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There was an error in the ePress version of Development 137, 2117-2126 published on May 26, 2010.

On p. 2119, eukaryotic translation initiation factor 4E is incorrectly defined as 4eIF4E, instead of eIF4E. The online issue and print versions are correct.

We apologise to the authors and readers for this mistake.
Specific roles of Target of rapamycin in the control of stem cells and their progeny in the *Drosophila* ovary

Leesa LaFever\(^1,2\), Alexander Feoktistov\(^1\), Hwei-Jan Hsu\(^1,2\) and Daniela Drummond-Barbosa\(^1,2,3,*,\)

**SUMMARY**

Stem cells depend on intrinsic and local factors to maintain their identity and activity, but they also sense and respond to changing external conditions. We previously showed that germline stem cells (GSCs) and follicle stem cells (FSCs) in the *Drosophila* ovary respond to diet via insulin signals. Insulin signals directly modulate the GSC cell cycle at the G2 phase, but additional unknown dietary mediators control both G1 and G2. Target of rapamycin, or TOR, is part of a highly conserved nutrient-sensing pathway affecting growth, proliferation, survival and fertility. Here, we show that optimal TOR activity maintains GSCs but does not play a major role in FSC maintenance, suggesting differential regulation of GSCs versus FSCs. TOR promotes GSC proliferation via G2 but independently of insulin signaling, and TOR is required for the proliferation, growth and survival of differentiating germ cells. We also report that TOR controls the proliferation of FSCs but not of their differentiating progeny. Instead, TOR controls follicle cell number by promoting survival, independently of either the apoptotic or autophagic pathways. These results uncover specific TOR functions in the control of stem cells versus their differentiating progeny, and reveal parallels between *Drosophila* and mammalian follicle growth.

**KEY WORDS:** TOR, Stem cells, Cell cycle, Cell growth, Oogenesis, *Drosophila*

**INTRODUCTION**

Stem cells self-renew and produce differentiating progeny for tissue integrity and function (Potten and Loeffler, 1990). Local and intrinsic factors maintain stem cell properties (Li and Xie, 2005) but external and circulating factors also affect stem cells. *Drosophila* intestinal and mammalian neural stem cells increase proliferation upon damage via insulin-like signals (Amcheslavsky et al., 2009; Yan et al., 2006; Zhang et al., 2001). Hormones modulate mammary stem cells (LaMarca and Rosen, 2008). Dietary factors stimulate mouse embryonic and hematopoietic stem cell activity (Hinge et al., 2009; Kim et al., 2009). It remains largely unknown, however, whether stem cells and their progeny respond to systemic changes uniformly or more specifically.

The *Drosophila* ovary houses stem cells in the germarium, the anterior-most portion of each ovariole (Fig. 1A) (Li and Xie, 2005). Two or three germline stem cells (GSCs) in a specialized niche self-renew and produce cystoblasts, which divide four times with incomplete cytokinesis to form germline cysts containing one oocyte and fifteen nurse cells. Follicle stem cells (FSCs) self-renew and produce follicle cells that envelop each cyst to form an egg chamber, or follicle. After leaving the germarium, each follicle develops through fourteen stages, forming a mature oocyte. As the cyst grows, follicle cells divide mitotically until stage 7, when they begin endoreplicating. Yolk uptake or vitellogenesis initiates at stage 8 (Spradling, 1993). The control of distinct stem cell populations and their differentiating progeny can thus be probed in this system.

Ovarian stem cells and their progeny respond to diet. On a protein-rich diet, GSCs and FSCs proliferate rapidly and their descendents divide and grow robustly. On a protein-poor diet, proliferation and growth are slowed, early germline cysts die and vitellogenesis is blocked (Drummond-Barbosa and Spradling, 2001). Insulin signaling is required for all responses, except early cyst viability (Drummond-Barbosa and Spradling, 2001; L.L. and D.D.-B., unpublished results). Insulin-like peptides directly promote GSC division, cyst growth and vitellogenesis (LaFever and Drummond-Barbosa, 2005) and indirectly control GSC maintenance (Hsu and Drummond-Barbosa, 2009). Insulin-like peptides promote GSC G2 progression through PI3 kinase and FOXO; however, additional diet mediators control both G1 and G2 (Fig. 1B) (Hsu et al., 2008).

The conserved TOR kinase regulates cell survival, growth and proliferation downstream of growth factors, amino acids, hormones and energy status (Wang and Proud, 2009). Tuberculosis complex 1 (TSC1) and TSC2 inhibit TOR activity (Pan et al., 2004), and the TOR and insulin pathways cross-talk, but also have independent functions (Hietakangas and Cohen, 2009). *Drosophila* hypomorphic Tor mutants have small ovaries with frequent cell death and absent vitellogenic follicles (Zhang et al., 2006), although specific oogenesis processes requiring Tor have remained unclear.

This study reveals specific Tor roles in *Drosophila* GSCs versus FSCs. Although Tor is required for proper proliferation of GSCs and FSCs, it plays a major role in GSC, but not FSC, maintenance. TOR also differentially regulates stem cells versus their progeny. Tor is necessary for early cyst proliferation, growth and survival by preventing apoptosis. By contrast, TOR does not regulate follicle cell proliferation and controls follicle cell growth and survival independently of apoptosis or autophagy. Follicle cell TOR activity also affects underlying cyst growth. Finally, TOR regulates these processes via insulin-dependent and -independent mechanisms. These studies uncover specific roles for TOR in the control of stem...
cells and their differentiating progeny in the Drosophila ovary. TOR is a known nutrient sensor in many systems (Wang and Proud, 2009); we therefore speculate that TOR is part of a broadly conserved mechanism that ties stem cell maintenance and function, and the survival, proliferation and growth of their descendents, to diet-dependent factors.

