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Plzf regulates germline progenitor self-renewal by opposing mTORC1

Robin M. Hobbs[1], **Marco Seandel**[2], **Ilaria Falciatori**[3], **Shahin Rafii**[3], and **Pier Paolo Pandolfi**[1,*]

[1]Cancer Genetics Program, Beth Israel Deaconess Cancer Center, Departments of Medicine and Pathology, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, Massachusetts 02115, USA

[2]Department of Surgery, Weill Cornell Medical College, New York 10065, USA

[3]Howard Hughes Medical Institute, Department of Genetic Medicine, Weill Cornell Medical College, New York 10065, USA

SUMMARY

Hyperactivity of mTORC1, a key mediator of cell growth, leads to stem cell depletion although the underlying mechanisms are poorly defined. Using spermatogonial progenitor cells (SPCs) as a model system, we show that mTORC1 impairs stem cell maintenance by a negative feedback from mTORC1 to receptors required to transduce niche-derived signals. We find that SPCs lacking Plzf, a transcription factor essential for SPC maintenance, have enhanced mTORC1 activity. Aberrant mTORC1 activation in *Plzf* $^{-/-}$ SPCs inhibits their response to GDNF, a growth factor critical for SPC self-renewal, via negative feedback at the level of the GDNF receptor. Plzf opposes mTORC1 activity by inducing expression of the mTORC1 inhibitor Redd1. Thus, we identify the mTORC1-Plzf functional interaction as a critical rheostat for maintenance of the spermatogonial pool, and propose a model whereby negative feedback from mTORC1 to the GDNF receptor balances SPC growth with self-renewal.

INTRODUCTION

Maintenance of a wide array of adult tissues is dependent on the presence of a resident stem cell pool with self-renewal potential that generates differentiating progeny. Factors regulating the balance between stem cell self-renewal and differentiation ensure tissue homeostasis while disruption of these regulatory mechanisms can lead to tissue degeneration or cancer (Ito et al., 2009). One factor central to stem cell homeostasis is mammalian TOR complex 1 (mTORC1), a signaling complex that promotes protein translation and cell growth by phosphorylating components of the translation machinery (Ma and Blenis, 2009). mTORC1 is regulated in response to diverse stimuli including nutrient availability, energy status, growth factors and cellular stress. Persistent mTORC1 activation in certain tissues leads to increased proliferation but subsequent exhaustion of the stem cell compartment, demonstrating that aberrantly activated mTORC1 is detrimental to stem cell maintenance (Castilho et al., 2009; Gan and DePinho, 2009; Yilmaz et al., 2006). It is proposed that

*Correspondence: ppandolf@bidmc.harvard.edu.

inappropriate mTORC1 activation drives stem cell depletion through aberrant translation of downstream targets and subsequent activation of tumor suppressive/fail-safe mechanisms resulting in cellular senescence or apoptosis (Ito et al., 2009). However, the molecular mechanisms and targets of mTORC1 in this context are currently unknown. Interestingly, inhibition of mTORC1 also extends organism lifespan (Harrison et al., 2009; Schieke and Finkel, 2006), consistent with the notion that declining stem cell potential underlies aging (Rossi et al., 2008).

Undifferentiated germline cells of the testis (spermatogonial progenitor cells; SPCs) are formed from gonocytes during postnatal development of the mouse testis and possess self-renewal potential (de Rooij and Russell, 2000). A major advance in the study of male germline biology was the development of culture systems allowing long-term SPC expansion *in vitro* while maintaining stem cell potential. Key to this was the observation that mice heterozygous for the glial cell - derived neurotrophic factor (GDNF) cytokine gene had a depletion of SPC activity (Meng et al., 2000). GDNF is produced by Sertoli cells within the testis, and signals via the GFRα1/c-Ret receptor to promote SPC self-renewal and growth through activation of Src family kinases and Akt (Lee et al., 2007b; Oatley et al., 2007). Culture of SPCs with GDNF plus a variety of additional factors (including basic fibroblast growth factor; bFGF) preserves self-renewal capabilities and allows essentially unlimited cell expansion while maintaining *in vivo* differentiation potential; assessed by the ability to repopulate depleted recipient testis (Kanatsu-Shinohara et al., 2003; Kubota et al., 2004; Seandel et al., 2007). Although some cellular signaling pathways involved in SPC self-renewal have been described, it remains unclear how SPCs integrate signals from general mitogenic stimuli with those required for self-renewal to balance stem cell maintenance and differentiation.

A limited number of cell intrinsic factors have also been implicated in SPC function, foremost amongst which is Promyelocytic Leukemia Zinc Finger (PLZF). *PLZF* was identified from the translocation breakpoint in t(11;17) acute promyelocytic leukemia (Chen et al., 1993) and encodes a transcription factor belonging to the POZ-*Krüppel* (POK) family. PLZF binds DNA through carboxy-terminal *Krüppel*-type Zinc fingers and recruits histone deacetylases (HDACs) via an amino-terminal POZ domain (David et al., 1998). Recruitment of HDACs to target promoters can result in gene repression although PLZF is also able to activate gene expression (Doulatov et al., 2009; Labbaye et al., 2002). Male mice lacking *Plzf* expression undergo progressive germ cell loss and testis atrophy with age causing infertility (Buaas et al., 2004; Costoya et al., 2004). Plzf is expressed by SPCs and is needed in a cell autonomous fashion for maintenance of the germ lineage. A male patient with biallelic PLZF loss-of-function and gonad hypoplasia has been recently reported (Fischer et al., 2008), emphasizing the role played by PLZF in germ cell biology.

SPC maintenance is dependent on Plzf plus key growth factors such as GDNF. As mTORC1 is activated in response to growth factor signaling we hypothesized that both Plzf and mTORC1 are involved in the GDNF response of SPCs and that Plzf and mTORC1 may crosstalk. To assess the roles of Plzf and mTORC1 in GDNF-dependent SPC maintenance, we developed systems for isolation and culture of SPCs from wildtype and *Plzf* $^{-/-}$ prepubertal mice (subsequent to formation of the SPC pool and prior to overt germ cell depletion in the *Plzf* $^{-/-}$ mouse). We find that Plzf opposes mTORC1 activity and define a cellular signaling network controlling SPC homeostasis whereby mTORC1 integrates mitogenic signals received by SPCs and determines their sensitivity to self-renewal stimuli.

