Specific roles of Target of rapamycin in the control of stem cells and their progeny in the Drosophila ovary

Leesa LaFever, Alexander Feoktistov, Hwei-Jan Hsu and Daniela Drummond-Barbosa

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\textsuperscript{1,2}, Alexander Feoktistov\textsuperscript{1}, Hwei-Jan Hsu\textsuperscript{1,2} and Daniela Drummond-Barbosa\textsuperscript{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 descendants 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). Tuberous sclerosis 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 cell proliferation downstream of growth factors, amino acids, hormones and energy status (Wang and Proud, 2009). Tuberous sclerosis 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. *TorQ87X*, *TorP2293L*, *TorE161K*, *Foxo25*, *Foxo21*, *InR169*, *InR350*, *Thor* and *Atp4d* 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, perurdance 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 germlarum 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 *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 Biochemicals; 1:500), rabbit anti-phospho-4E-BP1 (Thr37/46; Developmental Studies Hybridoma Bank [DSHB]; 1:10), mouse α-spectrin (DSHB; 1:500), mouse anti-Cyclin B (CycB; DSHB; 1:20), mouse anti-Lamin C (LamC) (DSHB; 1:100), mouse anti-fascin III (FASIII; DSHB; 1:25), Alexa 488-, 568-, or 633-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). A apoptosis 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 cycle 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). The FSC number were calculated. Follicle cell cycle in *Tor* mosaics was analyzed using CycB, BrdU, PHH3 and DUB (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 ImageJ 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
To test if the nutrient-sensor TOR (Wang and Proud, 2009) controls GSC proliferation at G2 largely independently of insulin signaling

In insulin-like peptides partially mediate the G2 effects of diet, but TOR controls GSC proliferation at G2 largely

RESULTS

To determine if TOR controls GSCs via a predominant effect on either G1 or G2, we analyzed fusome morphology in genetic mosaics. As a control experiment, we analyzed germline cysts for an insulin receptor mutation, InR, (see Fig. 1B in the supplementary material), and found an increased frequency of ‘G2 and M’ fusomes, in accordance with the known requirement for InR in GSC proliferation via G2 (Hsu et al., 2008). Similarly, Tor, GSCs in mosaics had an increased frequency of ‘G2 and M’ fusomes relative to neighboring control GSCs (see Fig. S1C in the supplementary material), consistent with an intrinsic requirement for G2 progression. Despite the similar trend, however, the frequencies of ‘G2 and M’ fusomes in Tor, and control GSCs in mosaics were both reduced relative to frequencies in whole Tor mutant and control females, respectively, suggesting either communication between GSCs in mosaics or background differences.

Both the insulin pathway and TOR are intrinsically required for normal GSC G2 progression. Thus, TOR regulation of G2 could be insulin-dependent or -independent (Fig. 1B). In InR mutants, removal of the downstream negative regulator foxo rescues the G2 delay (Hsu et al., 2008). We therefore reasoned that if TOR acts through insulin signaling to regulate G2, Tor, foxo double mutants should reverse the Tor mutant G2 delay. Because Tor, foxo double mutants showed that foxo does not rescue the reduced proportions observed in a wild-type background (Table 1; see Fig. 3A). As expected, loss of foxo rescues the low InR mutant to control GSC progeny ratio of InR

