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Tumor suppressor gene *Rb* is required for self-renewal of spermatogonial stem cells in mice

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The retinoblastoma tumor suppressor gene *Rb* is essential for maintaining the quiescence and for regulating the differentiation of somatic stem cells. Inactivation of *Rb* in somatic stem cells typically leads to their overexpansion, often followed by increased apoptosis, defective terminal differentiation, and tumor formation. However, *Rb*'s roles in germ-line stem cells have not been explored. We conditionally disrupted the *Rb* gene in mouse germ cells in vivo and discovered unanticipated consequences for GFRa1-protein-expressing A_{single} ($\text{GFRa1}^+ A_s$) spermatogonia, the major source of male germ-line stem cells. *Rb*-deficient $\text{GFRa1}^+ A_s$ spermatogonia were present at normal density in testes 5 d after birth, but they lacked the capacity for self-renewal, resulting in germ cell depletion by 2 mo of age. *Rb* deficiency did not affect the proliferative activity of $\text{GFRa1}^+ A_s$ spermatogonia, but their progeny were exclusively transit-amplifying progenitor spermatogonia and did not include $\text{GFRa1}^+ A_s$ spermatogonia. In addition, *Rb* deficiency caused prolonged proliferation of progenitor spermatogonia, transiently enlarging this population. Despite these defects, *Rb* deficiency did not block terminal differentiation into functional sperm; offspring were readily obtained from young males whose germ cell pool was not yet depleted. We conclude that *Rb* is required for self-renewal of germ-line stem cells, but contrary to its critical roles in somatic stem cells, it is dispensable for their proliferative activity and terminal differentiation. Thus, this study identifies an unexpected function for *Rb* in maintaining the stem cell pool in the male germ line.

spermatogenesis | testis | fertility

The retinoblastoma tumor suppressor gene *Rb* encodes the multifunctional protein RB, which actively controls multiple cellular processes, including cell-cycle progression and differentiation (1–3). Somatic stem cells, which possess the ability to self-renew and differentiate into specialized cells, are critical for maintaining tissue homeostasis as well as for repairing tissues after injury. It has been shown that somatic stem cells are largely held in quiescence through a process that involves *Rb* (3, 4). Mice that are conditionally deficient in *Rb* or *Rb* gene family members in the stem cell compartments of somatic tissues—such as blood (5), liver (6), muscle (7), and skin (8)—show a common phenotype: stem cells exit quiescence and proliferate. However, this defect does not appear to affect the stem cells' self-renewal capacity (5, 9).

In addition to maintaining the quiescence of somatic stem cells, RB also plays critical roles in their differentiation (see review in ref. 3). Somatic stem/progenitor cells that lack RB are unable to undergo terminal differentiation, reflecting RB's function in controlling somatic cell fate through modulating the transcriptional activity of master differentiation regulators (10–12). Cell death resulting from *Rb* deficiency also contributes to the absence of terminally differentiated somatic cells (5, 13). Moreover, *Rb* deficiency in stem/progenitor cells can lead to tumor formation in somatic tissues (6, 13, 14).

RB's important function in somatic stem cells raises the question of whether it plays a similar role in the regulation of germ-line stem cells. However, this question has remained unexplored. Male germ-line stem cells, which are located in the

testis, are also known as spermatogonial stem cells (SSCs). As with somatic stem cells, SSCs must undergo both self-renewal to sustain the stem cell pool and differentiation to give rise to terminally differentiated cells: spermatozoa (sperm). Spermatogenesis follows a differentiation scheme similar to that for somatic cell lineages. SSCs undergo mitotic divisions to generate progenitor (transit-amplifying) spermatogonia, followed by a series of differentiation events, including meiosis and spermiogenesis, to form highly specialized sperm cells. Despite the similarities between somatic stem cells and SSCs, they differ fundamentally in their cell-cycle status. Somatic stem cells are largely quiescent (for example, ~95% of adult hematopoietic stem cells in bone marrow), whereas SSCs are actively cycling throughout an animal's reproductive life (15–17). This difference poses interesting questions about how RB functions in SSCs and how this function compares with its role in somatic stem cells. We therefore decided to explore RB function in the germ line throughout the various stages of spermatogenesis.

Spermatogenesis normally begins from single, isolated germ cells called A_{single} or A_s spermatogonia, the population of which is thought to be, or at least contain, SSCs (18–23). A_s spermatogonia divide, with incomplete cytokinesis, to form chains of 2 (A_{paired} or A_{pr}) and then 4, 8, 16, or even 32 (A_{aligned} or A_{al}) spermatogonia. A_{al} spermatogonia then differentiate into spermatozoa in a wave-like manner (once every 8.6 d), moving synchronously through several phases: differentiating spermatogonia, spermatocytes (meiotic), and spermatids (postmeiotic) (24). In the mouse, the synchronized passage of spermatogenic cells through these phases results in 12 recognizable associations, known as seminiferous epithelial stages I–XII (24).

