Distinct germline progenitor subsets defined through Tsc2–mTORC1 signaling

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Abstract

Adult tissue maintenance is often dependent on resident stem cells; however, the phenotypic and functional heterogeneity existing within this self-renewing population is poorly understood. Here, we define distinct subsets of undifferentiated spermatogonia (spermatogonial progenitor cells; SPCs) by differential response to hyperactivation of mTORC1, a key growth-promoting pathway. We find that conditional deletion of the mTORC1 inhibitor Tsc2 throughout the SPC pool using Vasa-Cre promotes differentiation at the expense of self-renewal and leads to germline degeneration. Surprisingly, Tsc2 ablation within a subset of SPCs using Str8-Cre did not compromise SPC function. SPC activity also appeared unaffected by Amh-Cre-mediated Tsc2 deletion within somatic cells of the niche. Importantly, we find that differentiation-prone SPCs have elevated mTORC1 activity when compared to SPCs with high self-renewal potential. Moreover, SPCs insensitive to Tsc2 deletion are preferentially associated with mTORC1-active committed progenitor fractions. We therefore delineate SPC subsets based on differential mTORC1 activity and correlated sensitivity to Tsc2 deletion. We propose that mTORC1 is a key regulator of SPC fate and defines phenotypically distinct SPC subpopulations with varying propensities for self-renewal and differentiation.

Keywords differentiation; germline stem cells; mTORC1

Subject Category Stem Cells

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Introduction

It is becoming apparent that the stem cell populations of tissues are heterogeneous in nature [1]. Discrete subpopulations of stem cells have been identified within the same cell lineage that possess the defining stem cell property of self-renewal but differ in their lifespan, cell cycle status and contribution to tissue regeneration [2,3]. The existence of functionally distinct stem cell subtypes within the same tissue is suggested to underlie effective homeostasis and regeneration following injury [4]. The hierarchical relationship between these stem cell subsets is of great interest, as are the molecular mechanisms responsible for defining their divergent functional properties. Key to identification of stem cell subpopulations is the availability of transgenic reporter strains showing restricted expression within the phenotypically distinct stem cell subsets that allows cellular fate mapping [1,2].

Continual production of spermatozoa in the mouse testis is dependent on a resident population of undifferentiated spermatogonia with self-renewal potential (referred to here as spermatogonial stem/progenitor cells; SPCs) [5,6]. The SPC pool of adult mice is composed of isolated single cells (A-single spermatogonia or A[s]) and cysts of cells that remain interconnected by cytoplasmic bridges after cell division; 2-cell cysts are referred to as A-paired (A[p]) spermatogonia while chains of four or more cells as A-aligned (A[a]). SPC differentiation is marked by induction of the receptor tyrosine kinase c-Kit and a series of rapid mitotic divisions prior to meiosis. While most cells within the SPC pool are proposed to have stem cell potential, heterogeneity at the levels of cellular morphology and gene expression predicts differing propensities for self-renewal and differentiation. SPCs expressing Neurogenin 3 (Ngn3), typically A[a], are predisposed to differentiate [7]. Conversely, SPCs expressing Gfra1, a component of the glial cell line-derived neurotrophic factor (GDNF) receptor, predominantly A[s] and A[p], are more likely to act as stem cells [7,8]. In the steady-state, self-renewal is largely restricted to Gfra1⁺ cells, which then generate Ngn3⁺ cysts that in turn generate c-Kit⁺ differentiating spermatogonia. However, Ngn3⁺ A[a] cells can fragment to shorter cysts or A[s] cells and revert these characteristic gene expression patterns [7]. Thus, although a large fraction of the SPC pool is primed to differentiate, the cells retain the capacity to generate a long-lived stem cell population, a potential that is most evident upon transplantation or during testis regeneration [7,9]. However, the mechanisms that dictate the varying self-renewal and differentiation propensities of SPC subsets remain poorly understood.
The promyelocytic leukemia zinc finger (Plzf) gene, encoding a POZ-Krüppel (POK) family transcription factor, is essential for germ-line maintenance of the mouse and SPC self-renewal in vivo [10,11]. Importantly, Plzf is expressed by all cells within the SPC pool and is a well-established marker of this cell population [6,12]. Through study of the Plzf−/− mouse, we have previously implicated the mTORC1 signaling pathway as a critical regulator of SPC fate decisions [6]. Specifically, SPCs lacking Plzf display an aberrant tendency to differentiate rather than self-renew, an effect at least partially dependent on the ability of Plzf to inhibit mTORC1 through transcriptional modulation of the upstream regulator Redd1. This study highlights a common feature of tissue-specific stem cell compartments, the detrimental effects of aberrant mTORC1 activation on self-renewal [13–16].

The mTORC1 signaling complex is a central positive regulator of cell growth and is regulated by diverse stimuli including growth factor signaling, nutrient availability and cellular stress in order to balance anabolic and catabolic processes appropriate to the cellular environment [17]. While the mTORC1 pathway induces protein synthesis by stimulating cap-dependent mRNA translation, it can also regulate discrete gene expression programs at both transcriptional and translational levels [18–20]. In addition, mTORC1 activation drives specific bio-energetic and metabolic pathways in order to support increased cell growth [17]. Critically, mTORC1 has emerged as a key regulator of stem cell function plus aging and is commonly involved in the development of cancer.

In order to fully assess the role played by mTORC1 in control of SPC fate, and whether cell-autonomous and non-cell-autonomous contributions could be relevant to its role in SPC fate determination, we have developed mouse models with conditional deletion of Tsc2, a critical upstream inhibitor of mTORC1, within distinct cell populations of the testis. In combination with a detailed assessment of mTORC1 activity within the progenitor pool, our data conclusively define the functional role played by mTORC1 in control of SPC heterogeneity and differentiation commitment.