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

<table>
<thead>
<tr>
<th>Strain</th>
<th>Time</th>
<th>Germline cyst proportion</th>
<th>% germlia with dying cysts</th>
<th>Cyst growth rate</th>
<th>Presence of vitellogenic follicles</th>
</tr>
</thead>
<tbody>
<tr>
<td>FRT40A control</td>
<td>6</td>
<td>0.9 (773)***</td>
<td>16% (69)**</td>
<td>100% (27)**</td>
<td>Yes (24)**</td>
</tr>
<tr>
<td>TorE161K mutant females</td>
<td>10</td>
<td>1.0 (396)</td>
<td>18% (50)</td>
<td>100% (26)</td>
<td>Yes (14)</td>
</tr>
<tr>
<td>TorE161K/TorE161K</td>
<td>10</td>
<td>0.4 (206)***</td>
<td>28% (25)</td>
<td>27% (6)**</td>
<td>No (6)</td>
</tr>
<tr>
<td>TorE161K/TorE161K</td>
<td>10</td>
<td>0.3 (161)***</td>
<td>31% (72)**</td>
<td>27% (6)**</td>
<td>No (5)</td>
</tr>
<tr>
<td>TorE161K/TorE161K</td>
<td>10</td>
<td>0.3 (61)*****</td>
<td>37% (52)**</td>
<td>26% (10)**</td>
<td>No (5)</td>
</tr>
<tr>
<td>TorE161K/TorE161K</td>
<td>10</td>
<td>0.4 (115)***</td>
<td>15% (34)</td>
<td>48% (25)**</td>
<td>No (22)</td>
</tr>
<tr>
<td>TorE161K/TorE161K</td>
<td>10</td>
<td>0.4 (453)***</td>
<td>37% (43)**</td>
<td>27% (12)**</td>
<td>No (66)</td>
</tr>
<tr>
<td>TorE161K/TorE161K</td>
<td>10</td>
<td>0.3 (162)</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>FRT40A control (foxo bkgd)</td>
<td>10</td>
<td>1.0 (72)</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>TorE161K/TorE161K (foxo bkgd)</td>
<td>10</td>
<td>0.4 (89)***</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>InR19/foxo25</td>
<td>10</td>
<td>0.6 (159)</td>
<td>40% (9)</td>
<td>No (6)</td>
<td></td>
</tr>
<tr>
<td>InR19/foxo25</td>
<td>10</td>
<td>1.2 (257)*****</td>
<td>50% (25)</td>
<td>No (19)</td>
<td></td>
</tr>
<tr>
<td>FRT828B control</td>
<td>4</td>
<td>0.7 (318)</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Tsc1Q87X</td>
<td>10</td>
<td>0.6 (329)</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Tsc1Q87X</td>
<td>10</td>
<td>0.4 (225)**</td>
<td>160% (15)**</td>
<td>Yes (14)</td>
<td></td>
</tr>
<tr>
<td>Tsc1Q87X</td>
<td>10</td>
<td>0.4 (225)**</td>
<td>160% (37)**</td>
<td>Yes (37)</td>
<td></td>
</tr>
</tbody>
</table>

#Number of days after clone induction. #Ratio of GFP- or β-gal-negative cystoblasts or cysts to GFP- or β-gal-positive cystoblasts or cysts. #Percentage of germlia with at least one Apoptag-positive cyst. See Materials and methods for cyst growth rate measurements. #GFP- or β-gal-negative cysts past stage 7. Total number of cystoblasts and cysts analyzed is shown in parentheses. #Number of germlia analyzed is shown in parentheses. #Number of GFP- or β-gal-negative cysts analyzed is shown in parentheses. #Number of ovarioles containing vitellogenic stages analyzed. Statistically significant difference relative to controls: *, P<0.05; **, P<0.002; ***, P<0.001. Wild-type or Tor mutant clones generated in foxo/foxo background.

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.
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 W1251R/Tor E161K 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 and Drummond-Barbosa, 2009; Wang and Lin, 2005; Xie and Spradling, 1998). By contrast, TorP2293L and Drummond-Barbosa, 2009; Wang and Lin, 2005; Xie and Spradling, 1998). By contrast, TorP2293L and TorW1251R GSCs were lost significantly faster (Fig. 2B,D). No TUNEL-positive GSCs were detected in 226 geraria with a mosaic Tor germline, suggesting that Tor mutant GSCs are not lost by apoptosis. TOR activity is known to control autophagy (Chang et al., 2009), but inactivation of the autophagy pathway by an Aig7 mutation failed to rescue the Tor mutant GSC loss (see Table S1 in the supplementary material). Thus, the Tor GSC maintenance defect is probably caused by differentiation, although we cannot exclude the possibility that GSCs die by a distinct mechanism.

Given the increased loss of Tor mutant GSCs, we reasoned that high TOR activity might promote GSC maintenance. We generated germline clones mutant for Tsc1, which encodes an upstream negative regulator of TOR (Tapon et al., 2001). Surprisingly, Tsc1Q87X GSCs exhibited an even greater loss rate than Tor mutant GSCs, such that within a week from the initial measurement, no Tsc1Q87X 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 survival independently of 4E-BP and FOXO.