Hereafter we use the term A_{spa} spermatogonia to refer, collectively, to A_s , A_{pr} , and A_{al} spermatogonia (23). A_{spa} spermatogonia consist of both SSCs and progenitor spermatogonia, express specific protein markers [for example, promyelocytic leukemia zinc finger (PLZF)], and maintain the potential to self-renew or revert to self-renewing cells (21, 25, 26).

To explore the function of RB in the mouse germ line, we used a Cre recombinase-*loxP* conditional knockout strategy to remove *Rb* in germ cells before birth. Loss of *Rb* in germ cells resulted in rapid exhaustion of the SSC pool. Specifically, GFRa1-protein-expressing A_{single} ($\text{GFRa1}^+ A_s$) spermatogonia, which are thought to be the major source of SSCs (19–21, 27), were defective in self-renewal, and production of germ cells in the testis was quickly depleted. Thus, our study indicates that *Rb* is required for stem cell maintenance in the male germ line.

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Results and Discussion

Germ Cell Expression of RB Protein Ends Before Birth in Conditional Knockout Males. We crossed $Rb^{fl/fl}$ conditional mutant mice (28) with mice carrying a newly generated *mouse vasa homolog* ($Mvh^{Cre-mOrange}$, also known as *Ddx4*) allele (Fig. S1) in which the endogenous *Mvh* promoter drives expression of a Cre-mOrange fusion protein. The $Mvh^{Cre-mOrange/+}$ line showed Cre recombination activity in the germ line starting from ~embryonic day (E) 15.5, as assessed by crossing into the ROSA26-LacZ reporter strain (29) (Fig. S1F). To assay whether RB expression was subsequently lost in germ cells of homozygous $Rb^{fl/fl}; Mvh^{Cre-mOrange/+}$ male mice (hereafter referred to as *Rb* cKO), we examined RB expression in testes. We found that, at postnatal day 0 (P0), RB was expressed in control ($Rb^{fl/+}$) but not in *Rb* cKO germ cells (Fig. 1). Therefore, RB expression ceases in *Rb* cKO germ cells before birth.

Sterility by 2 mo of Age. Mature *Rb* cKO mice were grossly normal except that the testes were small (Fig. 2A). During the first 3–4 wk of age, testis weight in *Rb* cKO animals increased much like controls (including $Rb^{fl/+}$ and $Rb^{fl/+}; Mvh^{Cre-mOrange/+}$), but testis weight declined thereafter and remained low throughout adulthood (Fig. 2A). To assess the fertility of *Rb* cKO mice, each male was housed with a single wild-type female for 21 wk, beginning at 4 wk of age. Each control male sired four to six litters throughout the test period. By contrast, each *Rb* cKO male sired only one litter, born when the males were 9–11 wk of age (Fig. 2B); impregnation would have occurred 3 wk earlier, when the males were 6–8 wk of age. The *Rb* deletion allele was transmitted at normal Mendelian ratios. The average size of litters sired by *Rb* cKO males was modestly but not significantly reduced compared with controls (6.4 ± 2.9 vs. 8.1 ± 1.4 pups, respectively; $P > 0.05$). Taken together, these data suggest that *Rb*-deficient germ cells can develop into spermatozoa and produce offspring, but *Rb* cKO males become sterile by about 2 mo of age.

Limited Rounds of Spermatogenesis. As wild-type males develop, their germ cells undergo a G1/G0 cell-cycle arrest from E14.5 until 1–2 d after birth (30). In the experiments reported here, RB was ablated in the germ cells while they were arrested, but we observed no difference in germ cell numbers between control and *Rb* cKO testes at P0 (Fig. 3A). We also observed that both control and *Rb* cKO germ cells remained quiescent at P0, as determined by BrdU incorporation assay (>99% germ cells negative for BrdU in both groups). These results suggest that *Rb* is not required to maintain the G1/G0 arrest, consistent with a previous report (31).

To seek explanations for the loss of fertility in early adulthood in *Rb* cKO males, we examined the testicular histology of these mice from P7 to 2 mo of age. To ensure consistency in our analysis,

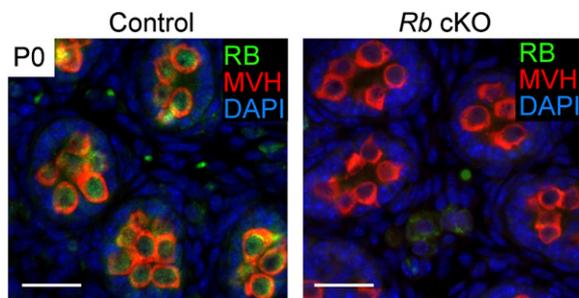


Fig. 1. Immunohistochemical staining for RB (green) and MVH (red) proteins in sections of control and *Rb* cKO testes at P0. MVH marks germ cells. Nuclei counterstained with DAPI (blue). (Scale bar, 25 μ m.)