RESULTS

Isolation, culture and comparative analysis of *Plzf* $^{+/+}$ and *Plzf* $^{-/-}$ SPCs

The testis is composed of a heterogeneous mix of mitotic and meiotic germ cells plus multiple types of somatic cells. SPCs are rare within the testis and systems for their purification are poorly developed. We therefore sought to take advantage of the expression of Plzf in SPCs to enrich for this cell type. Plzf-expressing cells were identified from juvenile testis by intracellular staining and flow cytometry using a newly developed monoclonal antibody (Figure 1A). Our aim was to correlate the Plzf-expressing population to sets of cell surface markers that would allow subsequent purification of those live cells. By adapting markers able to enrich for stem cell activity from cryptorchid testis (Kubota et al., 2003; Shinohara et al., 2000), we found that the Plzf-positive population was αv-integrin negative and expressed low levels of Thy-1 (Figure 1B). Negative selection for αv-integrin combined with Thy-1 positive selection allowed isolation of Plzf-positive cells and minimized somatic cell contamination (Figures S1A and S1B) (Virtanen et al., 1986). *c-Kit* expression is associated with SPC differentiation (Schrans-Stassen et al., 1999) and the αv^{neg} Thy-1^{low} population was composed primarily of c-Kit negative cells with a smaller c-Kit positive fraction (Figure 1C, Left). The c-Kit^{neg} cells were largely in G_1/G_0 phase of cell cycle while the c-Kit^{pos} population contained more cells in S plus G_2 phases (Figure 1C, Right). As SPCs are more quiescent than differentiating spermatogonia (Takubo et al., 2008), the αv^{neg} Thy-1^{low} population contains both SPCs (c-Kit^{neg}, quiescent) and cells undergoing differentiation (c-Kit^{pos}, proliferating). To confirm that the αv^{neg} Thy-1^{low} c-Kit^{neg} fraction was enriched for stem cells, we compared the ability of αv^{neg} Thy-1^{low} c-Kit^{neg} and unfractionated cells to repopulate recipient depleted testis (Ogawa et al., 1997). Donor cells were isolated from transgenic mice expressing GFP from the β-actin promoter, allowing visualization of donor-derived colonies (Figure 1D, Left). Numbers of GFP-positive colonies were scored 2 months post-transplant (Figure 1D, Right), representing numbers of engrafted stem cells. Stem cell activity was enriched ~25-fold in the αv^{neg} Thy-1^{low} c-Kit^{neg} population compared to unfractionated cells. As the αv^{neg} Thy-1^{low} c-Kit^{neg} fraction represents 3–5% of total cells (~1/25th of testis cellularity) from 10–14d postnatal testis (Figures 1B, 1C and 1G), a 25-fold enrichment of stem cell activity indicates that almost all stem cells are contained within this fraction. Given the number of colonies obtained from αv^{neg} Thy-1^{low} c-Kit^{neg} cells (Figure 1D) and an estimated 10% engraftment efficiency (Nagano et al., 1999), there are 1265 stem cells per 10^5 cells in this fraction (~1 in 80 cells have stem cell potential).

We next analyzed prepubertal *Plzf* $^{+/+}$ and *Plzf* $^{-/-}$ testis by flow-cytometry to determine if *Plzf* $^{-/-}$ mice display aberrant SPC activity prior to overt germ cell depletion. While the characteristic αv/Thy-1 flow profile was present in juvenile *Plzf* $^{-/-}$ testis (2 and 3 weeks postnatal), the αv^{neg} Thy-1^{low} population was depleted relative to the wildtype (Figures 1E and S1C) and the percentage of c-Kit^{pos} cells within the *Plzf* $^{-/-}$ αv^{neg} Thy-1^{low} fraction was increased (Figures 1F and S1D). The decreased frequency and numbers of αv^{neg} Thy-1^{low} c-Kit^{neg} cells in *Plzf* $^{-/-}$ testis (Figure 1G) confirms that Plzf loss is detrimental to SPCs while the increase in c-Kit^{pos} cells within the αv^{neg} Thy-1^{low} fraction is suggestive of increased differentiation commitment. As Plzf directly represses *c-Kit* expression (Filipponi et al., 2007), this can be reflective of a de-repression of the *c-Kit* locus. However, the *Plzf* $^{-/-}$ αv^{neg} Thy-1^{low} fraction still contains c-Kit^{neg} cells (Figures 1F and S1D), indicating that aberrant *c-Kit* expression is unlikely to fully explain the *Plzf* $^{-/-}$ phenotype. Additionally, both *Plzf* $^{+/+}$ and *Plzf* $^{-/-}$ αv^{neg} Thy-1^{low} c-Kit^{neg} cells show substantial enrichment in expression of the SPC-associated factors *Pou5f1/Oct4*, *Ngn3*, *Bcl6b* and *Pou3f1* (Figures 1H and S1E) (Oatley et al., 2006; Ohbo et al., 2003; Wu et al., 2010; Yoshida et al., 2006), confirming the identity of this fraction. We also noted that *Plzf* $^{-/-}$ αv^{neg} Thy-1^{low} cells had decreased levels of β1 and α6 integrins but equivalent levels of CD9 compared to controls

(Figures S1F and S1G) (Kanatsu-Shinohara et al., 2004b; Shinohara et al., 1999). Our data indicate that SPCs from juvenile *Plzf* $^{-/-}$ mice have an impaired ability to maintain an undifferentiated state, which precedes overt germ cell loss.

We next attempted *in vitro* culture of isolated *Plzf* $^{+/+}$ and *Plzf* $^{-/-}$ SPCs, adapting previously developed culture systems (Kubota et al., 2004; Seandel et al., 2007). As anticipated, germ cell colony-forming activity under SPC culture conditions was essentially entirely contained within the αv^{neg} Thy-1^{low} fraction (Figure S2A). We successfully derived SPC lines from both *Plzf* $^{+/+}$ and *Plzf* $^{-/-}$ αv^{neg} Thy-1^{low} cells, which grew as discrete colonies on mouse embryonic fibroblast (MEF) feeder cells (Figure 2A) and maintained their cell surface marker identity during exponential growth over 1 year of culture (Figure S2B). Wildtype SPC lines expressed Plzf (Figure 2B) while *Plzf* $^{-/-}$ SPC lines were more difficult to establish (not shown) consistent with a role for Plzf in SPC function. Early passages of *Plzf* $^{-/-}$ SPCs had a slower growth rate than wildtype cells (Figures S2C and S2D) but could be maintained long-term and growth became more comparable to wildtype cells at later passages (Figure 5C). The ability to culture *Plzf* $^{-/-}$ SPCs seemed counterintuitive, but the generation of these cultures became critical for further dissection of Plzf function and to the solution of this apparent contradiction.