MATERIALS AND METHODS

Drosophila culture and genetic mosaic analyses

Fly stocks were maintained at 22-25°C on standard medium, yw is a wild-type control. TorA948V, TorW1251R, TorE161K, TorP2293L, TorR248X alleles and other genetic elements are described (Ashburner and Drysdale, 1994; Bernal and Kimbrell, 2000; Hsu et al., 2008; Juhasz et al., 2007; LaFever and Drummond-Barbosa, 2005; Tapon et al., 2001; Zhang et al., 2006). Mosaic analyses of flipase (FLP)/FLP recognition target (FRT)-mediated stem-cell-derived clones, including cyst growth and GSC relative division rate measurements, were performed as described (LaFever and Drummond-Barbosa 2005; Hsu et al., 2008). Many rounds of stem cell division occur prior to our analyses (with the exception of the initial daughter cells from a newly mutant GSC); therefore, perdurance of wild-type products is not a concern. Early germline cysts were staged based on fusome morphology (de Cuevas and Spradling, 1998). Egg chambers were staged based on size and nuclear morphology (Spradling, 1993). GSC and FSC maintenance was measured as described (Song and Xie, 2003; Xie and Spradling, 1998). The fraction of germaria containing a GFP-negative GSC or FSC relative to total germarium number was measured at different times after heat-shock, starting at four days (T0). T0 values were set at 100% and remaining values normalized to T0. Results were subjected to a Student's t-test or Chi-square analysis.

Immunostaining and microscopy

Ovaries were fixed and stained with 4',6-diamidino-2-phenylindole (DAPI) and antibodies as described (Hsu et al., 2008). Antibodies used were: mouse anti-β-Gal (Promega; 1:500), rabbit anti-GFP (Torrey Pines; 1:5000), rabbit anti-phosphohistone H3 (PHH3; Upstate Biotechnology; 1:250), guinea pig anti-Double-parked (DUP) (gift from T. Orr-Weaver; 1:5000) (Whittaker et al., 2000), rat anti-BrdU (Accurate Chemicals; 1:500), rabbit anti-phospho-4E-BP1 (Thr37/46; Cell Signaling Technology; 1:200), rabbit anti-Cyclin B (CycB; DSHB; 1:20), mouse anti-Lamin C (LamC) (DSHB; 1:100), mouse anti-fasciclin III (FASIII; DSHB; 1:25), Alexa 488-, 568- or 647-conjugated goat anti-mouse, -rabbit, -guinea pig or -rat secondaries (Molecular Probes; 1:400). 5-bromo-2-deoxyuridine (BrdU) incorporation and detection were performed as described (Lilly and Spradling, 1996). ApopTag Fluorescein Direct In Situ Apoptosis Detection Kit (Millipore) was used as described (Drummond-Barbosa and Spradling, 2001). Samples mounted in Vectashield (Vector Lab) were analyzed using a Zeiss Axioplan 2, LSM 510 or LSM 700 confocal microscope.

Cell cycle analyses

GSC division analyses were performed in 0- to 2-day-old females maintained for five days on yeast standard medium as described (Hsu et al., 2008). Briefly, GSCs, identified by fusome morphology and cap cell juxtaposition, were scored using BrdU (S), PHH3 (M) and CycB (G2, M). CycE was not used to mark G1 because it is expressed during most of the GSC cell cycle (Hsu et al., 2008). Experiments were performed at least in triplicate and results subjected to Student’s t-test.

To measure proliferation of FSCs, optical confocal sections 1.5 μm apart along the z-axis of germaria containing FSC-derived clones were analyzed. We identified the FSC as the anterior-most, marker-negative follicle cell in the region immediately anterior to the germarium 2A-2B border (Margolis and Spradling, 1995). FSCs and their immediate daughters typically lie just anterior to the bright FASIII-staining region (Nystul and Spradling, 2009). Each marker-negative FSC was scored as BrdU-positive or BrdU-negative, and percentages of BrdU-positive FSCs relative to total marker-negative FSC number were calculated. Follicle cell cycle in Tor mosaics was analyzed using CycE, BrdU, PHH3 and DUP (late G1 and S) (Thomer et al., 2004). Chi-square statistical analyses were performed.

Follicle cell size analysis

Relative follicle cell size was measured in mosaic follicle cell monolayers at stages when follicle cells normally undergo mitosis (stages 2-6) or endoreplication (stages 7-10). Using Image 1.40g, Tor mutant or control GFP-negative follicle cell clones ranging from 2-16 cells were measured in arbitrary area units. For each measured GFP-negative clone, a similar
measurement was made for an adjacent wild-type, GFP-positive follicle cell group of equal number, and the ratio between GFP-negative and GFP-positive areas obtained. Ten to sixty pairs of measurements were made for each mosaic genotype, and average ratios were expressed as a percentage and subjected to Student’s t-test.

RESULTS

TOR controls GSC proliferation at G2 largely independently of insulin signaling

Insulin-like peptides partially mediate the G2 effects of diet, but additional mediators control both G1 and G2 (Hsu et al., 2008). To test if the nutrient-sensor TOR (Wang and Proud, 2009) controls GSC proliferation, we analyzed Tor\textsuperscript{W1251R}/Tor\textsuperscript{E161K} and Tor\textsuperscript{P2293L}/Tor\textsuperscript{E161K} hypomorphic females, identifying GSCs by position and fusome morphology (Fig. 1C). Frequencies of 5-bromo-2-deoxyuridine (BrdU) and phosphohistone H3 (PHH3) labeling were significantly lower in control cells. Alleles of increasing severity were used: Tor\textsuperscript{W1251R}, a kinase-dead; and Tor\textsuperscript{P2293L}, a null. In mosaic germaria, the ratio of the number of progeny (i.e. cystoblasts and cysts) from the Tor mutant GSC to the number of progeny from the control GSC was markedly reduced relative to the corresponding ratio in mock mosaic germaria (Table 1; see Fig. 3A), consistent with the slower proliferation of Tor mutant GSCs.