**Results and Discussion**

**Distinct cell types of testis exhibit mTORC1 activation**

As the role played by the mTORC1 signaling pathway in male germ-line regulation remains poorly defined, we first analyzed adult wild-type testis for evidence of mTORC1 activation. To this end, we used phosphorylation of ribosomal protein S6 (RPS6) as an indirect readout of mTORC1 activity [6,17]. Immunohistochemistry (IHC) for P-RPS6 revealed that both germ cells and somatic cells within the testis activate mTORC1 in the steady-state adult tissue (Fig 1A). P-RPS6 was present in a characteristic cytoplasmic staining pattern and more evident at certain stages of the cycling seminiferous epithelium than others [21] (Fig 1A and unpublished observations). P-RPS6-positive cells that were present on the basement membrane of the seminiferous tubules and identified as spermatogonia by morphology were most apparent in stage II–VI tubules when populations of differentiating spermatogonia are abundant. Further, P-RPS6 was detected in the cytoplasm of Sertoli cells, the critical somatic supportive cell of spermatogenesis, extending from the basement membrane toward the tubule lumen. P-RPS6-positive Sertoli cells were concentrated in late-stage VIII tubules, where one generation of mature spermatids has just been released into the tubule lumen and differentiating spermatogonia undergo their first mitotic division. These data suggest that coordinated mTORC1 activation can play a role in spermatogenesis.

In order to determine whether the SPC pool within testis showed evidence of mTORC1 activation, we performed whole-mount immuno-staining of adult seminiferous tubules for P-RPS6 and Plzf, an established marker of SPCs [6,10,11]. Importantly, P-RPS6 was mostly detected in differentiating spermatogonia, characterized by low Plzf expression and long cyst length (Fig 1B). These data suggested that mTORC1 activation was limited within the undifferentiated spermatogonial pool, consistent with the ability of Plzf to negatively regulate this pathway [6]. However, mTORC1-dependent RPS6 phosphorylation is indirect and dependent on the activation of downstream RPS6 kinases (S6K1 and 2) that integrate additional regulatory inputs besides mTORC1 [22]. Moreover, S6K is a relatively poor substrate for the mTORC1 kinase complex when compared to other direct targets such as eukaryotic translation initiation factor 4E binding protein 1 (4EBP1), a key downstream regulator of mRNA translation [22,23]. Therefore, we next assessed 4EBP1 phosphorylation as a more sensitive and direct measure of mTORC1 activity within the SPC pool. Whole-mount analysis of adult seminiferous tubules revealed a striking heterogeneous staining for phospho-4EBP1 within the Plzf-positive SPC population (Fig 1C). Phospho-4EBP1 was also detected in populations of differentiating spermatogonia (unpublished observations). Importantly, we observed a significant and positive correlation between 4EBP1 phosphorylation and increasing SPC cyst lengths (Fig 1C and D). While a limited fraction of shorter cysts (Aa and Ab) were phospho-4EBP1 positive (25–36% respectively), longer Aa cysts were frequently labeled and approximately 90% of Ass cysts containing 8 or more cells were phospho-4EBP1 positive. Conversely, an antibody specific for non-phosphorylated 4EBP1 labeled Plzf-positive Aa and Aac cells while longer Aa cysts were generally negative (Fig 1E). Interestingly, we noted that germ cells at later differentiation stages (B spermatogonia/pre-leptotene cells) were also positive for non-phosphorylated 4EBP1, indicating dynamic regulation of this pathway during spermatogenesis (Fig 1E). Given that increasing SPC cyst length is associated with an enhanced tendency to differentiate [7], our results suggested that mTORC1 activity was correlated with differentiation commitment within the progenitor pool. In an effort to confirm this observation, we assessed the phosphorylation status of 4EBP1 within the Gfrα1-positive fraction of SPCs containing the majority of self-renewing cells under steady-state conditions [7,8]. Noticeably, Gfrα1-positive cells (typically Aa and Ap) were rarely positive for phospho-4EBP1, with only approximately 15% labeled (Fig 1F; Supplementary Fig S1A and B). In contrast, expression of Lin28a, suggested to preferentially mark differentiation-committed cells within the SPC pool, was strongly associated with 4EBP1 phosphorylation levels (Supplementary Fig S1C) [24]. Together, these data demonstrate that mTORC1 activity is suppressed in the self-renewing population but induced in committed progenitors, indicating a potential role for this pathway in SPC fate decisions.

**Hyperactivation of mTORC1 by germ cell-specific deletion of Tsc2**

In order to dissect a potential cell-autonomous role for mTORC1 in SPC function, we next sought to genetically perturb mTORC1
Figure 1. Analysis of mTORC1 pathway activation in testis germ cells.

A Immunohistochemistry (IHC) of adult wild-type testis for P-RPS6. Left and right panels show P-RPS6-positive spermatogonia (Spg, indicated by arrowheads) and Sertoli cells (SCs), respectively. Seminiferous tubules stages are shown.

B Representative whole-mount images of wild-type adult seminiferous tubules stained for PLZF and phospho-RPS6. Arrowheads indicate P-RPS6-positive differentiating spermatogonia with low or absent Plzf expression. Duplicate animals were analyzed.

C Representative whole-mount images of wild-type adult seminiferous tubules stained for PLZF and phospho-4EBP1. Dashed outlines indicate Plzf-positive cysts of varying lengths.

