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Insulin and Target of rapamycin signaling orchestrate the development of ovarian niche-stem cell units in *Drosophila*

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SUMMARY

Tissue-specific stem cells and their niches are organized into functional units that respond to external cues in order to maintain organ homeostasis. Insulin and Target of rapamycin (Tor) signaling mediate external cues that control adult niches and stem cells. Whether these pathways play a role in the establishment of niche-stem cell units during organogenesis has been little explored. We show that during larval development both Insulin-like receptor (InR) and Tor participate in the establishment of ovarian niches and germline stem cells (GSCs) in *Drosophila melanogaster*. Tor and InR are required cell-autonomously for the proliferation of precursors for both somatic niches and GSCs. These pathways also promote the formation of terminal filaments (part of the somatic niche). Significantly, InR, but not Tor, signaling non-autonomously promotes primordial germ cell (PGC) differentiation. Somatic attenuation of the pathway retards PGC differentiation, whereas its activation results in their precocious differentiation. We also show that InR-mediated PGC differentiation is independent of somatic ecdysone signaling, but that further differentiation into cysts requires an ecdysone input. These results demonstrate that Tor and InR signaling actively participate in the formation of ovarian niches and stem cells by affecting both cell numbers and differentiation. The dual influence of Tor and InR on both somatic cells and PGCs ensures that these two cell populations develop coordinately. Our work further identifies a novel step in the regulation of germ cell differentiation by demonstrating that following *bag of marbles* expression, cyst formation requires an additional hormonal input.

KEY WORDS: Stem cells, Ovary, *Drosophila*, Nutrition, InR, Germ cells

INTRODUCTION

Stem cell units, which are composed of stem cells and their supportive microenvironment (or niche), are responsible for the homeostatic function of many organs (Arwert et al., 2012; Blanpain and Fuchs, 2009; Nakada et al., 2011; Spradling et al., 2008). Extensive evidence shows that the regulation of these units involves local signals as well as systemic factors such as diet and hormones (Ables et al., 2012; Amcheslavsky et al., 2009; Chell and Brand, 2010; Kapuria et al., 2012; McLeod et al., 2010; Shim et al., 2012; Sousa-Nunes et al., 2011; Yilmaz et al., 2012). How these stimuli affect the formation of niche-stem cell units remains largely unknown.

One of the major systemic cues is nutritional input conveyed by the Insulin/IGF and Target of rapamycin (Tor) signaling pathways (Garofalo, 2002). Ligand binding to the Insulin-like receptor (InR) causes recruitment and phosphorylation of the Insulin receptor substrate (encoded by the *Drosophila chico* gene) and subsequent activation of Phosphoinositide 3-kinase (PI3K) and Akt (Garofalo, 2002; Grewal, 2009; Oldham and Hafen, 2003). Tor activity is stimulated by an array of extracellular cues such as amino acids, glucose and oxygen (Arsham and Neufeld, 2006; Dann and Thomas, 2006; Foster and Fingar, 2010).

The InR and Tor pathways have been shown to affect germ cell biology. In *C. elegans* these pathways promote germ cell proliferation, repress precocious germ cell differentiation and participate in the establishment of an appropriately sized progenitor pool (Korta et al., 2012; Michaelson et al., 2010). In the *Drosophila* ovary, Insulin-like peptides (DILPs) directly regulate germline stem cell (GSC) division, cyst growth and vitellogenesis, while Tor is required for proper proliferation, maintenance and growth of GSCs and early cysts (Drummond-Barbosa and Spradling, 2001; LaFever and Drummond-Barbosa, 2005; LaFever et al., 2010; Richard et al., 2005; Sun et al., 2010). Insulin signaling also controls GSC maintenance indirectly by promoting niche cell maintenance via Notch signaling and niche-GSC interaction via E-Cadherin (Hsu and Drummond-Barbosa, 2009; Hsu and Drummond-Barbosa, 2011). Whereas the role of nutritional signals in the maintenance and function of the *Drosophila* adult niche-GSC unit has been thoroughly explored, much less is known about how InR and Tor signaling affect germ cells and their somatic support cells during ovary formation.

The adult *Drosophila* ovary contains 16-20 somatic niches and their resident GSCs (collectively termed niche-stem cell units). These units develop from a very small embryonic gonad composed of both somatic and germline precursors (Santos and Lehmann, 2004). During early larval stages, primordial germ cells (PGCs), which are the precursors of GSCs, and somatic precursors proliferate (Fig. 1A). The extent of precursor cell proliferation determines the number of precursors that are available for niche formation, and hence determines the number of niches that form during late larval stages (Gancz et al., 2011; Sarikaya et al., 2012).

Beginning at mid-larval third instar (ML3), niches differentiate in a graded fashion from the medial to the lateral side of the gonad. Terminal filament (TF) formation concludes at late larval third instar (LL3) (Sahut-Barnola et al., 1995; Song et al., 2002; Zhu and Xie, 2003). Toward the end of larval development, as larvae leave their food source to find a location in which to pupate, the first wave of PGC differentiation occurs in response to a signal induced by the steroid hormone ecdysone, which signals the initiation of oogenesis (Gancz et al., 2011). PGCs that are attached to the newly formed niches are protected from this differentiation signal and are maintained as adult GSCs (Fig. 1A).

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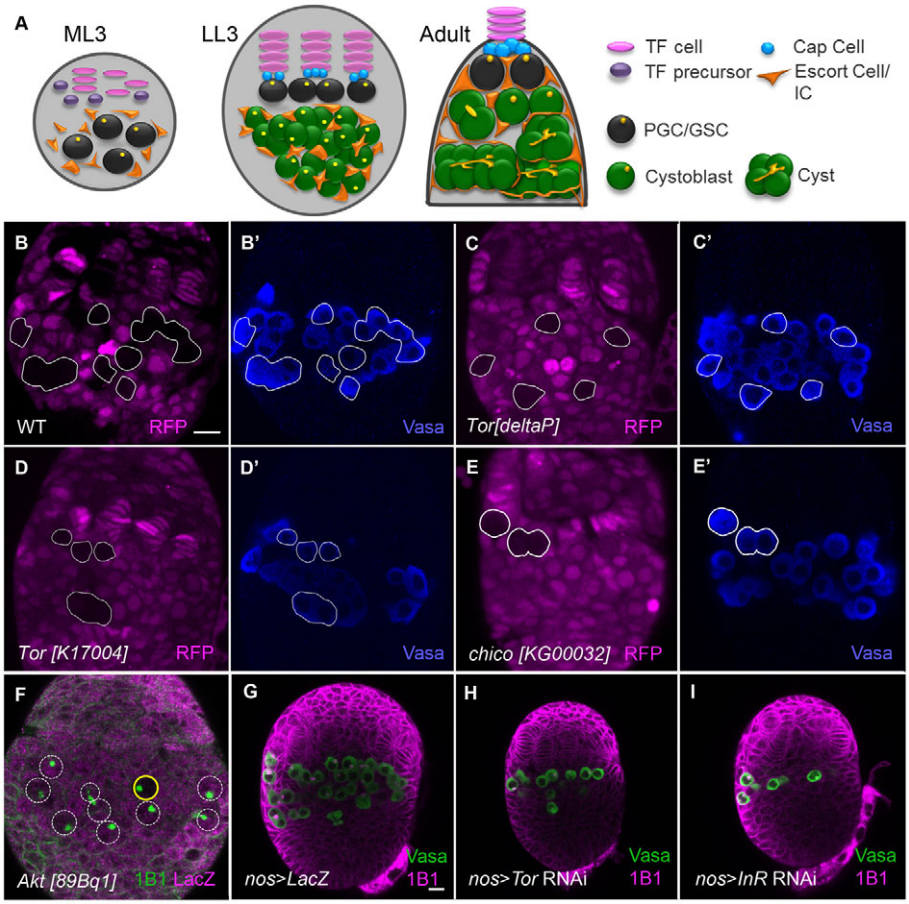

