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 *Stra8-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_{pr}) spermatogonia while chains of four or more cells as A-aligned (A_{al}). 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_{al} , 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_{pr} , 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_{al} 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.

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The *promyelocytic leukemia zinc finger* (*Plzf*) gene, encoding a POZ-Krüppel (POK) family transcription factor, is essential for germline 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 germline 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 read-out 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 immunostaining 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 (*A_s* and *A_{pr}*) were phospho-4EBP1 positive (25–36% respectively), longer *A_{al}* cysts were frequently labeled and approximately 90% of *A_{al}* cysts containing 8 or more cells were phospho-4EBP1 positive. Conversely, an antibody specific for non-phosphorylated 4EBP1 labeled *Plzf*-positive *A_s* and *A_{pr}* cells while longer *A_{al}* 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 *Gfra1*-positive fraction of SPCs containing the majority of self-renewing cells under steady-state conditions [7,8]. Noticeably, *Gfra1*-positive cells (typically *A_s* and *A_{pr}*) 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 $\pm$ 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 short Plzf-positive cysts containing non-phospho-4EBP1. Arrowheads indicate longer A_{al} 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 μ m. 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 germline and is active in a substantial fraction of the *Plzf*-expressing SPC pool plus differentiating spermatogonia and pre-meiotic cells [12,27].

Strikingly, analysis of juvenile (3 weeks postnatal) *Tsc2^{F/F} Stra8-Cre* testis 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 testis, while in *Tsc2^{F/F} Stra8-Cre* testis, *Tsc2* appeared entirely absent from germ cells but retained in Sertoli and interstitial cells. Subsequently, however, we noticed that a fraction of spermatogonia 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 *Tsc2^{F/F} Stra8-Cre* testis when compared to controls (Fig 1H). *Tsc2* depletion in the testis 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 *Tsc2^{F/F} Stra8-Cre* adults for defects in germline maintenance and function. As the testes of young *Tsc2^{F/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 *Tsc2^{F/F} Stra8-Cre* testes appeared similar to controls and comparable numbers of mature spermatozoa were found in the epididymides (Fig 2A). Furthermore, there was no significant change in the number of cells expressing the SPC marker *Plzf* in sections of *Tsc2^{F/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 *Tsc2^{F/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 *Tsc2^{F/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 *Plzf^{pos}* cells also express c-Kit

and represent differentiating SPCs [6,12]. However, there was no significant difference in the fraction of *Plzf^{pos}* cells that expressed c-Kit in control and *Tsc2^{F/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 *Tsc2^{F/F} Stra8-Cre* and control mice was comparable (Fig 2E).

Importantly, we noticed that the physical size of SPCs from *Tsc2^{F/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 *Tsc2^{F/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 *Plzf^{pos}* 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 (*Plzf^{pos} c-Kit^{neg}*) and ~60% of cells at early differentiation stages (*Plzf^{pos} c-Kit^{pos}*) from *Tsc2^{F/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 *Tsc2* floxed 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 *Tsc2^{F/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 *Tsc2^{F/A} Vasa-Cre* testis 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 *Tsc2^{F/A} Vasa-Cre* testis 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 *Tsc2^{F/A} Vasa-Cre* adults for potential testis phenotypes. Strikingly, we found that by 2 months postnatal, the testis of *Tsc2^{F/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 *Tsc2^{F/A} Vasa-Cre*

Figure 2. Assessment of SPC status in *Tsc2*^{F/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 μ m.

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; *Tsc2*^{F/F}) and conditional knockout (cKO; *Tsc2*^{F/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 $\pm$ SEM.

F Analysis of cell size by flow cytometry. Representative overlay of forward scatter (FSC) plots of the SPC fractions from 2 weeks postnatal littermate mice. The mean FSC of *Tsc2*^{F/F} *Stra8-Cre* (cKO) SPCs is significantly increased compared to *Tsc2*^{F/F} (Ctrl) cells. Duplicate mice were analyzed per genotype and *P*-value is indicated.

G Representative flow cytometric analysis of *Tsc2* and *c-Kit* expression by the *Plzf*-positive testis cell population from 2 weeks postnatal mice of the indicated *Tsc2*; *Stra8-Cre* genotypes. Percentage of cells within each quadrant gate is indicated.

