Rho, Eggless and Epidermal growth factor receptor signaling suppress expression of Dally (Kirilly et al., 2011; Liu et al., 2010; Wang et al., 2011), which restricts Dpp to GSC niche cells (Akiyama et al., 2008) and prevents diffusion from ECs in the GSC niche to ECs in the differentiation niche (Guo and Wang, 2009). Second, differentiation niche ECs express high levels of Thickveins, a Dpp receptor, to

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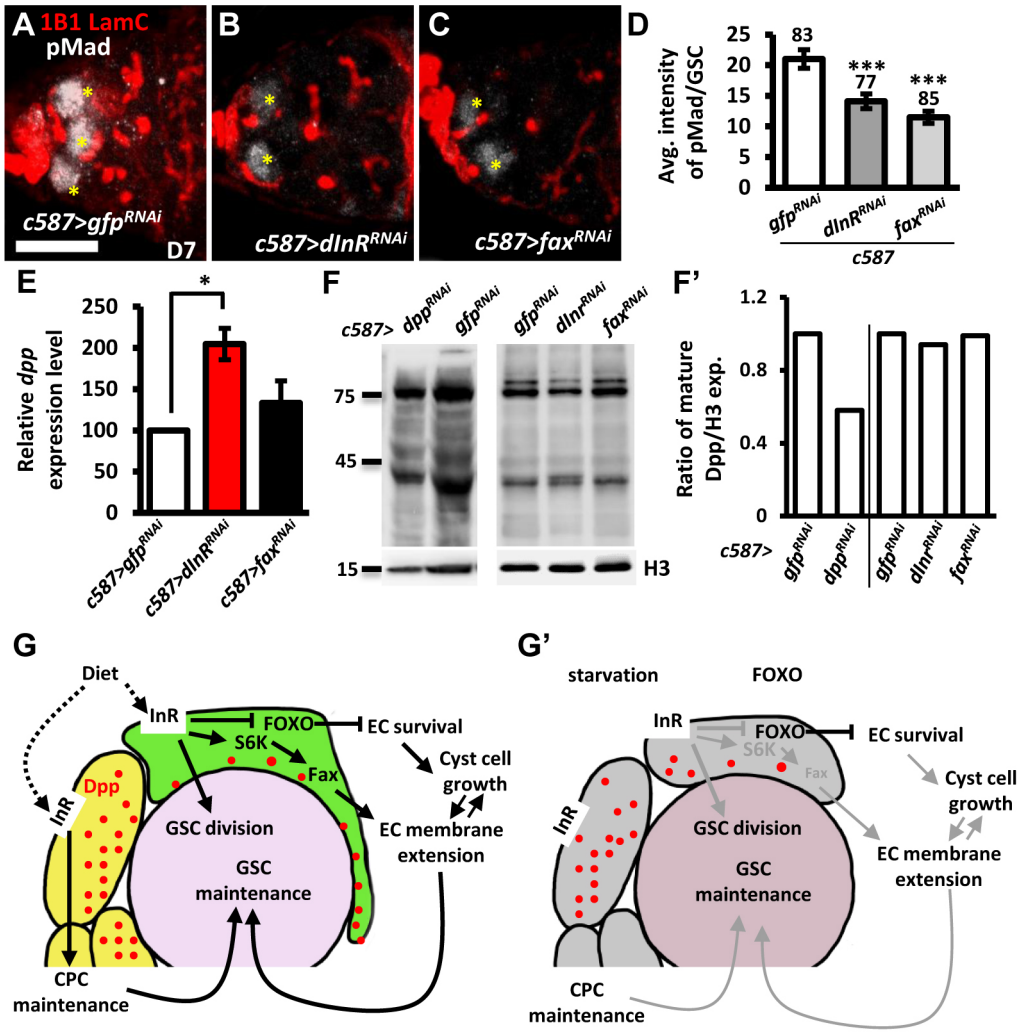