D Quantification of whole-mount analysis from (C). Graph displays percentage of Plzf-positive cysts of indicated lengths that are positive for P-4EBP1. Mean values from three independent animals are shown ± SEM. More than 450 cysts were scored per sample and significant differences are indicated. *P < 0.05; **P < 0.01; ***P < 0.001.

E Representative whole-mount images of wild-type adult seminiferous tubules stained for PLZF and non-phospho-4EBP1. Dashed outlines indicate Plzf-positive cysts containing non-phospho-4EBP1. Arrowheads indicate longer Aα chains negative for non-phospho-4EBP1. Asterisks mark non-phospho-4EBP1-positive differentiating B spermatogonia/pre-leptotene cells.

F Representative whole-mount images of wild-type adult seminiferous tubules stained for Gfrα1 and phospho-4EBP1. Dashed outlines indicate Gfrα1-positive cysts.

G Representative IHC for Tsc2 on sections of testis from 3 weeks postnatal mice of the indicated genotypes. Higher magnification inset shows an example spermatogonium retaining Tsc2 expression.

H Representative IHC for P-RPS6 on testis sections as in (G).

Data information: Scale bars are 50 μm. Dotted lines in whole mounts indicate the seminiferous tubule profile.
activity in a germ cell-specific fashion. To this end, we developed a conditional knockout mouse model for Tuberous sclerosis 2 (Tsc2), which encodes a key negative regulator of the mTORC1 pathway. Tsc2 functions in a heterodimeric complex with the unrelated protein Tsc1 and possesses GTPase-activating protein (GAP) activity toward the small G-protein Rheb, a potent activator of mTORC1 when in a GTP-bound state. As whole-body knockout of either Tsc1 or Tsc2 results in embryonic lethality [25,26], we at first crossed mice carrying floxed alleles of Tsc2 with transgenic mice expressing Cre recombinase from proximal elements of the Stra8 promoter [27]. Stra8-Cre drives efficient floxed (F) gene deletion in the postnatal male germ line and is active in a substantial fraction of the Plzf-expressing SPC pool plus differentiating spermatagonia and pre-meiotic cells [12,27].

Strikingly, analysis of juvenile (3 weeks postnatal) Tsc2F/F Stra8-Cre testes revealed no obvious phenotype; thus, we performed IHC for Tsc2 to confirm efficient gene deletion (Fig 1G). Tsc2 was ubiquitously expressed in the cytoplasm of both germ and somatic cell components of control testes, while in Tsc2F/F Stra8-Cre testes, Tsc2 appeared entirely absent from germ cells but retained in Sertoli and interstitial cells. Subsequently, however, we noticed that a fraction of spermatagonia adjacent to the tubule basement membrane still expressed Tsc2, consistent with the fact that Stra8-Cre is inactive in some SPCs [12]. Importantly, immunostaining for P-RPS6 indicated robust activation of the mTORC1 pathway in germ cells at different stages of maturation in Tsc2F/F Stra8-Cre testes when compared to controls (Fig 1H). Tsc2 depletion in the testes was also associated with increased phospho-4EBP1 levels (unpublished observations). Thus, Tsc2 is an important negative regulator of mTORC1 in male germ cells but appears dispensable for the spermatogenic process.

Given that aberrant activation of mTORC1 in SPCs is proposed to be detrimental to their function [6], we analyzed cohorts of Tsc2F/F Stra8-Cre adults for defects in germline maintenance and function. As the testes of young Tsc2F/F Stra8-Cre adults (1–2 months postnatal) did not display any consistent or obvious phenotype when compared to controls (unpublished observations), we analyzed older (6 months postnatal) animals. However, even at this age, sections of hematoxylin and eosin (H&E)-stained Tsc2F/F Stra8-Cre testes appeared similar to controls and comparable numbers of mature spermatocytes were found in the epididymes (Fig 2A). Furthermore, there was no significant change in the number of cells expressing the SPC marker Plzf in sections of Tsc2F/F Stra8-Cre testes compared to controls (Fig 2B and Table 1). We conclude that the hyperactivation of mTORC1 in response to Stra8-Cre-mediated deletion of Tsc2 does not result in germline maintenance defects.

**SPCs from Tsc2F/F Stra8-Cre testis display larger cell size**

Conditional deletion of Tsc2 with Stra8-Cre did not result in a gross testis phenotype; however, some spermatogonia still expressed Tsc2 in this model. As a subset of SPCs does not express Stra8-Cre [12,27], we next analyzed in detail SPC status from Tsc2F/F Stra8-Cre testis and the efficiency of Tsc2 deletion within this cell population. Fixed and permeabilized testis cells from pre-pubertal (2 weeks postnatal) mice were stained for Plzf, c-Kit and Tsc2 and analyzed by flow cytometry; allowing identification of the Plzf-expressing cell pool (Fig 2C). A minor fraction of Plzfpos cells also express c-Kit and represent differentiating SPCs [6,12]. However, there was no significant difference in the fraction of Plzfpos cells that expressed c-Kit in control and Tsc2F/F Stra8-Cre testis (Fig 2D), suggesting that the balance between SPC differentiation and self-renewal was not perturbed. Accordingly, the percentage of SPCs within the testis cell populations from Tsc2F/F Stra8-Cre and control mice was comparable (Fig 2E).