H 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 *Tsc2*^{F/F} (Ctrl) and 5 *Tsc2*^{F/F} *Stra8-Cre* (cKO) animals were analyzed.

adults compared to controls (Fig 3E). Critically, many tubules of adult *Tsc2*^{F/A} *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 *Tsc2*^{F/A} *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 *Tsc2*^{F/A} *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 *Tsc2*^{F/A} *Vasa-Cre* testis in a comparable manner to our assessment of SPC activity in the *Tsc2*^{F/F} *Stra8-Cre* model (see above). Importantly, flow cytometric analysis of fixed, permeabilized and immunostained cells from *Tsc2*^{F/A} *Vasa-Cre* testis (2 weeks postnatal) revealed a reduction in the *Plzf*^{pos} population compared to controls (Fig 4A). Importantly, the fraction of *Plzf*^{pos} cells that co-expressed the differentiation marker *c-Kit* was significantly

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

Cre driver	Plzf ^{pos} cells/tubule cross-section ^a		
	Control ^b	<i>Tsc2</i> cKO ^c	P-value
<i>Stra8-Cre</i> ^d	2.35 ± 0.19	2.15 ± 0.26	0.57
<i>Vasa-Cre</i> ^e	2.10 ± 0.15	0.68 ± 0.05	9.4 × 10 ⁻⁵
<i>Amh-Cre</i> ^e	2.24 ± 0.14	2.09 ± 0.22	0.59

^aMean 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 +/F *Cre*+ for *Stra8-Cre* cohort; +/F *Cre*+ for *Vasa-Cre* and *Amh-Cre* cohorts.

^cConditional knockout (cKO) genotypes were F/F *Cre*+ with *Stra8-Cre* and *Amh-Cre* drivers and F/Δ *Cre*+ with *Vasa-Cre* driver.

^dSix months postnatal.

^eTwo months postnatal.

increased in *Tsc2*^{F/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 *Tsc2*^{F/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 *Tsc2*^{F/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* expression within the Plzf^{pos} population, irrespective of c-Kit expression, from *Tsc2*^{F/F} *Vasa-Cre* testis (Fig 4F and G). Together, these data indicate that complete loss of *Tsc2* expression throughout the SPC pool disrupts self-renewal and leads to SPC exhaustion in adults.

To confirm that the effects of *Tsc2* ablation on SPCs are dependent on increased mTORC1 activation, we next tested whether treatment of juvenile *Tsc2*^{F/F} *Vasa-Cre* mice with the mTORC1 inhibitor rapamycin could rescue the changes in SPC function. Strikingly, upon rapamycin treatment, the fraction of Plzf^{pos} testis cells from *Tsc2*^{F/F} *Vasa-Cre* mice that expressed c-Kit was now comparable to vehicle-treated controls (Fig 4H and I). The significant increase in percentage of Plzf^{pos} testis cells expressing c-Kit previously detected in *Tsc2*^{F/F} *Vasa-Cre* versus control mice was still apparent in vehicle-treated animals (Fig 4I; Supplementary Fig S3B). Thus, rapamycin rescues the disrupted balance between self-renewal and differentiation commitment of *Tsc2*^{F/F} *Vasa-Cre* SPCs. Importantly, this regimen of rapamycin treatment was able to normalize both the fraction of SPCs found within *Tsc2*^{F/F} *Vasa-Cre* testis cells when compared to vehicle-treated controls and the increased cell size of *Tsc2*^{F/F} *Vasa-Cre* SPCs (Fig 4I; Supplementary Fig S3C). We conclude that the detrimental effects of *Vasa-Cre*-dependent *Tsc2* deletion on SPC function are mediated through aberrant activation of the mTORC1 pathway.

Transient cell non-autonomous SPC regulation by *Tsc2*/mTORC1 in the niche

It has recently become apparent that stem cell behavior can be affected in a cell non-autonomous manner by the level of 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-Müllerian 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) *Tsc2*^{F/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 hypertrophic 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 *Tsc2*^{F/F} *Amh-Cre* adults (2 months postnatal), the testis histology appeared grossly normal compared to controls (Supplementary Fig S4B).

Immunostaining testis sections of young *Tsc2*^{F/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 *Tsc2*^{F/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 Plzf^{pos} spermatogonial population appeared unaffected in young *Tsc2*^{F/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 Sox9^{pos} Sertoli cells from *Tsc2*^{F/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 Plzf^{pos} cells from *Tsc2*^{F/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 *Tsc2*^{F/F} *Amh-Cre* testis compared to controls (Supplementary Fig S4C). Together, these data suggest that while some relative expansion of the Plzf^{pos} cell

Figure 3. Vasa-Cre-mediated *Tsc2* deletion in germ cells drives SPC exhaustion.

