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# TSC1/2 tumour suppressor complex maintains *Drosophila* germline stem cells by preventing differentiation

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## SUMMARY

Tuberous sclerosis complex human disease gene products TSC1 and TSC2 form a functional complex that negatively regulates target of rapamycin (TOR), an evolutionarily conserved kinase that plays a central role in cell growth and metabolism. Here, we describe a novel role of TSC1/2 in controlling stem cell maintenance. We show that in the *Drosophila* ovary, disruption of either the *Tsc1* or *Tsc2* gene in germline stem cells (GSCs) leads to precocious GSC differentiation and loss. The GSC loss can be rescued by treatment with TORC1 inhibitor rapamycin, or by eliminating S6K, a TORC1 downstream effecter, suggesting that precocious differentiation of *Tsc1/2* mutant GSC is due to hyperactivation of TORC1. One well-studied mechanism for GSC maintenance is that BMP signals from the niche directly repress the expression of a differentiation-promoting gene *bag of marbles* (*bam*) in GSCs. In *Tsc1/2* mutant GSCs, BMP signalling activity is downregulated, but *bam* expression is still repressed. Moreover, *Tsc1 bam* double mutant GSCs could differentiate into early cystocytes, suggesting that TSC1/2 controls GSC differentiation via both BMP-Bam-dependent and -independent pathways. Taken together, these results suggest that TSC prevents precocious GSC differentiation by inhibiting TORC1 activity and subsequently differentiation-promoting programs. As TSC1/2-TORC1 signalling is highly conserved from *Drosophila* to mammals, it could have a similar role in controlling stem cell behaviour in mammals, including humans.

**KEY WORDS: *Drosophila* ovary, Target of rapamycin, Tuberous sclerosis complex, Differentiation, Germline stem cell, Maintenance**

## INTRODUCTION

Stem cells are characterized by the ability of self-renewal and generating daughters that give rise to one or more types of differentiated cells. These properties enable them to maintain homeostasis of many adult tissues throughout life. Stem cells in adult tissues are commonly regulated by extrinsic factors from the micro-environment or niche, and intrinsic factors that function cell-autonomously in stem cells (for reviews, see Li and Xie, 2005; Morrison and Spradling, 2008). Elucidating how the self-renewal and differentiation of stem cells are balanced by extrinsic and intrinsic factors is crucial in understanding tissue homeostasis control, the dysregulation of which is linked to many human diseases.

The *Drosophila* ovarian germline stem cells (GSCs) provide an attractive system in which to study the molecular mechanisms underlying stem cell self-renewal and differentiation (for a review, see Kirilly and Xie, 2007). A single stem cell-niche unit is found at the anterior tip of each germarium, in which terminal filament cells, cap cells and escort cells together form a functional GSC niche to host two or three GSCs (Fig. 1A) (Xie and Spradling, 2000). The niche physically anchors GSCs by forming DE-cadherin-mediated adherens junctions (Song et al., 2002). Additionally, the niche cells produce crucial signal molecules to govern GSC self-renewal and prevent differentiation. Previous studies have demonstrated a central role of BMP signalling in maintaining GSC self-renewal. Two ligands of BMP signalling, Decapentaplegic (Dpp) and Glass bottom boat (Gbb), which are expressed in the niche cells, directly act on GSCs through canonical pathway components to regulate the transcription of target genes (Song et al., 2004; Xie and Spradling, 1998). A major target is *bag of marbles* (*bam*) (Chen and McKearin, 2003a; Song et al., 2004), a differentiation-promoting gene (McKearin and Ohlstein, 1995; Ohlstein and McKearin, 1997). In GSCs, the activated/phosphorylated transcriptional factor Mad forms complexes with Medea (the *Drosophila* Smad4) and subsequently translocates to nucleus where they recognize a silencer element on the *bam* promoter and maintain transcriptional silencing of *bam* (Chen and McKearin, 2003a; Chen and McKearin, 2003b; Song et al., 2004). In cystoblasts, where *bam* repression is relieved, Bam functions together with Bgcn to inhibit the translation of Nanos (Nos), a translational repressor that prevents GSC differentiation; as a consequence, differentiation can proceed (Forbes and Lehmann, 1998; Li et al., 2009; Wang and Lin, 2004). Other factors are also required in the GSCs, such as Pelota (Pelo), a translational release factor-like protein that essentially helps in preventing GSC differentiation by modulating both BMP-Bam-dependent and -independent pathways (Xi et al., 2005). Furthermore, the microRNA pathway components, including Dcr-1, Ago1 and Loquacious are also cell-autonomously required for GSC division and maintenance (Hatfield et al., 2005; Jin and Xie, 2007; Park et al., 2007; Yang et al., 2007), whereas Mei-P26, a Trim-NHL containing protein, functions to antagonize the microRNA pathway and allow germline differentiation (Neumuller et al., 2008; Page et al., 2000). Therefore, in addition to transcriptional regulation by BMP signalling, post-transcriptional regulation is crucial for GSC self-renewal, although functional relationships among those regulators and pathways are not yet well understood.

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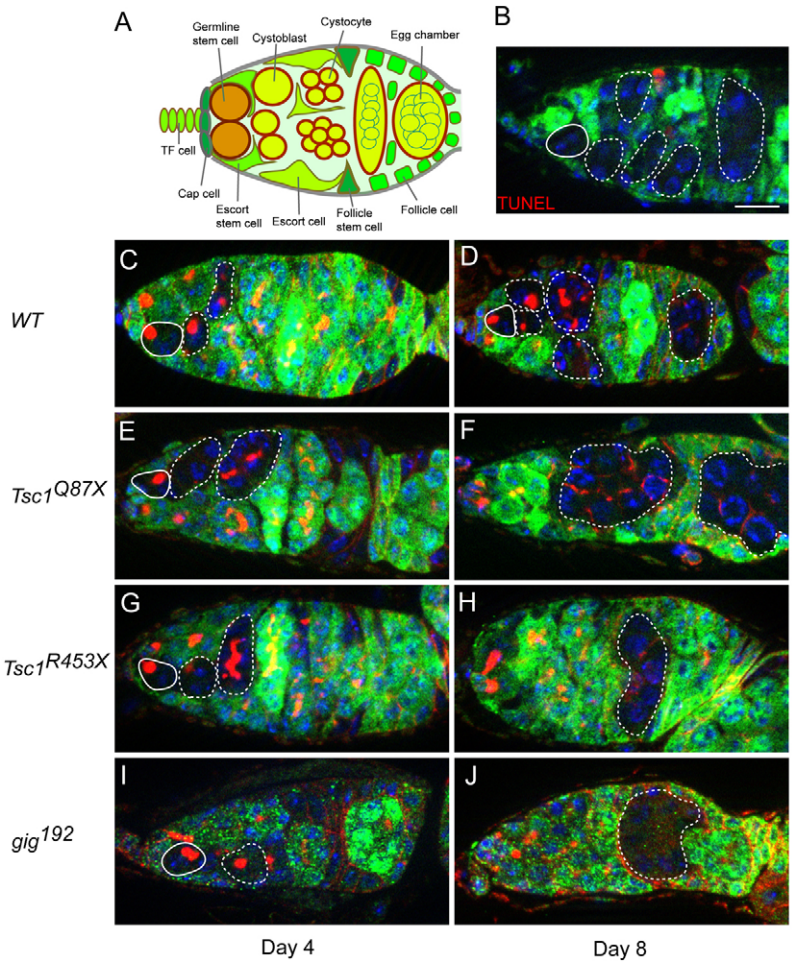


**Fig. 1. *Tsc1/2* is required for GSC maintenance but not survival.** (**A**) A schematic diagram shows a cross-section of the *Drosophila* germarium. (**B**) A germarium with a *Tsc1* mutant GSC clone marked by the absence of *lacZ* expression (green, solid line). The mutant clone shows negative staining for TUNEL (red). (C-J) Germaria with wild-type or *Tsc1/2* mutant GSC clones at a given time ACI. The GSCs are recognized by the absence of *lacZ* expression (green, solid lines), by their position (directly contacting the cap cells) and by the presence of round fusome (anti-α-Spectrin, red). (**C**,**D**) Germaria with marked wild-type GSCs at day 4 and 8 ACI. The marked GSCs are properly maintained in the niche during this period and produced many differentiating germline cysts (broken lines). (**E-J**) Germaria carrying *Tsc1/2* mutant GSC clones show that GSCs are no longer maintained at day 8 ACI, but the mutant germ cells are able to develop into germline cysts and egg chambers (broken lines). In all images, DAPI stain is in blue. Scale bar: 10 μm.