Importantly, we noticed that the physical size of SPCs from Tsc2F/F Stra8-Cre testis, as measured by forward scatter (FSC), was significantly larger than that of control SPCs (Fig 2F). Increased cell size and growth are a hallmark of elevated mTORC1 activity [6,28], further indicating that while SPC status was not perturbed in Tsc2F/F Stra8-Cre testis, Tsc2 deletion should have occurred to some extent. To confirm whether this was indeed the case, we compared Tsc2 protein levels in Plzfpos testis cells by flow cytometry (Fig 2G). Interestingly, essentially all the Plzf-expressing cells from control testis were positive for Tsc2, in agreement with the ubiquitous expression pattern of Tsc2 observed from IHC analysis of testis sections (see above). However, on average, ~40% of SPCs (Plzfpos c-Kitneg) and ~60% of cells at early differentiation stages (Plzfpos c-Kitpos) from Tsc2F/F Stra8-Cre testis had lost Tsc2 expression (Fig 2G and H). That the differentiating SPC population is found to contain more Tsc2-ablated cells than the undifferentiated pool suggests that Stra8-Cre is most active in differentiating cells and/or in SPCs primed for differentiation, consistent with previous data (see below) [12]. In addition, we confirmed that both mTOR itself and the key downstream target 4EBP1 are ubiquitously expressed within the Plzf-positive pool of juvenile testis, indicating that all SPCs would have the capacity to activate the pathway upon Tsc2 deletion (Supplementary Fig S2).

**Male adult germline maintenance is Tsc2 dependent**

Conditional deletion of Tsc2 in a subset of SPCs primed for differentiation using Stra8-Cre did not result in any apparent phenotype. To assess the effect of more extensive Tsc2 deletion within the SPC pool, we therefore crossed Tsc2flox mice with transgenic animals expressing Cre from the Vasa promoter. Vasa-Cre is induced in embryonic gonocytes prior to formation of the SPC pool and can drive efficient floxed gene deletion within the germline [12,29]. Importantly, from the analysis of pre-pubertal (2 weeks postnatal) testis sections, no gross germ cell phenotype was evident, suggesting that postnatal germline development of Tsc2F/A Vasa-Cre mice was unaffected (Supplementary Fig S3A). In addition, the presence of Plzf-expressing cells on the seminiferous tubule basement membrane of juvenile Tsc2F/A Vasa-Cre testes indicated that SPC populations were successfully established (Fig 3A). Immunostaining for P-RPS6 did, however, indicate a striking increase in mTORC1 pathway activity in germ cells of juvenile Tsc2F/A Vasa-Cre testes and a corresponding loss of Tsc2 expression (Fig 3B).

Having confirmed that induction of Tsc2 deletion with a Cre transgene that is active from embryonic germ cell stages did not appear to disrupt postnatal testis development and generation of SPCs, we next examined Tsc2F/A Vasa-Cre adults for potential testis phenotypes. Strikingly, we found that by 2 months postnatal, the testis of Tsc2F/A Vasa-Cre mice contained a significant number of degenerating tubules that had lost or were in the processing of losing germ cell components (Fig 3C and D). This germline degeneration correlated with reduced testis weight of Tsc2F/A Vasa-Cre.
adults compared to controls (Fig 3E). Critically, many tubules of adult Tsc2F/D Vasa-Cre testis, while sometimes still containing spermatocytes and/or spermatids, lacked cells expressing SPC markers including Plzf and Lin28a (Fig 3F) [6,24,30]. This significant and substantial reduction in the number of Plzf-positive cells in adult Tsc2F/D Vasa-Cre testis indicates an SPC maintenance defect (Table 1), consistent with the observed deterioration of germ cell components. Additionally, mRNA levels of the GDNF receptor components Gfra1 and Ret, critical for SPC maintenance and highly enriched within self-renewing SPC populations, were significantly decreased in Tsc2F/D Vasa-Cre versus control adult testis (Fig 3G) [8,31,32]. Together, these data indicate that Tsc2 deletion driven by the Vasa-Cre transgene results in an SPC maintenance defect and accompanying germline degeneration.

Tsc2/mTORC1 controls self-renewal versus differentiation fates of SPCs

In contrast to Stra8-Cre-mediated Tsc2 deletion, use of Vasa-Cre to drive conditional knockout of Tsc2 results in depletion of the SPC pool. To better understand why these two models give distinct phenotypes, we next performed a detailed analysis of SPC status in pre-pubertal Tsc2F/D Vasa-Cre testis in a comparable manner to our assessment of SPC activity in the Tsc2F/F Stra8-Cre model (see above). Importantly, flow cytometric analysis of fixed, permeabilized and immunostained cells from Tsc2F/D Vasa-Cre (2 weeks postnatal) revealed a reduction in the Plzfpos population compared to controls (Fig 4A). Importantly, the fraction of Plzfpos cells that co-expressed the differentiation marker c-Kit was significantly