- A** Representative IHC for Plzf on testis sections from 2 weeks postnatal mice. Scale bar: 50 μ m.
- B** IHC for P-RPS6 on testis sections from 2 weeks postnatal mice. Insets show corresponding IHC for *Tsc2*. Scale bar: 50 μ m.
- C** H&E-stained sections of testis from 2 months postnatal mice of the indicated *Tsc2*; *Vasa-Cre* genotypes. Asterisks indicate degenerating tubules displaying substantial germ cell loss. Lower panels show examples of degenerating tubules from a *Tsc2*^{F/Δ} *Vasa-Cre* adult at higher magnification. Arrowheads indicate Sertoli cells in a tubule containing maturing spermatozoa but lacking preceding germ cell stages. Tubules at initial stages of degeneration with some remaining germ cell components are indicated (#). Scale bar: 50 μ m.
- D** Quantification of the percentage of degenerating (Dgn) seminiferous tubules displaying germ cell loss from 2 months postnatal *Tsc2*^{F/+} *Vasa-Cre* (Ctrl) and *Tsc2*^{F/Δ} *Vasa-Cre* (cKO) mice. Tubules were scored from H&E-stained sections as indicated in (C). Four mice were analyzed per genotype and over 70 tubule cross-sections were scored per animal. Graph indicates mean values $\pm$ SEM. ***P* < 0.01.
- E** Testis weights of 2 months postnatal *Tsc2*^{F/+} *Vasa-Cre* (Ctrl) and *Tsc2*^{F/Δ} *Vasa-Cre* (cKO) mice. A total of four control and 2 cKO mice were analyzed. Mean value is shown $\pm$ SEM. ****P* < 0.001.
- F** IHC for Plzf (top panels) and Lin28a (bottom panels) on testis sections from 2 months postnatal mice of the indicated genotypes. Asterisks indicate tubules lacking Plzf- or Lin28a-expressing cells. Scale bar: 50 μ m.
- G** Quantitative RT-PCR for GDNF receptor components from total testis cells of 2 months postnatal *Tsc2*^{F/+} *Vasa-Cre* (Ctrl) and *Tsc2*^{F/Δ} *Vasa-Cre* (cKO) mice. mRNA levels are corrected to those of β -actin and normalized to a control sample. Testes from four controls and two cKO mice were analyzed. Horizontal bars indicate mean values. ***P* < 0.01; ****P* < 0.001.

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 germline degeneration [6]. In contrast, *Stra8*-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 germline maintenance can explain the lack of phenotype in *Tsc2*^{F/F} *Stra8*-Cre animals.

Figure 4. Analysis of SPC status in *Tsc2^{F/A} Vasa-Cre* testis.

- A Flow cytometric analysis of testis cells from 2-week postnatal mice of the indicated *Tsc2; Vasa-Cre* genotypes. Percentage of Plzf-positive cells is shown.
- B Analysis of c-Kit expression by the Plzf-positive testis cells of control (Ctrl; *Tsc2^{F/+} Vasa-Cre*) and conditional knockout (cKO; *Tsc2^{F/A} Vasa-Cre*) mice from A. Filled histogram (gray) indicates isotype control.
- C Testis cells from 2-week postnatal control and cKO mice were analyzed by flow cytometry as in A and B. Graph indicates percentage of Plzf-positive cells expressing c-Kit. Mean values are shown $\pm$ SEM. A total of six control and four cKO animals were analyzed. ** $P < 0.01$.
- D Percentage of SPCs (Plzf positive, c-Kit negative) within the testis cell populations from C. Mean values are shown $\pm$ SEM. ** $P < 0.01$.
- E FSC plot overlays of the SPC fractions from 2-week postnatal littermate mice. The mean FSC of *Tsc2^{F/A} Vasa-Cre* (cKO) SPCs is significantly increased compared to control SPCs (*Tsc2^{F/+} Vasa-Cre* or *Tsc2^{F/+} Vasa-Cre*). A total of four control and two cKO mice were analyzed and P value is indicated.
- F Flow cytometric analysis of Plzf-positive testis cells from 2-week postnatal mice. Percentage of cells within gates is indicated.
- G Quantification of analysis in F. Graph shows mean percentage of Plzf-positive cells with the indicated *Tsc2* and c-Kit expression. A total of six control (*Tsc2^{F/+} Vasa-Cre* or *Tsc2^{F/+} Vasa-Cre*) and 4 *Tsc2^{F/A} Vasa-Cre* (cKO) animals were analyzed.
- H Flow cytometric analysis of c-Kit expression by Plzf-positive testis cells from vehicle (Veh)-treated control (*Tsc2^{F/+} Vasa-Cre*) and rapamycin (Rapa)-treated *Tsc2^{F/A} Vasa-Cre* (cKO) juvenile mice.
- I Testis cells from Veh- and Rapa-treated control (*Tsc2^{F/+} Vasa-Cre* or *Tsc2^{F/+} Vasa-Cre*) and cKO juvenile mice were analyzed by intracellular staining and flow cytometry as in H. The fraction of testis cells identified as SPCs (Plzf positive, c-Kit negative) is indicated in the left panel, while the percentage of Plzf-positive cells expressing c-Kit is on the right. A total of four control and three cKO mice were analyzed for each treatment group. Horizontal bars indicate mean values and selected P values are included. * $P < 0.05$; *** $P < 0.001$.