An additional indicator of SPC potential is the ability to derive embryonic stem-like cells from the cultured lines (multipotent adult spermatogonial-derived stem cells; MASCs) (Kanatsu-Shinohara et al., 2004a; Seandel et al., 2007). We observed spontaneous formation of MASC colonies from 2 out of 7 wildtype lines (Figure S2E). MASC formation was not observed in 4 *Plzf* $^{-/-}$ SPC lines, possibly indicating a defective capability to generate MASCs. The multipotent capabilities of MASC lines were verified by teratoma formation assay (Figure S2F). Upon SPC to MASC conversion, expression of the pluripotency-associated factors *Pou5f1/Oct4*, *Sox2* and *Nanog* were substantially increased while *Plzf* expression was lost (Figure S2G and data not shown) (Seandel et al., 2007). Formation of MASCs indicates that SPC potential is maintained in our culture system, while the apparent inability of *Plzf* $^{-/-}$ SPCs to form MASCs can be consistent with their defective function.

Plzf $^{-/-}$ SPCs show enhanced mTORC1 activity

From analysis of cultured SPC lines, we noticed that *Plzf* $^{-/-}$ cells were physically larger than wildtype SPCs, measured by the FSC parameter of flow cytometry (Figures 2C and 2D). Alterations in cell size are associated with changes in activity of mTORC1 acting through its downstream targets S6Kinase1 (S6K1), which phosphorylates ribosomal S6 protein (RPS6), and 4EBP1 (Fingar et al., 2002; Ruvinsky et al., 2005). Given the role of mTORC1 in hematopoietic stem cells (Gan and DePinho, 2009), we considered that aberrant mTORC1 activity in *Plzf* $^{-/-}$ SPCs could contribute to their defective maintenance. Importantly, *Plzf* $^{-/-}$ SPCs had elevated levels of phosphorylated RPS6 compared to wildtype cells, confirming increased mTORC1 activity (Figures 2E and 3C). Inhibition of mTORC1 with Rapamycin decreased the size of *Plzf* $^{-/-}$ SPCs to that of wildtype cells suggesting that elevated mTORC1 activity was responsible for the size increase (Figure 2F). Rapamycin was confirmed to inhibit RPS6 phosphorylation in *Plzf* $^{-/-}$ SPCs (Figure 2E). Increased mTORC1 activity in *Plzf* $^{-/-}$ SPCs was associated with elevated levels of cellular protein (Figure S2H), consistent with the role of mTORC1 in regulating protein translation. Importantly, freshly isolated *Plzf* $^{-/-}$ SPCs were also physically larger (Figures 2G and 2H) and Rapamycin treatment of *Plzf* $^{-/-}$ mice normalized SPC size (Figures 2G and 2H). Together, our data indicates that Plzf inhibits mTORC1 activation in SPCs.

Growth factor-mediated regulation of mTORC1 in SPCs

Given the possibility that mTORC1 activity can regulate SPC function, we next defined key regulatory inputs of mTORC1 in wildtype SPCs. Growth factor signaling represents a major activating input of mTORC1 (Ma and Blenis, 2009; Shaw and Cantley, 2006) and mTORC1 inhibition interferes with mitogenic stimuli causing cell cycle arrest (Brown et al., 1994). Indeed, Rapamycin suppressed SPC colony growth and caused an accumulation of cells in G1 phase of the cell cycle (Figure S3A and S3B).

Growth factor receptors activate mTORC1 by signaling through phosphoinositide 3-kinase (PI3K)/Akt and Ras/Erk MAPK, which inhibit the TSC1/TSC2 complex (Huang and Manning, 2008); TSC1/TSC2 negatively regulates mTORC1 via its GTPase-activating protein activity towards the small G-protein Rheb. Treatment of wildtype SPCs with PI3K/Akt or Erk MAPK pathway inhibitors significantly reduced levels of phosphorylated RPS6 (Figure 3A), indicating that efficient mTORC1 activation in SPCs requires both PI3K/Akt and Erk MAPK. Inhibition of Src family kinases, implicated in the GDNF response of SPCs (Oatley et al., 2007), had modest effects on phospho-RPS6 (Figure 3A). Therefore, despite the ability of Src family kinases to activate PI3K/Akt downstream GDNF (Encinas et al., 2001; Oatley et al., 2007), this SPC self-renewal pathway couples inefficiently to mTORC1.

SPCs are maintained with a cocktail of growth factors (Kanatsu-Shinohara et al., 2003; Seandel et al., 2007) and are responsive to others (Hamra et al., 2007); thus we next assessed contributions of these factors to mTORC1 activation in wildtype SPCs. We found that GDNF triggered a modest phosphorylation of RPS6 while other mitogens (including bFGF) induced much higher levels of phospho-RPS6 (Figure 3B). The relative abilities of GDNF and bFGF to activate mTORC1 correlated to their efficiency of Akt and Erk activation (Figure 3B). However, this correlation did not hold true for epidermal growth factor (EGF) stimulation, possibly reflecting additional pathways by which EGF activates mTORC1 (Fan et al., 2009). In summary, the self-renewal signal GDNF poorly activates mTORC1 when compared to more general mitogens for SPCs (bFGF, EGF), in agreement with the Src kinase inhibition data (see above). However, we note that while GDNF is considered the key SPC self-renewal signal, SPC expansion and self-renewal *in vitro* requires a combination of GDNF plus other factors (e.g. bFGF) that stimulate mTORC1 more efficiently (Lee et al., 2007b).

Plzf uncouples mTORC1 activity from growth factor signaling in SPCs through Redd1 modulation

Given that mTORC1 activity in SPCs is regulated by growth factors present in the culture medium and resultant PI3K/Akt and Erk-MAPK activation, we next assessed whether the elevated mTORC1 activity of *Plzf* $^{-/-}$ SPCs correlated with an enhanced growth factor response. However, in comparison to wildtype cells, the Akt and Erk activities of steady state *Plzf* $^{-/-}$ SPCs were decreased while phospho-RPS6 was increased (Figure 3C), indicating that the elevated mTORC1 activity is not due to increased activity of growth factor pathways.