To determine if Tor controls GSCs via a predominant effect on either G1 or G2, we used fusome morphology as a cell cycle marker (Fig. 1C) (de Cuevas and Spradling, 1998; Hsu et al., 2008). Tor mutant females had a higher frequency of GSCs with ‘G2 and M’ fusomes, a lower frequency of GSCs with ‘G1 and S’ fusomes relative to controls (Fig. 1D; see Fig. S1A in the supplementary material), indicating reduced proliferation rates.

To determine if Tor controls GSCs via a predominant effect on either G1 or G2, we used fusome morphology as a cell cycle marker (Fig. 1C) (de Cuevas and Spradling, 1998; Hsu et al., 2008). Tor mutant females had a higher frequency of GSCs with ‘G2 and M’ fusomes, a lower frequency of GSCs with ‘G1 and S’ fusomes relative to controls (Fig. 1D; see Fig. S1A in the supplementary material). Thus, Tor mutant GSCs progress more slowly through G2. Interestingly, wild-type GSCs and dividing cysts display increased levels of phosphorylation of 4E-BP, the eukaryotic translation initiation factor 4E (eIF4E) binding protein, which serves as a TOR activity reporter (Miron et al., 2003) during M but not interphase (Fig. 1E-G), further suggesting that an increase in TOR activity might be necessary for the G2 to M transition.

To determine if TOR is required intrinsically or indirectly (e.g. through the niche) for GSC proliferation, we generated genetic mosaic females in which Tor mutant ovarian cells [recognized by the absence of a β-galactosidase (β-gal) or green fluorescent protein (GFP) marker] are present in the context of surrounding control cells. Alleles of increasing severity were used: Tor\textsuperscript{W1251R}, a hypomorph; Tor\textsuperscript{P2293L}, a kinase-dead; and Tor\textsuperscript{P2293L}, a null. In mosaic germaria, the ratio of the number of progeny (i.e. cystoblasts and cysts) from the Tor mutant GSC to the number of progeny from the control GSC was markedly reduced relative to the corresponding ratio in mock mosaic germaria (Table 1; see Fig. 3A), consistent with the slower proliferation of Tor mutant GSCs.

Table 1. Tor is required for germline cyst division, growth and vitellogenesis

<table>
<thead>
<tr>
<th>Strain</th>
<th>Time(^a)</th>
<th>Germline cyst proportion(^b)</th>
<th>% germaria with dying cysts(^c)</th>
<th>Cyst growth rate(^d)</th>
<th>Presence of vitellogenic follicles(^e)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FRT40A control</td>
<td>6</td>
<td>0.9 (773)(^f)</td>
<td>16% (69)(^g)</td>
<td>100% (27)(^h)</td>
<td>Yes (24)(^i)</td>
</tr>
<tr>
<td>10</td>
<td>1.0 (369)</td>
<td>18% (50)</td>
<td>100% (26)</td>
<td>Yes (14)</td>
<td></td>
</tr>
<tr>
<td>Tor\textsuperscript{P2293L}</td>
<td>6</td>
<td>0.4 (206)(^i)</td>
<td>28% (25)</td>
<td>27% (6)(^j)</td>
<td>No (6)</td>
</tr>
<tr>
<td>10</td>
<td>0.3 (161)(^i)</td>
<td>31% (72)(^i)</td>
<td>26% (10)(^i)</td>
<td>No (5)</td>
<td></td>
</tr>
<tr>
<td>Tor\textsuperscript{W1251R}</td>
<td>6</td>
<td>0.3 (81)(^i) (***)</td>
<td>37% (52)(^i)</td>
<td>27% (12)(^j) (***)</td>
<td>No (22)</td>
</tr>
<tr>
<td>10</td>
<td>0.4 (115)(^i) (***)</td>
<td>15% (34)</td>
<td>48% (25)(^i) (**)</td>
<td>No (22)</td>
<td></td>
</tr>
<tr>
<td>Tor\textsuperscript{W1251R}/Thor\textsuperscript{2}</td>
<td>6</td>
<td>0.4 (453)(^i) (***)</td>
<td>37% (43)(^i)</td>
<td>27% (12)(^j) (***)</td>
<td>No (66)</td>
</tr>
<tr>
<td>FRT40A control (foxo bkgd)(^k)</td>
<td>10</td>
<td>0.3 (162)</td>
<td>–</td>
<td>26% (5)</td>
<td>No (8)</td>
</tr>
<tr>
<td>Tor\textsuperscript{W1251R}/Tor\textsuperscript{P2293L} (foxo bkgd)(^k)</td>
<td>10</td>
<td>1.0 (72)</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>InR\textsuperscript{E19}</td>
<td>10</td>
<td>0.4 (89)(^i) (\textit{k})</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>InR\textsuperscript{E19}/foxo\textsuperscript{25}</td>
<td>10</td>
<td>0.6 (159)</td>
<td>–</td>
<td>40% (9)</td>
<td>No (6)</td>
</tr>
<tr>
<td>FRT82B control</td>
<td>4</td>
<td>0.7 (318)</td>
<td>–</td>
<td>50% (25)</td>
<td>No (19)</td>
</tr>
<tr>
<td>Tsc\textsuperscript{1Q87X}</td>
<td>4</td>
<td>0.6 (329)</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>10</td>
<td>0.7 (257)(^i) (***)</td>
<td>–</td>
<td>160% (15)(^j) (***)</td>
<td>Yes (14)</td>
<td></td>
</tr>
<tr>
<td>Tsc\textsuperscript{1Q87X}/InR\textsuperscript{E19}</td>
<td>4</td>
<td>0.4 (225)(^i) (\textit{k})</td>
<td>–</td>
<td>160% (37)(^j) (***)</td>
<td>Yes (37)</td>
</tr>
</tbody>
</table>

