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Quantitative analysis of male germline stem cell differentiation reveals a role for the p53-mTORC1 pathway in spermatogonial maintenance

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Abbreviations: mTORC1, mammalian target of rapamycin complex 1; FACS, flow cytometry; GDNF, glial cell line-derived neurotrophic; Plzf, promyelocytic leukemia zinc finger; hHLH, Basic helix-loop-helix; Stra8, stimulated by the retinoic acid gene 8; p-RPS6, phosphorylated ribosomal protein S6.

p53 protects cells from DNA damage by inducing cell-cycle arrest upon encountering genomic stress. Among other pathways, p53 elicits such an effect by inhibiting mammalian target of rapamycin complex 1 (mTORC1), the master regulator of cell proliferation and growth. Although recent studies have indicated roles for both p53 and mTORC1 in stem cell maintenance, it remains unclear whether the p53-mTORC1 pathway is conserved to mediate this process under normal physiological conditions. Spermatogenesis is a classic stem cell-dependent process in which undifferentiated spermatogonia undergo self-renewal and differentiation to maintain the lifelong production of spermatozoa. To better understand this process, we have developed a novel flow cytometry (FACS)-based approach that isolates spermatogonia at consecutive differentiation stages. By using this as a tool, we show that genetic loss of p53 augments mTORC1 activity during early spermatogonial differentiation. Functionally, loss of p53 drives spermatogonia out of the undifferentiated state and causes a consistent expansion of early differentiating spermatogonia until the stage of preleptotene (premeiotic) spermatocyte. The frequency of early meiotic spermatocytes is, however, dramatically decreased. Thus, these data suggest that p53-mTORC1 pathway plays a critical role in maintaining the homeostasis of early spermatogonial differentiation. Moreover, our FACS approach could be a valuable tool in understanding spermatogonial differentiation.

Introduction

Stem cells undergo both self-renewal and differentiation to maintain a lifelong production of mature cells for tissue regeneration. Imbalance between these processes results in the loss of tissue homeostasis that has been implicated in tissue degeneration, aging, and cancer. mTORC1 is a signaling complex that positively mediates cell growth, primarily through phosphorylation of components of the translation machinery, thus promoting protein translation.[1,2] Previous studies suggest a critical role for mTORC1 signaling in determining the stem cell self-renewal and differentiation decision.[3-5] Aberrant activation of mTORC1 leads to enhanced stem cell activity, activating the tumor suppressive mechanism in somatic stem cells and leading to cellular senescence or apoptosis.[2,6] In germline stem cells, genetic deletion of an inhibitor of mTORC1 activity, e.g., Plzf or Tsc2, can lead to spermatogonial stem cell differentiation at the expense of self-renewal, and thereby results in a premature exhaustion of the spermatogonia pool and a testicular aging-like phenotype.[7-10] Therefore, it is critical to understand how mTORC1 activity is precisely regulated, particularly under normal physiological conditions.

p53 has been extensively studied as the guardian of the genome for its tumor suppressor function.[11] Now, it is well characterized that, under genotoxic stress, elevated level of p53 results in cell cycle arrest to allow time for DNA repair. If unsuccessful, p53 activation initiates cell death programming to eliminate cells with damaged or mutated DNA.[12] Recently, mounting evidence suggests that p53 also controls the proliferation, self-renewal, and differentiation of embryonic and adult stem cells to maintain tissue or organ homeostasis.[13] This raises the question of whether the molecular pathways that p53 employs under genotoxic stress might also function under normal physiological conditions. For example, upon DNA damage, p53 negatively regulates mTORC1 activity to limit metabolic support, prevent cell division, and cause cellular quiescence, thereby exerting its function

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as a tumor suppressor.[14–16] Interestingly, under normal conditions, mTORC1 activity can be increased in tissue from p53-deficient mice such as the heart.[17] However, it remains unclear whether the p53-mTORC1 pathway is conserved in regulating stem cell activity during tissue homeostasis.

Spermatogenesis is a classic stem cell dependent process by which the undifferentiated spermatogonia undergo both mitotic and meiotic divisions to produce spermatozoa.[18] Morphological studies have classified spermatogonia into several subtypes.[19] Specifically, spermatogonia in the single-cell state are known as A single (A_{single}) spermatogonia, some of which are considered to be spermatogonial stem cells (SSCs). SSCs are traditionally regarded as the foundation of the spermatogonial differentiation hierarchy and give rise to paired A (A_{paired}) spermatogonia, which are connected by an intercellular bridge. A_{paired} cells then divide into chains of 4 – 32 aligned ($A_{aligned}$) spermatogonia. These three types of cells all maintain stem cell properties and are considered undifferentiated spermatogonia. After a differentiation step in which $A_{aligned}$ spermatogonia are committed irreversibly to gamete production, the cells are classified as differentiating type A spermatogonia. This is followed by a series of divisions that culminate in B spermatogonia, which then divide to yield premeiotoic (preleptotene) spermatocytes that transition from the mitotic cell cycle to the meiotic cell cycle. While the transition from undifferentiated $A_{aligned}$ spermatogonia to differentiating type A spermatogonia occurs every 8.6 d in mice, it takes an A_{single} spermatogonia 35 d to fully differentiate into spermatozoa. In addition, recent studies have also characterized genes whose expressions are associated with specific stages during spermatogonial differentiation. For example, Gfra1, a component of the glial cell line-derived neurotrophic (GDNF) factor receptor, is predominately expressed in the A_{single} spermatogonia.[20] Functionally, GDNF stimulates the self-renewal of undifferentiated spermatogonia.[21] The promyelocytic leukemia zinc finger (Plzf) is expressed in most of undifferentiated type A spermatogonia.[7,8] Importantly, Plzf is essential for spermatogonia maintenance, as loss of Plzf promotes differentiation at the expense of self-renewal and results in the exhaustion of the spermatogonia. Plzf might mediate this effect by suppressing mTORC1 activity via transcriptionally activating *Redd1*.[9] Basic helix-loop-helix (hHLH) protein Sohlh1 then appears in $A_{aligned}$ spermatogonia and is essential for spermatogonial development.[22,23] Preleptotene spermatocytes entering meiosis are characterized by their intense expression of stimulated by the retinoic acid gene 8 (Stra8), a protein indispensible for premeiotic DNA synthesis.[24-26] Despite these progresses, the process of early spermatogonial differentiation is still not well understood.