Fig. 8. Insulin signaling and Fax in ECs promote Dpp transfer to GSCs. (A-C) 7-day-old (D7) *c587>gfp*^{RNAi}, *c587>dInR*^{RNAi} and *c587>fax*^{RNAi} germaria with 1B1 (red: fusomes), LamC (red: TF and CPC nuclear envelopes) and pMad (gray: Dpp signaling) staining. Yellow asterisks indicate GSCs. Scale bar: 10 µm. (D) Average intensity of pMad in GSCs of *c587>gfp*^{RNAi}, *c587>dInR*^{RNAi} and *c587>fax*^{RNAi} germaria 7 days after eclosion. Number of GSCs analyzed are shown above each bar. Data are means±s.e.m. ***P<0.001. (E) qRT-PCR analysis of *dpp* transcript levels in control, *c587>dInR*^{RNAi} and *c587>fax*^{RNAi} ovaries 7 days after eclosion. The mRNA levels of *dpp* were normalized to *RPL19*. (F) Western blots for Dpp levels (47 kDa) in 7-day-old *c587-GAL4*-driven *dpp*-KD, *gfp*-KD, *dInR*-KD and *fax*-KD ovaries. Histone H3 (17 kDa) was used as an internal control. Molecular weight markers are indicated to the left of the blots. (F′) Ratio of mature Dpp to H3 expression (analyzed from the blot in F). (G,G′) Model showing how insulin signaling in ECs controls GSCs and their progeny. On a protein-rich diet (G), insulin signaling is activated in the GSC niche, including cap cells (CPCs) and anterior ECs (green). Insulin signaling in cap cells promotes their survival, which serves to maintain GSCs (Hsu and Drummond-Barbosa, 2009). In ECs, insulin signaling promotes membrane extension via S6K and Fax to wrap GSCs, probably increasing the efficiency of Dpp delivery for GSC maintenance. The survival of ECs is also regulated via Foxo. Insulin signaling in ECs facilitates germ cell proliferation and growth, which may provide feedback to further promote EC membrane protrusion. Under starvation conditions (G′), CPC numbers are decreased, and diminished Fax expression impairs EC membrane extension, together resulting in GSC loss. The combination of EC loss and diminished membrane extension in ECs also slows germ cell cyst proliferation and growth.

sequester any excess Dpp (Luo et al., 2015). Finally, Lsd1, Rho and Piwi directly repress *dpp* mRNA expression in differentiation niche ECs (Eliazer et al., 2011; Jin et al., 2013; Kirilly et al., 2011; Ma et al., 2014). Disrupting these mechanisms of Dpp silencing results in accumulation of GSC-like cells and is accompanied by defects in EC membrane extension. Interestingly, mutations in Bam, a germ cell differentiation factor, cause similar phenomena (Kirilly et al., 2011), indicating that germ cells may play a role in the development of EC membrane protrusions. Moreover, posterior ECs have a greater number of highly elaborated membrane extensions than anterior ECs, further suggesting that EC membrane extension is heavily influenced by differentiated germ cells. Because of this complex regulation, previous studies have not been able to address the role of EC membrane extension per se on GSCs and their progeny.

In the present study, disruption of insulin signaling and Fax expression in ECs affected EC membrane extension but not differentiation of GSC progeny, providing an opportunity to investigate the role of EC membrane extension in GSCs and their progeny. Clonal analyses revealed that membrane extension in anterior ECs was more sensitive to insulin signaling and Fax expression than in posterior ECs. As such, knockdown of *dInR* or *fax* in the anterior-most ECs severely blocked membrane extension of ECs, whereas the effect was more moderate in posterior ECs. This finding is in agreement with a previous study showing that membrane extension of posterior ECs is jointly controlled by the EC and the associated differentiated germ cells (Kirilly et al., 2011). Knockdown of *dInR* or *fax* in the entire EC lineage caused GSC loss with decreased proliferation and growth rates in germ cell cysts

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(although the reduction of cyst proliferation in *fax*-KD flies did not reach statistical significance), suggesting a role for EC membrane extension in GSC maintenance and cyst cell proliferation and growth. In addition, GSCs wrapped by *dInR*-KD or *fax*-KD ECs displayed decreased BMP signaling, suggesting that membrane extensions of anterior-most ECs may increase the efficiency of transfer for Dpp from the GSC niche cells to the GSCs.