Fig. 1. Tor and InR signaling increase PGC numbers cell-autonomously. (**A**) Illustration of *Drosophila* mid-larval third instar (ML3), late larval third instar (LL3) ovary and an adult germarium. At ML3, terminal filaments (TFs, magenta) begin to form from TF precursors (purple). Primordial germ cells (PGCs, black) are located at the posterior and associate with intermingled cells (ICs, orange). At LL3, TF cells are organized in stacks. GSCs (black) are established close to the niches. Away from niches, PGCs initiate differentiation and some have become cystoblasts (green). Towards the end of LL3, cap cells (blue) begin forming. During pupal stages, TFs and their attached GSCs separate to form the adult germarium. (**B-E′**) RFP labels WT cells (B-E, magenta) and germ cells are labeled by anti-Vasa (B′-E′, blue). PGCs mutant for $Tor^{\Delta P}$ (C,C′), Tor^{K17004} (D,D′) or $chico^{KG00032}$ (E,E′) are recognized by lack of RFP (outlined) and form fewer clones than WT PGCs (B,B′). (**F**) 1B1 antibody (green) outlines somatic cells and labels fusomes within PGCs (outlined). Anti-β-gal labels WT cells (magenta). Few Akt^{89Bq1} mutant PGCs (yellow line) are observed. (**G-I**) Germ cells are labeled by anti-Vasa (green). Germ cell-specific removal of either Tor (H) or InR (I) by RNAi leads to fewer PGCs compared with WT (G). Scale bars: 10 μm in B for B-F and in G for G-I.

To form the correct number of niche-stem cell units, which will include fixed numbers of both somatic niche cells and GSCs, the rate of proliferation and differentiation of both somatic and germ cell populations must be coordinated. Two signaling pathways are currently known to actively coordinate the somatic and germ cell lineages. The first is Epidermal growth factor (EGF), which coordinates PGC proliferation rates with intermingled cell (IC) numbers in the ovary (Gilboa and Lehmann, 2006). The second is ecdysone, which initiates both somatic niches and PGC differentiation (Gancz et al., 2011; Hodin and Riddiford, 1998).

Although ecdysone signaling determines the temporal window for precursor cell proliferation, the extent of this proliferation could be determined by additional signals. Here we demonstrate that InR and Tor signaling promote the proliferation of both somatic and germ cell lineages in the ovary. We also show that the status of PGC differentiation is connected to the somatic growth of the ovary via InR, but not Tor, signaling. Therefore, hormonal cues other than ecdysone are required for ovary morphogenesis and for the coordination of the somatic and germline lineages.

MATERIALS AND METHODS

Fly stocks

tj-Gal4 is an NP insertion line [P(GawB)NP1624] into the *traffic jam* gene, and was obtained from the Drosophila Genetic Resource Center. *nos*-Gal4-VP16 was obtained from Dr Ruth Lehmann (HHMI, New York University). *bamP*-GFP is a GFP reporter fused to a fragment of the *bam* promoter. The transgene located on the X chromosome was obtained from Dr Dennis McKearin (HHMI, Washington DC, USA). FRT82B, Akt^{89Bq1} was obtained from Dr Tian Xu (HHMI, Yale University, USA). FRT82B, InR^{339} was obtained from Dr Ernst Hafen (ETH, Zurich, Switzerland). The RNAi line directed against *EcR* (1765R-4) was obtained from NIG-Fly. *UAS*-EcRA.W650A, FRT40A, $Tor^{\Delta P}$ and RNAi lines directed against *Tor* (GL00156, HMS01114, HMS00904), *InR* (GL00139), *chico* (JF02964), *Akt* (HMS0007), *raptor* (JF01087, JF01088), UAS-Tor.wt and UAS-Rheb.Pa were obtained from the Bloomington Stock Center. $chico^{KG00032}$ and Tor^{K17004} were obtained from the Bloomington Stock Center and recombined with FRT40A. Tsc1 (22252GD), Tsc2 (6313GD, 103417), *Akt* (2902GD) and *Rps11* (23475GD, 23477GD) were obtained from the Vienna Drosophila RNAi Center (VDRC). *UAS*-InR and *UAS*-lacZ were provided by Dr Jessica Treisman (New York University, USA).

Germline clones were generated using the line *bamP*-GFP,*hs*-Flp^{122}; FRT40A, RFPnls or *bamP*-GFP,*hs*-Flp^{122};; FRT82B, *arm*-lacZ. Clones were induced by heat shock 48 hours after egg laying (AEL) for 30 minutes at 37°C.

Larval staging

To obtain flies at similar developmental stages, care was taken to work with undercrowded cultures. Flies were transferred into a fresh vial to lay eggs for 2 hours, and were then removed. Vials were left at 25°C for 72 hours (early larval third instar, EL3), 96 hours (mid-larval third instar, ML3) or 120 hours (late larval third instar, LL3). Under these conditions the development of wild-type gonads is uniform. The terminology we use is according to Ashburner et al. (Ashburner et al., 2005), and is different from that used by Zhu and Xie (Zhu and Xie, 2003), who go by King (King, 1970).

Staining

Ovary staining was performed as previously described (Maimon and Gilboa, 2011).

The following monoclonal antibodies were obtained from the Developmental Studies Hybridoma Bank (developed under the auspices of the NICHD and maintained by the University of Iowa, Department of Biology): monoclonal 1B1 antibody directed against an Adducin (Hu li tai shao – FlyBase) (1:20), developed by Dr Howard Lipshitz; Z1.3C11.OA1 anti-Broad

Z1 (1:10), developed by Dr Greg Guild; 4D9 anti-Engrailed (1:20), developed by Dr Corey Goodman; and anti-Bam (1:10), developed by Dr Dennis McKearin. Rabbit anti-Vasa (1:5000) was a gift from Dr Ruth Lehmann. Rabbit anti-pMad (1:1000) was a gift from Dr Ed Laufer (Columbia University). Guinea pig anti-Tj (1:7000) was a gift from Dr Dorothea Godt (University of Toronto). Rabbit anti-β-gal (1:15,000) was from Cappel. Rabbit anti-GFP (1:1000) was from Invitrogen. Rabbit anti-phospho-Akt (D9E) and rabbit anti-phospho-p44/42 (D13.14.4E) were from Cell Signaling. Secondary antibodies were from Jackson ImmunoResearch or Invitrogen.