Here, we report the development of a novel flow cytometry (FACS)-based approach that allows for separation and isolation of spermatogonia at consecutive differentiation stages. By using this as a tool, we show that loss of p53 amplifies mTORC1 signaling and promotes spermatogonial differentiation, suggesting a role for the p53-mTORC1 pathway in stem cell maintenance under tissue homeostasis.

Results

Quantitative analysis of spermatogonial differentiation

Meiosis can be viewed as a differentiation process unique to the germ line. Transcriptional activation of *Stra8* that triggers meiotic initiation is perhaps the earliest event known to date during germline stem cell differentiation of both sexes.[27] Therefore, we considered the possibility that *Stra8* promoter activity might be useful in monitoring early germline stem cell differentiation. Toward this end, we have reported the successful development of a transgenic mouse line in which the 1.4-kb *Stra8* promoter (−1400 to +11) drives the expression of the reporter gene, *green fluorescence protein* (GFP) (referred hereafter as the *pStra8-GFP* transgenic mouse line).[28] The *pStra8-GFP* transgenic mice exhibit gonad-specific GFP expression in post-pubertal testes in males and sex-specific GFP expression in female embryonic ovaries during the developmental window of meiotic initiation. We combined the *pStra8-GFP* reporter with an established strategy of 2 additional cell surface markers to characterize spermatogonial differentiation:[29] α6-integrin, which is a marker of undifferentiated spermatogonia with stem cell capacity, and c-Kit, which is turned on upon spermatogonial differentiation. These two cell surface markers allowed us to first identify the c-Kit^{negative} α6-integrin^{high}, undifferentiated spermatogonia compartment and the c-Kit^{positive}α6-integrin^{low}, differentiating spermatogonia compartment (**Fig. 1A**). EpCAM was not used as a pan-spermatogonia marker as previously reported,[29] as recent studies as well as our own investigation suggest that EpCAM level is low in undifferentiated spermatogonia but increases along with early spermatogonial differentiation (data not shown).[30]

Within the c-Kit^{negative} α6-integrin^{high} compartment that has been shown to be composed mostly of undifferentiated spermatogonia, we were able to further identify a GFP-negative population (population 1 or P1) and a GFP-positive population (population 2 or P2) (**Fig. 1A**). Markers for undifferentiated spermatogonia (namely, *Gfra1, Nanos2, Id4, Ngn3*, and *Plzf*) were highly enriched in both P1 and P2 populations when compared with cells from the c-Kit^{positive}α6-integrin^{low}, differentiating spermatogonia compartment (**Fig. 1L-P**).[7,8,31-34] Since the *Stra8* promoter activation may indicate commitment to meiotic initiation, we considered cells in the P1 population to be further enriched for the most primitive undifferentiated spermatogonia due to their complete lack of *Stra8* promoter activity. We considered cells in the P2 population to represent the earliest differentiating spermatogonia within this undifferentiated spermatogonia compartment. This is because, while their c-Kit levels still remained negative, *Stra8* promoter activity was already initiated (**Fig. 1A**). Expression of Gfra1 is present in earlier stages of spermatogonia development than that of Plzf. Indeed, we found cells in P1 expressed higher levels of *Gfra1*, while cells in P2 population expressed higher levels of *Plzf* mRNA (**Fig. 1L and P**). In addition, 80.3% (49 out of 61 cells examined) of cells from P1 expressed Gfra1 and this number dropped to 36.1% (26 out of 72 cells examined) in P2, while only 45.2% (42 out of 93) of cells from P1 expressed Plzf and this number increased to 90.6% (75 out of 83) in P2 (**Fig. 1C and D**). Moreover, to locate cells

in P1 and P2 in juvenile testicular sections, we identified cells that expressed Gfra1 but not GFP (cells from P1) or expressed Plzf together with low levels of GFP (cells from P2) (**Fig. G and H**). Taken together, these molecular features suggest that, by using the *Stra8* promoter-driven GFP as a reporter, we can further separate the previously reported undifferentiated spermatogonia population into the more primitive P1 population and the P2 population, which is on the verge of initial differentiation.

Within the c-Kit^{positive} α6-integrin^{low} differentiating spermatogonia compartment, we identified 4 separable populations based on their distinct levels of GFP expression (**Fig. 1A**). We refer to them hereafter as population 3 to population 6 (P3 to P6) based on their progressively decreasing levels of GFP expression with cells in P3 exhibiting the highest GFP and cells in P6 the lowest (**Fig. 1A and B**). We considered P3 to P6 populations to be differentiating spermatogonia that may include early spermatocytes at consecutive stages toward maturation. Interestingly, α6-integrin levels from P3 to P6 decrease concomitantly together with those of GFP expression (**Fig. 1A**). The differential GFP intensities are not due to various copies of transgenes, in that we "knocked" our *pStra8-GFP* transgenic cassette into the *Hprt* neutral locus on the X chromosome. Therefore, our male *pStra8-GFP* mice carry 1 copy of *pStra8-GFP* reporter per cell.[28] Importantly, we identified P5 as the preleptotene spermatocytes, which were characterized by their intense Stra8 staining (80 out of 89 cells examined) and their doublet morphology, consistent with previous reports for preleptotene spermatocytes (**Fig. 1F**).[35] Thus, cells in P3 and P4 populations are differentiating spermatogonia, an identification supported by their expression of Sohlh1 in cells from P3 populations (83 out of 93 cells examined) (**Fig. 1E**). Cells in P6 population are probably early meiotic spermatocytes. Consistently, cells from P3 to P6 population showed progressive features of meiosis initiation and progression by the expression of *Stra8, Spo11, Dmc1*, and *Sycp3* mRNAs (**Fig. 1R–U**).[24,36-38] We identified these cells in testicular sections as cells that exhibited decreasing levels of GFP expressions but showed Sohlh1 (P3), Stra8 (P5), and Sycp3 (P6) expression (**Fig. 1J and K**).