We next considered alternative mechanisms by which mTORC1 activity would be increased in *Plzf* $^{-/-}$ SPCs. Cellular stress-response pathways provide additional regulatory inputs resulting in mTORC1 inhibition when conditions such as low energy availability or hypoxia require temporary arrest of cell growth (Huang and Manning, 2008). We hypothesized that mTORC1 hyperactivity in *Plzf* $^{-/-}$ SPCs could be due to reduced activity of stress response pathways. Accordingly, we first assessed levels of upstream regulatory proteins as stress pathways converge on mTORC1 at the level of TSC1/TSC2. However, we did not find

significant differences in levels of Tsc1, Tsc2, Rheb or the mTOR kinase itself in *Plzf* $^{-/-}$ SPCs compared to wildtype cells (Figure 4A).

By contrast, we noticed that levels of Redd1 (also known as Ddit4, Rtp801 and Dig2) were substantially lower in *Plzf* $^{-/-}$ SPCs compared to controls, at both protein and mRNA levels (Figure 4A and 4B). Redd1 is induced by multiple types of cell stress (e.g. hypoxia, DNA damage) and by developmental signals and inhibits mTORC1 through regulation of TSC1/TSC2 (Brugarolas et al., 2004; Corradetti et al., 2005; Ellisen et al., 2002). As a role for Redd1 in SPCs is not described, we analyzed the distribution of *Redd1* expression in isolated spermatogonial fractions and found a relative enrichment of *Redd1* mRNA in SPCs (Figure 4C). We also confirmed that freshly isolated *Plzf* $^{-/-}$ SPCs had lower *Redd1* expression compared to controls (Figure 4C). Furthermore, shRNA knockdown of Redd1 in wildtype SPCs increased mTORC1 activity (Figure 4D and S4). Together, our data suggest that Plzf opposes mTORC1 by maintaining *Redd1* expression and that Redd1 is a key mTORC1 regulator in SPCs.

Plzf is a transcriptional activator of the mTORC1 inhibitor Redd1 in SPCs

Redd1 expression was decreased at the mRNA level in *Plzf* $^{-/-}$ SPCs. We therefore tested whether Plzf could directly induce *Redd1* expression. We first performed a chromatin immunoprecipitation (ChIP) assay to assess whether Plzf was bound to the *Redd1* promoter in SPCs. We scanned a 2kb region upstream *Redd1* that contains binding sites for multiple transcription factors known to regulate *Redd1* expression (Figure 4E) (Ellisen et al., 2002; Lin et al., 2005; Shoshani et al., 2002). Importantly, we could detect Plzf association with distal promoter (DP) regions but not proximal promoter (PP) regions (Figure 4E), indicating that Plzf regulates *Redd1* expression through recruitment to the DP. To confirm this we performed luciferase assays with REDD1 promoter constructs containing both DP+PP elements and the PP element alone (Figure 4F). In agreement with our ChIP results, PLZF activated the DP+PP construct but not the PP construct. Further, deletion of the PLZF POZ domain prevented PP+DP reporter activation, indicating a requirement for this protein-protein interaction domain. We conclude that Plzf directly activates *Redd1* through the DP in order to inhibit mTORC1 in SPCs.

Active mTORC1 inhibits SPC self-renewal pathways via a negative feedback loop

As *Plzf* $^{-/-}$ SPCs show increased mTORC1 but decreased Akt activities (Figure 3C), we considered that negative feedback from mTORC1 could prevent *Plzf* $^{-/-}$ cells from activating Akt in response to growth factors. In cells lacking TSC1/TSC2, increased mTORC1-signaling induces a negative feedback loop from the mTORC1 downstream target S6K to upstream signaling components causing inhibition of PI3K/Akt (Harrington et al., 2004; Shah et al., 2004). Therefore, we tested whether aberrant mTORC1 activity inhibited the response of *Plzf* $^{-/-}$ SPCs to GDNF, a growth factor required for SPC self-renewal, which could explain the maintenance defect of *Plzf* $^{-/-}$ SPCs. We compared the response of starved *Plzf* $^{+/+}$ and *Plzf* $^{-/-}$ SPCs to GDNF in the absence or presence of Rapamycin to vary mTORC1 activity (Figures 5A and 5B). Consistent with previous data, *Plzf* $^{-/-}$ SPCs showed lower levels of basal and GDNF-stimulated Akt activity when compared to wildtype SPCs, indicating a substantially reduced ability of *Plzf* $^{-/-}$ cells to activate PI3K/Akt. However, upon Rapamycin treatment, the ability of *Plzf* $^{-/-}$ SPCs to activate Akt in response to GDNF was significantly increased and became comparable to that of wildtype cells. This rescue of GDNF responsiveness by Rapamycin indicates that a negative feedback response from aberrantly activated mTORC1 suppresses the response of *Plzf* $^{-/-}$ SPCs to GDNF. Indeed, if GDNF levels in the media were reduced, *Plzf* $^{-/-}$ SPC growth was inhibited more substantially than that of the wildtype cells (Figure 5C). A decreased sensitivity to GDNF due to negative feedback from mTORC1 can explain the defect in

Plzf $^{-/-}$ SPC maintenance *in vivo* when levels of GDNF are limiting (Meng et al., 2000) and how *Plzf* $^{-/-}$ SPCs can be cultured *in vitro* when GDNF is supplied to excess. Importantly, GDNF expression in *Plzf* $^{-/-}$ testis was equivalent to that of the wildtype (Figure S5), ruling out non-cell autonomous defects in production of niche factors.

Activated mTORC1 suppresses expression of GDNF receptor components

We next sought to determine the mechanism by which activated mTORC1 inhibits response of *Plzf* $^{-/-}$ SPCs to GDNF. Negative feedback from mTORC1 to PI3K/Akt was originally characterized in the context of insulin responsiveness and signaling through IRS1/2 proteins (Harrington et al., 2005). Although IRS proteins have been implicated in activation of PI3K by the GDNF receptor component c-Ret (Hennige et al., 2000), we noticed that expression of the GDNF receptor components GFRα1 and c-Ret in SPCs were responsive to Rapamycin (Figure 5D and 5E). Cultured *Plzf* $^{-/-}$ SPCs expressed lower levels of GFRα1 and c-Ret compared to wildtype cells (correlating with reduced GDNF-responsiveness) but upon mTORC1 inhibition, expression of these GDNF receptor components was increased back to wildtype levels. Expression of GFRα1 and c-Ret were also lower in freshly isolated *Plzf* $^{-/-}$ SPCs compared to wildtype SPCs (Figure 5F), confirming that Plzf loss disrupts GDNF receptor expression *in vivo*. Our data suggest that aberrantly activated mTORC1 inhibits the response of *Plzf* $^{-/-}$ SPCs to GDNF by opposing expression of the receptor. Interestingly, mTORC1 inhibits upstream signaling events in MEFs through transcriptional inhibition of the PDGF receptor (Zhang et al., 2007). In SPCs, negative feedback between mTORC1 and the GDNF receptor will lead to loss of self-renewal when mTORC1 is hyperactivated, such as occurs upon loss of *Plzf* expression (Figure S6A). This feedback loop would inversely couple cell growth to self-renewal, so that upon expansion of the stem cell pool by mitogenic stimulation and resultant mTORC1 activation, response of the cells to self-renewal signals is inhibited, restricting stem cell numbers. Interestingly, GDNF activates mTORC1 weakly in SPCs when compared to other mitogens (e.g. bFGF) (Figure 3B), suggesting that GDNF would trigger a limited activation of the negative feedback response while bFGF could cause more extensive inhibition of GDNF signaling via mTORC1.