We used EdU (Invitrogen, C10337) to label cells in S phase and propidium iodide (Sigma, P4864) to label dead cells, both according to manufacturer's specifications.

Imaging and analysis

Confocal imaging was with a Zeiss LSM 710 on a Zeiss Observer Z1. For analysis of staining intensity, the Measure tool in ImageJ (NIH) was used for the selected cell area. When background staining levels differed between control and experimental ovaries, background intensity was subtracted. For ovary and cell size, the ImageJ Measure tool was also used.

Statistics

For statistical analyses, two-tailed Student's *t*-test or chi-squared test was performed. $P<0.05$ was considered significant. Precise *P* values are indicated in the text, figures or the supplementary tables.

RESULTS

InR and Tor signaling cell-autonomously regulate the number of GSC precursors

To generate functional niche-GSC units, sufficient PGCs must exist in the larval ovary to fill all the forming somatic niches. Initially, the embryonic gonad contains ~12 PGCs, which proliferate during larval stages (Gilboa and Lehmann, 2006; Poirié et al., 1995). To investigate the cell-autonomous role of the InR and Tor pathways in PGC proliferation during larval stages, we removed different components of these pathways from PGCs using mosaic (clonal) analysis (Xu and Rubin, 1993). A significant reduction in the number of clones, as compared with wild type (WT), was observed when PGCs were mutant for Tor, the Insulin receptor substrate homolog Chico, or the downstream InR effector Akt (Akt1 – FlyBase) (Fig. 1B-F; supplementary material Table S1). Consistent with the clonal analysis results, germline-specific depletion of either Tor or InR by RNAi resulted in a marked decrease in PGC numbers (Fig. 1G-I; supplementary material Table S2).

A reduction in PGC numbers could be the result of cell death or could indicate a reduction in cell proliferation. Using the vital dye propidium iodide, we observed no PGC death in WT, *Tor* or *InR* RNAi ML3 ovaries ($n=20$, $n=26$ and $n=21$, respectively), suggesting that the reduction in PGC numbers is a result of a reduced proliferation rate. PGC proliferation was directly evaluated by EdU, which is incorporated into the DNA of cells undergoing S phase. Germline depletion of either Tor or InR by RNAi resulted in a significant decrease in the percentage of EdU-positive PGCs at ML3 (supplementary material Table S3).

It has previously been demonstrated that *Drosophila* adult GSCs and their progeny adjust their proliferation rates in response to nutrition through Tor and InR signaling (Drummond-Barbosa and Spradling, 2001; LaFever and Drummond-Barbosa, 2005; LaFever et al., 2010). Our results extend these findings to show that InR and Tor signaling are required cell-autonomously for PGC proliferation.

Cell-autonomous InR and Tor signaling do not affect GSC establishment or PGC differentiation

At the end of larval development, PGCs that are close to niches are established as the future GSCs, while the remaining PGCs differentiate (Zhu and Xie, 2003). We examined whether InR or Tor signaling might play a cell-autonomous role in GSC establishment. Similar to GSCs, PGCs that reside at the niche are maintained by Dpp signaling (Gilboa and Lehmann, 2004; Zhu and Xie, 2003), which results in phosphorylation of Mothers against dpp (pMad) and in the suppression of the major differentiation gene *bag of marbles* (*bam*) (Chen and McKearin, 2003a; Song et al., 2004). Normal levels of pMad were observed in germline clones mutant for *InR*, *chico* or *Tor*, as compared with their WT neighbors (Fig. 2A-C; supplementary material Table S4), indicating that neither InR nor Tor signaling disrupts GSC establishment in a cell-autonomous manner.

We next tested whether a reduction in the InR or Tor pathways might affect the ability of PGCs to differentiate. PGC differentiation was monitored using *bamP*-GFP, a GFP reporter that recapitulates Bam expression (Chen and McKearin, 2003b). In late larval third instar (LL3) WT ovaries, PGCs that are located away from the niche and display only background levels of pMad upregulate *bamP*-GFP ($n=29$; Fig. 2D). The expression of *bamP*-GFP was examined in germline clones that were mutant for *Tor* or *Akt*. PGC clones were scored as being *bamP*-GFP negative (Fig. 2E, yellow line) or *bamP*-GFP positive. The group of *bamP*-GFP-positive cells was divided into a low expressing subgroup (up to 50% of the maximal WT GFP levels) and a high expressing subgroup (50-100% of the maximal WT GFP intensity; Fig. 2E, solid and dotted lines, respectively). As expected, the GFP-negative cells were usually found in proximity to the niches (Fig. 2E). No significant change in GFP levels or in the subdivision of clones within the three subgroups was observed in germline clones mutant for *Tor* or *Akt*, as compared with WT clones (Fig. 2F). Together, these data suggest that InR and Tor signaling are required cell-autonomously within PGCs for their proliferation but not for GSC establishment or PGC differentiation into cystoblasts.

Ovary size is regulated by Tor and InR signaling

To form the adult niche-GSC unit, PGCs and niche precursors both proliferate. In light of the intrinsic role of InR and Tor in promoting PGC proliferation, we examined whether these pathways also control the proliferation of the somatic ovary. For specific expression in the somatic ovary we used the driver line *traffic jam*-Gal4 (*tj*-Gal4), which is expressed in the somatic cells of the ovary (Li et al., 2003). Somatic overexpression of InR resulted in a marked increase in ovary size (compare Fig. 3A with 3B; supplementary material Table S5). By contrast, somatic reduction of either Tor or Insulin signaling by expression of *Tor* RNAi or *Akt* RNAi resulted in much smaller ovaries (Fig. 3C,D; supplementary material Table S5). The reduction in ovary size was not caused by increased cell death, as assessed using the vital dye propidium iodide [0 or 1 dead cells for WT ($n=13$); 0 dead cells for *Akt* RNAi ($n=29$) and *Tor* RNAi ($n=22$)]. Interestingly, *chico* RNAi ovaries were not significantly smaller than WT ovaries (Fig. 3E; supplementary material Table S5). This could be due to a weaker effect of the RNAi construct. Alternatively, considering the strong effects of this construct on niche formation and PGC differentiation (see below), this result might indicate a Chico-independent signaling capacity of *Drosophila* InR (Garofalo, 2002; Marin-Hincapie and Garofalo, 1995).