Tuberous sclerosis complex is a human disease characterized by the presence of benign tumour cells termed hamartomas in multiple organs, and usually caused by mutations in either *Tsc1* or *Tsc2* genes (for a review, see Inoki et al., 2005). Studies led in *Drosophila* have demonstrated that TSC1 and TSC2 form a physical and functional complex to control cell growth (Gao and Pan, 2001; Ito and Rubin, 1999; Potter et al., 2001; Tapon et al., 2001). It functions downstream of PI3K and Akt, and upstream of TOR (target of rapamycin) and S6K. Biochemically, TSC1 and TSC2 function as a GAP (GTPase activating protein) to inhibit Rheb (Ras homolog enriched in brain), a small G protein that positively regulates TOR (for reviews, see Inoki et al., 2005; Kwiatkowski and Manning, 2005). TOR is a Ser/Thr kinase conserved from yeast to mammals that controls multiple cellular processes, such as cell growth, proliferation and metabolism. TOR functions via two complexes, TORC1, which is sensitive to rapamycin and performs most of TOR functions, and TORC2, which is not sensitive to rapamycin and regulates cytoskeleton organization. TORC1 has two well-studied targets, S6K and 4EBP, which can be phosphorylated by TORC1 and regulate protein synthesis. TSC1/2-TORC1 signaling has been implicated in mediating many environmental cues, such as growth factor signals, amino acids and ATP, and could function as a signal integration point to regulate cell growth and metabolism (for reviews, see Kwiatkowski and Manning, 2005; Wullschleger et al., 2006).

In this study, we have revealed a novel role of TSC1/2-TORC1 signaling in regulating GSC maintenance and differentiation in the *Drosophila* ovary. Our study suggests a model whereby, in addition to the general role in cell growth control, TSC1/2-TORC1 signaling could also orchestrate GSC self-renewal and differentiation through regulating both BMP-bam-dependent and -independent differentiation programs. These results underline the importance of translational regulation in controlling GSC behaviour.

## MATERIALS AND METHODS

### Fly stocks

Unless otherwise stated, flies were reared at 25°C on standard media with fresh yeast paste added to the food surface. The fly stocks used for this study include: $Tsc1^{Q87X}$, $Tsc1^{R453X}$, $gig^{192}$ (Tapon et al., 2001), $gig^{56}$ (Ito and Rubin, 1999), $Tsc1^{29}$ (Gao and Pan, 2001), *bam-GFP* (Chen and McKearin, 2003b), $bam^{\Delta 86}$ (McKearin and Ohlstein, 1995), $Tor^{P1}$ (*P[lacw]l(2)k17004*), $Tor^{\Delta P}$ (Zhang et al., 2000) and $S6k^{l-1}$ (Montagne et al., 1999).

### Clonal analysis

Wild-type and mutant GSC clones were generated by FLP/FRT-mediated mitotic recombination (Xu and Rubin, 1993), and the genotypes of flies studied are as follows:

*hsflp/+; FRT82B arm-lacZ/ FRT82B*;
*hsflp/+; FRT82B arm-lacZ/ FRT82B* $Tsc1^{Q87X}$;
*hsflp/+; FRT82B arm-lacZ/ FRT82B* $Tsc1^{R453X}$;
*hsflp/+; FRT82B arm-lacZ/ FRT82B* $Tsc1^{29}$;
*hsflp/+; arm-lacZ FRT 80B/ FRT80B*;
*hsflp/+; arm-lacZ FRT 80B/* $gig^{192}$ *FRT80B*;
*hsflp/+; arm-lacZ FRT 80B/* $S6k^{l-1}$ *FRT80B*;
*hsflp/+; arm-lacZ FRT 80B/* $S6k^{l-1}$ $gig^{56}$ *FRT80B*;
*hsflp/+; bam-GFP/+; FRT82B arm-lacZ/ FRT82B* $Tsc1^{Q87X}$;

*hsflp/+; bam-GFP/+; FRT82B arm-lacZ/ FRT82B Tsc1*$^{R453X}$;
*hsflp/+; FRT82B arm-lacZ/ FRT82B bam*$^{\Delta 86}$;
*hsflp/+; FRT82B arm-lacZ/ FRT82B Tsc1*$^{29}$ *bam*$^{\Delta 86}$;
*hsflp/+; arm-lacZ FRT 40A/ Tor*$^{P1}$*T40A*;
*hsflp/+; arm-lacZ FRT 40A/ Tor*$^{\Delta P}$ *FRT40A*;
*hsflp/+; Tor*$^{\Delta P}$ */+; FRT82B arm-lacZ/ FRT82B Tsc1*$^{R453X}$.

Flies with FRT80B were treated with a stronger heat-shock regime because the recombination rate is relatively low for FRT80B. Flies were heat shocked in a 37°C water bath three times per day for 3 days for 80 minutes each time. Flies with other FRT sites were heat shocked twice a day for 3 days for 60 minutes each time, as previously described (Song and Xie, 2002; Xie and Spradling, 1998). For time-course clonal analysis, flies were randomly grouped and were analyzed at days 4, 6, 8 and 11 after clone induction (ACI), respectively. To examine pMAD and Bam expression level in *Tsc1* mutant GSCs, flies were analyzed at day 6 ACI. To examine the effect of *Tor/+* on the efficiency of rapamycin treatment in *Tsc1* mutant GSCs, flies were analyzed at days 4, 8 and 20 ACI, respectively. For pMad detection in the imaginal discs, larvae at the first or second instars were heat shocked for 120 minutes and dissected in 2-5 days.

### Rapamycin feeding

Different doses of rapamycin were used for different sets of experiments. For all experiments, rapamycin dissolved in ethanol was directly added to the food surface and the liquid was air-dried before used. In *Tsc1* mutant rescue experiment, 100 μl of 500 μM rapamycin was added to each vial. For TOR inhibition in wild-type flies, 100 μl of 4 mM rapamycin was added to each vial. For experiments to test the effect of *Tor/+* on rapamycin treatment, 100 μl of 100 μM rapamycin was added to each vial. Fresh food with rapamycin was replaced every other day until dissection.

### Immunostaining

Antibody staining of ovaries was performed as previously described (Zhao et al., 2008). Briefly, ovaries were fixed in 4% Paraformaldehyde for 15 minutes, and blocked in 5% normal goat serum in PBT [10 mM $NaH_2PO_4/Na_2HPO_4$, 175 mM NaCl (pH 7.4), plus 0.1% Triton X-100]. Primary and secondary antibodies were then added according to standard procedures. The following antisera and dilutions were used: rabbit anti-galactosidase (1:6000; Cappel); mouse anti-galactosidase (1:100; Molecular Probes); rabbit anti-GFP (1:300; Molecular Probes); mouse anti-α-spectrin (1:50; DSHB); rat anti-DE-cadherin (1:10; DSHB); mouse anti-orb (1:50; DSHB); mouse anti-HtsRC (1:50; DSHB); rabbit anti-Bam (1:100, a gift from D. Chen); and rabbit anti-pMad (1:3000; a gift from E. Laufer). Secondary antibodies, including goat anti-rabbit or anti-mouse IgG conjugated to Alexa 488 or Alexa 568 (Molecular Probes), were used at a dilution ratio of 1:300. For DAPI (4′, 6′-diamidino-2-phenylindole) staining, ovaries were incubated in PBT with 0.2 μg/ml DAPI (Sigma) for 6 minutes. All images were taken by Zeiss Imager Z1 equipped with ApoTome system, then processed in Adobe Photoshop and Illustrator.