**TOR controls 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 foxo1+/foxo2− 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 GSCs in mosaics. Numbers above bars indicate the number of cystoblasts and cysts (A), mosaic germlina (B) or cysts (D) analyzed. *, P<0.05; **, P<0.005; ***, P<0.001. Scale bar: 50 μm.

As described above, Tor mutant GSCs produce markedly fewer progeny relative to control GSCs in mosaic (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 mosiacs, 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). Stages were identified by fusome morphology, which becomes progressively more branched as cyst cell number increases (de Cuevas and Spradling, 1998). Although the frequencies of cystoblasts and 2-
cell cysts per Tor mutant GSC were indistinguishable from those of controls, no Tor<sup>P2293L</sup> 8- or 16-cell cysts were observed in mosaic germlaria, 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>P2293L</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 escaper 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>1<sup>67X</sup></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; Telemav 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-Karatza 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> to 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; Telemav 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 (Telemav 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 InRE<sup>19</sup> to control GSC progeny (which reflects slower proliferation of InRE<sup>19</sup> GSCs) is reversed by the foxo<sup>−</sup> mutation, but not by the Tsc<sup>1<sup>67X</sup></sup> mutation, in double-mutant mosaics (Table 1). The reverse occurs in cyst growth control. InRE<sup>19</sup> cysts have markedly reduced growth rates (Table 1) (LaFever and Drummond-Barbosa, 2005), whereas InRE<sup>19</sup> Tsc<sup>1<sup>67X</sup></sup> cysts have higher growth rates than control cysts in mosaic ovarioles (Table 1). By contrast, InRE<sup>19</sup> foxo<sup>−</sup> cysts had cyst growth rates comparable with those of InRE<sup>19</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>P2293L</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<sup>P2293L</sup> or Tor<sup>W1251R</sup> FSCs (Fig. 4D,E) indicated 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.
To directly test if Tor mutant follicle cells have reduced proliferation, we analyzed TorW1251R 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 TorW1251R 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 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 TorW1251R mosaic follicle cells in Atg7 homozygotes. Well-fed Atg7d4 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 autophagy being required for clearance of dying follicles (Pritchett et al., 2009). Despite these clear indications that autophagy is disrupted in Atg7d4 females, the ratio of TorP2293L or TorW1251R to control follicle cells remained unchanged in the Atg7d4 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 TorW1251R, 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 follicles (15/22 for TorP2293L mosaics and 14/22 for TorW1251R 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, TorP2293L or TorW1251R 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, and 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 BrdU- or PHH3-positive TorW1251R follicle cells 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 Tsc1Q87X 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.
Development 137 (13)

recent studies uncovered dietary effects on Drosophila external and physiological inputs (Drummond-Barbosa, 2008). Our Stem cells support multiple adult tissues, and they also respond to DISCUSSION (2124 and Proud, 2009) that converge on G2 to regulate Both insulin signals and TOR are nutrient-sensing factors (Wang diet-dependent pathways G2 is a major point of GSC proliferation control by nutrient-responsive factor might contribute to the coordination between different stem cell populations and their descendents. 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. 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 (Gan 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.

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 (Narbonne and Roy, 2006; Ueishi et al., 2009). Starvation promotes deleterious mutations during Saccharomyces cerevisae 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. cerevisae, 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.

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
secrete 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 mosaic 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 germline 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 Inr-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; Telemann et al., 2005). Furthermore, Thor mutation has no obvious phenotype in Drosophila except for increased sensitivity to stress and impaired innate immunity (Bernal and Kimberl, 2000; Telemann 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 Testis mutant GSCs.

**Acknowledgements**

L.L., A.F., H.-J.H. and D.D.-B. designed and interpreted the experiments. L.L. and D.D.-B. wrote the paper. L.L., A.F. and H.-J.H. performed the experiments: L.L. contributed to all figures and tables; A.F. contributed to Figs 3-5 and 7, and Tables S1 and S3 in the supplementary material; H.-J.H. contributed to Fig. S1 in the supplementary material. We thank J. Von Stetina for her initial observations. We are grateful to E. Ables and M.-Y. Kim for valuable comments on this manuscript. We thank T. Neufeld, T. Orr-Weaver, J. Bateman, I. Hanirahani, E. Matunis, Flybase, Cell Signaling Technology (anti-p4E-BP sample) and the Bloomington and Kyoto Stock Centers for stocks and reagents. This work was supported by National Institutes of Health R01 GM069675 and American Cancer Society RSG-DCC-112316, and training grants T32 HD007502 and T32HD007043 (support for L.L.). Depostited in PMC for release after 12 months.