Changes in ovary size could be partially attributed to changes in cell number (supplementary material Tables S7, S9; see below) and partially to changes in cell size (supplementary material Table S6). Changes in cell size varied between the various ovarian somatic lineages (supplementary material Table S6), suggesting a differentially regulated response. Combined, these results show that the proliferation and growth of both components of the adult

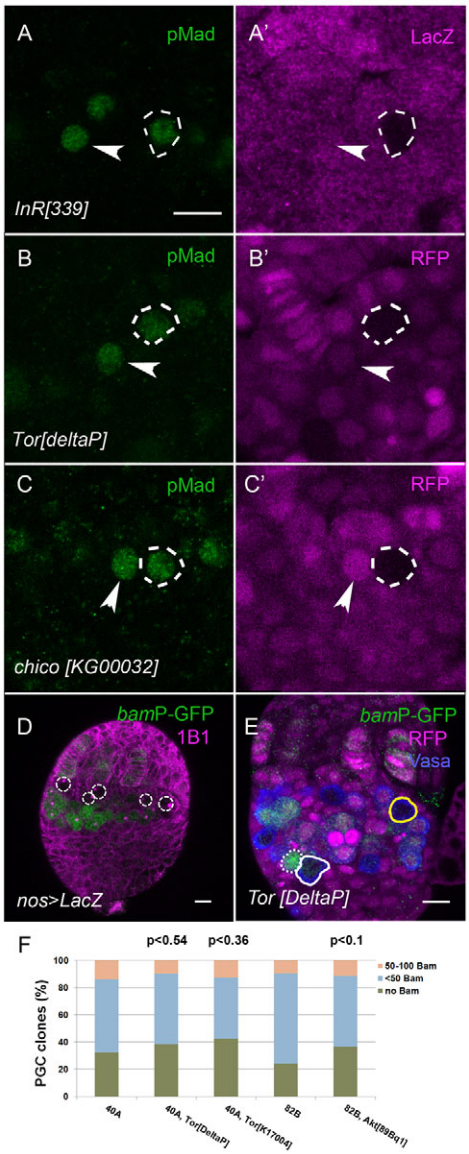

Fig. 2. Cell-autonomous Tor and InR signaling do not affect GSC establishment or differentiation. (**A-C′**) Anti-pMad (A-C, green) labels PGCs with active BMP signaling; anti-β-gal or RFP (A′-C′, magenta) labels WT cells. pMad levels in *InR* (A,A′), *Tor* (B,B′) or *chico* (C,C′) mutant PGCs (outlined) are similar to that of their WT neighbors (arrowheads). (**D**) WT LL3 ovaries. *bamP*-GFP (green) is expressed in PGCs that are located far from the niche but not in PGCs close to the niche (outlined). (**E**) Germ cells are labeled by anti-Vasa (blue). Germline clones were recognized by lack of RFP (magenta) and scored as *bam* negative or as expressing low levels of *bam* (<50% of WT) or high levels of *bam* (50-100% of WT) (yellow, solid and dotted lines, respectively). (**F**) *bamP*-GFP is expressed normally in LL3 germ cells mutant for $Tor^{\Delta P}$, Tor^{K17004} or Akt^{89Bq1}. Results are presented as percentage of PGC clones. At least 70 germ cells were scored in each genotype, and *P*-values of a chi-square test are presented. Scale bars: 10 μm (in A for A-C′).

niche-GSC unit depend on the same signals, contributing to their coordination.

InR and Tor signaling affect somatic cell specification

We next examined whether InR and Tor control somatic cell specification as well as proliferation. Two somatic cell lineages were examined: ICs, which directly contact PGCs; and TF cells, which constitute part of the niche for GSCs. ICs were identified by anti-Tj antibodies, which label all somatic cells until ML3, but are restricted to ICs by LL3 (Gilboa and Lehmann, 2006; Li et al., 2003). A significant increase in the number of Tj-expressing cells at LL3 was observed following InR overexpression (supplementary material Table S7). These extra cells were also detected at the posterior of the ovary where Tj is not normally expressed (compare Fig. 3A with 3B). By contrast, somatic depletion of Tor, Chico or Akt by RNAi led to a decrease in the number of Tj-positive cells (supplementary material Table S7). In addition, ICs in these ovaries surrounded the medial region, where PGCs are localized, but failed to intermingle with PGCs, an important hallmark of late larval stage patterning (Fig. 3C-E). ICs are known to be a location of active MAPK signaling, which plays a role in coordinating IC and PGC numbers (Gilboa and Lehmann, 2006). We therefore examined the effect of perturbing Insulin and Tor signaling on the levels of activated MAPK (supplementary material Table S8). Depletion of either Chico, Akt or Tor by RNAi resulted in reduced levels of nuclear pMAPK. Therefore, Tor and InR signaling regulate not only proliferation but also somatic patterning and cell behavior.

To examine TF formation, we used anti-Engrailed (En) antibodies (Fig. 3; supplementary material Table S9) or the enhancer trap line *hedgehog*-lacZ (supplementary material Fig. S1). In WT ovaries, En and Hedgehog are expressed in all TF stacks, and by LL3 an average of 18 stacks (n=41; Fig. 3F; supplementary material Table S9) could be observed. This value fits well with the known number of niches in adult *Drosophila*, and indicates that, in the WT, TF formation is complete by LL3. Somatic overexpression of InR resulted in a remarkable increase in TF numbers (an average of almost 50 niches, n=36; Fig. 3G). Conversely, somatic reduction of Tor, Chico or Akt by RNAi significantly reduced the number of TF stacks as well as the number of TF cells per stack (Fig. 3H-J; supplementary material Table S9). Significantly, despite their normal size, *chico* RNAi ovaries contained an average of only five TFs (supplementary material Table S9). This suggests that InR signaling might be required for TF specification independently of its role in cell proliferation.

Once formed, TFs induce the formation of cap cells, the second component of the niche (Li et al., 2003; Song et al., 2007). Cap cell formation and maintenance are also affected by InR signaling and nutrition (Hsu and Drummond-Barbosa, 2009; Hsu and Drummond-Barbosa, 2011). Therefore, InR and Tor modulate not only the number of PGCs but also the number of niches and their composition.