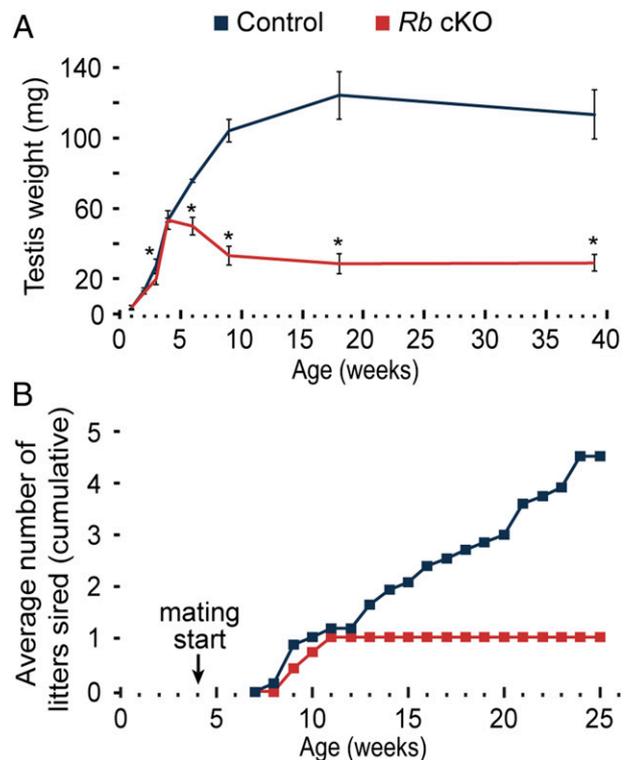


Fig. 2. *Rb* cKO male mice are initially fertile, but become sterile by about 2 mo of age. (A) Time-course study of testis weight in control and *Rb* cKO mice (mean \pm SD, $n \geq 3$ for all points). * $P < 0.001$ (two-tailed Student *t* test). (B) Fertility test for male control and *Rb* cKO mice ($n = 7$ for each group). Starting at 4 wk of age, each male was housed with a mature wild-type female for 21 wk. Numbers of litters sired were binned by week and averaged.

we focused on seminiferous tubule cross-sections at stage VII in both control and *Rb* cKO testes (except at P7, when seminiferous tubule staging is not possible). By P28, *Rb* cKO tubules displayed a striking depletion of “early” (less advanced differentiating) spermatogenic cell types (Fig. 3B and Fig. S2). In P28 control tubules, we observed four types of germ cells (spermatogonia, preleptotene spermatocytes, pachytene spermatocytes, and round spermatids), corresponding to four rounds of spermatogenesis. By contrast, many *Rb* cKO tubules contained only the more advanced differentiating cell types (pachytene spermatocytes and round spermatids) and lacked the earlier cell types (spermatogonia and preleptotene spermatocytes). In quantitative terms, only 28% of *Rb* cKO stage VII tubule cross-sections contained spermatogonia, compared with 91% of controls (Fig. 3C). At P40, this number dropped to 17%, compared with 81% of controls (Fig. 3C). Consistent with these findings, the number of preleptotene spermatocytes per 1,000 Sertoli cells was dramatically reduced in *Rb* cKO testes by P40, compared with controls (Fig. S3). By 2 mo of age, most tubules in *Rb* cKO testes were devoid of germ cells, and the few germ cells that remained were mostly elongated spermatids (highly advanced differentiating cells; Fig. 3B, *Inset*). These results demonstrate that *Rb* cKO testes initiate a very limited number of rounds of spermatogenesis, but that these limited rounds are histologically like those observed in controls. These findings suggest that the principal defect in *Rb* cKO testes is premature exhaustion of the SSC pool.