**Competing interests statement**

The authors declare no competing financial interests.

**Supplementary material**

Supplementary material for this article is available at http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.050351/-/DC1

**References**


Fingar, D. C., Salama, S., Tsou, C., Harlow, E. and Blenis, J. (2002). Mammalian cell size is controlled by mTOR and its downstream targets S6K1 and 4EBP1/eIF4E. Genes Dev. 16, 1472-1487.


Table S1. Tor mosaic GSC loss and low follicle cell numbers are independent of Atg7

<table>
<thead>
<tr>
<th>Strain</th>
<th>% GSC loss</th>
<th>% GFP-negative follicle cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>FRT40A control</td>
<td>4.8% (21)</td>
<td>46.2% (4673)</td>
</tr>
<tr>
<td>FRT40A control (Atg7&lt;sup&gt;d4&lt;/sup&gt; bkgd)&lt;sup&gt;f&lt;/sup&gt;</td>
<td>5.0% (20)</td>
<td>42.4% (5565)</td>
</tr>
<tr>
<td>Tor&lt;sup&gt;W125R&lt;/sup&gt;</td>
<td>27.5% (40)</td>
<td>12.1% (3629)</td>
</tr>
<tr>
<td>Tor&lt;sup&gt;W125R&lt;/sup&gt; (Atg7&lt;sup&gt;d4&lt;/sup&gt; bkgd)&lt;sup&gt;f&lt;/sup&gt;</td>
<td>20.0% (40)&lt;sup&gt;g&lt;/sup&gt;</td>
<td>6.0%* (4368)&lt;sup&gt;h&lt;/sup&gt;</td>
</tr>
<tr>
<td>Tor&lt;sup&gt;P2293L&lt;/sup&gt;</td>
<td>44.1% (34)</td>
<td>17.1% (6401)</td>
</tr>
<tr>
<td>Tor&lt;sup&gt;P2293L&lt;/sup&gt; (Atg7&lt;sup&gt;d4&lt;/sup&gt; bkgd)&lt;sup&gt;f&lt;/sup&gt;</td>
<td>35.0% (23)&lt;sup&gt;g&lt;/sup&gt;</td>
<td>5.0%* (2945)&lt;sup&gt;h&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup>Clones were analyzed 10 days after clone induction.
<sup>b</sup>Percentage of germaria with GFP-negative cystoblasts or cysts but lacking their GFP-negative GSC mother (which indicates recent GSC loss) relative to the total number of ovarioles with a mosaic germline.
<sup>c</sup>Percentage of GFP-negative follicle cells relative to the total number of follicle cells analyzed.
<sup>d</sup>Total number of germaria with mosaic germline analyzed is shown in parentheses.
<sup>e</sup>Total number of follicle cells analyzed is shown in parentheses.
<sup>f</sup>Clones were generated in Atg7<sup>d4</sup> homozygous background.
<sup>g</sup>There is no statistically significant difference between results in wild-type versus Atg7<sup>d4</sup> background.
<sup>h</sup>The Atg7<sup>d4</sup> mutation does not rescue of the Tor mutant phenotype, but the percentage of GFP-negative follicle cells is significantly smaller in the Atg7<sup>d4</sup> background. *, P<0.001.
Table S2. Tor does not appear to regulate FSC maintenance