InR and Tor signaling do not regulate the timing of niche formation

Niche numbers are determined by two factors: the number of TF precursors and the timing of TF differentiation (Gancz et al., 2011; Green and Extavour, 2012; Sarikaya et al., 2012). Since InR and Tor activities control precursor proliferation, we tested whether they can also affect the timing of the initiation of TF differentiation. In WT ML3 ovaries, when TF differentiation initiates, only a few cells express En. These cells are still disorganized, and very few short filaments can be detected at this stage (Gancz et al., 2011) (Fig. 4A). We found that the timing of niche formation was not altered by changes in Insulin signaling (Fig. 4B,C). Similar precursor numbers initiated differentiation in InR-overexpressing or WT ML3 ovaries (supplementary material Table S10), and the filaments themselves did not form precociously (Fig. 4B). As expected, only a few TF cells (~2-10 cells) were specified in ML3 *chico* RNAi ovaries

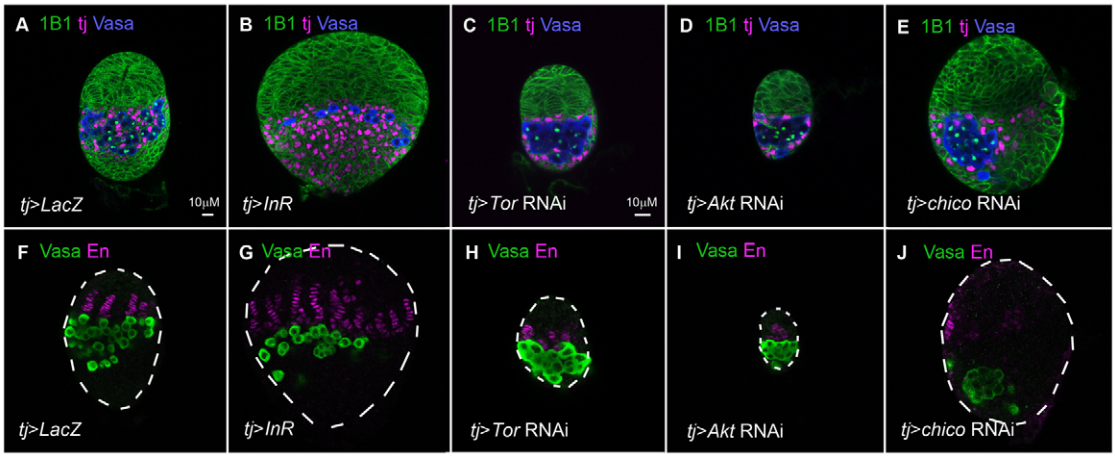

Fig. 3. Tor and InR determine ovary size, patterning and niche numbers. (**A-E**) LL3 ovaries. 1B1 antibody outlines somatic cells and labels fusomes within germ cells (green). ICs are labeled by anti-Tj (magenta) and PGCs with anti-Vasa (blue). Somatic expression of InR (B) results in ovary growth and increased IC numbers compared with WT (A). *Tor* RNAi (C) and *Akt* RNAi (D) ovaries are smaller and contain fewer ICs, which are outside the PGC area. *chico* RNAi ovaries (E) are not smaller but ICs are fewer in number and do not intermingle with PGCs. (**F-J**) TFs are labeled with anti-En (magenta). Germ cells are labeled with anti-Vasa (green). (F) In WT LL3 ovaries TF stacks form throughout the anterior of the ovary. (G) Somatic expression of InR results in an increase in TF numbers. Somatic depletion of either Tor (H), Akt (I) or Chico (J) by RNAi results in fewer and shorter TF stacks. Scale bars: 10 μm in A for A,B,F,G and in C for C-E,H-J.

(n=12; Fig. 4C); however, the timing of their first appearance (ML3) was normal.

Since fewer TF structures were formed and fewer TF cells were present per stack upon reduction in InR and Tor somatic signals, we tested how these altered niches function in larval PGC maintenance. pMad labeling was observed in *chico*, *Akt* and *Tor* RNAi ovaries (Fig. 4D-H). However, the percentage of pMad-positive germ cells was lower than in WT. Additionally, in *Akt* and *Tor* RNAi ovaries, pMad staining intensity was reduced (supplementary material Table S11). This lower intensity could be the result of a reduced function of each TF cell, or of the lower numbers of TF cells per stack.

In summary, both the Tor and InR signaling pathways affect niche formation and function. However, they do not affect the timing of niche formation, which is controlled by ecdysone signals (Gancz et al., 2011).

InR signaling non-autonomously regulates PGC numbers and promotes PGC differentiation

InR and Tor signaling affect ICs and TFs, which control PGC proliferation and differentiation. We therefore tested whether these pathways might affect PGCs in a non-cell-autonomous manner. Indeed, somatic expression of *Tor*, *chico* or *Akt* RNAi non-autonomously reduced PGC numbers, as compared with WT ovaries (Fig. 3F,H-J; supplementary material Table S2). This might result from a reduced capability of these small ovaries to support PGCs, indicating that InR and Tor regulate the size of the GSC precursor pool both cell-autonomously and non-autonomously.

Next, we tested whether InR and Tor signaling can affect PGC maintenance/differentiation non-cell-autonomously. In WT ML3 ovaries, BMP signaling in PGCs prevents their differentiation (Gilboa and Lehmann, 2004) and all PGCs are pMad positive (Fig. 5A). Accordingly, WT PGCs at this stage do not express the differentiation marker *bamP*-GFP (Fig. 5B). By contrast, pMad staining could not be detected in most PGCs of InR-overexpressing ML3 ovaries (Fig. 5C), suggesting that these PGCs are not maintained. Indeed, somatic overexpression of InR or of an activated form of InR resulted in precocious PGC differentiation, marked by the upregulation of *bamP*-GFP at ML3 (Fig. 5D,E). Less than 10% of PGCs remained GFP negative at this stage (n=12). By LL3, differentiation was advanced and cysts with up to 16 cells, marked by branched and extended fusomes, could be observed (n=20; Fig. 5F, inset). Such developed cysts are not observed in WT LL3 ovaries (Gancz et al., 2011) (Fig. 2D). At the pre-pupal stage, 20% of the cysts (n=21) expressed the late differentiation marker Orb, which is not expressed at all in WT ovaries at this stage (Gancz et al., 2011; Lantz et al., 1994). Since GSCs are established in InR-overexpressing LL3 ovaries (Fig. 4E) from a population of cells that is mostly *bamP*-GFP positive at ML3, this suggests that the new niches that form in the second half of the third instar might be inducing the de-differentiation of Bam-expressing cells. Such a phenomenon has been recorded previously (Kai and Spradling, 2004).

In the WT, PGC differentiation commences at the wandering stage, when PGCs that are not located close to the niche upregulate *bam* (Fig. 2D) (Gancz et al., 2011; Zhu and Xie, 2003). Reduction of Insulin signaling by somatic expression of either *chico* or *Akt* RNAi resulted in a reduction in PGC differentiation at LL3; fewer PGCs upregulated *bamP*-GFP in these ovaries. In addition, the intensity of *bamP*-GFP expression was lower than in WT ovaries (Fig. 5G,H; supplementary material Table S12). Since the Bam expression level correlates with the differentiation status of germ cells (Chen and McKearin, 2003b), the lower intensity of *bamP*-GFP suggests that PGC differentiation in *chico* and *Akt* RNAi ovaries either starts later or progresses more slowly.