### Cell apoptosis detection

Ovaries carry *Tsc1* mutant GSCs were dissected at 6 days ACI, and in situ cell death detection kit (Roche) was used to detect apoptosis according to the manufacturer's manual and as previously described (Zhao et al., 2008).

## RESULTS

### TSC1/2 maintains GSCs by preventing their differentiation

As *Tsc1* or *Tsc2* (also named *gigas*, *gig*) homozygous mutants died at early larval stages (Ito and Rubin, 1999; Tapon et al., 2001), we used the Flipase (FLP)-mediated mitotic recombination technique to generate marked mutant GSCs and then studied their function in adult GSCs (Xu and Rubin, 1993). The marked wild-type or mutant GSCs were induced by heat-shock treatments, and were recognized by their position, the absence of *lacZ* expression (Fig. 1B-J, green), and the presence of spherical-shaped fusomes, a germline-specific organelle in cytoplasm (recognized by anti-α-spectrin, red) (Lin et al., 1994). *Tsc1*$^{Q87X}$, *Tsc1*$^{R453X}$, *Tsc1*$^{29}$ and *gig*$^{192}$ alleles were used, all of which are strong or genetic null alleles (Ito and Rubin, 1999; Tapon et al., 2001). Flies of appropriate genotypes were subjected to time-course clonal analysis after clone induction (ACI) as previously described (Xie and Spradling, 1998).

At day 4 ACI, marked GSCs and follicle cell clones were readily observed, with 31%, 30%, 32%, 33% and 34% of germaria containing marked GSCs, respectively for wild-type, *Tsc1*$^{Q87X}$, *Tsc1*$^{R453X}$, *Tsc1*$^{29}$ and *gig*$^{192}$ clones. The follicle cells in mutant clones of *Tsc1* and *gig* showed increased cell sizes (Fig. 5E and data not shown), consistent with their roles in cell growth control. However, the size of mutant GSCs did not show obvious enlargement (Fig. 1E-J). After a week, there was no significant drop in the percentage of germaria with wild-type GSC clones (Fig. 1C,D; Table 1), which is consistent with previous observations that wild-type GSCs have a slow turn over rate (Xie and Spradling, 1998). However, *Tsc1* and *gig* mutant GSCs showed rapid and progressive loss from their niches during this short period, with only about 2% to 3% of germaria containing marked GSCs for all mutant alleles at day 11 ACI (Fig. 1E-J; Table 1). *Tsc1* and *gig* mutant germline cysts and egg chambers were readily observed and seemed to be able to differentiate normally, as 15 nurse cells and one oocyte were developed within each developing germline cyst, although their sizes were larger compared with wild-type germline cysts and egg chambers at the same developmental stages (Fig. 5A,C; see Fig. S1 in the supplementary material), suggesting that TSC1/2 also controls

**Table 1. Disruption of TSC1/2 function causes GSC loss via TORC1 activation**

| | Percentage of GSC clones at various days ACI* | | | |
|---|---|---|---|---|
| Genotype | Day 4 | Day 6 | Day 8 | Day 11 |
| *FRT82B* | 0.31 (300) | 0.43 (299) | 0.41 (326) | 0.36 (319) |
| *FRT82B Tsc1*$^{Q87X}$ | 0.30 (293) | 0.21 (234) | 0.10 (280) | 0.02 (197) |
| *FRT82B Tsc1*$^{R453X}$ | 0.32 (247) | 0.23 (240) | 0.12 (233) | 0.03 (180) |
| *FRT82B Tsc1*$^{29}$ | 0.33 (208) | 0.21 (213) | 0.10 (232) | 0.02 (208) |
| *FRT80B* | 0.36 (209) | 0.43 (212) | 0.38 (222) | 0.36 (219) |
| *gig*$^{192}$ *FRT80B* | 0.34 (207) | 0.27 (248) | 0.07 (212) | 0.02 (152) |
| *S6k*$^{l-1}$*FRT80B* | 0.42 (207) | N.D. | 0.43 (143) | 0.50 (136) |
| *S6k*$^{l-1}$*gig*$^{56}$*FRT80B* | 0.41 (189) | N.D. | 0.38 (224) | 0.33 (227) |
| *FRT82B Tsc1*$^{Q87X}$ + *r*$^{\dagger}$ | 0.39 (217) | 0.38 (261) | 0.43 (244) | 0.38 (254) |
| *FRT82B Tsc1*$^{R453X}$ + *r*$^{\dagger}$ | 0.40 (220) | 0.35 (217) | 0.32 (133) | 0.26 (201) |

*The percentage of GSC clones at a given time point is calculated by the number of germaria with marked GSCs divided by the total number of germaria examined (in parentheses).
†*r*, rapamycin treatment.

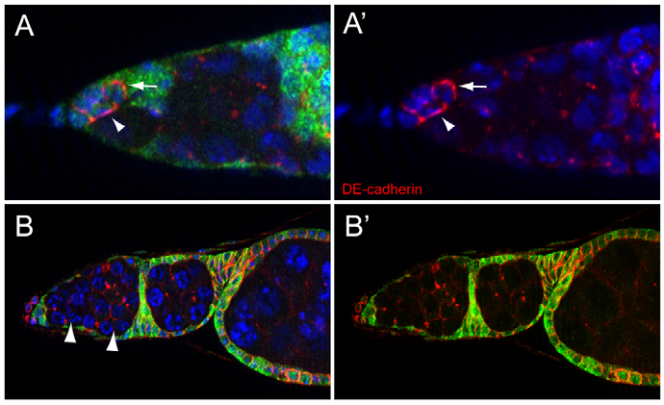


**Fig. 2. Loss of *Tsc1* mutant GSC is not caused by cell competition.** (**A**,**A′**) A germarium that contains one marked *Tsc1* mutant GSC (lack of *lacZ* expression, green) and one unmarked wild-type GSC stained with DE-Cadherin (red). The cadherin level at the interface between *Tsc1* mutant GSC and cap cell (arrowhead) is similar to that between the wild-type GSC and cap cell (arrow). (**B**,**B′**) A germarium that contains only *Tsc1* mutant germline cysts (arrowheads, lack of *lacZ* expression) without wild-type GSCs. The mutant germline cysts at the niche region have polyploid nuclei and lack fusomes (anti-α-Spectrin, red); DAPI stain is in blue.

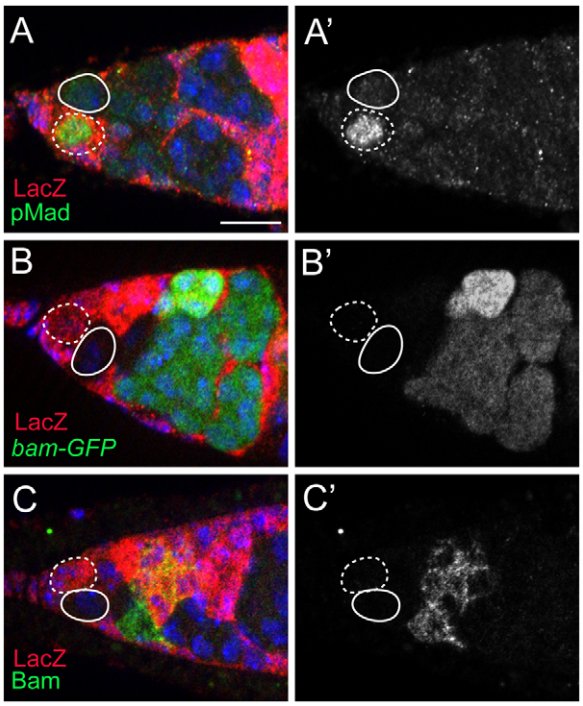


**Fig. 3. TSC1 maintains BMP signaling activity in GSCs.** (**A**,**A′**) Anterior part of a germarium that contains one marked *Tsc1* mutant GSC (solid line, absence of *lacZ* expression, red) and one wild-type GSC (dashed line). In *Tsc1* mutant GSC, pMAD expression (green, white in A′) is significantly downregulated compared with that in the wild-type GSC. (**B**,**B′**) A *Tsc1* mutant GSC (solid line, absence of *lacZ* expression, red) and one wild-type GSC (broken line). In mutant GSC, *bam-GFP* expression (green, white in B′) is not obviously upregulated compared with wild-type GSC. (**C**,**C′**) A *Tsc1* mutant GSC (solid line, absence of *lacZ* expression, red) also shows no obvious upregulation of Bam protein (green, white in C′) levels compared with the neighboring wild-type GSC (broken line). In all images, DAPI stain is in blue. Scale bars: 10 μm.

the growth of developing germline cysts. By calculating the number of germline cysts produced by *Tsc1* mutant GSCs that were still maintained at day 8 ACI, we found that *Tsc1* mutant GSCs have a similar cell division rate compared with wild-type GSCs (see Fig. S2 in the supplementary material).