Insulin-PI3K signaling alters the cytoskeleton, probably via Fax, in response to diet

Insulin has multiple effects on cells, including stimulation of glucose import, effects on gene expression and alteration of cell morphology (Tsakiridis et al., 1999). In cultured cells, insulin induces rapid actin filament reorganization and ruffling of the plasma membrane for pinocytosis (Tsakiridis et al., 1999). This reorganization requires activation of the insulin receptor-insulin receptor substrate-PI3K pathway (Tsakiridis et al., 1999), which results in the production of phosphatidylinositol-3,4,5-trisphosphate [PtdIns(3,4,5)P_3, PIP3] and recruitment of PIP3-binding proteins to the plasma membrane to initiate signaling events that control glucose metabolism, cell growth and movement (Cantley, 2002; Hu et al., 2016; Rathmell et al., 2003). It has been shown that actin remodeling requires the PIP3-dependent activation of guanine nucleotide exchange factors in the Rho/Rac/CDC42 family (Hanna and El-Sibai, 2013). Although we did not rescue Fax levels in *dInR*-KD ECs to reverse the phenotype, our genetic evidence strongly suggests that Fax is a novel target of the insulin-PI3K signaling pathway, which may participate in cytoskeleton rearrangement. Fax was first identified by its genetic enhancement of phenotypes caused by the Abelson tyrosine kinase (Abl) mutation (Hill et al., 1995). Abl is an actin-binding protein that regulates axonal guidance in the developing *Drosophila* and mammalian central nervous system (Hoffmann, 1991; Lanier and Gertler, 2000; Moresco and Koleske, 2003). Structurally, *Drosophila* Fax is a membrane protein without an identified transmembrane domain (Fig. S8; Figs 5, 6) (Hill et al., 1995). It is possible that Fax, acting downstream of insulin signaling, regulates the cytoskeleton via Abl or another actin regulator. In support of this idea, we found that knockdown of Abl in ECs also results in incomplete wrapping of germ cell cysts (Fig. S8). Given that Fax is highly conserved across species, we suggest that the same regulatory axis is present in higher animals, although the role of Fax in other species has not been explored.

MATERIALS AND METHODS

Drosophila stocks and culture

Drosophila stocks were maintained at 22-25°C in standard medium, unless otherwise indicated. The w^{1118} strain was used as a wild-type control. The null Inr^{339} and hypomorphic Inr^{E19}, $chico^{1}$, $foxo^{25}$, $foxo^{21}$ and fax^{M7} mutant alleles have been previously described (Böhni et al., 1999; Brogiolo et al., 2001; Choi et al., 2011; Hill et al., 1995; Hsu et al., 2008). fax^{CRISP} is a genetic null allele in which exons 1 and 5 of the *fax* gene are replaced by the CRISPR cassette (WellGenetics, Taipei, Taiwan). *fax-gfp* (YCO036) is a GFP-protein trap line (Buszczak et al., 2007). *UAS-RNAi* lines against *InR* (VDRC992), *Abl* (B35327), *fax* (VDRC103929 and B33687), dpp^{RNAi} (B25782) and GFP (B103929) were obtained from the Vienna *Drosophila* RNAi Center or Bloomington Stock Center. Efficiencies of InR^{RNAi} and Abl^{RNAi} have been previously reported (Choi et al., 2011; Kannan et al., 2014; Liu et al., 2015), and the efficiency of fax^{RNAi} was examined in this study. The *bab1-GAL4*, *c587-GAL4*, *ptc-GAL4*, *UAS-dInR*^{WT}, *UAS-p110*^{CAAX}, *UAS-S6K*^{DN} and *UAS-foxoA3* lines have been previously described (Barcelo and Stewart, 2002; Hsu and Drummond-Barbosa, 2009, 2011; Lai et al., 2017; Leevers et al., 1996; Puig et al., 2003). The *UAS-p110*^{CAAX} construct encodes p110, and the C-terminus includes the polybasic region and CAAX box of mammalian K-Ras, producing a membrane-targeted p110. The *UAS-S6K*^{DN} construct encodes a dominant-negative form of S6K with a glutamine substitution for lysine (K109Q). The *UAS-foxoA3* construct encodes a mutant form of dFoxo, in which all three putative dAkt phosphorylation sites (T44, S190 and S259) are mutated to alanine. Flies expressing *RNAi* or other transgenes with or without *tub-GAL80*^{ts} were cultured at 18°C to suppress GAL4 expression during development, and then switched to 29°C to allow GAL4 expression. Other genetic elements are described in FlyBase. Flies were cultured in standard medium (normal diet), in standard medium with wet yeast paste (protein-rich diet) or in a vial containing only a Kimwipe soaked in 5% molasses (protein-poor diet).