The inhibition of PGC differentiation in *chico* or *Akt* RNAi ovaries might result directly from reduced somatic InR signaling or indirectly from their reduced size. To distinguish between these two possibilities, we depleted the ribosomal protein Rps11 from the ovary soma using RNAi. Although *Rps11* RNAi ovaries were small and had almost no niches, PGC differentiation and *bamP*-GFP expression at LL3 were normal, suggesting that reduced ovary size per se does not inhibit PGC differentiation (Fig. 5I). Importantly, although somatic expression of *Tor* or *raptor* (a part of the Tor complex) RNAi resulted in small ovaries, *bamP*-GFP expression in

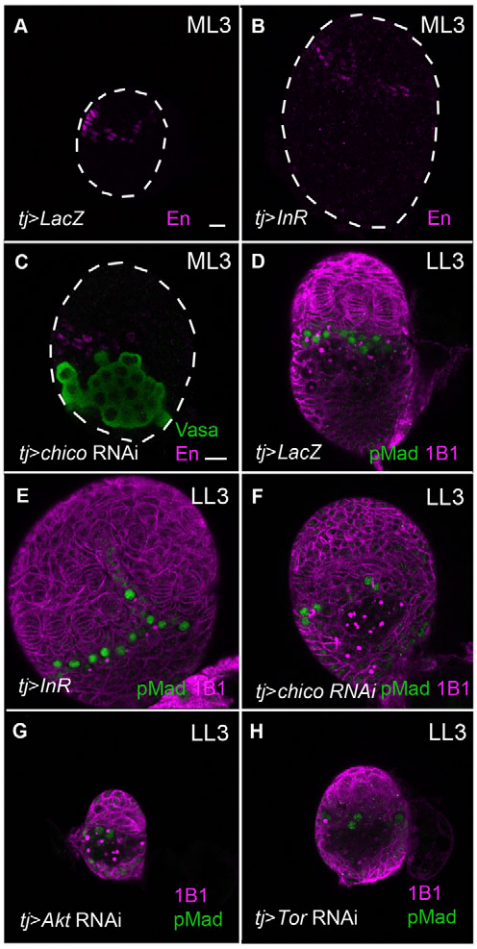

Fig. 4. InR and Tor signaling do not change the timing of niche formation. (**A-C**) TFs are labeled with anti-En (magenta). (A) In ML3 ovaries, only a few En-expressing cells are present and TF stacks have just begun to form. (B) TF stacks do not form precociously following somatic overexpression of InR. (C) Germ cells are labeled with anti-Vasa (green). TF cell specification is initiated normally in ML3 *chico* RNAi ovaries. (**D-H**) 1B1 antibody outlines somatic cells and labels fusomes within germ cells (magenta). Anti-pMad (green) labels PGCs close to formed niches. In WT LL3 ovaries (D), only PGCs closest to the niche retain pMad labeling. (E,F) pMad staining following somatic expression of InR (E) or *chico* RNAi (F). (G,H) Reduced niche maintenance in *Akt* RNAi (G) or *Tor* RNAi (H) ovaries. Scale bars: 10 μm (in A for A,B,D-H).

PGCs was not reduced (Fig. 5J-L; supplementary material Table S12). Similarly, activation of the Tor pathway by overexpressing Tor, Rheb (a positive regulator of the Tor pathway) or by depletion of the inhibitory complexes Tsc1 or Tsc2 (Gigas – FlyBase) by RNAi, had no effect on PGC differentiation or on ovary size (data not shown). In conclusion, our results show that PGC differentiation is regulated specifically by somatic InR, but not Tor, signaling.

InR signaling requires somatic ecdysone signaling to promote cyst formation

To further evaluate how somatic Insulin signaling might regulate PGC differentiation, we closely examined the timing of *bam* expression in InR-overexpressing ovaries. In the WT, the first PGCs expressing *bamP*-GFP can be seen at 112 hours AEL, as larvae start wandering. These cells are still single and harbor a spherical fusome (Gancz et al., 2011). Germline cysts form only a few hours later, at the beginning of pupation, when Bam-expressing PGCs divide to form two- and then four-cell cysts (Fig. 6A, inset; 100% of ovaries, $n=19$). Therefore, less than 12 hours pass between the first appearance of *bam* expression and the first observed cysts.

To define the earliest time point of PGC differentiation in InR-overexpressing ovaries, we examined gonads prior to ML3 and found that 45% of ovaries exhibited *bamP*-GFP expression in some PGCs already at early larval third instar (72 hours AEL; $n=22$; Fig. 6B). However, despite the early initiation of *bam* expression, PGCs remained single and harbored spherical fusomes at ML3 (Fig. 6C). Differentiating cysts were only observed at LL3 (Fig. 5F). Therefore, PGCs arrest their development following *bam* expression for a significant time period (over 40 hours) in InR-overexpressing ovaries.

These results highlight two novel features of PGC differentiation. First, PGCs can express *bam* in response to somatic InR signaling. Second, the transition from PGCs to cysts is a two-stage process (from PGC to *bam*-expressing cells and from these to cysts), and each step might require particular signals. We have previously established that PGCs in WT ovaries differentiate in response to a specific ecdysone pulse occurring after ML3 and prior to pupation (Gancz et al., 2011; Warren et al., 2006). This suggests that ecdysone, in addition to InR, might control one or both steps. To test the involvement of ecdysone signaling in the various stages of PGC differentiation, and the possible connection between the InR and ecdysone pathways, we examined whether *bam*-expressing PGCs in InR-overexpressing ovaries could progress to form cysts in the absence of ecdysone signaling. As previously reported, PGCs in ovaries overexpressing the dominant-negative form of EcR isoform A (EcR-DN) in the soma did not upregulate *bamP*-GFP even at LL3 (Gancz et al., 2011) (Fig. 6D). Surprisingly, PGCs in ML3 ovaries expressing both InR and EcR-DN did express *bamP*-GFP, suggesting that somatic InR signals can induce *bam* expression independently of ecdysone signaling (Fig. 6E). However, these PGCs could not progress to form cysts even at the white pupa stage (Fig. 6F, inset), suggesting that somatic ecdysone signaling is required for the second stage of cyst formation. These epistasis experiments indicate that the two hormonal pathways, InR and ecdysone, act in parallel to promote PGC differentiation (Fig. 6G). A parallel role is also supported by our inability to establish a direct molecular link between the two pathways in the ovary (supplementary material Fig. S2).

In summary, our results connect InR signaling within the somatic ovary to the differentiation status of PGCs, and uncover a novel regulatory step in germ cell differentiation, showing that cyst differentiation does not directly follow *bam* upregulation.