\(^a\)Number of days after clone induction. \(^b\)Ratio of GFP- or β-gal-negative cystoblasts or cysts to GFP- or β-gal-positive cystoblasts or cysts. \(^c\)Percentage of germaria with at least one Apoptag-positive cyst. \(^d\)See Materials and methods for cyst growth rate measurements. \(^e\)GFP- or β-gal-negative cysts past stage 7. \(^f\)Total number of cystoblasts and cysts analyzed is shown in parentheses. \(^g\)Number of germaria analyzed is shown in parentheses. \(^h\)Number of GFP- or β-gal-positive cystoblasts or cysts. \(^i\)Number of ovarioles containing vitellogenic stages analyzed. \(^j\)Statistically significant difference relative to controls: *, P<0.05; **, P<0.002; ***, P<0.0001. \(^k\)Wild-type or Tor mutant clones generated in foxo\textsuperscript{1}/foxo\textsuperscript{25} background.
mosaics (Table 1). Cyst death contributes to the low Tor cyst proportion ratio (see below); however, the fact that removal of foxo does not result in a partial cyst proportion rescue suggests that Tor controls GSC proliferation largely independently of the insulin pathway, although insulin signaling upstream of FOXO might provide a minor contribution to TOR activation.

**TOR activity is required for GSC maintenance**

Five- to seven-day-old Tor<sup>W1251R</sup> or Tor<sup>E161K</sup> females had fewer GSCs per gerarium (1.8 ± 0.7, n=472) than controls (2.8 ± 0.7, n=557). To test if Tor is intrinsically required for GSC maintenance, we performed a relative GSC maintenance assay in Tor mosaics (Fig. 2). Control GSCs were lost slowly (Fig. 2A,D), as expected (Hsu et al., 1998). Asterisk, GFP-negative GSC; arrows, TorP2293L or Tsc<sup>Q87X</sup> cysts derived from GSCs subsequently lost. (D,E) Quantification of relative GSC maintenance in control, TorP2293L, Tsc<sup>W1251R</sup> and Tsc<sup>Q87X</sup> mosaic germaria. The fraction of mosaics containing a GFP-negative GSC was arbitrarily set at 100% at time point T<sub>0</sub> (4 days after clone induction) and data from one week (T<sub>1 week</sub>), two weeks (T<sub>2 weeks</sub>) or three weeks (T<sub>3 weeks</sub>) later were normalized to T<sub>0</sub>. Error bars, s.e.m. *, P<0.01; **, P<0.005; ***, P<0.001. Scale bar: 10 μm.

**Fig. 2. Normal TOR activity promotes GSC maintenance.** (A–C) Control, TorP2293L and Tsc<sup>Q87X</sup> mosaic germaria. GFP (green), control cells; 181 (red), fusomes and follicle cell membranes; LamC (red), cap cell nuclear membranes (arrowhead). GSCs are outlined.

**TOR controls germline cyst proliferation and survival**

As described above, Tor mutant GSCs produce markedly fewer progeny relative to control GSCs in mosaics (Fig. 3A; Table 1), partially owing to reduced proliferation (Fig. 1). Tor, however, can also regulate cell survival (Chang et al., 2009). Indeed, compared with control mosaics, Tor mosaics show increased frequency of TUNEL-positive cysts (Fig. 3B; Table 1), suggesting apoptosis of Tor mutant cysts.

To test whether Tor mutant cysts die at specific stages, we quantified the frequency of control and Tor mutant cystoblasts and 2-, 4-, 8- and 16-cell cysts (normalized per GSC). (C) Average number of marker-negative cystoblasts (CB) or cysts (2CC, 2-cell cyst; 4CC, 4-cell cyst; 8CC, 8-cell cyst; 16CC, 16-cell cyst) analyzed. *, P<0.04; **, P<0.02; ***, P<0.001. (E) Control, Tor<sup>W1251R</sup> or Tsc<sup>Q87X</sup> cysts (arrowheads) in mosaics. 1B1 (red), cell membranes; GFP (green), wild-type cells. Scale bar: 50 μm.

**Fig. 3. TOR regulates germline cyst proliferation, growth and survival independently of 4E-BP and FOXO.** (A) Ratios of marker-negative to marker-positive GSC progeny in mosaic germaria. foxo bkgd indicates clones induced in foxo<sup>21</sup>/foxo<sup>25</sup> females. (B) Percentage of germaria with TUNEL-marked dying cystoblasts and/or cysts. (C) Average number of marker-negative cystoblasts (CB) or cysts (2CC, 2-cell cyst; 4CC, 4-cell cyst; 8CC, 8-cell cyst; 16CC, 16-cell cyst) normalized per marker-negative GSC in control and Tor mosaics. (D) Growth rates (normalized to control) for marker-negative cysts in mosaics. Numbers above bars indicate the number of cystoblasts and cysts (A), mosaic germaria (B) or cysts (D) analyzed. *, P<0.04; **, P<0.02; ***, P<0.001. (E) Control, Tor<sup>W1251R</sup> or Tsc<sup>Q87X</sup> cysts (arrowheads) in mosaics. 1B1 (red), cell membranes; GFP (green), wild-type cells. Scale bar: 50 μm.

Tsc<sup>Q87X</sup> GSCs exhibited an even greater loss rate than Tor mutant GSCs, such that within a week from the initial measurement, no Tsc<sup>Q87X</sup> GSC remained in the niche (Fig. 2C,E). Optimal levels of TOR activity might therefore be required for proper GSC maintenance. Alternatively, TSC1 might be required for GSC maintenance independently of TOR.

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cell cysts per Tor mutant GSC were indistinguishable from those of controls, no Tor<sup>P225SL</sup> 8- or 16-cell cysts were observed in mosaic germaria, and the Tor<sup>W1251R</sup> allele yielded fewer 8-cell cysts and no 16-cell cysts at 10 days after clone induction (Fig. 3C). Thus, most Tor mutant cysts apparently die at the 8- and 16-cell stages. Occasionally, we observed follicles in which Tor mutant cysts with fewer than 16 cells were encapsulated with a neighboring wild-type 16-cell cyst, which might contribute to Tor mutant cyst reduction. Finally, the number of Tor mutant cystoblasts and 2-cell cysts was similar to controls despite reduced Tor mutant GSC proliferation rates, suggesting proportionately slowed Tor mutant cyst division.