The presence of differentiated germline clones in *Tsc1/2* mutants implies that the loss of *Tsc1/2* mutant GSC is caused by the commitment to differentiation without self-renewal, rather than to cell death. To verify this hypothesis, we performed TUNEL labelling to detect apoptosis in *Tsc1* mutant GSCs. None of the $Tsc1^{Q87X}$ ($n$=106) and $Tsc1^{R453X}$ mutant GSCs ($n$=98) examined were positive for TUNEL (Fig. 1B); thus, the mutant GSCs are not apoptotic. Potentially, GSC loss could also be the consequence of out-competition by the neighboring wild-type GSCs, where cadherin-mediated cell adhesion plays a crucial role (Jin et al., 2008). Nevertheless, there was no obvious difference in DE-cadherin level at the stem cell/niche cell interface between *Tsc1* mutant and wild-type GSCs (Fig. 2A). Besides, when a niche contains only *Tsc1* mutant GSCs, they still differentiated into germline cysts (Fig. 2B), suggesting that *Tsc1* mutant GSC loss is not caused by cell competition. Therefore, TSC1/2 functions cell-autonomously for GSC maintenance by preventing precocious differentiation.

## BMP signaling activity is downregulated in *Tsc1* mutant GSCs

As the BMP-Bam pathway is known to keep GSCs in undifferentiated state, we investigated whether BMP signaling is compromised in *Tsc1/2* mutant GSCs. We used antibodies against phosphorylated Mad (pMad) to detect BMP pathway activity in *Tsc1* mutant GSCs. Normally, high levels of nuclear pMad are detected in GSCs but not in the differentiating cystoblasts or early-stage cystocytes. Strikingly, pMad level was significantly decreased in $Tsc1^{Q87X}$ [86% of mutant clones showed downregulation (32/37)] and $Tsc1^{R453X}$ [73% (19/26)] mutant GSCs compared with the neighboring wild-type GSCs (Fig. 3A), indicating that TSC1/2 is required for BMP signaling activity in GSCs. We then tested whether TSC1/2 could also affect BMP signaling in other tissues. In imaginal wing discs, Dpp is expressed at the AP boundary and functions as a morphogen to pattern the AP axis. *Tsc1* mutant clones in the wing discs showed similar levels of pMad expression compared with wild-type cells (see Fig. S3A in the supplementary material). Similarly, pMad expression was not altered in *Tsc1* mutant clones in the eye discs (see Fig. S3B in the supplementary material). These observations suggest that TSC1 is required for BMP signaling specifically in GSCs.

To further test whether the reduced BMP signaling in *Tsc1* mutant GSCs could cause *bam* upregulation and subsequently GSC differentiation, we examined *bam* transcription using a GFP reporter driven by the *bam* promoter (*bam-GFP*) (Chen and McKearin, 2003b). *bam-GFP* can reliably reflect *bam* transcription level: GFP is undetectable in GSCs, whereas it is detectable in cystoblasts, will reach a high level in early-stage cystocyts and then decrease in the late-stage cysts (Chen and McKearin, 2003b). Interestingly, in $Tsc1^{Q87X}$ mutant GSCs, there was no obvious upregulation of *bam-GFP* (Fig. 3B) (38 out of 40 GSCs examined) compared with neighboring wild-type GSCs. For further confirmation, we also examined Bam protein expression in *Tsc1* mutant GSCs. Normally, Bam protein is

undetectable is the cytoplasm of GSCs, but accumulates as cystoblast differentiate, reaches highest level in eight-cell cysts, and then disappears dramatically in 16-cell cysts (McKearin and Ohlstein, 1995). Similarly, we did not observe Bam expression in all *Tsc1* mutant GSCs examined ($Tsc1^{Q87X}$, $n$=50; $Tsc1^{R453X}$, $n$=50) (Fig. 3C). In addition, both *bam*-GFP and Bam protein expression pattern seemed to be normal in *Tsc1* mutant cysts (Fig. 3B,C and data not shown), indicating that *Tsc1* mutation did not affect *bam* expression in the differentiating germ cells either. Therefore, *Tsc1* mutant GSCs display compromised BMP signaling activities, but *bam* transcription seems to be properly repressed.

## A function of TSC1 in maintaining GSCs independent of Bam

To evaluate further the contribution of BMP-Bam pathway on *Tsc1/2* mutant GSC loss, we tested whether *bam* elimination could rescue the precocious differentiation of *Tsc1/2* mutant GSCs by generating *Tsc1 bam* double mutant ($Tsc1^{29}$ $bam^{\Delta 86}$) GSC clones.

As previously reported, *bam* elimination in wild-type GSCs ($bam^{\Delta 86}$, a null allele) completely blocked differentiation, leading to the development of GSC-like tumours (McKearin and Ohlstein, 1995). We observed that the mutant GSCs were well maintained in the niche, consistent with a previous observation that *bam* mutant GSCs are very competitive in occupying the niche (Fig. 4A,B,E) (Jin et al., 2008). Intriguingly, many *Tsc1 bam* double mutant germ cells showed enlarged and branched fusomes (Fig. 4D). After staining with anti-Orb, an oocyte marker that is initially detected in the cytoplasm of eight-cell or early 16-cell cystocytes (Lantz et al., 1994), and with HtsRC, a ring canal marker that starts to be expressed at around 16-cell stage (Robinson et al., 1994) (Fig. 4F), we found that the expression of both markers was not observed in *Tsc1 bam* double mutant germline clones (Fig. 4G), suggesting that the double mutant germ cells could only undergo limited differentiation into early cystocytes. Moreover, *bam* mutation could not rescue the loss of *Tsc1* mutant GSCs, as double mutants still displayed the loss of GSC phenotype, although their loss was delayed compared with that of *Tsc1* mutant GSCs (Fig. 4E). Taken together, we conclude that, although required for BMP signaling, TSC1/2 also represses GSC differentiation independently of *bam*; this might represent the primary mechanism by which TSC1/2 maintains GSCs.

## TORC1 is downstream of TSC1/2 in controlling GSC maintenance

It is well established that TORC1 functions downstream of TSC1/2 in controlling cell growth and metabolism. To test whether TSC1/2 also represses GSC differentiation through TORC1, we treated flies with rapamycin, a specific TORC1 inhibitor. The follicle cells from mutant flies treated with rapamycin were comparable in size with wild-type follicle cells (Fig. 5F). However, follicle cells in untreated *Tsc1* mutant clones were enlarge, demonstrating that *Tsc1* mutant cell growth was effectively suppressed by rapamycin treatment. Strikingly, the loss of GSC phenotype was also significantly rescued. After rapamycin treatment, more *Tsc1* mutant GSCs were maintained from day 4 to day 11 ACI [97% (0.38/0.39) for $Tsc1^{Q87X}$ and 65% (0.26/0.40) for $Tsc1^{R453X}$], compared with fewer than 1% (0.03/0.32 and 0.02/0.30 respectively) in controls (Table 1, Fig. 5A-D).