Figure 2. Assessment of SPC status in Tsc2F/F Stra8-Cre testis.
A Representative images of testis sections from 6 months postnatal mice of the indicated genotypes stained with hematoxylin and eosin (H&E). Insets show higher magnification details of mature sperm present in the epididymis. Scale bar is 50 μm.
B Representative IHC for Plzf on testis sections as in (A).
C Representative flow cytometric analysis of fixed and permeabilized testis cells from 2 weeks postnatal mice for Plzf expression.
D Analysis of c-Kit expression by the Plzf-positive fractions of control (Ctrl; Tsc2F/F) and conditional knockout (cKO; Tsc2F/F Stra8-Cre) testis cells. Filled histogram (gray) indicates isotype-stained control. No significant difference was found in the percentage of Plzf-positive cells expressing c-Kit between six control and five cKO animals.
E Fraction of total testis cells identified as SPCs (Plzf positive, c-Kit negative) from the flow cytometric analysis of (D). Mean values are shown ± SEM.
F Analysis of cell size by flow cytometry. Representative overlay of forward scatter (FSC) plots of the SPC fractions from 2 weeks postnatal littersmate mice. The mean FSC of Tsc2F/F Stra8-Cre (cKO) SPCs is significantly increased compared to Tsc2F/F (Ctrl) cells. Duplicate mice were analyzed per genotype and P-value is indicated.
G Quantification of the flow cytometry analysis shown in (G). Mean percentage of Plzf-positive testis cells with indicated Tsc2 and c-Kit expression status is shown. A total of six Tsc2F/F (Ctrl) and 5 Tsc2F/F Stra8-Cre (cKO) animals were analyzed.
increased in Tsc2F/F Vasa-Cre versus control testis, suggesting that SPCs from this knockout model have an aberrant tendency to differentiate and/or reduction in self-renewal (Fig 4B and C). Indeed, the relative fraction of SPCs was significantly reduced in juvenile Tsc2F/F Vasa-Cre testis (Fig 4D), demonstrating that Vasa-Cre-mediated Tsc2 deletion is detrimental to the SPC population.

Consistent with successful Tsc2 ablation, SPCs from pre-pubertal Tsc2F/F Vasa-Cre mice demonstrated significant increases in cell size as measured by FSC, an indicator of increased mTORC1 activity (Fig 4E, see above). Critically, when assessing Tsc2 protein levels by flow cytometry, we observed a complete ablation of Tsc2 (Fig 4E, see above). Critically, when assessing Tsc2 protein levels as measured by FSC, an indicator of increased mTORC1 activity within cells contributing to the stem cell niche. In particular, mTORC1-dependent changes in production of paracrine factors by niche cells have been described to impact both positively and negatively on stem cell self-renewal in other systems [33,34]. While the contribution of different somatic cell types to the SPC niche remains an area of intense study, the Sertoli cell is understood to be a critical component through its production of growth factors required for SPC self-renewal and growth [5]. In wild-type adult testis, we find evidence of stage-specific mTORC1 activation in Sertoli cells suggesting a potential role for this pathway in niche function (Fig 1A, see above).

Thus, we next assessed the indirect niche-dependent effects of aberrant mTORC1 activation on SPC function. To this end, Tsc2 floxed mice were crossed with animals carrying the Amh-Cre transgene, which express Cre in a Sertoli cell-specific fashion from the anti-Mullerian hormone (Amh) promoter [35]. Amh-Cre expression is detectable by embryonic day (E) 11.5 in the developing testis [36]. Analysis of juvenile (3 weeks postnatal) Tsc2F/F Amh-Cre testis revealed an apparent disruption of the seminiferous epithelium compared to controls, the lumen of some tubules appearing full of the eosinophilic cytoplasm from hypertropic Sertoli cells (Supplementary Fig S4A). Meiotic cell degeneration and vacuolization could also be observed in these tubules (Supplementary Fig S4A and unpublished observations), consistent with a previous study [37]. However, these changes in tubule morphology were temporary, and in young Tsc2F/F Amh-Cre adults (2 months postnatal), the testis histology appeared grossly normal compared to controls (Supplementary Fig S4B).

Immunostaining testis sections of young Tsc2F/F Amh-Cre adults for the Sertoli cell marker Sox9 identified a typical population of Sertoli cells localized to the basal layer of the seminiferous tubules (Fig 5A) [38]. As anticipated, Sertoli cells of adults displayed striking increases in staining for P-RPS6, confirming successful deletion of Tsc2 in this cell population (Fig 5B). Importantly, however, numbers of Plzf-positive spermatogonia from adult Tsc2F/F Amh-Cre testis (2 months postnatal) were comparable to controls, indicating that SPC activity was unaffected by aberrant mTORC1 activation in Sertoli cells (Fig 5C and Table 1).

Although the Plzfpos spermatogonial population appeared unaffected in young Tsc2F/F Amh-Cre adults, we next assessed SPC status in a more detailed fashion in juvenile mice by flow cytometric analysis of fixed and permeabilized testis cells (see above). Immunostaining for the SPC marker Plzf together with the Sertoli cell marker Sox9 demonstrated minor, yet significant, alterations in the relative proportions of these cell types within the pre-pubertal testis (Fig 5D and E). An observed decrease in the fraction of Sox9pos Sertoli cells from Tsc2F/F Amh-Cre testis could also be inferred from decreased expression of the Sertoli cell-expressed gene Amh as detected by RT–PCR of testis RNA (Supplementary Fig S4C) [39]. Critically, however, the percentage of Plzfpos cells from Tsc2F/F Amh-Cre testis that expressed c-Kit was comparable to that of control testis, indicating that self-renewal versus differentiation decisions of SPCs was not substantially affected (Fig 5F and G). Indeed, expression of glial cell line-derived neurotrophic factor (GDNF), a key Sertoli cell-derived factor that drives SPC self-renewal, was not significantly altered in Tsc2F/F Amh-Cre testis compared to controls (Supplementary Fig S4C). Together, these data suggest that while some relative expansion of the Plzfpos cell

Table 1. Effects of conditional Tsc2 deletion on the Plzf-expressing spermatogonial pool.