Genetic mosaic analysis

Genetic mosaic EC clones were generated by FLP/FLP recognition target (FRT)-mediated mitotic recombination (Xu and Rubin, 1993). Flies of genotypes *hs-flp*^{122}/+; *act5c*>stop>*Gal4 UAS-gfp*/*UAS-rfp hs-flp*^{122}/+; *UAS-dinr*^{RNAi}/+; *act5c*>stop>*Gal4 UAS-gfp*/+ *hs-flp*^{122}/+; and *act5c*>stop>*Gal4 UAS-gfp*/*UAS-fax*^{RNAi} were generated from standard crosses. Newly eclosed flies were subjected to heat shock at 35°C for 10 min. After heat shock, the larvae were cultured at 25°C until dissection. Homozygous mutant cells were identified by the absence of GFP in conventional mosaic analyses, and RNAi-expressing cells were recognized by the presence of GFP in flip-out clones.

Immunostaining and fluorescence microscopy

Ovaries were dissected in Grace's insect medium (Lonza), fixed for 13 min at room temperature in Grace's medium supplemented with 5% formaldehyde (Alfa Aesar), washed and stained, as previously described (Hsu et al., 2008). The following antibodies were used: rabbit anti-Fax (1:1500, a gift from Dr Eric C. Liebl, Denison University, OH, USA), rabbit anti-GFP (1:2000, Torrey Pines Biolabs, ABIN110592), chicken anti-GFP (1:2000, Invitrogen, A10262), rabbit anti-Vasa (1:250, Santa Cruz, sc-30210), rabbit anti-pMad (1:200, Abcam, ab52903), mouse anti-1B1 [1:30, Developmental Studies Hybridoma Bank (DSHB) 7H9 1B1], mouse anti-LamC (1:20, DSHB, LC28.26), mouse anti-FasIII (1:25, DSHB, 7G10), mouse anti-Cora (1:100, DSHB, C615.16), guinea pig anti-Tj (1:10,000, a gift from Dr Dorothea Godt, University of Toronto, Canada), Alexa Fluor 488 anti-rabbit IgG (1:250, Invitrogen), Alexa Fluor 568 anti-mouse IgG (1:250, Invitrogen), Alexa Fluor 633 anti-rabbit IgG (1:250, Invitrogen), Alexa Fluor 488 anti-guinea pig IgG (1:250, Abcam), Alexa Fluor 568 anti-guinea pig IgG (1:250, Abcam) and Alexa Fluor 568 anti-chicken IgG (1:250, Jackson ImmunoResearch). The ApopTag Fluorescein *in situ* Apoptosis Detection Kit (Roche) was used as previously described (Hsu et al., 2008). EdU incorporation was performed as previously described (Tseng et al., 2016). In brief, dissected ovarioles were incubated in 10 µM EdU in Grace's medium for 30 min at room temperature, and then washed and fixed in 4% paraformaldehyde for 13-15 min. Samples were stained with one of the antibodies listed above, incubated in Click-iT reaction cocktail for 30 min and then washed in PBST twice for 10 min per wash. Samples were incubated in 0.5 µg/ml DAPI, mounted in mounting solution [2% N-propyl gallate (Sigma), 85% glycerol] and analyzed using a Zeiss LSM 700 confocal microscope.

GSCs were characterized by the anterior position of their fusome (labeled by 1B1 staining), which is directly adjacent to cap cells (cap cell nuclear envelopes were labeled by LamC staining) (Hsu et al., 2008). For Fax-GFP and pMad expression, we used ImageJ to measure the average fluorescence intensity (arbitrary units) in target cells in confocal *z*-sections at the largest germarial face. GSC nuclear diameter was measured in the same sections. Numbers of GSCs or cap cells were compared using a chi-square test. Student's *t*-test or one-way ANOVA with Holm-Sidak *post hoc* comparisons were used for all other analyses.