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 β geo cassette [12,41]. Importantly, from whole-mount analysis of adult and pre-pubertal 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 (Plzf^{pos} c-Kit^{pos}) were largely GFP^{pos}, consistent with previous data and the pattern of *Tsc2* deletion in *Tsc2^{F/F} Stra8-Cre* testis (see above) [12].

Strikingly, the cell size of GFP^{pos} (*Stra8-Cre*^{pos}) SPCs, as measured by FSC, was significantly increased compared to that of GFP^{neg} (*Stra8-Cre*^{neg}) SPCs from Z/EG; *Stra8-Cre* testis (Fig 6B and C). Further, the size of cells initiating differentiation (Plzf^{pos} c-Kit^{pos}) was significantly increased above both SPC fractions. Importantly,

Figure 5. *Tsc2*-deficient Sertoli cells support SPC function.

A IHC for the Sertoli cell marker Sox9 on testis sections from 2 months postnatal mice of the indicated genotypes. Scale bar: 50 μ m.
B IHC for P-RPS6 on testis sections from 2 months postnatal mice. Scale bar: 50 μ m.
C IHC for Plzf on testis sections from 2 months postnatal mice. Scale bar: 50 μ m.
D Representative flow cytometric analysis of Sox9 and Plzf expression by testis cells from 2 weeks postnatal mice of the indicated *Tsc2; Amh-Cre* genotypes. Plzf- and Sox9-positive cell populations are indicated.
E Testis cells from 2 weeks postnatal control (*Tsc2^{F/+}* or *Tsc2^{F/F}*) and cKO (*Tsc2^{F/F} Amh-Cre*) mice were analyzed for Plzf and Sox9 expression by flow cytometry as described in (D). Graph indicates the mean percentage of testis cells positive for Plzf and Sox9 $\pm$ SEM. Five mice were analyzed per genotype group. * $P < 0.05$; ** $P < 0.01$.
F Comparative flow cytometry analysis of c-Kit expression by Plzf-positive testis cells from control (*Tsc2^{F/F}*) and *Tsc2^{F/F} Amh-Cre* (cKO) 2 weeks postnatal mice. Filled histogram (gray) indicates isotype-stained control.
G Percentage of Plzf-positive cells from the flow cytometric analysis described in (E) that express c-Kit. Mean values are shown $\pm$ SEM.

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^{neg}* SPCs (low) to *Stra8-Cre^{pos}* 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 *A_{al}* subsets were GFP positive, while GFP-negative Plzf-positive cells were typically *A_s* and *A_{pr}* 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 Gfr α 1-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 Gfr α 1-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 (RAR γ), a marker specific for differentiation-prone *A_{al}* SPCs and spermatogonia at the very first step of the differentiation pathway (*A₁* cells), correlated strongly with GFP expression (Fig 6E; Supplementary Fig S5C) [42]. Specifically, only approximately 5% of RAR γ -positive cells in the juvenile testis were GFP negative (Fig 6F). This substantial difference in Cre activity between the Gfr α 1 and RAR γ 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

Figure 6.

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

Figure 6. *Stra8*-Cre expression delineates distinct spermatogonial populations.