Rapamycin treatment attenuates the SPC maintenance defect of *Plzf* $^{-/-}$ mice and increases SPCs in WT control mice

As inhibition of aberrant mTORC1 activity in *Plzf* $^{-/-}$ SPCs *in vitro* rescues their response to GDNF, we next asked whether mTORC1 inhibition *in vivo* could rescue the *Plzf* $^{-/-}$ SPC maintenance defect. We initiated Rapamycin treatment of *Plzf* $^{-/-}$ and wildtype control mice during early stages of postnatal development (10d postnatal) and continued daily treatment for 1 week, a regimen able to normalize the increased size of *Plzf* $^{-/-}$ SPCs (Figure 2G and 2H). Subsequent analysis of SPC compartment status demonstrated that Rapamycin increased both frequency and numbers of v^{neg} Thy-1^{low} c-Kit^{neg} cells in *Plzf* $^{-/-}$ testis, approaching those values found in vehicle-treated wildtype controls (Figures 6A and 6B). Rapamycin treatment of *Plzf* $^{-/-}$ mice increased both the frequency of αv^{neg} Thy-1^{low} cells and normalized the increased percentage of c-Kit^{pos} cells within this fraction compared to controls (Figure 6A). We conclude that aberrant mTORC1 activation can explain, at least in part, the SPC maintenance defect of *Plzf* $^{-/-}$ mice.

Intriguingly, Rapamycin also increased the frequency of SPCs in wildtype mice (Figure 6B), suggesting that mTORC1 inhibition stimulates SPC function in a wildtype setting. Indeed, in wildtype SPCs, Rapamycin enhanced Akt activation in response to GDNF (Figures 5A and 5B) and increased GDNF receptor expression (Figures 5D and 5E). Importantly, prolonged Rapamycin treatment of juvenile wildtype mice increased the number of cells with high levels of Plzf expression in the testis (Figures 6C, 6D and S6B) and enhanced GDNF

receptor expression (Figure S6C), suggesting that physiological mTORC1 activity controls size and function of the SPC pool *in vivo*.

DISCUSSION

Disruption of genes encoding for negative regulators of the PI3K/Akt/mTORC1 pathway (e.g. *Pten*, *Tsc1* and *Pml*) leads to loss of hematopoietic stem cell (HSC) quiescence and subsequent exhaustion. Rapamycin prevents HSC depletion, demonstrating that aberrant mTORC1 activation is responsible for stem cell loss (Chen et al., 2008; Gan et al., 2008; Ito et al., 2008; Yilmaz et al., 2006). The negative effects of mTORC1 on HSC maintenance have been attributed to its downstream targets in cell growth pathways (Chen et al., 2008) and aberrant mTORC1 activity elicits activation of tumor suppressive mechanisms that can contribute to stem cell failure (Figure S6D) (Alimonti et al., 2010; Lee et al., 2007a). By studying Plzf function in SPCs, we now implicate negative feedback responses from the mTORC1 pathway to receptors required to transduce niche-derived self-renewal signals in the loss of stem cell potential (Figure S6E).

HSC maintenance is also linked to niche signals acting through growth factor receptors (Arai et al., 2004; Yoshihara et al., 2007) thus it will be of interest to translate our findings into the hematopoietic system. In addition, the decreased sensitivity of *Plzf* $^{-/-}$ SPCs to GDNF mimics a reduced production of niche-signals; the decline in stem cell numbers in aging mouse testis is due partly to declining niche function and GDNF production (Ryu et al., 2006; Zhang et al., 2006), thus loss of *Plzf* expression can represent a premature aging phenotype. Our studies therefore provide further insight into the association between stem cell maintenance, mTORC1 and aging (Harrison et al., 2009; Rossi et al., 2008).

A correlation between aging and cancer is also apparent, likely due to the accumulation of multiple genetic hits over time for tumor development (Rossi et al., 2008). As stem cells are resident long-term in tissues and possess self-renewal capability (a feature of cancer stem cells), they are suggested to be the source of many cancers. However, loss of tumor suppressors (e.g. *Pten, Pml)* trigger stem cell depletion (Ito et al., 2009) and subsequent cancer development is associated with additional genetic hits (Guo et al., 2008). It is proposed that oncogenic hits in stem cells elicit activation of fail-safe mechanisms that have to be evaded to allow cancer development (Figure S6D). These fail-safe mechanisms prevent propagation of clones of cells derived from stem cells with oncogenic mutations and the consequent risk of acquiring additional mutations that lead to cancer. The mTORC1 hyper-activation that occurs in response to loss of tumor suppressors such as *Pten* and *Pml* is likely a trigger of this fail-safe mechanism as Rapamycin prevents stem cell exhaustion. Based on our new model, mTORC1 hyperactivation in response to oncogenic stimulation may also desensitize the cancer stem cell to niche-signals that are required for self-renewal; thus additional mutations supporting niche-independent self-renewal could allow cancer to develop (Figure S6E).

PLZF was first identified from involvement in acute promyelocytic leukemia (APL) (Chen et al., 1993). Thus, our model of Plzf-dependent SPC maintenance can have implications for leukemia pathogenesis. The ability of Plzf to inhibit mTORC1 through *Redd1* induction may be relevant in this context given that perturbed PLZF function and elevated mTORC1 activity are associated with leukemia development (He et al., 2000; Martelli et al., 2007; McConnell and Licht, 2007). In addition, REDD1 is involved in leukemic and myeloid cell differentiation (Gery et al., 2007; Nishioka et al., 2009), suggesting that this gene can be a relevant PLZF target in hematopoietic cells. *PML*, also identified from its involvement in chromosomal translocations with the *RARA* gene in APL, is required for HSC maintenance

and inhibits mTORC1 (Bernardi et al., 2006; Ito et al., 2008); thus illustrating a striking commonality of function between the *RARA* partners of APL.