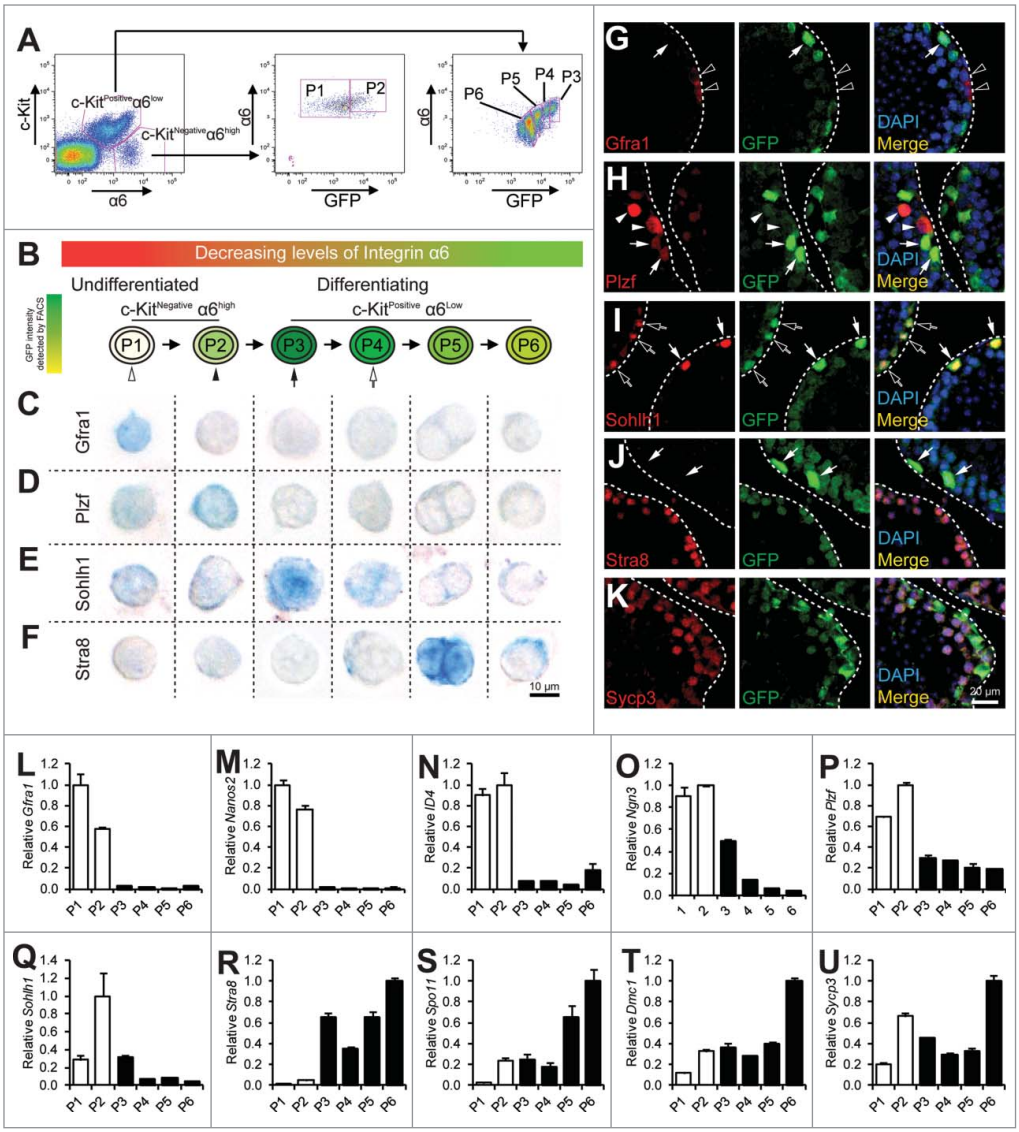

Figure 1. Analysis of spermatogonial differentiation by flow cytometry. (**A**) representative flow profile of separating spermatogonia differentiation into P1 to P6 by using a combination of markers for undifferentiated SSC (α6-integin), differentiating spermatogonia (c-Kit), and GFP driven by the Stra8 promoter. Note the concomitant decrease of α6-integrin level with GFP in the c-Kit^{Pos} α6-integrin^{low} population. (**B**) schematic diagram showing progression of spermatogonial development from P1 to P6. **C**–**F**, immunohistochemistry staining of cells from P1 to P6 populations isolated from 4-weeks old juvenile *pStra8-GFP* mice. Expression of Gfra1 (**C**), Plzf (**D**), Sohlh1 (**E**), and Stra8 (**F**) was detected by alkaline phosphatase (AP). Cells shown are magnified views of a single cell. **G**–**K**, dual-immunofluorescence staining of GFP (green) with Gfra1 (**G**), Plzf (**H**), Sohlh1 (**I**), Stra8 (**J**), and Sycp3 (**K**) (red) in 4-weeks old juvenile mouse testes. **L**–**U**, qPCR analysis of relative mRNA expression of the indicated genes in P1 to P6. Twenty,000 cells were isolated from 4-weeks old juvenile *pStra8-GFP* mice by FACS as shown in panel **A**. mRNA levels are normalized to those of β-*actin*. Data are representative of 3 independent set of experiment. Graphs represent mean ± standard deviations from duplicate reactions.