Western blot analysis

Western blot analyses were performed as previously described (Tseng et al., 2014). Ten pairs of ovaries were dissected from 1-week-old flies and lyzed in RIPA buffer supplemented with 2× EDTA-free Complete Protease Inhibitor

Cocktail (Roche) on ice for 1 h. Lysates (40 µg aliquots) were boiled in sample buffer for 10 min, separated by 10% SDS-PAGE, blotted onto a PVDF membrane, and then blocked with 1× Tris-buffered saline containing 0.1% Triton X-100 (TBST, pH 7.5) and 0.5% bovine serum albumin for 1 h at room temperature. The blots were incubated with guinea pig anti-Fax (1:1500, a gift from Dr Allan Spradling, Department of Embryology, Carnegie Institution for Science, MD, USA), rabbit anti-Fax (1:1500, a gift from Dr Eric C. Liebl), rabbit anti-GFP (1:1500, Torrey Pines Biolabs), mouse anti-Dpp (1:500, R&D Systems, MAB 159) or rabbit anti-Histone H3 (1:1000, Abcam) antibodies at 4°C overnight with shaking. After three 10 min washes with 1× PBST, the blots were incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (1:5000, Jackson ImmunoResearch), HRP-conjugated goat-anti-mouse IgG (1:5000, Millipore) and HRP-conjugated goat-anti-guinea pig IgG (1:5000, Millipore) for 1 h at room temperature, and then washed three times with 1× TBST. Signals were detected and measured using the ECL system (Perkin Elmer). The blot of ovary lysates from YC0036 (Fax-GFP) was first used to detect Fax expression with a guinea pig anti-Fax antibody. Then the blot was stripped at 60°C for 30 min with stripping buffer containing 62.5 mM Tris-HCl (pH 6.8), 2% (w/v) SDS and 100 mM 2-mercaptoethanol, and reprobed with an antibody recognizing GFP.

Quantitative PCR analysis

Ovaries were dissected and stored at −80°C until use. Total RNA was extracted using a standard Trizol (Invitrogen) extraction protocol. In brief, 10 pairs of frozen ovaries were homogenized in 500 µl of Trizol, followed by a phase separation step with 500 µl 1-bromo-3-chloropropane (Sigma). RNA was precipitated with isopropanol, washed with 75% ethanol and resuspended in 50 µl of RNase-free water. Total RNA (1 µg) was reverse transcribed with Moloney Murine Leukemia Virus Reverse Transcriptase (Invitrogen). Steady-state mRNA levels were determined using LightCycler 480 Probes Master mix combined with probes from the Universal ProbeLibrary (Roche). Primers used to amplify *fax* A/C, *fax* B/D, *fax* E/F and *dpp* transcripts are listed in Table S4.

In silico analyses for Fax

Alignments of predicted *fax* mRNA-coding sequences were generated in MEGA version 5.2 (Tamura et al., 2011) software. Fax amino acid sequence comparison was aligned using NCBI software (NCBI BLAST, http://blast.ncbi.nlm.nih.gov/Blast.cgi). Phylogenetic trees were generated using the Maximum Likelihood method with 1000 times bootstrap resampling in MEGA version 5.2. Protein domains were identified by MOTIF searches (http://www.genome.jp/tools/motif/) (Bateman et al., 2002).

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Methodology: H.-J.H.; Validation: H.-J.H.; Formal analysis: Y.-H.S., K.-Y.L., S.-H.K.; Investigation: Y.-H.S., E.R., C.-M.L., K.-Y.L., S.-H.K., H.-Y.L., M.-H.W., H.-J.H.; Data curation: Y.-H.S., C.-M.L., H.-J.H.; Writing - original draft: Y.-H.S., H.-J.H.; Writing - review & editing: H.-J.H.; Visualization: Y.-H.S., E.R., H.-Y.L., M.-H.W., H.-J.H.; Supervision: H.-J.H.; Project administration: Y.-H.S., H.-J.H.; Funding acquisition: H.-J.H.

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Supplementary information

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