<table>
<thead>
<tr>
<th>Strain</th>
<th>4 days(a)</th>
<th>8 days</th>
<th>11 days</th>
<th>12 days</th>
<th>18 days</th>
<th>25 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FRT40A control</td>
<td>57%(^b) (101)(^c)</td>
<td>55% (93)</td>
<td>--</td>
<td>47% (72)</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Tor(^P2293L)</td>
<td>95% (93)</td>
<td>70% (98)</td>
<td>--</td>
<td>69% (75)</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Tor(^W1251R)</td>
<td>87% (195)</td>
<td>54% (214)</td>
<td>--</td>
<td>39% (198)</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>FRT82B control</td>
<td>92% (100)</td>
<td>71% (108)</td>
<td>--</td>
<td>61% (81)</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Tsc1(^\Delta F)</td>
<td>81% (99)</td>
<td>84% (115)</td>
<td>--</td>
<td>66% (76)</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Experiment 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FRT40A control</td>
<td>--</td>
<td>--</td>
<td>40% (67)</td>
<td>--</td>
<td>33% (57)</td>
<td>13% (62)</td>
</tr>
<tr>
<td>Tor(^P2293L)</td>
<td>--</td>
<td>--</td>
<td>43% (28)</td>
<td>--</td>
<td>24% (51)</td>
<td>5.0% (40)</td>
</tr>
<tr>
<td>Tor(^W1251R)</td>
<td>--</td>
<td>--</td>
<td>30% (77)</td>
<td>--</td>
<td>20% (50)</td>
<td>5.5% (55)</td>
</tr>
<tr>
<td>Experiment 3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FRT40A control</td>
<td>--</td>
<td>--</td>
<td>25% (67)</td>
<td>--</td>
<td>41% (63)</td>
<td>9.1% (55)</td>
</tr>
<tr>
<td>Tor(^P2293L)</td>
<td>--</td>
<td>--</td>
<td>40% (35)</td>
<td>--</td>
<td>21% (67)</td>
<td>1.6% (61)</td>
</tr>
<tr>
<td>Tor(^W1251R)</td>
<td>--</td>
<td>--</td>
<td>20% (70)</td>
<td>--</td>
<td>3.4% (87)</td>
<td>13% (55)</td>
</tr>
<tr>
<td>Experiment 4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FRT40A control</td>
<td>--</td>
<td>--</td>
<td>46% (50)</td>
<td>--</td>
<td>37% (49)</td>
<td>11% (56)</td>
</tr>
<tr>
<td>Tor(^P2293L)</td>
<td>--</td>
<td>--</td>
<td>15% (47)</td>
<td>--</td>
<td>31% (51)</td>
<td>17% (41)</td>
</tr>
<tr>
<td>Tor(^W1251R)</td>
<td>--</td>
<td>--</td>
<td>41% (56)</td>
<td>--</td>
<td>16% (70)</td>
<td>14% (50)</td>
</tr>
<tr>
<td>Experiment 5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FRT40A control</td>
<td>62% (183)</td>
<td>--</td>
<td>--</td>
<td>45% (121)</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Tor(^P2293L)</td>
<td>66% (67)</td>
<td>--</td>
<td>--</td>
<td>58% (139)</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Tor(^W1251R)</td>
<td>41% (119)</td>
<td>--</td>
<td>--</td>
<td>23% (141)</td>
<td>--</td>
<td>--</td>
</tr>
</tbody>
</table>

\(^a\)Number of days after clone induction.  
\(^b\)Percentage of germaria containing at least one GFP-negative FSC.  
\(^c\)Total number of germaria analyzed is shown in parentheses.
<table>
<thead>
<tr>
<th>Strain</th>
<th>Time</th>
<th>% of wild-type cysts with altered growth</th>
</tr>
</thead>
<tbody>
<tr>
<td>FRT40A control</td>
<td>6</td>
<td>delayed-0% (15)</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>delayed-0% (13)</td>
</tr>
<tr>
<td>TorR248X</td>
<td>6</td>
<td>delayed-75% (4)</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>delayed-93% (15)</td>
</tr>
<tr>
<td>TorP229SL</td>
<td>6</td>
<td>delayed-85.7% (23)</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>delayed-93% (15)</td>
</tr>
<tr>
<td>TorW1251R</td>
<td>6</td>
<td>delayed-50% (2)</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>delayed-95% (19)</td>
</tr>
<tr>
<td>TorW1251R Thor2</td>
<td>10</td>
<td>delayed-100% (7)</td>
</tr>
<tr>
<td>Tsc1Q87X</td>
<td>10</td>
<td>accelerated-80% (5)</td>
</tr>
<tr>
<td>Tsc1Q87X InR154</td>
<td>10</td>
<td>accelerated-80% (5)</td>
</tr>
</tbody>
</table>

a Number of days after clone induction.
b Percentage of wild-type germline cysts surrounded by at least one-third GFP-negative follicle cells showing either delayed or accelerated growth (see Materials and methods).
c Number of wild-type cysts surrounded by at least one-third GFP-negative follicle cells analyzed.