**TOR controls germline cyst growth**

Although most Tor mutant cysts die early, a few of those cysts form a follicle. These are usually 16-cell cysts (or sometimes 8-cell cysts), and are more frequently observed at 6 rather than 10 days after clone induction. Tor mutant cysts grow at about 25% of the wild-type rate, suggesting a significant growth delay (Fig. 3D,E). In fact, follicles of Tor<sup>P225SL</sup> cysts do not grow past the stage 2 size, whereas follicles of hypomorphic Tor<sup>W1251R</sup> cysts reach the stage 3 or 4 size (Fig. 3E; Table 1). It is possible that these rare encaser follicles result from TOR protein perdurance in the initial progeny from a newly mutant GSC. In fact, Tor-null mutants can develop to larval stage 2 before death, presumably owing to perdurance of maternally derived TOR protein for about two to three days (Zhang et al., 2000). Conversely, Tsc<sup>1067X</sup> cysts have increased follicle growth rates (Fig. 3D,E; Table 1), consistent with the increased imaginal cell growth and proliferation observed upon TSC1/2 loss (Gao et al., 2002).

**TOR control of cyst proliferation, growth, and survival is 4E-BP-independent**

Many of the functions of Tor reflect its role in protein translation control via downstream targets 4E-BP (encoded by Thor in Drosophila) and S6 kinase (S6K) (Miron and Sonenberg, 2001; Teleman et al., 2008). 4E-BP is a conserved translational inhibitor that binds eIF4E, a cap-dependent translational activator, and 4E-BP phosphorylation by TOR releases eIF4E inhibition (Hay and Sonenberg, 2004). Accordingly, eIF4E overexpression leads to increased cell size in mammalian cells and Drosophila (Lachance et al., 2002; Lazaris-Karatzas et al., 1990). To test if reduced TOR activity affects cyst numbers and growth rates via 4E-BP-mediated translational inhibition, we analyzed Tor<sup>W1251R</sup> Thor<sup>+</sup> clones. Neither the proportion of Tor<sup>W1251R</sup> Thor<sup>+</sup> control germline cysts nor the growth rates of Tor<sup>W1251R</sup> Thor<sup>+</sup> cysts were statistically different from Tor<sup>W1251R</sup> mosaics (Fig. 3A,D; Table 1), indicating that Thor is dispensable for these effects. Instead, TOR might control translation in the germline primarily via S6K or MYC (Miron and Sonenberg, 2001; Teleman et al., 2008). In fact, mutation of S6K in mosaic ovarioles results in similar defects to those caused by Tor mutation (L.L. and D.D.-B., unpublished results), and MYC has been shown to act downstream of TOR (Teleman et al., 2008) and to control ovarian cell size (Maines et al., 2004).

**TOR mediates the effects of insulin signaling on germline cyst growth but also receives additional inputs**

Tor and the insulin pathway are required to control GSC proliferation and cyst growth (this study) (LaFever and Drummond-Barbosa, 2005; Hsu et al., 2008), and several studies showed that insulin signaling is among inputs integrated by TOR (Grewal, 2009; Hay and Sonenberg, 2004). Although the effects of insulin signaling on GSC division are mediated by foxo (Hsu et al., 2008), it is unknown whether foxo mutation suppresses the slow growth of InR mutant cysts, which could be potentially mediated via Tor and/or foxo. As expected, the low ratio of InR<sup>R19</sup> to control GSC progeny (which reflects slower proliferation of InR<sup>E19</sup> GSCs) is reversed by the foxo<sup>23</sup> mutation, but not by the Tsc1<sup>1067X</sup> mutation, in double-mutant mosaics (Table 1). The reverse occurs in cyst growth control. InR<sup>R19</sup> cysts have markedly reduced growth rates (Table 1) (LaFever and Drummond-Barbosa, 2005), whereas InR<sup>E19</sup> foxo<sup>23</sup> cysts have higher growth rates than control cysts in mosaic ovarioles (Table 1). By contrast, InR<sup>E19</sup> foxo<sup>23</sup> cysts had cyst growth rates comparable with those of InR<sup>E19</sup> cysts in mosaic ovarioles (Table 1). Thus, although the insulin pathway controls the proliferation of GSCs via foxo, it apparently controls the growth of their differentiating progeny via Tor. Nevertheless, null Tor mutant cysts have a more severe growth delay relative to null InR cysts (LaFever and Drummond-Barbosa, 2005), strongly suggesting that additional dietary factors besides insulin signaling modulate cyst growth via TOR.

**TOR is necessary for FSC proliferation, but not maintenance**

Tor is intrinsically required for GSC proliferation and maintenance; therefore, we wondered if Tor might similarly control FSCs as a mechanism to coordinate the response of both stem cell types to diet-dependent signals. To determine if Tor is required for FSC proliferation, we identified FSCs based on lineage tracing and measured S phase frequencies in control versus Tor mutant FSCs (Fig. 4A). Approximately half of control GFP-negative FSCs are BrdU-positive, whereas this frequency is drastically reduced for Tor<sup>P225SL</sup> or Tor<sup>W1251R</sup> FSCs (Fig. 4B), indicating that Tor is required for normal FSC proliferation.

To determine if Tor activity controls FSC maintenance, we performed an FSC maintenance assay in mosaic ovarioles analyzed at different times after clone induction (see Materials and methods). Although there was considerable experimental variability (see Table S2 in the supplementary material), Tor and Tsc1 do not appear to play a major role in FSC maintenance.