DISCUSSION

Organogenesis is a complex process that results in structures that contain different cells at specific spatial and numerical ratios. Organ construction must therefore involve coordination between many local signals that specify the proliferation and differentiation of several cell types. How such coordination is achieved and how systemic factors might affect these processes are still not fully understood. The fly ovary, with its 16-20 repetitive niche-stem cell units, each containing somatic cells and germ cells, is a good model system with which to investigate this problem. In this work we show that Tor and InR signaling participate in *Drosophila* gonadogenesis and in the establishment of GSCs. In particular, InR signaling affects germ cell proliferation and differentiation on three different levels: cell-autonomously, non-cell-autonomously via the somatic part of

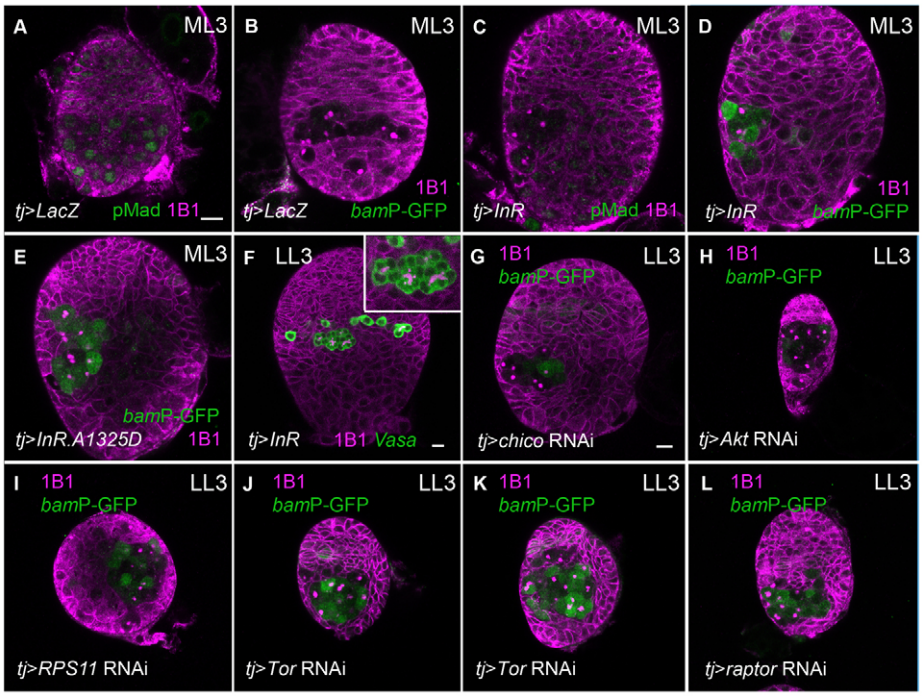

Fig. 5. InR signaling non-cell-autonomously promotes PGC differentiation. In all images, 1B1 antibody outlines somatic cells and labels fusomes within germ cells (magenta). (**A**) At ML3 all PGCs contain pMad (anti-pMad staining, green) in their nuclei. (**B**) The differentiation marker *bamP*-GFP (anti-GFP, green) is not expressed at this stage. (**C-E**) pMad is not expressed in most PGCs of ML3 ovaries overexpressing InR (C), whereas *bamP*-GFP is precociously expressed in ovaries overexpressing either InR (D) or an activated form of InR (E). (**F**) PGCs are labeled with anti-Vasa (green). By LL3, InR-overexpressing ovaries have developed differentiating cysts marked by branched and extended fusomes (inset). (**G-L**) PGC differentiation is marked by *bamP*-GFP (anti-GFP, green). Reduced GFP expression and PGC differentiation following somatic expression of *chico* RNAi (G) or *Akt* RNAi (H). Somatic expression of *Rps11* RNAi (I), *Tor* RNAi (J,K) or *raptor* RNAi (L) has no effect on PGC differentiation. Scale bars: 10 µm (in A for A-E and in G for G-L).

the ovary, and systemically. This study also highlights PGC differentiation as a multiple step process, and shows that the conversion of a PGC to a cyst requires sequential hormonal signals.

Regulation of precursor cell proliferation by the InR and Tor signaling pathways

Cell growth and proliferation in the larva require energy and metabolites. Accordingly, these processes are controlled by the InR and Tor pathways, which are sensors of the metabolic state of the organism (Arsham and Neufeld, 2006; Foster and Fingar, 2010; Goberdhan and Wilson, 2003; Grewal, 2009). It was previously demonstrated that Insulin and Tor signaling promote the proliferation of germline precursors in *C. elegans* (Korta et al., 2012; Michaelson et al., 2010). We extend this finding and show that, in *Drosophila*, both somatic cells and PGCs require Tor and InR signaling cell-autonomously for their proliferation. This response is not limited to the larval growth period. The ovary is an active organ that maintains growing populations of cells. Accordingly, in the adult, somatic follicle cells, GSCs and germline cysts respond to nutrition by changing their proliferation rate (Drummond-Barbosa and Spradling, 2001; LaFever and Drummond-Barbosa, 2005; LaFever et al., 2010; Sun et al., 2010).

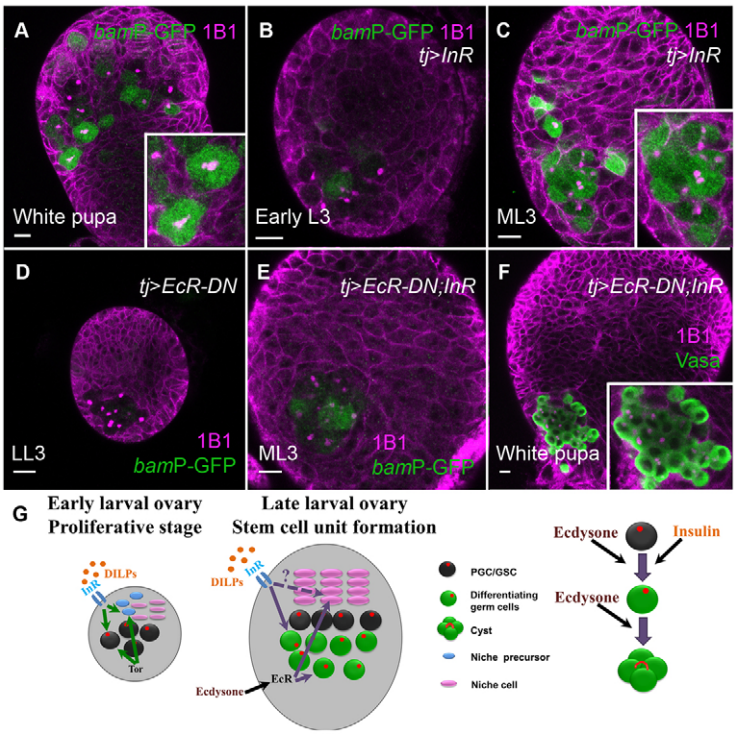

Fig. 6. Somatic InR signaling cannot promote cyst formation in the absence of ecdysone signaling. In all images, 1B1 antibody outlines somatic cells and labels fusomes within germ cells (magenta). (A-E) PGC differentiation is marked by *bamP*-GFP (green). (**A**) At the white pupa stage, differentiating cysts are formed, marked by the increase in *bamP*-GFP levels and by the branched and extended fusomes (inset). (**B**) *bamP*-GFP (green) is precociously upregulated in early L3 InR-overexpressing ovaries. (**C**) PGCs in ML3 InR-overexpressing ovaries remain single, with a spherical fusome (inset). (**D**) PGCs fail to upregulate *bamP*-GFP in LL3 ovaries overexpressing the dominant-negative form of EcRA (EcR-DN). (E,F) Ovaries expressing both EcR-DN and InR. (**E**) *bamP*-GFP is precociously upregulated at ML3. (**F**) PGCs are labeled by anti-Vasa (green). Only single fusomes are observed even at the white pupae stage (inset). (**G**) During early larval stages Tor and InR signaling cell-autonomously regulate the proliferation of both PGCs and somatic niche precursors (green arrows). At late larval stages ecdysone signaling induces the differentiation of somatic niches (purple arrow). InR might also be required for TF specification (purple dashed arrow). InR and ecdysone independently promote *bam* expression in PGCs, and ecdysone input is required for further differentiation into germline cysts. Scale bars: 10 µm.