<table>
<thead>
<tr>
<th>Cre driver</th>
<th>Plzfpos cells/tubule cross-section*</th>
<th>Controlb</th>
<th>Tsc2 KOc</th>
<th>P-value</th>
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<tr>
<td>Stra8-CreA</td>
<td>2.35 ± 0.19</td>
<td>2.15 ± 0.26</td>
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<tr>
<td>Vasa-CreB</td>
<td>2.10 ± 0.15</td>
<td>0.68 ± 0.05</td>
<td>9.49 × 10⁻⁵</td>
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<tr>
<td>Amh-CreC</td>
<td>2.24 ± 0.14</td>
<td>2.09 ± 0.22</td>
<td>0.59</td>
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</tbody>
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*Mean values are shown ± SEM. Three mice per genotype were analyzed for Stra8-Cre and Amh-Cre cohorts, and four mice per genotype were analyzed for the Vasa-Cre group. Over 50 tubule cross-sections were scored per animal. bLittermate controls were of the following genotypes: +/+ Cre+ or +/+ Cre+ for Stra8-Cre cohort; +/+ Cre+ for Vasa-Cre and Amh-Cre cohorts. cConditional knockout (cKO) genotypes were Tsc2F/F Amh-Cre and Amh-Cre drivers and F/Cre+ with Vasa-Cre driver. dSix months postnatal. eTwo months postnatal.
pool occurs in the pre-pubertal testis in response to aberrant mTORC1 activation in Sertoli cells, SPC fate and the SPC population of young adults are both unaffected.

**Stra8-Cre preferentially marks mTORC1 active committed progenitors**

Through efficient *Vasa-Cre*-mediated deletion of Tsc2 in SPCs, we have confirmed that aberrant mTORC1 activation in SPCs promotes differentiation commitment and results in germ line degeneration [6]. In contrast, *Stras8-Cre*-driven Tsc2 deletion within a subset of SPCs has no apparent functional consequences. Given that *Stra8* itself is associated with germ cell differentiation [40], we considered that the *Stra8-Cre* transgene might exhibit selective expression in differentiation-committed SPCs. Deleting Tsc2 in those SPCs that already show evidence of mTORC1 activation (see above) and that make limited contributions to steady-state germ line maintenance can explain the lack of phenotype in *Tsc2<sup>F/F</sup> Stra8-Cre* animals.
To test whether Stra8-Cre is expressed in discrete subsets of SPCs as predicted, we crossed Stra8-Cre mice with transgenic Z/EG reporter animals that express enhanced green fluorescent protein (GFP) upon Cre-dependent deletion of an upstream bgeo cassette [12,41]. Importantly, from whole-mount analysis of adult and prepubertal Z/EG; Stra8-Cre testis, we confirmed that a fraction of Plzf-positive SPCs was GFP negative, thus indicating that they neither expressed Cre nor were derived from Cre-expressing cells (Supplementary Fig S5A). Correspondingly, analysis of juvenile Z/EG; Stra8-Cre testis by flow cytometry demonstrated that while a majority of SPCs were GFP positive, a discrete GFP-negative population was observed (Fig 6A). In addition, cells initiating differentiation (Plzfpos c-Kitpos) were largely GFPpos, consistent with previous data and the pattern of Tsc2 deletion in Tsc2F/F Stra8-Cre testis (see above) [12].

Strikingly, the cell size of GFPpos (Stra8-Crepr) SPCs, as measured by FSC, was significantly increased compared to that of GFPneg (Stra8-Creneg) SPCs from Z/EG; Stra8-Cre testis (Fig 6B and C). Importantly,
inhibition of mTORC1 in vivo by rapamycin treatment of Z/EG; Stra8-Cre juvenile mice essentially eliminated differences in cell size between the cell fractions, confirming that size changes are mediated by differential mTORC1 activation (Fig 6B). These results indicated that mTORC1 activity increases in a graded fashion from Stra8-Cre-negative SPCs (low) to Stra8-Cre-positive SPCs (intermediate) to cells initiating differentiation (high) and suggested a positive correlation between Stra8-Cre expression, mTORC1 activation and SPC differentiation. That Stra8-Cre was preferentially expressed in mTORC1-active SPC populations was further suggested by whole-mount analysis of juvenile and adult Z/EG; Stra8-Cre seminiferous tubules (Fig 6D). The majority of mTORC1-active A1a subsets were GFP positive, while GFP-negative Plzf-positive cells were typically A1a and A1g and P-4EBP negative/low.

Together, our data indicated that within the SPC pool Stra8-Cre is predominantly expressed by differentiation-committed cells that have increased mTORC1 activity (see above). To further define this expression pattern, we compared GFP expression in self-renewing and committed SPC fractions of Z/EG; Stra8-Cre testis, taking advantage of selective markers for these populations. Interestingly, within the Gfrα1-positive self-renewing SPC subpopulation of both juvenile and adult Z/EG; Stra8-Cre mice, GFP expression was markedly heterogeneous (Fig 6E; Supplementary Fig S5B). Accordingly, more than 20% of Gfrα1-positive cells were GFP negative in the juvenile, demonstrating that the transgene was inactive in a substantial fraction of the self-renewing SPC pool (Fig 6F). Conversely, expression of retinoic acid receptor γ (RARγ), a marker specific for differentiation-prone A1a SPCs and spermatogonia at the very first step of the differentiation pathway (A1 cells), correlated strongly with GFP expression (Fig 6E; Supplementary Fig SSC) [42]. Specifically, only approximately 5% of RARγ-positive cells in the juvenile testis were GFP negative (Fig 6F). This substantial difference in Cre activity between the Gfrα1 and RARγ populations confirms that Stra8-Cre is preferentially active in committed progenitors although is still evident within a portion of the self-renewing pool. Considering that recombination of both floxed Tsc2 alleles in a cell would probably
be less efficient than recombination of a single ZEG reporter, we conclude that Stra8-Cre-driven Tsc2 deletion would be inefficient within the self-renewing SPC pool but significant within mTORC1-active committed progenitors.