To further confirm the specificity of rapamycin in inhibiting TORC1 activity, we tested whether eliminating one copy of *Tor* could help to alleviate GSC loss in *Tsc1* mutants. When using a low concentration of rapamycin, $Tsc1^{R453X}$ mutant GSCs with only one copy of wild-type *Tor* were better maintained than those with two copies (Table 2). Thus, the loss of GSC

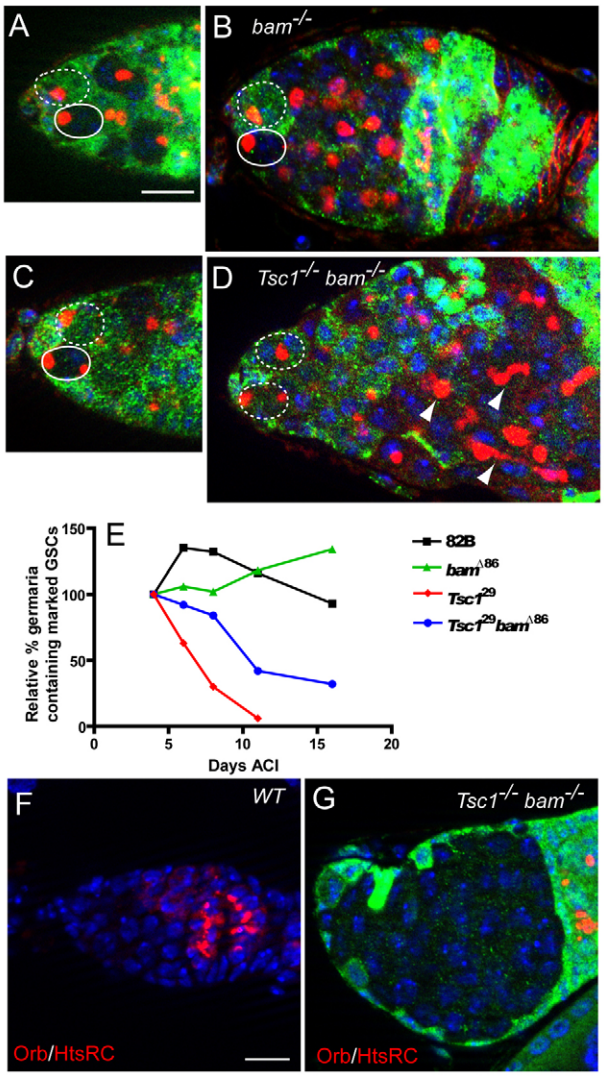


**Fig. 4. Loss of *bam* could not prevent differentiation of *Tsc1* mutant GSCs.** (**A**,**B**) Germaria containing *bam* mutant GSC clones (absence of *lacZ* expression, green, solid lines) as well as wild-type GSCs (broken lines) at day 4 (A) and day 11 (B) ACI. All the daughter germ cells that derived from *bam* mutant GSCs exhibit GSC-like characters (anti-α-Spectrin, red). (**C**) A germarium that contains a marked *Tsc1 bam* double-mutant GSC (solid line) and an unmarked wild-type GSC (broken line) at day 4 ACI. (**D**) A germarium at day 11 ACI that contains two wild-type GSCs (broken line), but no mutant GSCs. Many *Tsc1 bam* mutant germ cells have enlarged or branched fusome structures (arrowheads), indicating that they undergo differentiation. (**E**) A plot showing time course analysis of the maintenance rate for wild type, *Tsc1*, *bam* and *Tsc1 bam* double-mutant GSC clones. For each genotype, the percentage of germaria with marked GSCs is normalized to 100% at day 4 ACI. (**F**) A wild-type germarium stained with anti-Orb (red, cytoplasmic) and anti-HtsRC (red, dots/ring-like structure). (**G**) A germarium with *Tsc1 bam* double-mutant germline clones marked by absence of *lacZ* expression (green) also stained with anti-Orb and anti-HtsRC (red). There is no detectable expression of Orb and HtsRC markers in the mutant cells. In all images, DAPI stain is in blue. Scale bars: 10 μm.

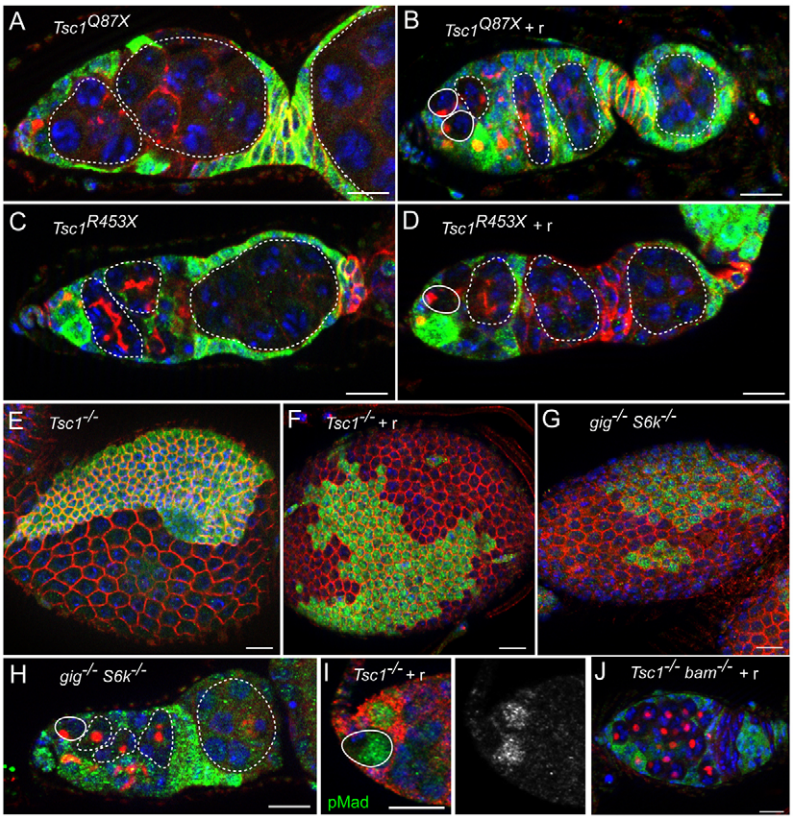


**Fig. 5. *Tsc1/2* mutant GSC loss is caused by TORC1 hyperactivation.** (**A**) Part of an ovariole from the untreated females at day 11 ACI showing *Tsc1*$^{Q87X}$ mutant germline cysts and egg chambers (broken lines) marked by the absence of *lacZ* expression (green). The germline cysts are abnormally large in size. (**B**) With rapamycin treatment, *Tsc1*$^{Q87X}$ mutant GSCs (solid lines) are maintained in the niche, and the derived germline cysts (broken lines) do not show enlargement in cyst size. (**C**,**D**) Similarly, rapamycin treatment (D) also rescues GSC loss and cell growth phenotypes of *Tsc1*$^{R453X}$ mutant GSC clones. (**E**) A stage 7 egg chamber with a *Tsc1*$^{Q87X}$ mutant follicle cell clone (absence of *lacZ* expression, green) shows that the mutant follicle cells have enlarged cell sizes. (**F**) The mutant follicle cells (absence of *lacZ*, green) from rapamycin-treated females show similar sizes to the neighboring wild-type follicle cells. (**G**) *gig*$^{56}$ *S6k*$^{l-1}$ double-mutant follicle cells (absence of *lacZ*, green) show similar sizes as the neighboring wild-type follicle cells. (**H**) *gig*$^{56}$ *S6k*$^{l-1}$ double mutant GSCs (solid line) are maintained in the niche, and the derived germline cysts (broken lines) also show relatively normal sizes. (**I**) A germarium from rapamycin-treated females containing *Tsc1* mutant GSCs (absence of *lacZ*, red) stained with pMad (green). pMad has a similar expression level in the mutant GSC (solid circle, see also in a separate channel for pMad on the right) and in the neighboring wild-type GSC. (**J**) *Tsc1*$^{29}$ *bam* double mutant GSC clones from rapamycin-treated females at day 11 ACI do not show enlarged or branched fusome phenotype. In all images, DAPI stain is in blue. Scale bars: 10 μm.

phenotype caused by *Tsc1/2* mutations is probably due to TORC1 hyperactivation. As S6K is a well-studied target of TORC1 that can be phosphorylated by TORC1 in order to regulate protein synthesis, we asked whether mutation in *S6k* could rescue the loss of GSCs in *Tsc2* mutants. *S6k* mutant (*S6k*$^{l-1}$, a null allele) GSCs did not show any obvious defects in GSC maintenance (Table 1), although the mutant follicle cells were relatively smaller in size (not shown), which is consistent with its role in the control of cell growth (Montagne et al., 1999). Strikingly, *gig S6k* double mutant (*S6k*$^{l-1}$ *gig*$^{56}$, both null alleles) GSCs were properly maintained, with about 80% (0.33/0.41) of mutant GSCs maintained from day 4 to day 11 ACI, compared with fewer than 1% (0.02/0.34) of *gig* mutant GSCs maintained (Fig. 5H and Table 1). Similarly, *S6k* mutation also efficiently suppressed the overgrowth of both germline cysts and somatic follicle cells (Fig. 5G,H). Therefore, TORC1 functions downstream of TSC1/2 in both maintaining GSCs and controlling cell growth in the *Drosophila* ovary.