**TOR does not affect follicle cell proliferation**

Given that Tor controls FSC division, we asked whether Tor also regulates proliferation of their progeny. First, we compared Tor mutant to control follicle cell numbers in mosaic ovarioles (Fig. 4C-E). In control mosaics containing wild-type GFP-positive and GFP-negative FSCs, approximately equal numbers of follicle cells derived from each FSC result in a one-to-one ratio of progeny (Fig. 4C,E) (see also Margolis and Spradling, 1995). By contrast, Tor mosaics have a significantly reduced ratio of Tor<sup>P225SL</sup> or Tor<sup>W1251R</sup> to control follicle cells (Fig. 4D,E). Similar results were observed for InR<sup>E19</sup> or InR<sup>E19</sup> foxo<sup>23</sup> follicle cells (L.L. and D.D.-B., unpublished results), suggesting that insulin signaling controls follicle cell numbers independently of foxo. Although we had concluded that InR is not required cell autonomously in follicle cells (LaFever and Drummond-Barbosa, 2005), retrospective analyses revealed our previous misinterpretation due to weak GFP staining (heterozygous InR follicle cells with one copy of GFP mistaken for GFP-negative InR mutant follicle cells). These data suggest that Tor controls follicle cell survival and/or proliferation, given that the reduced Tor mutant FSC proliferation cannot account for the dramatic reduction in Tor mutant follicle cell number (Nystul and Spradling, 2009).
To directly test if Tor mutant follicle cells have reduced proliferation, we analyzed Tor\(^{W1251R}\) follicle cells using Cyclin B (G2 and M), BrdU (S), PHH3 (M) and Double parked (DUP, late G1 and S) (Thomer et al., 2004; Whittaker et al., 2000). Surprisingly, the frequencies of cells positive for these cell cycle markers were indistinguishable between control and Tor mutant follicle cells (Fig. 4F), indicating that Tor does not modulate follicle cell proliferation, unlike for FSCs. Thus, Tor controls follicle cell numbers by modulating their survival.

**TOR promotes follicle cell survival independently of suppression of apoptotic or autophagic cell death**

Tor modulates apoptosis and autophagy in many systems (Chang et al., 2009; Diaz-Troya et al., 2008). We therefore examined apoptosis incidence in control versus Tor mutant follicle cells in mosaics using TUNEL labeling and activated Caspase-3 antibody staining. Negligible numbers of either TUNEL-positive or activated Caspase-3-positive follicle cells were observed in both control or Tor\(^{W1251R}\) mosaics (9-41 mosaic ovarioles analyzed for each genotype and condition), suggesting that Tor mutant follicle cells are not eliminated by apoptosis.

If Tor mutant follicle cell number reduction is a result of autophagic death, then blocking autophagy in Tor mosaics should increase Tor mutant follicle cell numbers to wild-type levels. Null mutations in \(\text{Atg7}\) result in an 85-95% reduction in autophagy at the ultrastructural level, but homozygous females are still viable and fertile (Juhasz et al., 2007; Juhasz and Neufeld, 2008). We therefore analyzed Tor\(^{W1251R}\) mosaic follicle cells in \(\text{Atg7}\) homozygotes. Well-fed \(\text{Atg7}\) females exhibited vitellogenic follicle degeneration and stage-14 oocyte accumulation, which normally occur under starvation and are consistent with impaired autophagy-dependent nutrient mobilization from the fat body (a storage tissue) (Drummond-Barbosa and Spradling, 2001; Grewal and Saucedo, 2004; Hou et al., 2008). Furthermore, degenerating follicles accumulate, in agreement with apoptosis being required for clearance of dying follicles (Pritchett et al., 2009). Despite these clear indications that autophagy is disrupted in \(\text{Atg7}\) females, the ratio of \(\text{Tor}\)\(^{P2293L}\) or Tor\(^{W1251R}\) to control follicle cells remained unchanged in the \(\text{Atg7}\) background (see Table S1 in the supplementary material), suggesting that reduction in Tor mutant follicle cell numbers does not require autophagic death.

Alternative mechanisms might explain the reduced survival of Tor mutant follicle cells. When the entire follicle cell monolayer is homozygous for Tor\(^{W1251R}\), follicle development is supported through stage 9 (see Fig. S2 in the supplementary material), suggesting that surrounding wild-type cells might contribute to the reduced Tor mutant follicle cell numbers. Intriguingly, Tor mutant follicle cells surrounded by wild-type follicle cells often appeared to be undergoing extrusion from the mosaic follicle cell layer. Approximately 65% of mosaic ovarioles containing Tor mutant follicle cells (15/22 for Tor\(^{W1251R}\) mosaics and 14/22 for Tor\(^{P2293L}\) mosaics) displayed at least one mutant cell above or below the wild-type monolayer, which was never observed in control mosaics (Fig. 4G,H). As described above, extruded Tor mutant follicle cells were negative for activated Caspase-3. These results suggest that wild-type neighbors eliminate Tor mutant follicle cells without apparent apoptosis.

**TOR regulates follicle cell size and timely exit from the follicle cell mitotic program**

We also examined whether Tor controls follicle cell size. Follicle cells undergo mitotic cell divisions until stage 6, then transition to an endoreplicative program and greatly increase in size (Royzman and Orr-Weaver, 1998). During mitotic stages, Tor\(^{P2293L}\) or Tor\(^{W1251R}\) follicle cells are significantly smaller than neighboring control cells, whereas in endoreplicative stages, this difference is more pronounced (Fig. 5), demonstrating that Tor controls not only follicle cell number, but also size.

The more pronounced difference in Tor mutant follicle cell size in endoreplicative stages led us to hypothesize that Tor might control the mitosis-to-endoreplication transition. Mitotic follicle
cells go through G1, S, G2, M, whereas endoreplicating follicle cells alternate between G1 and S (Lee and Orr-Weaver, 2003; Wu et al., 2008). To directly determine whether Tor mutant follicle cells continue to divide mitotically beyond stage 6, we examined the PHH3 (M) and CycB (G2 and M) markers in Tor mutant follicle cells (Fig. 6A-C). We analyzed BrdU-incorporation as a control (S phase present in both programs) and no significant difference was observed between control and TorW1251R follicle cells (Fig. 6C). As expected, neighboring control follicle cells at stages 7 and 8 were all negative for PHH3 and CycB (Fig. 6A-C), unlike earlier mitotic follicle cells (see Fig. 4F). By contrast, some of the TorW1251R follicle cells at stages 7 and 8 were positive for PHH3 (4.4%) and CycB (26%; Fig. 6A-C), suggesting that TorW1251R follicle cells divide mitotically past stage 6.