- A Representative flow cytometric analysis of GFP and c-Kit expression by the Plzf-positive testis cell population from 2 weeks postnatal Z/EG; *Stra8*-Cre mice. Quadrant gates were set according to wild-type control mice. The fraction of cells within quadrant gates is indicated.
- B Representative FSC plot overlay of the indicated testis cell fractions from 2 weeks postnatal Z/EG; *Stra8*-Cre mice as in (A) (left panel; Ctrl). FSC plots of the equivalent populations from rapamycin-treated Z/EG; *Stra8*-Cre mice are shown (right panel; Rapa).
- C Quantification of the flow cytometry analysis shown in (B). Graph shows mean FSC values of the indicated testis cell fractions from Z/EG; *Stra8*-Cre mice. Horizontal bars represent mean values; three mice were analyzed. * $P < 0.05$; ** $P < 0.01$.
- D Representative whole-mount images of Z/EG; *Stra8*-Cre juvenile (upper panels) and adult (lower panels) seminiferous tubules illustrating positive association between GFP expression and phospho-4EBP1 staining within the Plzf-positive population. Dashed outlines indicate Plzf-positive cysts of varying lengths. A_{hi} are generally positive for both GFP and P-4EBP, while GFP-negative Plzf-positive cells are often A_{lo} and P-4EBP negative/low. Arrowheads indicate occasional Plzf-positive cells that are P-4EBP positive but GFP negative. Duplicate samples from independent animals at both ages were analyzed.
- E Representative whole-mount images of juvenile mice assessing GFP expression in Gfra1-positive (upper panels) and RAR γ -positive (lower panels) spermatogonial populations. Arrowheads indicate Gfra1-positive GFP-negative cells.
- F Quantification of whole-mount analysis from (E). Graph indicates percentage of cells within the indicated spermatogonial populations negative for GFP. Mean values are shown from three independent juvenile mice $\pm$ SEM. A total of 12–15 mm of tubule length was analyzed per sample. ** $P < 0.01$.
- G FSC plot overlay of Plzf^{pos} c-Kit^{neg} SPC testis cell fractions from 2 weeks postnatal wild-type mice divided according to RAR γ expression. Testis cells were pooled from 2 littermate mice for analysis. Plot is representative from two independent experiments.
- H Model depicting subdivision of SPCs according to mTORC1 activity status. These subpopulations have distinct self-renewal capabilities (curved arrows) and differentiation tendencies. Levels of mTORC1 activity in the different cell fractions are indicated, and the predicted hierarchy between the populations under steady-state conditions is shown. Markers associated with the distinct populations are indicated, as is the expression pattern of the *Stra8*-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 μ m. Dotted lines in whole mounts indicate the seminiferous tubule profile.

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 germline 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 are major cell-extrinsic regulatory inputs of the mTORC1 pathway, of which a variety have described expression in the testis and can activate mTORC1 through upstream PI3Kinase-Akt and Erk MAPK pathways [5,6,43]. Indeed, SPC maintenance and function is dependent on appropriate regulation of the PI3Kinase-Akt pathway [44]. Physical constraints of the SPC niche coupled with the localized production of growth factors by cells within the niche may underlie the observed differential mTORC1 activation within the SPC pool [45]. Notably, however, we find that mTORC1 activation within cells of the SPC niche (Sertoli cells) does not affect expression of key niche growth factors or appear to affect stem cell function, in contrast to other systems [33,34]. Further, local variations in the concentration of retinoic acid (RA), an important regulator of germ cell differentiation [46], may result in differential mTORC1 activation in SPCs; RA-dependent activation of mTORC1 is directly associated with differentiation in other cell systems [47]. Interestingly, *Stra8* was originally identified as an RA-responsive gene and RA-response elements are present within the proximal *Stra8* promoter used in the *Stra8*-Cre transgene [27,48,49]. Given that we find *Stra8*-Cre to be preferentially active within mTORC1-active SPC subsets, RA may represent an important extrinsic factor driving mTORC1 activation in SPCs. However, whether the mTORC1 pathway is a

critical downstream target for RA-dependent induction of SPC differentiation remains to be determined.

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*^{-/-} 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* ovary 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 response 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*^{-/-} 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*^{F/A} *Vasa-Cre* mice from mating *Tsc2*^{F/+} *Vasa-Cre* males with *Tsc2*^{F/F} 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 α 1 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-RAR γ 1 (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 Cytotfix buffer and permeabilized in Phosflow Perm buffer III (BD Biosciences) prior to immunostaining. Primary antibodies were as follows: Alexa 488- or Alexa 647-conjugated anti-Plzf [12], phycoerythrin (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 RAR γ 1 (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:
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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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