As previously stated, *Tsc1* mutant GSCs showed reduced BMP signaling activity. We therefore asked whether TORC1 inhibition could restore the activity back to its normal level, in *Tsc1* mutant GSCs. After rapamycin treatment, *Tsc1* mutant GSCs were properly maintained and levels of pMad expression were also comparable with those in neighboring wild-type GSCs (Fig. 5I). This suggest that TSC1/2 also functions by inhibiting TORC1 in order to maintain BMP signaling activity in GSCs. Consistent with the notion that TSC1/2 controls both *bam*-dependent and -independent differentiation pathways via TORC1, rapamycin treatment prevented differentiation of *Tsc1 bam* double mutant GSCs, as branched fusomes in the mutant germ cells were no longer observed in the treated flies (Fig. 5J).

## TOR is not required for proper BMP signaling or for germline differentiation

To test whether TOR is required to negatively regulate BMP signaling activity in GSCs, we treated wild-type flies (*w*$^{1118}$) with a high concentration of rapamycin to repress TORC1 activity and examined its effect on BMP signaling activity. Late oogenesis from treated females was blocked because few late-stage egg chambers or eggs were observed in the treated ovaries, suggesting that the rapamycin treatment inhibits ovary growth. In the germaria from treated flies, GSCs and their niches seemed to be morphologically normal and no obvious defect in GSC maintenance was found (Fig. 6A,B). Similar to GSCs in untreated flies, pMad was specifically enriched in the anterior tip of GSCs, but not in differentiating germ cells (Fig. 6A). Consistently, *bam-GFP* was fully repressed in GSCs, and derepressed in cystoblasts and in differentiating germline cysts (Fig. 6B).

**Table 2. Removing a copy of *Tor* increases the efficiency of rapamycin in preventing *Tsc1* mutant-GSC loss**

| | Percentage of GSC clones at various days ACI | | |
|---|---|---|---|
| Genotype | Day 4 | Day 8 | Day 20 |
| *Cyo/+; FRT82B Tsc1*$^{R543X}$ clone + *r* | 0.32 (212) | 0.24 (223) | 0.20 (196) |
| *Tor*$^{\Delta P}$*/+; FRT82B Tsc1*$^{R543X}$ clone + *r* | 0.33 (198) | 0.30 (216) | 0.27 (128) |

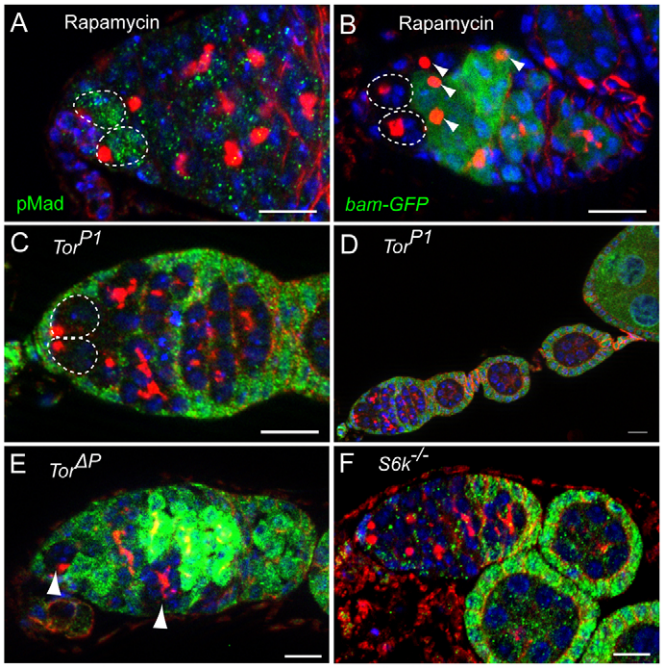


**Fig. 6. TOR is not required for BMP signaling or for germline differentiation.** In all images, anti-α-Spectrin stain is in red and DAPI stain is in blue. (**A**) A germarium from rapamycin-treated flies labelled with α-Spectrin (red) and pMAD (green) shows that GSCs (broken circles) are pMAD (green) positive, but differentiating germ cells are not. (**B**) A germarium from rapamycin-treated flies labelled with α-Spectrin (red) and *bam-GFP* (green) shows that GSCs (broken circles) do not express *bam-GFP*, and *bam-GFP* is visible in cystoblasts and differentiating germline cysts. There are four cystoblast-like cells (arrowheads) in this germarium. (**C**) A germarium with two *Tor*$^{P1}$ mutant GSCs (broken lines), which are marked by absence of *lacZ* expression (green) expression. The mutant GSC-derived germline cysts differentiate normally in the germarium. (**D**) An ovariole with *Tor*$^{P1}$ mutant egg chambers (marked by the absence of *lacZ* expression, green) shows that *Tor*$^{P1}$ mutant egg chambers are growth-arrested at mid oogenesis. (**E**) A germarium with one *Tor*$^{\Delta P}$ mutant GSC and one *Tor*$^{\Delta P}$ mutant germline cyst (arrowheads) marked by the absence of *lacZ* expression (green). (**F**) *S6k* mutant GSCs and the derived differentiating germline cysts are marked by the absence of *lacZ* expression (green). The mutant germline cysts differentiate normally. Scale bars: 10 μm.

Interestingly, there were more cystoblasts accumulated in the germarium of the treated flies than in untreated flies (Fig. 6B), indicating that rapamycin treatment could cause delay in cystoblast differentiation. We then examined *Tor* mutant flies to test directly the functional requirement for TOR in germline differentiation. Reducing TOR function by *Tor*$^{P1}$ mutation, which carries a P-element insertion at the 5′-UTR that causes reduced *Tor* expression (Zhang et al., 2000), did not significantly affect germline differentiation. The mutant germline cysts showed relatively normal morphology (Fig. 6C), although the development of mutant egg chambers was arrested in mid-oogenesis (Fig. 6D). The daughters generated by *Tor*$^{\Delta P}$ GSCs, a null allele of *Tor*, could also differentiate into cysts, but the mutant cysts soon arrested in growth and degenerated (Fig. 6E; data not shown). These observations indicate that TOR is not required for germline cyst differentiation, but may be essential for germline growth and survival. Consistent with the notion that TOR is not required for germline differentiation, *S6k* mutant GSCs were also able to produce daughters that could properly differentiate into germline cysts and egg chambers (Fig. 6F). Therefore, the accumulated cystoblasts in rapamycin-treated flies could be caused by non-specific effects of rapamycin. We conclude that TOR is not required for the normal BMP signaling pattern in the germline or for germline differentiation; however, it essential for germline growth and survival.

## DISCUSSION

TSC1 and TSC2, the gene products of tuberous sclerosis complex human disease, are evolutionarily conserved cell growth regulators in metazoans. In this study, we have illustrated that, in the *Drosophila* ovary, in addition to controlling the growth of germline cysts and somatic follicle cells, TSC1/2 also regulates GSC maintenance. Clonal analysis demonstrates that TSC1/2 functions intrinsically in GSCs for their maintenance by preventing precocious differentiation. Mechanistically, TSC1/2 maintains BMP signaling activity and represses differentiation programs independently of *bam* in the GSCs, via inhibition of TORC1-S6K. Therefore, this study reveals a novel and potentially general mechanism of stem cell maintenance by TSC1/2, which may also help in better understanding the pathology of tuberous sclerosis complex and other human diseases associated with TOR dysregulation.