We have identified a role for Plzf-mediated *Redd1* expression in inhibiting mTORC1 activation in SPCs. As mTORC1 activity has a significant impact on the response of SPCs to GDNF, the tight regulation of this pathway is critical. *Redd1* is also induced in response to cellular stress and in particular, by hypoxia (Brugarolas et al., 2004; Shoshani et al., 2002). Interestingly, the HSC niche has been suggested to exist in a hypoxic state (Kubota et al., 2008; Parmar et al., 2007) that, if the SPC niche had similar characteristics, could be responsible for *Redd1* induction. However, SPCs reside close to the vasculature *in vivo* (Yoshida et al., 2007) and *Redd1* expression is maintained when SPCs are cultured at atmospheric oxygen levels. Thus *Redd1* expression in this case depends on a Plzf tonic transcriptional activity and its induction is seemingly independent of hypoxia.

As mTORC1 inhibition enhances SPC function even in a wildtype setting, our data also have important therapeutic implications. It is tempting to speculate that Rapamycin could be used to enhance SPC activity in humans; potentially allowing improved SPC culture from testicular biopsies and generation of therapeutically relevant MASCs (Figure 6E). Furthermore, our findings provide a novel and straightforward explanation for the requirement of Plzf in germline maintenance.

EXPERIMENTAL PROCEDURES

Mouse maintenance and manipulation

Plzf $^{-/-}$ mice are previously described (Costoya et al., 2004). Mice were treated with Rapamycin (LC Laboratories) at a daily dose of 4mg/kg as described (Yilmaz et al., 2006). Transgenic mice expressing EGFP from the β-actin promoter were from The Jackson Laboratories. Testis transplant assays were performed as described (Seandel et al., 2007) with 50×10^3 unfractionated or 5×10^3 αv^{neg} Thy-1^{low} c-Kit^{neg} testis cells injected per recipient testis. Teratoma formation assays in NOD/SCID mice (Jackson Laboratories) were performed as described (Seandel et al., 2007).

Antibody generation

Monoclonal anti-PLZF antibody (clone 9E12) was raised in Armenian hamster against a pool of Keyhole Limpet Hemocyanin (KLH)-conjugated peptides at the Memorial Sloan-Kettering Cancer Center (MSKCC) antibody facility and reacts to a peptide within the hinge domain of mouse Plzf (RSKEGPGTPTRRSVITSARE). Antibody was directly conjugated to Alexa 488 or Alexa 647 for use in flow cytometry (MSKCC antibody facility).

Flow cytometry

Single cell suspensions were prepared from testis as described (Ogawa et al., 1997) and resuspended in Phosphate Buffered Saline (PBS) containing 2% fetal bovine serum (FBS) for subsequent staining and analysis (Filipponi et al., 2007). For a detailed description of flow cytometry methods and antibodies used refer to the Supplemental Experimental Procedures.

Cell culture and treatment

SPC fractions from testis were plated directly onto mitotically inactivated MEF feeder cells. Medium for SPC culture was as previously described (Seandel et al., 2007) supplemented with 10ng/ml IGF-I (Peprotech). MASCs and mouse ESCs (v6.5) were cultured in embryonic stem cell medium on MEF feeders (Seandel et al., 2007). Knockdown of Redd1 expression in SPCs by shRNA was performed using pLKO.1 lentiviral vectors (Open

Biosystems). A detailed description of methods is included in the Supplemental Experimental Procedures.

Immunofluorescence (IF), immunohistochemistry (IHC) and Western blot

Tissue and cultured cells were fixed in 4% paraformaldehyde prior to processing for IF and IHC as described (Costoya et al., 2004; Filipponi et al., 2007). For Western blot analysis, cells were lysed in RIPA buffer and processed as described (Costoya et al., 2008). Detailed methods are included in the Supplemental Experimental Procedures.

Chromatin immunoprecipitation (ChIP)

SPCs were trypsinized and resuspended in SPC medium then incubated in tissue culture dishes for 30 minutes to remove contaminating (adherent) MEFs. Preparation and immunoprecipitation of chromatin was performed using a SimpleChIP Enzymatic Chromatin IP kit (Cell Signaling Technology) according to manufacturers instructions and monoclonal anti-PLZF antibody (Calbiochem). DNA samples were analyzed by quantitative PCR using a QuantiTect SYBR Green PCR kit (Qiagen) and Light Cycler (Roche). Primer sequences for the Redd1 promoter are included in the Supplemental Experimental Procedures.

Luciferase assay

REDD1 promoter constructs are described (Lin et al., 2005) and were cloned from U2OS genomic DNA into the pGL3-enhancer vector (Promega). Luciferase assays were performed with 293HEK cells as described (Barna et al., 2002). Please refer to Supplemental Experimental Procedures for details.

Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

RNA was harvested using Trizol reagent (Invitrogen) then used for first strand cDNA synthesis and subsequent quantitative PCR analysis as described (Filipponi et al., 2007). For primer sequences and detailed methodology please see Supplemental Experimental Procedures.

Statistical analysis—Results from cell growth assays, testis transplants and flow cytometry analysis were assessed for statistical significance by a standard two-tailed t-test.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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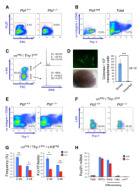

Figure 1. Isolation and analysis of *Plzf* $^{+/+}$ and *Plzf* $^{-/-}$ SPCs

(A) Detection of Plzf-expressing spermatogonia from juvenile testis (10–14d postnatal) by intracellular staining and flow cytometry using anti-Plzf antibody.

(B) Plzf expressing cells correlate to a discrete αv-integrin negative, Thy-1 low population by flow cytometry. CD45 marker excludes contaminating leukocytes.

(C) Left panel: Flow cytometric analysis of c-Kit within the Plzf-expressing αv-integrin negative, Thy-1 low population. Right panels: Cell cycle status of c-Kit negative and positive populations plus percentage of cells in G1/S/G2 cell cycle phases from a representative sample.