We note that in the absence of appropriate antibodies to other committed SPC markers such as Ngn3, expression of RARγ effectively labels those SPCs and spermatogonia at the earliest stages of differentiation commitment (Supplementary Fig S5D). Moreover, RARγ-pos SPCs are significantly larger than RARγ-neg SPCs, indicating that RARγ expression successfully marks mTORC1-active, differentiation-committed cells (Fig 6G; Supplementary Fig S5E).

Role of the mTORC1 pathway in defining SPC heterogeneity and fate

The SPC population can be envisaged as being composed of discrete cell subsets, distinguishable by morphology and gene expression, which exhibit varying propensities for self-renewal and differentiation (Fig 6H). Our data demonstrate that SPC subsets with high self-renewal potential suppress mTORC1 activity, while those populations with high differentiation tendencies have an activated mTORC1 pathway. Given that we find aberrant mTORC1 activation to inhibit SPC self-renewal, we propose that differential
activation of this signaling complex defines the distinct functional characteristics of these SPC subsets (Fig 6H). Therefore, in response to Vasa-Cre-driven Tsc2 deletion, the mTORC1-low self-renewing subsets would be converted into an mTORC1-active state characterized by a high propensity for differentiation. This would cause a switch in the overall balance of cell fate from self-renewal to differentiation and ultimately lead to SPC exhaustion and germ-line degeneration.

Our study demonstrates that the TSC complex is an important cell-intrinsic regulator of the mTORC1 signaling pathway in SPCs and would act to control the equilibrium between mTORC1-low and mTORC1-active populations (Fig 6H). However, whether the observed disparity in mTORC1 activation by SPC subsets is due to intrinsic mechanisms or extrinsic cues is not clear. Growth factors and would act to control the equilibrium between mTORC1-low and mTORC1-active, differentiation-prone state. The fraction of cells within quadrant gates is indicated. Markers associated with the distinct populations are indicated, as is the expression pattern of the Str8-Cre transgene. Dashed arrow represents proposed capability of differentiation-prone SPC subsets to contribute to self-renewing pool during regeneration. Balanced self-renewal and differentiation of the SPC pool is dependent on the TSC1/2 complex, which inhibits adoption of an mTORC1-active, differentiation-prone state.

Data information: Scale bars are 50 μm. Dotted lines in whole mounts indicate the seminiferous tubule profile.

**Conserved function of the mTORC1 pathway in adult stem cell regulation**

A role for mTORC1 in SPC maintenance was originally proposed from studies of the Plzf<sup>-/-</sup> mouse [6]. By conditional deletion of Tsc2 in SPCs, we now confirm a key role for this signaling pathway in SPC fate decisions. Interestingly, disruption of either Tsc1 or Tsc2 genes in germline stem cells (GSCs) of the *Drosophila* ovaries results in their differentiation and loss in an mTORC1-dependent manner [50,51], indicating a conserved role for the TSC complex in regulation of GSC fate. Increased activation of mTORC1 in SPCs/GSCs of both mouse and *Drosophila* inhibits responses of the cells to key niche-derived signals required for self-renewal [6,51], providing a novel mechanism by which mTORC1 can control the functional properties of stem cells. Specifically, in SPCs, mTORC1 can inhibit expression of GDNF receptor components, thus preventing maintenance of the undifferentiated state [6,31,52]. Transcriptional profiling of SPCs upon mTORC1 inhibition in *vivo* has revealed numerous changes in expression of genes involved in the oxidative stress response, signal transduction pathways and SPC regulation [53]. Indeed, many cellular processes controlled by mTORC1, including mRNA translation, autophagy and metabolism [17], are implicated in stem cell regulation [14,54,55]. Thus, besides the GDNF receptor, multiple downstream targets of mTORC1 may be relevant in controlling SPC fate.

Intriguing parallels can also be drawn between the roles of mTORC1 in spermatogenesis and neurogenesis, since a direct association between mTORC1 activation and neural differentiation is evident from studies in multiple model systems. For example, neuronal progenitor differentiation in developing *Drosophila* is induced by mTORC1 acting downstream insulin receptor signaling [56]. In the mouse, Tsc1 deletion and aberrant mTORC1 activation in embryonic neural stem cells (NSCs) triggers a reduction in
self-renewal capacity and premature differentiation [16]. Remarkably, negative feedback between mTORC1 and the upstream PI3Kinase-Akt pathway was proposed to underlie this reduction in NSC self-renewal [16], similar to the effects of chronic mTORC1 activation on GDNF-dependent Akt activation in SPCs from the Plzf<sup>−/−</sup> model [6]. Interestingly, in rodent models of neuroprogenitor differentiation, mTORC1 pathway inhibition through Redd1 induction is reported to oppose differentiation onset [57]. Importantly, in this latter study, differentiation stimuli transiently induce Redd1, thus limiting mTORC1 activation and reducing progenitor depletion through differentiation. It can be significant that we find Redd1 to be regulated in an mTORC1-dependent fashion at both mRNA and protein levels in cultured SPCs (Supplementary Fig S6) [6]. Thus, in response to mTORC1-activating stimuli, Redd1 can act as part of a negative feedback circuit to limit the fraction of SPCs that transition to an mTORC1-high state and which are ultimately likely to differentiate.