The occurrence of PHH3- and CycB-positive TorW1251R follicle cells at stages 7 and 8 could reflect a consistent defect of TorW1251R follicle cell mitotic exit, or a defect in a subset of TorW1251R follicle cells. We therefore analyzed expression of DUP, a conserved pre-replicative complex component (Thomer et al., 2004; Whittaker et al., 2000), in mosaic follicle cell layers. Dup has a robust and dynamic G1 and S pattern in mitotic follicle cells but becomes more diffuse during endoreplication, when it is confined to G1 (Thomer et al., 2004). TorW1251R follicle cells in stages 7 and later exhibit the dynamic DUP pattern characteristic of a mitotic cell cycle in contrast to the diffuse pattern of neighboring control follicle cells (Fig. 6D,D′), suggesting that all TorW1251R follicle cells have a defect in endoreplication entry. We could not determine if TorW1251R follicle cells eventually endoreplicate owing to prohibitively low frequencies of Tor mutant follicle cell clones beyond stage 8. Thus, reduced Tor activity leads to either a delay or a block in the mitosis to endoreplication switch.

**Follicle cell TOR activity influences underlying cyst growth and vitellogenesis**

Germline cyst growth and surrounding follicle cell proliferation are coordinated (LaFever and Drummond-Barbosa, 2005; Maines et al., 2004; Wang and Riechmann, 2007). As Tor intrinsically controls follicle cell number, we asked whether Tor mutant follicle cells influence underlying wild-type cyst growth. If TorP2293L or TorW1251R follicle cells cover at least one-third of a wild-type cyst, there is a significant growth delay, evident by larger wild-type follicles positioned anteriorly (Fig. 7A-C; see Table S3 in the supplementary material). Conversely, wild-type cysts surrounded by TorP2293L follicle cells have accelerated growth (Fig. 7D; see Table S3 in the supplementary material). These results indicate that, although Tor does not control follicle cell proliferation, follicle cell TOR activity affects growth of the underlying germline, presumably via effects on follicle cell number and/or growth.

Follicle cell TOR activity also influences vitellogenesis progression of underlying oocytes. We rarely found mosaic ovarioles with a fully Tor mutant follicle cell monolayer and wild-type germline. Vitellogenesis was supported in only 2 out of 12 examples of such ovarioles containing TorW1251R follicle cells, which could reach a small stage-10 follicle before degenerating (see Fig. S2 in the supplementary material). For TorP2293L follicle cells, no vitellogenic follicles were observed in 14 ovarioles examined. These data suggest that follicle cells communicate with germ cells downstream of TOR to regulate vitellogenesis. Among plausible mechanisms would be an effect of Tor on the production of yolk proteins by follicle cells (Hansen et al., 2004; Hansen et al., 2005) or a more indirect effect involving the coordination between follicle cell number and germ cell development.
DISCUSSION

Stem cells support multiple adult tissues, and they also respond to external and physiological inputs (Drummond-Barbosa, 2008). Our recent studies uncovered dietary effects on Drosophila ovarian stem cells (Drummond-Barbosa and Spradling, 2001; Hsu and Drummond-Barbosa, 2009; Hsu et al., 2008; LaFever and Drummond-Barbosa, 2005). Insulin signals control GSCs, their niche and differentiating progeny; however, additional dietary mediators remain obscure. Here, we reveal strikingly specific effects of TOR on GSCs, FSCs and their progeny. Coupled to studies showing the conserved role of TOR as a nutrient sensor (Wang and Proud, 2009), these results address how specific effects of a nutrient-responsive factor might contribute to the coordination between different stem cell populations and their descendents.

G2 is a major point of GSC proliferation control by diet-dependent pathways

Both insulin signals and TOR are nutrient-sensing factors (Wang and Proud, 2009) that converge on G2 to regulate Drosophila GSC proliferation (Hsu et al., 2008) (this study). G2 regulation in response to diet/insulin signals also occurs in Drosophila male GSCs and in Caenorhabditis elegans germline precursors (Marini et al., 1999). Starvation promotes deleterious mutations during Saccharomyces cerevisiae division (Marini et al., 1999), and cancer cells form repair foci during a delayed G2 upon DNA damage (Kao et al., 2001). The multitude of GSC G2 regulators might reflect a mechanism to ensure genomic integrity under poor dietary conditions.

Although TOR regulates the G1–S transition, it also modulates G2–M in S. cerevisiae, Schizosaccharomyces pombe and mammalian cells (Wang and Proud, 2009). Combined with the Tor role in GSC G2, the increased phosphorylation of 4E-BP specifically during M suggests that a TOR activity increase might precede the G2–M transition. Interestingly, activated TOR is highly enriched at the mitotic spindles of rat ovarian granulosa cells and TOR inhibition by rapamycin impairs their proliferation (Yaba et al., 2008). Marked increases in S6K activity and 4E-BP1 phosphorylation in M occur in HeLa cells (Boyer et al., 2008; Heesom et al., 2001), further suggesting TOR activity cell cycle regulation as part of a conserved mechanism to tie G2–M to nutrient availability.