(D) Left panels: Seminiferous tubules repopulated with donor GFP-positive αv integrin negative, Thy-1 low, c-Kit negative cells in testis transplant assay. Fluorescent image (top) and brightfield image of same recipient testis (bottom) are shown. Right panel: Numbers of GFP positive colonies obtained from αv integrin negative, Thy-1 low, c-Kit negative (sorted) and unsorted testis cells in recipient testes 2 months post-transplant. Data is presented as mean number of colonies per 1×10^5 donor cells together with standard error of the mean (SEM). Fold enrichment of stem cell activity in sorted populations is indicated (***P<0.0001). Values are averaged from two independent experiments. 13 recipient testes were analyzed for sorted populations and 12 for unsorted.

(E) Representative αv-integrin/Thy-1 flow profiles of *Plzf* $^{+/+}$ and *Plzf* $^{-/-}$ littermate mouse testes at 2 weeks postnatal age. The αv-integrin negative, Thy-1 low gate, containing Plzf-positive cells in WT testis is indicated.

(F) Representative flow cytometry analysis of c-Kit expression within the αv-integrin negative, Thy-1 low testis cell fractions from (E).

(G) Quantification of frequency and absolute numbers of αv integrin negative, Thy-1 low, c-Kit negative testis cells from mice of the indicated postnatal ages and *Plzf* genotypes (n=3 per genotype and age group, **P<0.002, *P<0.02).

(H) Quantitative RT-PCR analysis of Pou5f1 mRNA expression in testis cell populations from juvenile *Plzf* $^{+/+}$ and *Plzf* $^{-/-}$ littermate mice: Total (unfractionated testis cells), SPCs (αv-integrin negative, Thy-1 low, c-Kit negative), early differentiating spermatogonia (αv-integrin negative, Thy-1 low, c-Kit positive), late differentiating spermatogonia (αv-integrin negative, Thy-1 negative, c-Kit positive) and somatic cells (αv-integrin positive). mRNA levels are normalized to those of β-actin and standard deviations from duplicate reactions are shown.

See also Figure S1.

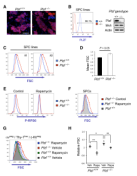

Figure 2. Increased mTORC1 pathway activity in *Plzf* $^{-/-}$ SPCs

(A) SPC lines established from culture of αv-integrin negative, Thy-1 low cells from juvenile *Plzf* $^{+/+}$ and *Plzf* $^{-/-}$ littermate mice immunostained for the germ cell marker Mvh and counterstained with DAPI to detect DNA. Scale bar is 100μm.

(B) Cultured SPCs of the indicated genotype analyzed for Plzf expression by intracellular staining and flow cytometry (left panel) and by Western blot (right panel). The germ cell marker Mvh plus actin are used as loading controls.

(C) Analysis of SPC size by flow cytometry. Forward-scatter (FSC) profiles of two independently derived sets of littermate *Plzf* $^{+/+}$ and *Plzf* $^{-/-}$ SPC lines (#1 and #2) are shown.

(D) Quantification of flow cytometry profiles from (C) showing averaged mean FSC with standard deviation and *P* value.

(E) Control (DMSO) and Rapamycin-treated *Plzf* $^{+/+}$ and *Plzf* $^{-/-}$ SPCs were fixed and permeabilized 48 hours post-treatment and levels of Phospho-RPS6 analyzed by flow cytometry.

(F) Normalization of increased *Plzf* $^{-/-}$ SPC size by mTORC1 inhibition. FSC profiles of *Plzf* $^{-/-}$ SPCs treated with DMSO (control) or Rapamycin for 48 hours compared to untreated *Plzf* $^{+/+}$ SPCs.

(G) Overlay of representative αv-integrin negative, Thy-1 low, c-Kit negative testis cell fraction FSC profiles from *Plzf* $^{+/+}$ and *Plzf* $^{-/-}$ littermate mice treated from 10 days postnatal age for a period of 1 week with Vehicle or Rapamycin.

(H) Quantification of relative FSC values of αv-integrin negative, Thy-1 low, c-Kit negative testis cells of mice treated as in (G) (n=4 per genotype and treatment group, **P*<0.05, ***P*<0.01).

See also Figure S2.

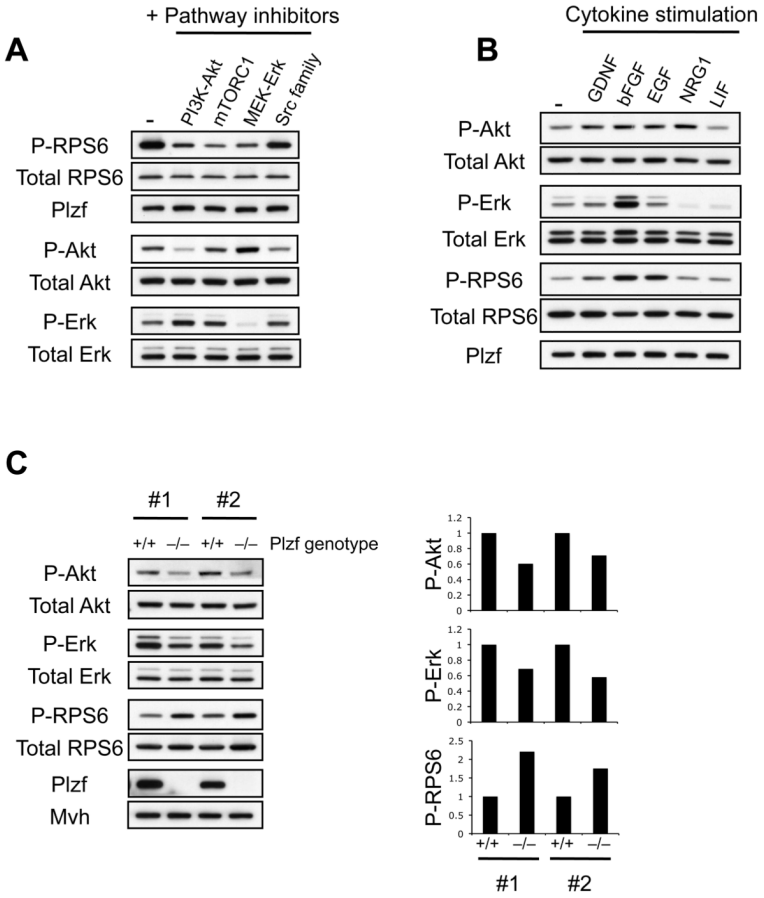

Figure 3. Upstream pathways and growth factors regulating mTORC1 in SPCs

(A) WT SPCs fed with complete SPC medium containing inhibitors to the indicated signaling pathways were harvested 5 hours after treatment and analyzed by Western blot for the indicated proteins and phospho (P)-proteins.