In summary, we characterize an unappreciated heterogeneity within the SPC pool based on differential mTORC1 activity and demonstrate the key instructive role played by the mTORC1 pathway in the regulation of SPC fate. Given that the mTORC1 pathway is regulated in a dynamic fashion, these studies also suggest a mechanistic basis for the differing yet reversible differentiation tendencies of discrete SPC subsets [7]. Further, as mTORC1 plays a seemingly conserved role in regulation of adult stem cell function, it will be interesting to assess whether activity of this pathway drives observed functional heterogeneity of stem cells in other tissues.

Materials and Methods

Mouse maintenance and treatments

Mice carrying floxed Tsc2 alleles are previously described [58]. Stra8-Cre, Vasa-Cre, Amh-Cre and Z/EG reporter mice were obtained from Jackson Laboratories. The Vasa-Cre transgene was carried from males of breeding pairs due to maternal effects that result in whole-body Cre-mediated recombination in offspring [12,29]. When Vasa-Cre is carried from males, low-level Cre expression during zygotic development can result in global Cre-mediated recombination in offspring [29], resulting in embryonic lethality with Tsc2 deletion. Expected frequency of obtaining Tsc2<sup>−/−</sup> Vasa-Cre mice from matings Tsc2<sup>−/+</sup> Vasa-Cre males with Tsc2<sup>−/+</sup> females is 1:4, and observed frequency was ~1:20. Both Stra8-Cre and Amh-Cre transgenes were carried from females. Juvenile mice were routinely analyzed at 2 weeks of age. Mice were treated with rapamycin or vehicle daily for 7 days starting at postnatal day 10 as described [6]. All animal experiments were subject to approval by the Institutional Animal Care and Use Committee of Beth Israel Deaconess Medical Center and the Monash University Animal Ethics Committee.

Immunohistochemistry (IHC) and histology

Testis tissue was fixed in 4% paraformaldehyde (PFA) prior to embedding in paraffin and processing for IHC and histology as described [12]. Primary antibodies used were as follows: polyclonal anti-phospho RPS6 (Ser235/236) and polyclonal anti-LIN28A (Cell Signaling Technology), monoclonal anti-Plzf (clone 9E12) [6], rabbit monoclonal anti-TSC2, C-term (Epitomics) and polyclonal anti-Sox9 (Chemicon). Nuclei counterstains were performed with hematoxylin or DAPI.

Whole-mount immunofluorescence

Testes were detunicated and seminiferous tubules teased apart and rinsed in phosphate-buffered saline (PBS) on ice. Tubules were fixed with 4% PFA for 6 h at 4°C and washed in PBS prior to blocking in 0.3% PBS Triton X-100 (PBSX) supplemented with 5% fetal bovine serum (FBS) and 1% bovine serum albumin (BSA). Tubules were incubated overnight at 4°C with primary antibodies diluted in PBSX containing 1% BSA. Samples were washed in PBSX and primary antibodies detected with appropriate Alexa Fluor-conjugated secondary antibodies (Jackson ImmunoResearch). Tubules were mounted in Vectashield mounting medium (Vector Labs) and analyzed with an Axio Imager Z1 m with Apotome (Zeiss) or Nikon Invert C1 confocal microscope at the Monash University Micro Imaging facility. Primary antibodies used were as follows: goat anti-PLZF, anti-Gfr alpha1 and anti-Lin28a (R&D Systems), chicken anti-GFP (Abcam), monoclonal rabbit anti-phospho RPS6 (Ser235/236), anti-phospho 4E-BP1 (Thr37/46), anti-non-phospho 4E-BP1 (Thr46), anti-Lin28a and anti-RARγ1 (Cell Signaling Technology).

Flow cytometry

Methodology for intracellular staining and flow cytometric analysis of testis cells has been detailed previously [12]. Briefly, single cell suspensions obtained from the sequential digestion of testes with collagenase and trypsin were fixed in Cytofix buffer and permeabilized in Phosflow Perm buffer III (BD Biosciences) prior to immunostaining. Primary antibodies were used as follows: Alexa 488- or Alexa 647-conjugated anti-Plzf [12], phycocerythrin (PE)-conjugated anti-c-Kit/CD117 (eBioscience), rabbit monoclonal anti-TSC2 and anti-mTOR (Epitomics), rabbit or chicken polyclonal anti-GFP (Invitrogen and Abcam) and monoclonal rabbit anti-4EBP1 and RARγ1 (Cell Signaling Technology). Primary antibodies were detected as appropriate with Alexa 488- and 647-conjugated secondary antibodies (Invitrogen and Jackson ImmunoResearch). Samples were run on an LSRII (BD Biosciences) and data were analyzed with FlowJo software (Tree Star).

qRT–PCR

RNA was extracted from testes and cultured SPCs using TRIzol reagent (Invitrogen) and treated with DNase prior to cDNA synthesis with a first-strand synthesis kit (Affymetrix). Cultured SPCs were washed off embryonic fibroblast feeder cells prior to RNA extraction [6]. Quantitative PCRs were run on a LightCycler (Roche) using a QuantiTect SYBR Green PCR kit (Qiagen). Primer sequences were previously described [6,36].

Statistical analysis

Assessment of statistical significance was performed using a 2-tailed t-test. P-values are indicated as follows: *P < 0.05; **P < 0.01; ***P < 0.001; not significant (ns) P > 0.05.
Supplementary information for this article is available online: http://embor.embopress.org

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Author contributions
RMH and PPP conceived and designed the study. RMH, HML and JM performed the experiments and data analysis. RMH, HML and PPP wrote the paper. TK and TN provided critical reagents.

Conflict of interest
The authors declare that they have no conflict of interests.

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