Specific effects of TOR on stem cell populations

Although both GSCs and FSCs require Tor for normal proliferation, only GSC maintenance requires optimal TOR activity. These distinctions do not reflect a fundamental difference between germline and somatic stem cells because TOR appears to control the maintenance of several, although probably not all, mammalian somatic stem cell types. In hematopoietic stem cells, Tsc1 or PTEN loss results in increased TOR signaling and short-term expansion, but also progressive stem cell depletion (Fan et al., 2008; Yilmaz et al., 2006). TOR activation downstream of Wnt1 overexpression leads to transiently increased hair follicle proliferation followed by stem cell loss (Castilho et al., 2009). By contrast, PTEN mutant ovarian granulosa cells do not become depleted (Fan et al., 2008), despite elevated TOR activity (Adhikari et al., 2010). Because granulosa cells might derive from stem cells (Lavranos et al., 1999), it is tempting to speculate that maintenance of these stem cells might not require precise TOR regulation, similar to Drosophila FSCs.

TOR differentially regulates stem cells and their progeny

Ovarian stem cells and their progeny respond to TOR differently. Reduced Tor activity leads to apoptosis of 8- and 16-cell cysts, but Tor mutant GSCs do not appear to undergo apoptotic or autophagic death. The niche might conceivably prevent GSC death. Indeed, we find no reports of GSC cell death within their in vivo niche. Consistent with the niche promoting GSC survival, GSCs die at higher rates when separated from somatic cells in culture (Niki, 2009). Laser ablation of the single apical niche cell causes death of Locusta migratoria male GSCs (Zahn et al., 2007). This model, however, does not account for a normal number of Tor mutant cystoblasts and 2-cell cysts. Perhaps a combination of niche displacement and growth defects leads to Tor mutant 8- and 16-cell cyst death.

Reduced Tor activity slows FSC proliferation, but has no effect on the cell cycle of follicle cells. This striking difference suggests that follicle cell proliferation might be largely insensitive to direct effects of diet. Follicle cells might instead receive their primary cue to divide from the underlying germline, perhaps via the actomyosin cytoskeleton, as recently suggested (Wang and Riechmann, 2007). Consistent with this idea, when germline cyst growth is slowed down by InR or Myc mutation, surrounding wild-type follicle cells adjust their numbers accordingly (LaFever and Drummond-Barbosa, 2005; Maines et al., 2004), although it remains to be determined if this reflects changes in follicle cell proliferation per se.

Tor mutant follicle cells are extruded in a competitive environment

Cell competition can occur when cell populations with different growth capacities coexist. It has been proposed that a cell senses the translational capacity of their neighbors and thus distinguishes ‘winner’ versus ‘loser’ cells. The ‘losers’ undergo apoptosis and
secretion of growth factors that stimulate ‘winner’ proliferation (Johnston, 2009). Although Tor regulates growth and translation (Wang and Proud, 2009), Tor mutant follicle cells do not exhibit apoptosis, but are instead extruded from ovarian monolayers. Apoptosis-independent extrusion of cells with compromised Decapentaplegic (DPP, a bone morphogenetic protein family member) signaling has been reported in mosaic Drosophila wing disc epithelia (Shen and Dahmann, 2005; Gibson and Perrimon, 2005). This similarity suggests a possible connection between DPP signaling and TOR.

Insulin-dependent and independent roles of TOR in cell growth and proliferation
TOR can be activated downstream of insulin signaling but also receives additional inputs (Grewal, 2009). Insulin signaling controls germine growth via TOR, whereas insulin (via FOXO) and TOR signaling regulate GSC proliferation in parallel. TOR-null ovarian cell defects are also more severe than Insr-null defects (see also LaFever and Drummond-Barbosa, 2005), implying that TOR receives additional inputs during oogenesis.

Amino acid transport activates TOR signaling in Drosophila and mammals (Avruch et al., 2009; Hietakangas and Cohen, 2009). The Drosophila genome predicts approximately 40 amino acid transporters (http://flybase.org) and recent evidence suggests that methionine is a key dietary amino acid for oogenesis in Drosophila (Grandison et al., 2009). Further studies should investigate how various classes of amino acid transporters affect ovarian TOR signaling and amino acid requirements for specific oogenesis processes.

4E-BP and translational control downstream of TOR
4E-BP, encoded by Thor, represses cap-dependent translation via eIF4E inhibition. TOR phosphorylates and inhibits 4E-BP, leading to translation de-repression (Fingar et al., 2002). 4E-BP, however, does not mediate TOR ovarian phenotypes, suggesting that TOR probably acts through S6K or MYC (Grewal, 2009; Hay and Sonenberg, 2004; Li et al. 2010). Indeed, S6K overexpression partially restores Tor mutant growth, viability and fertility (Zhang et al., 2000), whereas MYC loss causes germline growth phenotypes similar to Tor defects (Maines et al., 2004).

Whether or not 4E-BP is required in any other tissues to mediate the effects of reduced TOR activity remains unclear. Although overexpression of eIF4E increases cell growth rates and overexpression of 4E-BP results in smaller cell size, loss of 4E-BP does not phenocopy eIF4E overexpression (Fingar et al., 2002; Teelen et al., 2005). Furthermore, Thor mutation has no obvious phenotype in Drosophila except for increased sensitivity to stress and impaired innate immunity (Bernal and Kimbrell, 2000; Teelen et al., 2005). Although Thor is required for dietary restriction effects on lifespan (Zid et al., 2009), we found no reports of Tor Thor double mutants in the literature.

Parallels between the role of TOR in Drosophila and mammalian ovaries
Our results bring to light interesting parallels between the role of TOR in Drosophila and mammalian ovaries. Insulin and TOR signaling are active in mammalian ovaries (Adhikari et al., 2010; Yaba et al., 2008) and rapamycin inhibits follicle growth in cultured mouse ovaries (Yaba, 2008), suggesting similar regulation of oocyte growth and follicle cell numbers between Drosophila and mammals. Although adult mammalian ovaries do not contain GSCs, overexpression of either insulin or TOR signaling in mouse primordial germ cells leads to premature ovarian failure caused by the hyperactivation and subsequent depletion of the primordial germ cell pool (Adhikari et al., 2010; Reddy et al., 2008), a phenotype that is arguably reminiscent of the rapid loss of Tsc1 mutant GSCs.

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Competing interests statement
The authors declare no competing financial interests.

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