### TSC1/2 maintains GSCs via TORC1 inhibition

TSC1/2 is known to regulate cell growth via inhibition on TORC1. In this study, we have demonstrated that it also functions by inhibiting the activity of TORC1 to maintain GSCs. Treatment with rapamycin, a TORC1-specific inhibitor, can completely rescue GSC loss in *Tsc1* mutants. In addition, eliminating S6K, which functions downstream of TORC1 in regulating protein translation, could also completely rescue GSC loss in *Tsc2* mutants. Interestingly, the daughters of *Tor* mutant GSCs can differentiate into germline cyst properly, indicating that TOR is normally not required for differentiation, but its hyperactivation in *Tsc1/2* mutants drives precocious GSC differentiation. The simplest explanation of the delayed cystoblast differentiation in rapamycin-treated females might be a non-specific effect of drug treatment. However, it is also possible that TORC1 inhibition by rapamycin might cause repression of some, but not all, aspects of TOR function, which leads to uncoordinated development and/or differentiation of cystoblasts in response to GSC division. Consistently, we also observed accumulated cystoblasts when overexpressing both *Tsc1* and *Tsc2* in the germline (data not shown). Together with the observation that TSC1/2-TORC1 signaling controls cell growth of germline cysts, our study suggests that TSC1/2-TORC1 may serve as a signaling integration point that orchestrates germline division, differentiation and development in order to control egg production in response to the local micro-environment and the system environment of the animals.

### TSC1/2 is required to maintain BMP signaling activity in GSCs

In the *Drosophila* ovary, BMP signaling from the niche directly suppresses *bam* expression in GSCs to prevent differentiation. This signaling is crucial for GSC maintenance. As revealed by pMad expression, BMP signaling activity is significantly downregulated in *Tsc1* mutant GSCs. We also demonstrated that downregulation of pMad in *Tsc1* mutant GSCs is mediated by TORC1 hyperactivation, as rapamycin treatment is able to restore the downregulated pMad level. However, TOR is not required for

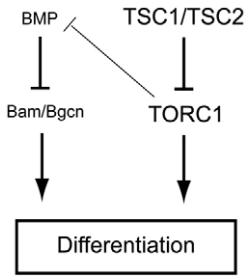


**Fig. 7. A working model for TSC1/2 function in GSC maintenance.** In GSCs, TSC1/2 complex is crucial for preventing hyperactivation of TORC1 in order to maintain GSCs in undifferentiated state. In *Tsc1/2* mutant GSC, TORC1 hyperactivation may trigger GSC differentiation through a pathway in parallel with the BMP-Bam pathway. In addition, TORC1 hyperactivation also negatively regulates BMP signaling activity through unknown mechanisms. Therefore, TSC1/TSC2 maintains GSC by simultaneously controlling both BMP-Bam-dependent and -independent differentiation pathways.

proper BMP signaling activity because pMad expression is not altered in rapamycin-treated germaria. Therefore, only TORC1 hyperactivation could inhibit BMP signaling in GSCs through unknown mechanisms, and this inhibitory effect occurs specifically in GSCs, as BMP signaling activity is not altered in *Tsc1* mutant imaginal disc cells.

Logically, *bam* expression could be derepressed in *Tsc1* mutant GSCs as a consequence of BMP pathway downregulation. Surprisingly, we could not detect significant upregulation of *bam-GFP* expression in mutant GSCs, although in other GSCs that were compromised by BMP signaling, such as *tkv* mutant and *mad* mutant GSCs, *bam* transcription is significantly upregulated (Song et al., 2004). Nevertheless, there might still be residual BMP signaling activities in *Tsc1/2* mutant GSCs that are sufficient to suppress *bam* expression. Consistent with this notion is the observation that *bam-GFP* is not obviously upregulated in aged GSCs, even if BMP signaling activity has been significantly reduced (Zhao et al., 2008). Together with the observation that *bam* mutation could not rescue the differentiation of *Tsc1* mutant germ cells, we suggest that the compromised BMP signaling activity may not be primarily responsible for *Tsc1/2* mutant GSC loss. It is not clear why the effect of TSC1/2 on BMP signaling occurs specifically in GSCs. Possibly, *Tsc1/2* mutant GSCs, once induced, have already primed for differentiation through a Bam-independent mechanism, which may trigger a positive feedback signal to inhibit BMP signaling activity, in order to facilitate differentiation.

## TSC1/2 regulates a Bam-independent differentiation pathway in GSCs

Our study also reveals a BMP-Bam-independent mechanism that probably underlies the major role of TSC1/2-TORC1 signaling in GSC maintenance. The phenotype of *Tsc1 bam* double mutant germ cells differs from the *bam* alone mutant germ cells, as the double mutant GSCs can still become lost from the niche over time and undergo further differentiation into early cystocytes. Interestingly, the phenotype of *Tsc1/2* mutant GSCs is similar to that of *pelota* (*pelo*) mutants. *Pelo* encodes a translational release factor-like protein and may regulate GSC maintenance at the translational level (Xi et al., 2005). In *pelo* mutant GSCs, there is also a downregulation of BMP signaling but no obvious upregulation of *bam* expression, and *bam pelo* double mutant germ cells are able to undergo similar limited differentiation into cystocytes, suggesting that TSC1/2 and Pelo might function in the same or parallel pathway to control GSC differentiation. We propose, as illustrated in Fig. 7, that similar to Pelo, TSC1/2 might function in a parallel pathway with the BMP-Bam pathway to control GSC differentiation, possibly by regulating the translation of differentiation-related mRNAs.

Pum and Nos, which are known to function together to repress translation of the target mRNAs in embryos, are also essential for GSC maintenance (Forbes and Lehmann, 1998; Wang and Lin, 2004). Recent genetic and biochemical studies suggest that Bam/Bgcn may directly inhibit the function of Pum/Nos to allow cystoblast differentiation (Chen and McKearin, 2005; Kim et al., 2009; Li et al., 2009; Szakmary et al., 2005). However, BMP signaling activation is able to prevent differentiation of *nos* mutant primordial germ cells, indicating that Pum/Nos could also function in parallel with the BMP-Bam pathway to control germ cell differentiation (Gilboa and Lehmann, 2004). In the future, it would be important to determine the functional relationships between the TSC1/2-TORC1 pathway, Pelo and Pum/Nos in regulating GSCs, and whether these factors, together with the microRNA pathway, target similar mRNAs to control GSC differentiation.

In this study, we have identified a novel role of TSC1/2 in controlling GSC maintenance and differentiation in the *Drosophila* ovary. Increasing evidence also suggests similar roles for TSC1/2-TOR signaling in regulating adult stem cell differentiation in mammals. For example, TSC1/2-mTOR signaling is also required for maintaining the quiescence of haematopoietic stem cells (HSCs), as *Tsc1* deletion drives HSCs from quiescence to rapid cycling, which compromises HSC self-renewal (Chen et al., 2008). Thus, TSC1/2-TOR signaling could have an evolutionarily conserved role in regulating stem cell maintenance and differentiation from *Drosophila* to mammals.

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**Competing interests statement**
The authors declare no competing financial interests.

**Supplementary material**
Supplementary material for this article is available at
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**References**

**Chen, C., Liu, Y., Liu, R., Ikenoue, T., Guan, K. L. and Zheng, P.** (2008). TSC-mTOR maintains quiescence and function of hematopoietic stem cells by repressing mitochondrial biogenesis and reactive oxygen species. *J. Exp. Med.* **205**, 2397-2408.
**Chen, D. and McKearin, D.** (2003a). Dpp signaling silences bam transcription directly to establish asymmetric divisions of germline stem cells. *Curr. Biol.* **13**, 1786-1791.
**Chen, D. and McKearin, D.** (2003b). A discrete transcriptional silencer in the bam gene determines asymmetric division of the Drosophila germline stem cell. *Development* **130**, 1159-1170.
**Chen, D. and McKearin, D.** (2005). Gene circuitry controlling a stem cell niche. *Curr. Biol.* **15**, 179-184.
**Forbes, A. and Lehmann, R.** (1998). Nanos and Pumilio have critical roles in the development and function of Drosophila germline stem cells. *Development* **125**, 679-690.
**Gao, X. and Pan, D.** (2001). TSC1 and TSC2 tumor suppressors antagonize insulin signaling in cell growth. *Genes Dev.* **15**, 1383-1392.
**Gilboa, L. and Lehmann, R.** (2004). Repression of primordial germ cell differentiation parallels germ line stem cell maintenance. *Curr. Biol.* **14**, 981-986.
**Hatfield, S. D., Shcherbata, H. R., Fischer, K. A., Nakahara, K., Carthew, R. W. and Ruohola-Baker, H.** (2005). Stem cell division is regulated by the microRNA pathway. *Nature* **435**, 974-978.