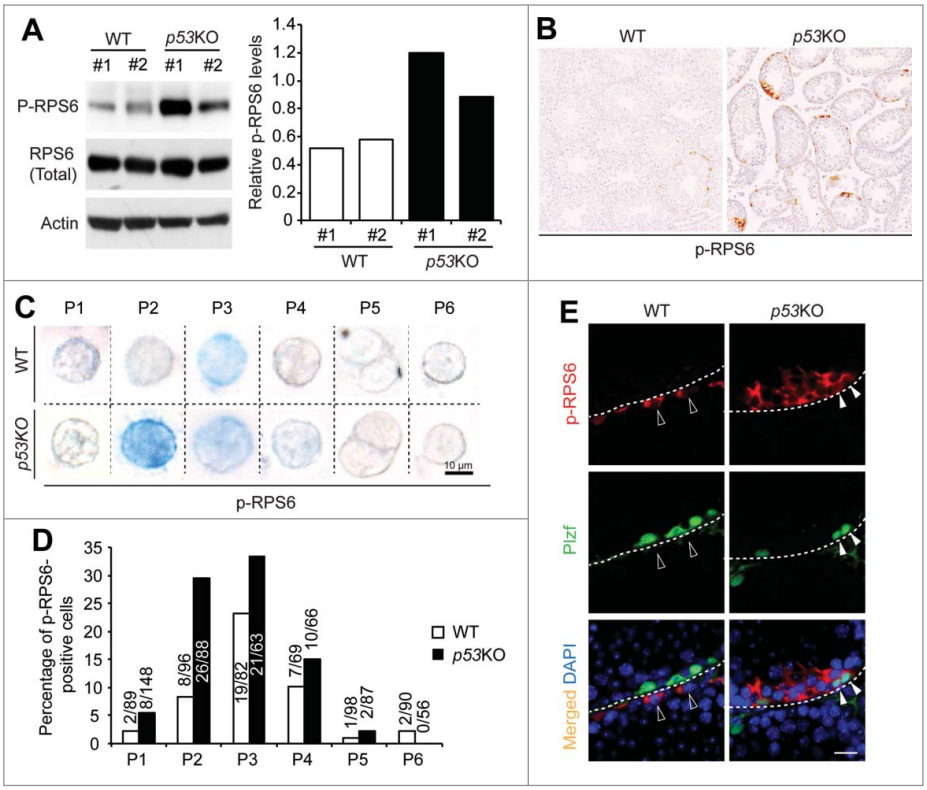

Figure 2. p53 loss amplifies mTORC1 activity in differentiating spermatogonia. (**A**) western blotting analysis of p-RPS6 from 2 mice per genotype (#1 and #2) (left). Quantification of the relative levels of p-RPS6 against total RPS6 (right). (**B**) immunohistochemistry staining of p-RPS6 in 4-weeks old juvenile WT and *p53*KO testis. Images shown are representative from 3 mice per genotype examined. (**C**) immunohistochemistry staining of p-RPS6 detected by alkaline phosphatase (AP) in cells from P1 to P6 populations isolated from 4-weeks old juvenile WT and *p53*KO *pStra8-GFP* mice. Cells shown are magnified views of a single cell. Number of positive cells detected and total number of cells examined are indicated. (**D**) comparison of the percentages of p-RPS6-positive cells in P1 to P6 populations. Graphs represent mean values ± SEM. n = 3 mice per genotype, $^{*}P < 0.05$. (**E**) dual-immunofluorescence staining of p-RPS6 (red) and Plzf (green) in 4-weeks old juvenile WT and *p53*KO testis.

p53 deletion augments mTORC1 signaling during spermatogonial differentiation

p53 is expressed at low levels under normal physiological conditions and sometimes its activation can only be reflected by the activation of its promoter.[39,40] To test whether p53 is an upstream suppressor of mTORC1 activity during spermatogonial differentiation, we compared the levels of the phosphorylated ribosomal protein S6 (p-RPS6) in wild-type (WT) and *p53*KO testes. We chose p-RPS6, because p-RPS6 is a downstream target of mTORC1 through ribosomal protein S6 Kinase 1 and has been previously used to monitor mTORC1 activity during spermatogonial differentiation.[9,41,42] In western blotting analysis of whole testicular lysate, we found that p-RPS6 levels were increased in *p53*KO testes when compared to WT testes (**Fig. 2A**). Consistently, sections from *p53*KO testes exhibited a higher level of p-RPS6 staining than those from WT testes (**Fig. 2B**).

We next introduced the *pStra8-GFP* reporter allele into *p53*KO mice. WT littermates with *pStra8-GFP* reporter were used as controls. Since p-RPS6 has also been reported to be present not only in germ cells but also in the somatic Sertoli cells, we confirmed that p53 deletion-induced upregulation of mTORC1 activity in germ cells by isolating cells from P1 to P6 from WT and *p53*KO testes (**Fig. 1A**). Consistent with recent previous reports that mTORC1 activation defines a subset of spermatogonia committed to differentiation,[10] p-RPS6 was mostly present during early spermatogonial differentiation at P3 stages in WT testes. However, in *p53*KO testes, there was a robust increase in the percentage of cells that expressed p-RPS6 at P2 stage of spermatogonial differentiation (**Fig. 2C and D**). Cells from P2 expressed high levels of Plzf (**Fig. 1D**), which has been reported to suppress mTORC1 activity to maintain self-renewal of spermatogonia.[9] Consistently, we found that, in WT testes, Plzf-expressing cells usually did not show p-RPS6 expression (**Fig. 2E**). However, in *p53*KO testes, we identified cells that showed both strong Plzf and p-RPS6 expressions (**Fig. 2E**). Taken together, these data indicate that p53 suppresses mTORC1 activity during normal spermatogonia development and suggest that this pathway is independent of Plzf.

p53 deletion results in the loss of spermatogonia maintenance and meiotic differentiation

To examine the functional consequence of p53 loss on early spermatogonia differentiation, FACS analysis showed that, while p53 deletion had no appreciable effect on the frequency of c-Kit^{negative} α6-integrin^{high}, undifferentiated spermatogonia compartment (**Fig. 3A**), the c-Kit^{positive}α6-integrin^{low}, differentiating spermatogonia compartment was significantly decreased when compared with WT controls (**Fig. 3B**). This initial analysis seems to be opposite to our speculation that elevated mTORC1 activity would trigger spermatogonial differentiation and the previous reports that *p53*KO testes contain significantly higher numbers of differentiating spermatogonia.[39]

We next examined the frequencies of P1 to P6 within these 2 compartments. We found that p53 deletion caused a 10.8% decrease in the percentage of P1 population but consistent increases in the percentages from P2 to P5 populations (27.5% in P2, 62.7% in P3, 52.9% in P4 and 17.2% in P5) (**Fig. 3C–E**). Thus, these data suggest that p53 deletion results in a phenotype resembling loss of the stem cell maintenance by driving spermatogonia out of the undifferentiated stage (P1) for differentiation (P2 to P5). The percentage of the P6 population,

however, was dramatically decreased by 27.7% (**Fig. 3C and E**), which is consistent with the reported role for p53 in promoting meiotic recombination.[40]