The cell-autonomous response of both soma and germline to InR and Tor signaling represents one mechanism by which the coordination of growth within an organ is achieved.

In addition, we find that the Tor and InR pathways affect PGC proliferation non-cell-autonomously. Smaller somatic ovaries correlate with reduced PGC proliferation, while overexpression of InR diverts PGCs from a proliferation to a differentiation program. Thus, coordination between somatic growth and germline division is monitored and corrected by more than one mechanism. It is as yet unclear how the state of somatic growth is communicated to the germline. The secondary signal might be a local ligand or might involve direct contact with the ICs. We have previously shown that the somatic cells of the ovary can control PGC proliferation via a feedback loop involving EGFR signaling in somatic cells and an unidentified signal that represses PGC proliferation (Gilboa and Lehmann, 2007). InR and Tor signaling might somehow affect this unknown signal.

Control of niche-stem cell unit differentiation by InR and Tor signaling

InR and Tor signaling are required for the differentiation of somatic ICs and TFs. ICs fail to integrate with PGCs when somatic cells have reduced InR or Tor signaling, suggesting these pathways affect IC behavior. Similarly, ovaries with reduced somatic InR and Tor signaling develop fewer TFs. This is consistent with previous observations that diet restriction (yeast deprivation) during the third instar results in reduced ovariole number (Hodin and Riddiford, 2000; Tu and Tatar, 2003). One explanation for this reduction is the reduction in TF precursors due to early proliferation defects (Green and Extavour, 2012; Sarikaya et al., 2012). However, the strong reduction in TF numbers in *chico*-deficient ovaries, despite the relatively normal gonad size, suggests a specific role of InR in TF cell determination. Although InR signaling has been mostly associated with cell proliferation, a role for this pathway in neuronal cell differentiation has been described (Bateman and McNeill, 2004). The ovary might be another organ in which InR signaling affects cell differentiation. In the ovary, InR signaling can increase the number of cap cells by modulating Notch signaling, which is required for the establishment of this cell type (Hsu and Drummond-Barbosa, 2009; Hsu and Drummond-Barbosa, 2011; Song et al., 2007; Ward et al., 2006). Thus, InR signaling acts at least twice in niche formation: first, it is required for TF formation, and then for cap cell establishment and maintenance.

We find that activation of InR signaling in the soma initiates PGC differentiation precociously, whereas its repression postpones PGC differentiation. Combined, our results show that InR signaling is required for the maturation of the two components of the stem cell unit: the somatic niches and the PGCs that will occupy them. This coordination might be important at times when nutrient availability is limited, and niche formation is retarded. If PGCs differentiated normally, prior to the formation of protective niches, this would have resulted in full germ cell differentiation and lack of GSCs. Retarding PGC differentiation at times of limited nutrient availability allows additional time for niche formation prior to full depletion of the stem cell precursors.

bam expression does not lead directly to cyst formation

In InR-overexpressing ovaries, PGCs initiate their differentiation and express *bam* as early as the beginning of third instar (72 hours AEL). They then arrest their development for nearly 2 days; germline cysts form only following the normal elevation in ecdysone signaling, and fail to do so in its absence (Fig. 6). One possibility is that somatic InR signaling is required (non-autonomously) for the initiation of *bam* transcription, while ecdysone initiates Bam translation, thereby transforming *bam*-expressing PGCs into proper cystoblasts. Alternatively, somatic InR might be sufficient for cystoblast formation, but further differentiation into germline cysts requires ecdysone signaling. We could not resolve this issue using the available anti-Bam antibody because this low-affinity reagent cannot recognize the naturally low levels of Bam protein in cystoblasts. It has previously been shown that PGCs can form cysts as early as second instar following *hs-bam* expression (Kai and Spradling, 2004). Therefore, the requirement for somatic ecdysone signaling can be overridden by ectopic, high Bam expression.

Irrespective of the mechanism by which somatic InR promotes PGC differentiation, our results suggest that the passage from a *bam*-expressing cell to a germline cyst might not be as direct as previously thought. Classical studies suggested that the major event in GSC differentiation is *bam* expression, and that Bam is both necessary and sufficient for GSCs to differentiate into germline cysts (Ohlstein and McKearin, 1997). However, these experiments were performed in ovaries in which the soma was WT. Our data suggest that the second signal required for cyst formation emanates from the soma. In support of this notion, somatic expression of a dominant-negative form of the Rho GTPase in adult germaria results in loss of contact between GSC daughter cells and escort cells. As a result, cystoblasts fail to differentiate into cysts and linger in the germarium (Kirilly et al., 2011). Thus, the second signal that emanates from the soma is required not only for PGC differentiation, but also continuously during adult oogenesis.

Hormonal regulation of organogenesis

Two hormonal pathways are required to promote PGC differentiation and the initiation of oogenesis: the ecdysone and the Insulin pathways. Both are required for proper somatic proliferation and lineage differentiation, and both act non-cell-autonomously to promote PGC differentiation. Our epistasis analysis shows that both InR and ecdysone are required independently in the somatic ovary for *bam* expression in PGCs, and that ecdysone is additionally required to prepare the soma for its role in promoting cyst development.

Of note, we could not detect a direct link between the ecdysone and InR pathways in the somatic cells of the ovary, suggesting that they act in parallel. However, the two pathways are linked systemically. In particular, Insulin and Tor signaling are required in the prothoracic gland for ecdysone synthesis (Caldwell et al., 2005; Colombani et al., 2005; Layalle et al., 2008; Mirth et al., 2005; Walkiewicz and Stern, 2009). Because the timing of ecdysone release is intimately connected to the timing of niche and PGC differentiation (Gancz et al., 2011), nutrition affects gonadogenesis in a systemic manner. Combined, these data suggest that InR signaling affects the ovarian stem cell precursors on multiple levels: cell-autonomously, non-cell-autonomously from the somatic ovarian cells, and systemically.

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Competing interests statement

The authors declare no competing financial interests.

Author contributions
D.G. carried out the experiments. L.G. and D.G. designed the experiments and wrote the manuscript.

Supplementary material
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