**Inoki, K., Corradetti, M. N. and Guan, K. L.** (2005). Dysregulation of the TSC-mTOR pathway in human disease. *Nat. Genet.* **37**, 19-24.
**Ito, N. and Rubin, G. M.** (1999). gigas, a Drosophila homolog of tuberous sclerosis gene product-2, regulates the cell cycle. *Cell* **96**, 529-539.
**Jin, Z. and Xie, T.** (2007). Dcr-1 maintains Drosophila ovarian stem cells. *Curr. Biol.* **17**, 539-544.
**Jin, Z., Kirilly, D., Weng, C., Kawase, E., Song, X., Smith, S., Schwartz, J. and Xie, T.** (2008). Differentiation-defective stem cells outcompete normal stem cells for niche occupancy in the Drosophila ovary. *Cell Stem Cell* **2**, 39-49.
**Kim, J. Y., Lee, Y. C. and Kim, C.** (2009). Direct inhibition of Pumilo activity by Bam and Bgcn in Drosophila germ line stem cell differentiation. *J. Biol. Chem.* **285**, 4741-4746.
**Kirilly, D. and Xie, T.** (2007). The Drosophila ovary: an active stem cell community. *Cell Res.* **17**, 15-25.
**Kwiatkowski, D. J. and Manning, B. D.** (2005). Tuberous sclerosis: a GAP at the crossroads of multiple signaling pathways. *Hum. Mol. Genet.* **14**, R251-R258.
**Lantz, V., Chang, J. S., Horabin, J. I., Bopp, D. and Schedl, P.** (1994). The Drosophila orb RNA-binding protein is required for the formation of the egg chamber and establishment of polarity. *Genes Dev.* **8**, 598-613.
**Li, L. and Xie, T.** (2005). Stem cell niche: structure and function. *Annu. Rev. Cell Dev. Biol.* **21**, 605-631.
**Li, Y., Minor, N. T., Park, J. K., McKearin, D. M. and Maines, J. Z.** (2009). Bam and Bgcn antagonize Nanos-dependent germ-line stem cell maintenance. *Proc. Natl. Acad. Sci. USA* **106**, 9304-9309.
**Lin, H., Yue, L. and Spradling, A. C.** (1994). The Drosophila fusome, a germline-specific organelle, contains membrane skeletal proteins and functions in cyst formation. *Development* **120**, 947-956.
**McKearin, D. and Ohlstein, B.** (1995). A role for the Drosophila bag-of-marbles protein in the differentiation of cystoblasts from germline stem cells. *Development* **121**, 2937-2947.
**Montagne, J., Stewart, M. J., Stocker, H., Hafen, E., Kozma, S. C. and Thomas, G.** (1999). Drosophila S6 kinase: a regulator of cell size. *Science* **285**, 2126-2129.
**Morrison, S. J. and Spradling, A. C.** (2008). Stem cells and niches: mechanisms that promote stem cell maintenance throughout life. *Cell* **132**, 598-611.
**Neumuller, R. A., Betschinger, J., Fischer, A., Bushati, N., Poernbacher, I., Mechtler, K., Cohen, S. M. and Knoblich, J. A.** (2008). Mei-P26 regulates microRNAs and cell growth in the Drosophila ovarian stem cell lineage. *Nature* **454**, 241-245.
**Ohlstein, B. and McKearin, D.** (1997). Ectopic expression of the Drosophila Bam protein eliminates oogenic germline stem cells. *Development* **124**, 3651-3662.
**Page, S. L., McKim, K. S., Deneen, B., Van Hook, T. L. and Hawley, R. S.** (2000). Genetic studies of mei-P26 reveal a link between the processes that control germ cell proliferation in both sexes and those that control meiotic exchange in Drosophila. *Genetics* **155**, 1757-1772.
**Park, J. K., Liu, X., Strauss, T. J., McKearin, D. M. and Liu, Q.** (2007). The miRNA pathway intrinsically controls self-renewal of Drosophila germline stem cells. *Curr. Biol.* **17**, 533-538.
**Potter, C. J., Huang, H. and Xu, T.** (2001). Drosophila Tsc1 functions with Tsc2 to antagonize insulin signaling in regulating cell growth, cell proliferation, and organ size. *Cell* **105**, 357-368.
**Robinson, D. N., Cant, K. and Cooley, L.** (1994). Morphogenesis of Drosophila ovarian ring canals. *Development* **120**, 2015-2025.
**Song, X. and Xie, T.** (2002). DE-cadherin-mediated cell adhesion is essential for maintaining somatic stem cells in the Drosophila ovary. *Proc. Natl. Acad. Sci. USA* **99**, 14813-14818.
**Song, X., Zhu, C. H., Doan, C. and Xie, T.** (2002). Germline stem cells anchored by adherens junctions in the Drosophila ovary niches. *Science* **296**, 1855-1857.
**Song, X., Wong, M. D., Kawase, E., Xi, R., Ding, B. C., McCarthy, J. J. and Xie, T.** (2004). Bmp signals from niche cells directly repress transcription of a differentiation-promoting gene, bag of marbles, in germline stem cells in the Drosophila ovary. *Development* **131**, 1353-1364.
**Szakmary, A., Cox, D. N., Wang, Z. and Lin, H.** (2005). Regulatory relationship among piwi, pumilio, and bag-of-marbles in Drosophila germline stem cell self-renewal and differentiation. *Curr. Biol.* **15**, 171-178.
**Tapon, N., Ito, N., Dickson, B. J., Treisman, J. E. and Hariharan, I. K.** (2001). The Drosophila tuberous sclerosis complex gene homologs restrict cell growth and cell proliferation. *Cell* **105**, 345-355.
**Wang, Z. and Lin, H.** (2004). Nanos maintains germline stem cell self-renewal by preventing differentiation. *Science* **303**, 2016-2019.
**Wullschleger, S., Loewith, R. and Hall, M. N.** (2006). TOR signaling in growth and metabolism. *Cell* **124**, 471-484.
**Xi, R., Doan, C., Liu, D. and Xie, T.** (2005). Pelota controls self-renewal of germline stem cells by repressing a Bam-independent differentiation pathway. *Development* **132**, 5365-5374.
**Xie, T. and Spradling, A. C.** (1998). decapentaplegic is essential for the maintenance and division of germline stem cells in the Drosophila ovary. *Cell* **94**, 251-260.
**Xie, T. and Spradling, A. C.** (2000). A niche maintaining germ line stem cells in the Drosophila ovary. *Science* **290**, 328-330.
**Xu, T. and Rubin, G. M.** (1993). Analysis of genetic mosaics in developing and adult Drosophila tissues. *Development* **117**, 1223-1237.
**Yang, L., Chen, D., Duan, R., Xia, L., Wang, J., Qurashi, A. and Jin, P.** (2007). Argonaute 1 regulates the fate of germline stem cells in Drosophila. *Development* **134**, 4265-4272.
**Zhang, H., Stallock, J. P., Ng, J. C., Reinhard, C. and Neufeld, T. P.** (2000). Regulation of cellular growth by the Drosophila target of rapamycin dTOR. *Genes Dev.* **14**, 2712-2724.
**Zhao, R., Xuan, Y., Li, X. and Xi, R.** (2008). Age-related changes of germline stem cell activity, niche signaling activity and egg production in Drosophila. *Aging Cell* **7**, 344-354.