We further confirmed these FACS-based observations through histological analysis. Comparing sections from the WT and *p53*KO testes, we observed a 39.4% increase in the numbers of cells that expressed intense levels of Plzf per tubule in the *p53*KO (**Fig. 4A and B**). Additionally, we found there was a 55.7% increase in the percentage of Stra8-expressing, stage VII seminiferous tubules (**Fig. 4C and D**). These observations are consistent with the increase in the P2 to P5 populations as shown through FACS. Furthermore, there was a 40.2% decrease in tubules containing Sycp3-expressing, meiotic spermatocytes (**Fig. 4E and F**), which matches the observed decrease in the P6 population shown through FACS (**Fig. 3C and E**).

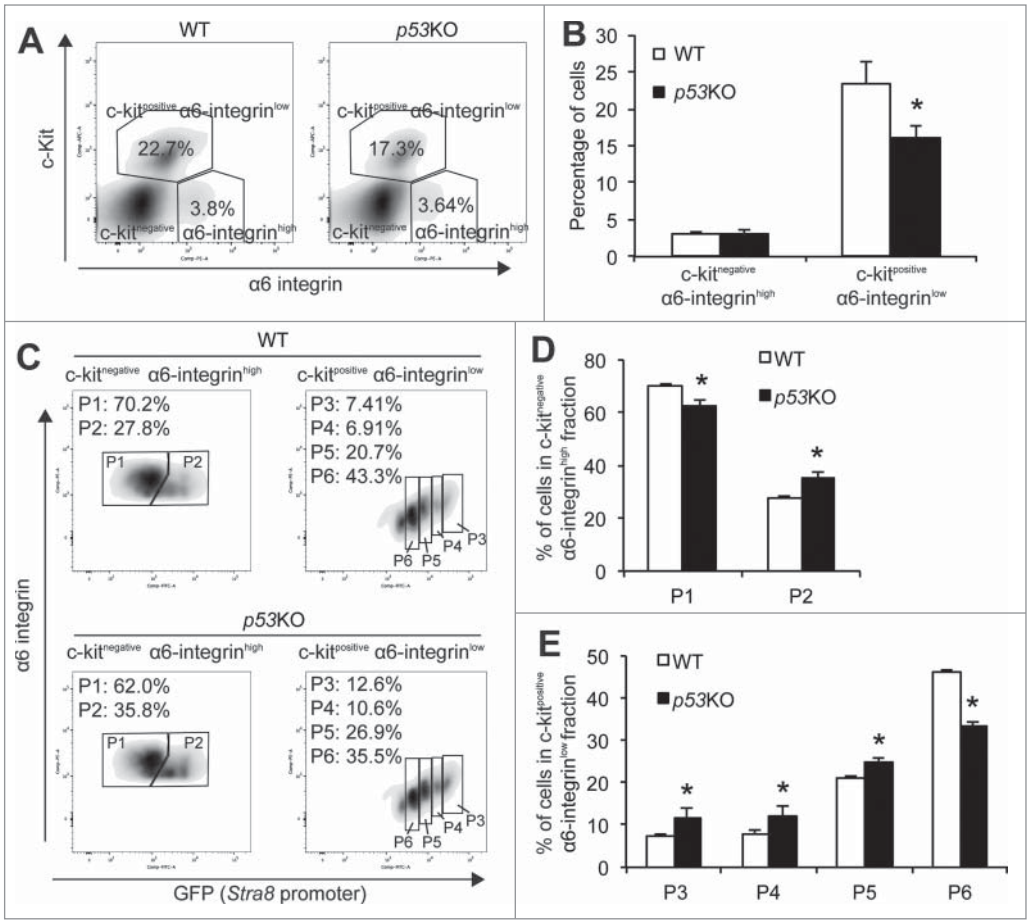

Figure 3. Quantitative analysis of the impact of p53 loss on spermatogonial differentiation. (**A**) representative flow profiles of the c-Kit^{negative} α6-integrin^{high} undifferentiated spermatogonia compartment and the c-Kit^{positive} α6-integrin^{low} differentiating spermatogonia compartment from 4-weeks old WT (left) and *p53*KO (right) testes. (**B**) quantification of frequency of c-Kit^{negative} α6-integrin^{high} undifferentiated spermatogonia compartment and the c-Kit^{positive} α6-integrin^{low} differentiating spermatogonia compartment in testes of WT and *p53*KO mice at 4-weeks of age. Graphs represent mean values ± SEM. n = 4 mice per genotype, $^*P < 0.05$. (**C**) representative flow profiles of P1 and P2 in the c-Kit-negative α6-integrin-high spermatogonia compartment (left panels) and P3 to P6 in the c-Kit-positive α6-integrin-low (right panels) from WT (upper panels) and *p53*KO (lower panels) testes. (**D** and **E**) quantification of frequency of P1 to P6 populations in testes of wild-type and *p53*KO mice at 4-weeks of age. Graphs represent mean values ± SEM. n = 4 mice per genotype, $^*P < 0.05$.

Discussions

Similar to its role as the guardian of the genome in somatic cells, p53 function in germ cells of the testes primarily involves removing cells with DNA damage due to genotoxic stress through apoptosis.[39] In addition, by using a traditional histological approach, a previous study shows that *p53*KO testes contain significantly higher numbers of differentiating spermatogonia.[39] Here, by using a different strain of *p53*KO mice paired with a quantitative approach to analyze spermatogonial differentiation from undifferentiated spermatogonia to early meiotic spermatocytes, we further explored the impact of p53 deletion on the homeostasis of early spermatogonial differentiation. Specifically, we found that p53 deletion amplifies the differentiating spermatogonia compartment by depleting the undifferentiated spermatogonia compartment (**Fig. 4G**). This phenotype is associated with enhanced mTORC1 activity, which has been shown to promote spermatogonial differentiation at the expense of self-renewal.[9] In addition, since mTORC1 activity is a primary determinant of cell size, enhanced mTORC1 may also contribute to the formation of the giant cells frequently observed in *p53KO* mice or mice with reduced levels of p53 protein.[39,43] Taken together, these data suggest a critical role for the p53-mTORC1 pathway in regulating spermatogonial differentiation.