(B) WT SPCs were starved overnight in basal SPC medium lacking cytokine supplements. The indicated cytokines were then added and SPCs harvested 20 minutes later then analyzed by Western blot. Cytokines were used at the concentrations present in complete SPC medium. Neuregulin1 (NRG1) is not a standard supplement for SPC medium although SPCs are responsive to it (see text).

(C) Western blot analysis of two independently derived sets of littermate *Plzf* $^{+/+}$ and *Plzf* $^{-/-}$ SPC lines (#1 and #2) under steady state conditions is shown to the left. Quantification of the relative levels of phospho-Akt, Erk and RPS6 (corrected to the total levels of respective proteins) are shown in panels on the right.

See also Figure S3.

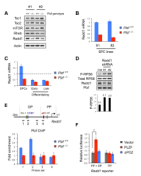

Figure 4. Plzf regulates expression of the mTORC1 pathway inhibitor Redd1

(A) Western blot analysis for components of the mTORC1 pathway in two independently derived sets of littermate *Plzf* $^{+/+}$ and *Plzf* $^{-/-}$ SPC lines (#1 and #2) under steady state conditions.

(B) Quantitative RT-PCR analysis of Redd1 mRNA expression in independently derived *Plzf* $^{+/+}$ and *Plzf* $^{-/-}$ SPC lines. mRNA levels are normalized to those of β-actin and standard deviations from duplicate reactions are shown.

(C) Quantitative RT-PCR analysis of Redd1 mRNA expression in spermatogonial fractions from juvenile *Plzf* $^{+/+}$ and *Plzf* $^{-/-}$ littermate mice: SPCs (αv-integrin negative, Thy-1 low, c-Kit negative), early differentiating spermatogonia (αv-integrin negative, Thy-1 low, c-Kit positive), late differentiating spermatogonia (αv-integrin negative, Thy-1 negative, c-Kit positive). mRNA levels are normalized to those of β-actin and standard deviations from duplicate reactions are shown. Dashed line indicates *Redd1* expression levels in unfractionated testis cells.

(D) WT SPCs infected with control shRNA (against GFP) or two independent shRNA constructs against Redd1 were analyzed by Western blot for the indicated proteins. Quantification of relative levels of phospho-RPS6 (corrected to total RPS6 levels) is shown under respective lanes. Cells were under steady-state culturing conditions (see also Figure S4).

(E) Top panel: *Redd1* promoter (from translation start site at +1 to 2kb upstream) with location of previously described transcription factor binding sites. The promoter is divided into proximal and distal regions (PP and DP respectively). Positions of ChIP amplicons are indicated. Bottom panel: ChIP assay. Quantitative PCR for *Redd1* promoter regions from WT SPC chromatin pulled down with Plzf antibody. Fold enrichment is shown relative to background of chromatin pulled down from *Plzf* $^{-/-}$ SPCs with the same antibody.

(F) Luciferase reporter assay with constructs containing the PP+DP Redd1 promoter regions or PP alone. 293HEK cells were transfected with the appropriate luciferase reporter together with PLZF constructs or vector control as indicated. Reporter activities were normalized to that of TK-Renilla and empty vector luciferase reporter controls (**P*<0.001).

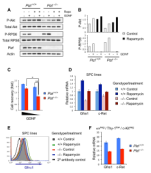

Figure 5. A negative feedback loop from mTORC1 to the GDNF receptor is activated in *Plzf* $^{-/-}$ SPCs

(A) *Plzf* $^{+/+}$ and *Plzf* $^{-/-}$ SPCs starved overnight in basal SPC medium supplemented with DMSO (vehicle control) or Rapamycin were stimulated with 10ng/ml GDNF for 20 minutes prior to harvesting for Western blot analysis.

(B) Quantification of relative levels of phospho-Akt and phospho-RPS6 (corrected to total levels of respective proteins) from (A).

(C) *Plzf* $^{+/+}$ and *Plzf* $^{-/-}$ SPCs were plated in medium containing decreasing concentrations of GDNF, harvested one week later and counted to determine fold cell recovery as an indicator of growth. The concentration of GDNF was varied from 4ng/ml (left bars) to 1ng/ml (right bars). Standard deviations from duplicate wells are indicated (**P<0.01, *P<0.02).

(D) Quantitative RT-PCR analysis of GDNF receptor components in *Plzf* $^{+/+}$ and *Plzf* $^{-/-}$ SPCs treated with DMSO (vehicle control) or Rapamycin for 48 hours. mRNA levels are normalized to those of β-actin and standard deviations from duplicate reactions are shown.

(E) Flow cytometry analysis of Gfr 1 levels from SPC lines treated as in (D).

(F) Quantitative RT-PCR analysis of GDNF receptor components in αv-integrin negative, Thy-1 low, c-Kit negative testis cell fractions pooled from 2 weeks-old *Plzf* $^{+/+}$ and *Plzf* $^{-/-}$ mice. mRNA levels are normalized to those of β-actin and standard deviations from duplicate reactions are shown.

See also Figure S5.

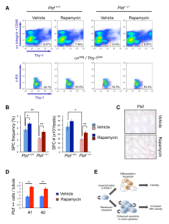

Figure 6. mTORC1 inhibition attenuates SPC maintenance defect of *Plzf* $^{-/-}$ mice

(A) Top panels: Representative αv-integrin/Thy-1 flow profiles of testis cells from *Plzf* $^{+/+}$ and *Plzf* $^{-/-}$ mice treated with Vehicle or Rapamycin from 10 days postnatal age for a period of 1 week and analyzed the day after completing treatment. Bottom panels: Flow cytometry analysis of c-Kit expression within the corresponding αv-integrin negative, Thy-1 low fractions.

(B) Quantification of frequency and absolute numbers of αv integrin negative, Thy-1 low, c-Kit negative testis cells from mice of the indicated genotypes treated with Vehicle or Rapamycin as in (A) (n≥5 per genotype and treatment group, ***P*<0.02, **P*<0.05).

(C) WT juvenile mice (2–3 weeks postnatal) were treated daily for 2 weeks with Rapamycin or Vehicle. Testes were harvested and subject to immunohistochemistry for Plzf. Representative images are 20×.

(D) Quantification of cells showing strong positivity for Plzf in seminiferous tubules of testis from (C), representing SPCs. Results from duplicate experiments (#1, #2) are shown together with SEM (***P*<0.01).

(E) Illustration of the effects of aberrant mTORC1 activity or inhibition of physiological mTORC1 activation with Rapamycin on SPC numbers and function.

See also Figure S6.