Age-related upregulation of p53 expression is frequently linked to cellular senescence and loss of tissue or organ function.[44,45] Here, we also observed that *p53* levels were significantly elevated in aged testes (**Fig. S1**). Since p53 is a negative regulator of mTORC1 activity, this raises the possibility that higher levels of p53 in aged testes may cause insufficient mTORC1 activity required for normal spermatogonial differentiation. In fact, lower levels of mTORC1 activity has been observed in germ cells from aged testes when compared with those from young testes.[46]

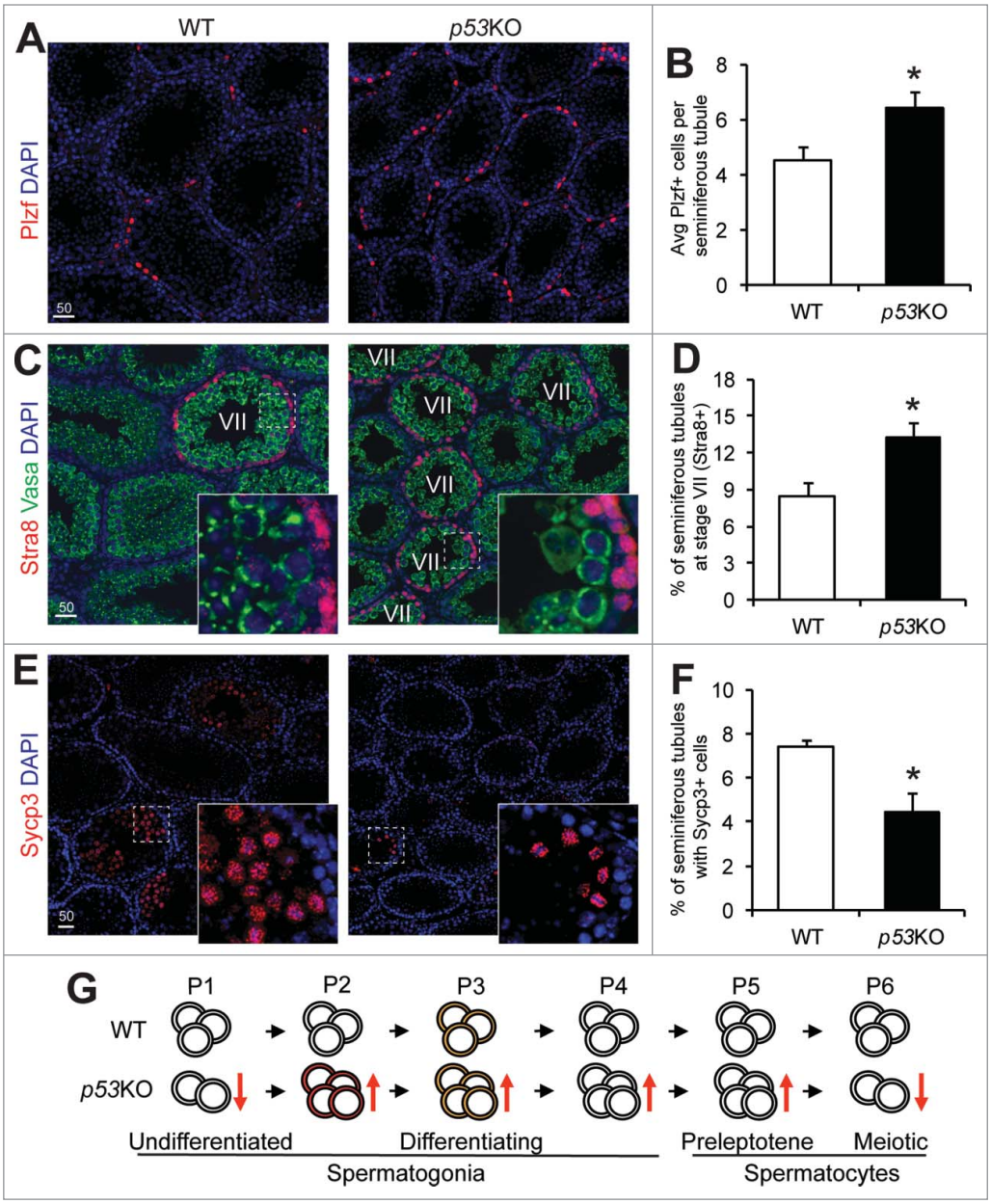

Figure 4. p53 loss expands the spermatogonia pool but causes a loss of meiotic spermatocytes. **A**) immunofluorescence staining of Plzf in 4-weeks old juvenile WT and *p53*KO testis. (**B**) quantification of cells showing strong positivity for Plzf in seminiferous tubules of testis from (**A**) representing early differentiating spermatogonia. Graphs represent mean values ± SEM. n = 4 mice per genotype, $^{*}P < 0.05$. (**C**) dual-immunofluorescence staining of Stra8 (red) and Vasa (green) in 4-weeks old juvenile WT and *p53*KO testis. (**D**) quantification of percentage of stage VII seminiferous tubules in cross sections as shown in (**C**). Graphs represent mean values ± SEM. n = 4 mice per genotype, $^{*}P < 0.05$. (**E**) immunofluorescence staining of Sycp3 in 4-weeks old juvenile WT and *p53*KO testis. (**F**) quantification of the percentage of seminiferous tubules containing meiotic spermatocytes showing strong positivity for Sycp3 from panel E. Graphs represent mean values ± SEM. n = 4 mice per genotype, $^{*}P < 0.05$. (**G**) schematic diagram showing the effect of p53 deletion on spermatogonial differentiation. Orange and red colors in cells at P2 and P3 populations indicate mTORC1 activity.

There has been a continuous interest to enrich undifferentiated spermatogonia by using genetic or cell surface markers. For example, the αv-integrin^{negative} Thy-1^{low} c-Kit^{negative} fraction is reportedly enriched for spermatogonia with stem cell capacity.[9] Thy-1 and Gfra1 have been used in previous studies to isolate undifferentiated spermatogonia.[47] Recently, it is reported that cells transcriptionally active for *Id4* defines a subset of the male germline stem cell population.[48] In our current study, by combining the existing strategy of α6-integrin and c-Kit with the *pStra8-GFP* reporter, we were able to not only isolate an undifferentiated spermatogonia population (P1) but also a series of their progeny at consecutive differentiating stages toward maturation (P2 to P6). We consider cells in the P1 population to be enriched for more primitive undifferentiated spermatogonia than cells in the P2 population. This conclusion is supported by this population's complete lack of the *Stra8* promoter activity, whose activation is among the earliest events known to date during meiotic initiation in germ cells of both sexes.[24,25]

This FACS-based approach may have many applications in the study of spermatogonial development or even provide insight into the general multi-stepped process during early stem cell differentiation. For example, transplantation of cells from P1 to P6 populations into chemotherapy-depleted recipient testes to test their repopulation capacity may be valuable in characterizing the progressive loss of stemness during spermatogonial differentiation as well as the molecular mechanisms responsible for this event. Moreover, since dedifferentiation has been reported to occur during early spermatogonial differentiation, transplantations of cells from P1 to P6 populations would be critical to elucidate the homeostasis. Since the *Stra8* promoter activity is indicative of early spermatogonial differentiation, comparative gene expression analysis between P1 and P2 populations that share similar α6-integrin^{high} c-Kit^{negative} profiles but show distinct *Stra8* promoter activity may help reveal

genes uniquely expressed in the most primitive undifferentiated spermatogonia or spermatogonial stem cells (P1). So far, most genes that have been reported to mark undifferentiated spermatogonia, e.g., *Ngn3, Id4, Nanos2*, are present in both P1 and P2 populations. So identification of genes uniquely expressed by cells in P1 population might help identify the most primitive undifferentiated spermatogonia that still remain elusive. On the other hand, genes uniquely enriched in P2 population may relate to differentiation commitment and therefore provide insight into the intrinsic cyclicity of spermatogenesis. In our initial analysis, we already identified certain discrete features for P1 and P2 populations. For example, P1 is highly enriched for *p21* mRNA expression (data not shown), consistent with the role for p21 in keeping stem cells at a quiescence state, while cells in P2 are highly enriched for *Sohlh1* mRNA expression (**Fig. 1Q**), a gene required for spermatogonial development (although Sohlh1 protein appears at P3 stage, highlighting a critical role for transcription/translation control during spermatogonial differentiation).[22,49,50] In addition to identifying genes for the most primitive undifferentiated spermatogonia, comparative gene expression analysis between P4, the latest stage of differentiating spermatogonia, and P5, preleptotene premeiotic spermatocytes, will be helpful to identify genes involved in the process of mitosis to meiosis transition, the defining feature of germ cells. Therefore, an integrated transcriptome, proteome, and methylome analysis of cells from P1 to P6 populations may provide a comprehensive molecular landscape of spermatogonial differentiation.

Materials and Methods

Animals

*p53*KO were obtained from Taconic (TSG-p53).[51] *pStra8-GFP* mice are generated as previously described.[28] All procedures and care of animals were carried out according to the Massachusetts General Hospital Institutional Animal Care and Use Committee (IACUC).

Isolation of mouse testicular germ cells and FACS analysis

Dissociated testicular cells from wild-type or *p53*KO;*pStra8-GFP* mouse testes were generated by sequential digestion of dissected and minced seminiferous tubules with 1 mg/ml Type IV collagenase (Worthington) in the presence of 20 μg/ml DNAse I (Sigma) and 0.05% Trypsin (Gibco). The testes were dissected out and the capsules removed. Prior to mincing, tubules were washed twice with Hank's balanced salt solution (HBSS) to remove interstitial cells. After mincing, cells were resuspended in Type IV collagenase and DNase I solution and shaken vigorously at 37°C for 30 minutes. Cells were then passed through 20 G needles and washed with HBSS containing 0.2% FBS. An equivalent amount of Trypsin (to previous collagenase solution) was used to resuspend cells which were again shaken vigorously at 37°C for 30 minutes. Trypsin was neutralized by 10% fetal bovine serum (FBS) in HBSS and the cells were passed through a 70 μm strainer to remove clumps. Cells were collected by centrifugation and then resuspended in Cell Staining Buffer (Biolegend) for counting and subsequent staining. After blocking for 10 minutes with a 1:100 dilution of TruStain fcX^{TM} anti-mouse CD16/32 (BioLegend), the following antibodies to cell surface markers were used: 1:40 dilution of APC-conjugated c-Kit antibody (CD117, Bioscience Catalog #553356) and 1:10 dilution of PE-conjugated α6-integrin (CD49f, Bioscience Catalog #555736). Cells were stained for 30 minutes, washed twice, and then resuspended with HBSS containing 0.2% FBS for sorting.

Immunocytochemistry and immunofluorescence

For frozen sections, testes were fixed in 4% paraformaldehyde for one hour at 4°C, dehydrated in 15% sucrose, and embedded in 7.5% gelatin. For paraffin sections, testes were fixed in 4% paraformaldehyde overnight at 4°C, embedded in paraffin. Tissues were then sectioned at 10 μm (frozen) or 6 μm (paraffin) for analysis. Antibodies used include: Gfra1 (AF560, R&D systems), Plzf (clone D-9, Santa Cruz Biotechnology Catalog# 28319), Sohlh1 (abcam), Stra8 (ab49602, Abcam), and Sycp3 (ab15093, Abcam). For immunofluorescence, detection was performed using Alexa fluor 488-conjugated donkey anti-mouse and Alexa fluor 546-conjugated donkey anti-rabbit secondary antibodies. Frozen sections stored in −80°C were dried at room temperature and then washed 3 times for 10 min each in PBS warmed to 37°C. Paraffin sections were hydrated 3 times in Xylene, 2 times in 100% Ethanol, 95% Ethanol, 80% Ethanol, 70% Ethanol, and H_2O, all for 5 minutes each. Antigen retrieval was performed by boiling slides for 15 minutes in sodium citrate heated to 100°C. Slides were cooled to room temperature and then washed 3 times for 10 min each with TBST/PBST. Slides were blocked at room temperature for 1 hour with DAKO protein block and then incubated overnight at 4°C with primary antibodies in blocking solution (5% normal serum, 1% BSA, 0.3% Triton X-100 in PBST). After washing 3 times with PBST, slides were incubated at room temperature for 30 minutes with secondary antibodies in blocking solution. Slides were then washed, stained with DAPI, and mounted with ProLong(R) Gold antifade reagent (Life Technologies). For immunocytochemistry, detection was performed using Alkaline Phosphatase goat anti-rabbit, horse anti-goat, and horse anti-mouse IgG and the Alkaline Phosphatase Substrate Kit III from Vector Laboratories. Cells were sorted directly onto slides coated with poly-lysine-D, dried overnight at room temperature, and stored at 4°C. Prior to staining, slides were rinsed in PBS and fixed in 4% PFA in PBS for 15 minutes at room temperature. Slides were then washed 3 times in ice cold PBS and then permeabilized with 0.3% Triton X-100 for 10 minutes at room temperature. After washing, cells were incubated at room temperature for 1 hour with DAKO protein blocked and then incubated overnight at 4°C with primary antibody in blocking solution. After washing, slides were incubated at room temperature for 30 minutes with alkaline phosphatase-conjugated goat anti-rabbit, horse anti-mouse, or horse anti-goat secondary antibodies in blocking solution. Slides were washed again and developed with Vector Blue substrate working solution for 5–10 minutes at room temperature. Slides were rinsed and mounted in Vecta-Mount AQ Aqeous Mounting Medium (Vector Laboratories).

Gene expression analysis

Total RNA from each sample was reverse transcribed by using SuperScript III from Invitrogen. qPCR was conducted by using SsoAdvanced™ Universal SYBR® Green Supermix (BioRad) with the following primers:

Plzf forward, 5′-AAACGGTTCCTGGACAGTTTGCGAC-3′
reverse, 5′-CCAGTATGGGTCTGTCTGTGTGTCTCC-3′
Stra8 forward, 5′-CCTGGTAGGGCTCTTCAACA-3′
reverse, 5′-GGCTTTCTTCCTGTTCCTGA-3′
Gfra1 forward, 5′-CACTCCTGGATTTGCTGATGT-3′
reverse, 5′-AGTGTGCGGTACTTGGTGC-3′
β-*actin* forward, 5′-CTGCCGCATCCTCTTCCTC-3′
reverse, 5′-GCCACAGGATTCCATACCCA-3′
Nanos2 forward, 5′-CTGCAAGCACAATGGGGAGT-3′
reverse, 5′-CGTCGGTAGAGAGACTGCTG-3′
ID4 forward, 5′-CAGTGCGATATGAACGACTGC-3′
reverse, 5′-GACTTTCTTGTTGGGCGGGAT-3′
Ngn3 forward, 5′-ACGACTTCCAGACGCAATTT-3′
reverse, 5′-CCATCCTAGTTCTCCCGACTC-3′
Sohlh1 forward, 5′-ACAGCTTGCAGACCTCATCACAAAG-3′
reverse, 5′-ACACAGACATCTCCAGGACAGATGC-3′
Spo11 forward, 5′-AACAATCAGATGGCTTGGTCTCCTC-3′
reverse, 5′-GGCTTGGATCTCTGCCTTCATCTTA-3′
Dmc1 forward, 5′-ATCGGGATTCCAAGATGATG-3′
reverse, 5′-TGCAGAGGGCTCTTCTTGTT-3′
Sycp3 forward, 5′-GGGGCCGGACTGTATTTACT-3′
reverse, 5′-GGAGCCTTTTCATCAGCAAC-3′
p53 forward, 5′-TGGAAGACTCCAGTGGGAAC-3′
reverse, 5′-TCTTCTGTACGGCGGTCTCT-3′
Oct4 forward, 5′-CACGAGTGGAAAGCAACTCA-3′
reverse, 5′-AGATGGTGGTCTGGCTGAAC-3′
CyclinD1 forward, 5′-AGAGGCGGATGAGAACAAGC-3′
reverse, 5′-CATGGAGGGTGGGTTGGAAA-3′
CyclinD2 forward, 5′-GCTGGAGTGGGAACTGGTAG-3′
reverse, 5′-CGGATCAGGGACAGCTTCTC-3′
p21 forward, 5′-CCTGGTGATGTCCGACCTG-3′
reverse, 5′-CCATGAGCGCATCGCAATC-3′

Western blotting analysis

Total protein was isolated in RIPA buffer supplemented with 1 mM PMSF (Sigma) and a protease inhibitor cocktail (Sigma P8340). Lysates were cleared by centrifugation at 14,000 × *g* for 10 min at 4°C, and protein concentrations in supernatants were determined (DC protein assay; BioRad). Equal amount of protein from each sample was mixed with LDS sample buffer (Invitrogen) plus sample reducing agent (Invitrogen), and denatured for 10 min at 70°C. Proteins were resolved in 4−12% Bis-Tris gels (Invitrogen), and transferred to PDVF membranes. Blots were probed with antibodies against p-RPS6 (Ser236/236, Cell Signaling Catalog#2211), total RPS6 (Cell Signaling Catalog#2217), or pan-Actin (NeoMarkers Catalog# MS-1295), washed and reacted with horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse IgG (BioRad). Detection was performed with the Clarity™ ECL Western Blotting Substrate (BioRad).

Data analysis and presentation

All experiments were independently replicated at least 3 times, using different mice, tissues or cells for each replicate. Where possible, assignment of mice to experimental groups was made randomly. Quantitative data from replicate experiments were pooled, analyzed by ANOVA or Student *t*-test in GraphPad Prism software, and are presented as the means ± SEM. Statistical analysis was performed. Representative images obtained from immunohistochemical and immunofluorescence assays are provided for qualitative assessment.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Material

Supplemental data for this article can be accessed on the publisher's website.

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