Transient Increase in Number of Spermatogonia. Next, we examined the kinetics of germ cell depletion and were surprised to find that, in *Rb* cKO tubules, germ cell depletion (resulting from SSC exhaustion) is preceded by a transient increase in the number

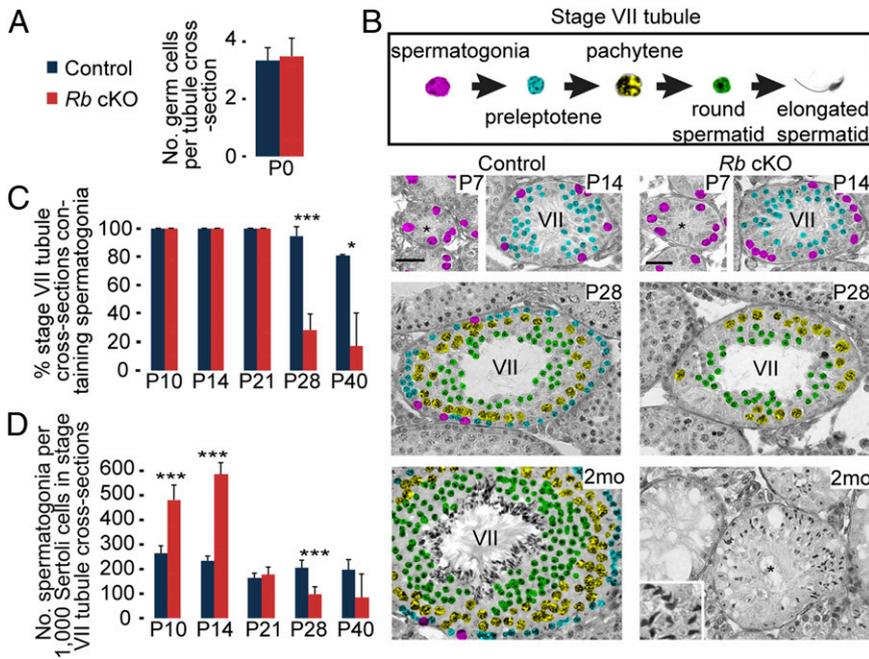


Fig. 3. *Rb* cKO mice exhibit limited rounds of spermatogenesis. (A) Number of germ cells per tubule cross-section in control and *Rb* cKO testes at P0 ($n = 4$ for each group, $P > 0.05$). (B) Histology of stage VII seminiferous tubule cross-sections of control and *Rb* cKO testes. (Upper) Spermatogenic cell types present in stage VII tubule. Each cell type is shaded with a different color. Unmarked images can be seen in Fig. S2. Asterisks indicate tubule cross-sections that cannot be staged. (Inset) Elongated spermatids at higher magnification. (Scale bar, 25 μm .) (C) Frequency of stage VII tubule cross-sections containing at least one spermatogonium in control and *Rb* cKO testes. (D) Number of spermatogonia per 1,000 Sertoli cells in stage VII tubule cross-sections of control and *Rb* cKO testes ($n \geq 3$ for all points, except $n = 2$ for P40 controls, in C and D). Error bars indicate SD. * $P < 0.05$; *** $P < 0.001$ (two-tailed Student *t* test).

of spermatogonia (particularly progenitor spermatogonia). We discovered this by examining the number of spermatogonia per 1,000 Sertoli cells in stage VII tubule cross-sections, where nearly all spermatogonia are progenitor spermatogonia (32). In control tubules, the number of spermatogonia was relatively constant across the time points examined (Fig. 3D). In contrast, the number of spermatogonia in *Rb* cKO testes was markedly increased at P10 and P14, compared with controls, followed by the previously described reductions at P28 and P40 (Fig. 3D). We postulated that this transient increase in the number of spermatogonia in *Rb* cKO testes might reflect a second role for *Rb* (in addition to that of maintaining SSCs). Specifically, *Rb* might limit proliferation of progenitor spermatogonia. If so, we might expect that, in *Rb* cKO testes, progenitor spermatogonia would continue to overproliferate at P28 and P40, even as the total numbers of spermatogonia declined because of SSC exhaustion. To test this prediction, we measured the number of spermatogonia per 1,000 Sertoli cells in the stage VII tubule cross-sections

that still retained spermatogonia at P28 and P40. We observed that the number of spermatogonia per 1,000 Sertoli cells was increased in these tubule cross-sections of *Rb* cKO testes compared with their counterparts in controls (Fig. S4). This result corroborates the hypothesis that *Rb* restricts the number of progenitor spermatogonia. Taken together, the histological analyses allow us to identify two germ cell phenotypes in *Rb* cKO testes: premature SSC exhaustion and increased expansion of progenitor spermatogonia.

Progressive Loss of GFRa1⁺ A_s Spermatogonia with Proliferation Unaffected. To substantiate the finding that SSCs are prematurely exhausted in *Rb* cKO testes, we measured the abundance of GFRa1⁺ A_s spermatogonia between P5 and P28 by staining whole-mount tubules for PLZF and GFRa1. PLZF is expressed in all A_s spermatogonia, whereas GFRa1 is expressed mainly in A_s and A_{pr} spermatogonia (21, 33, 34). To ensure that *Rb* deficiency does not affect the GFRa1 expression pattern, we examined PLZF⁺ A_s spermatogonia in control and *Rb* cKO testes

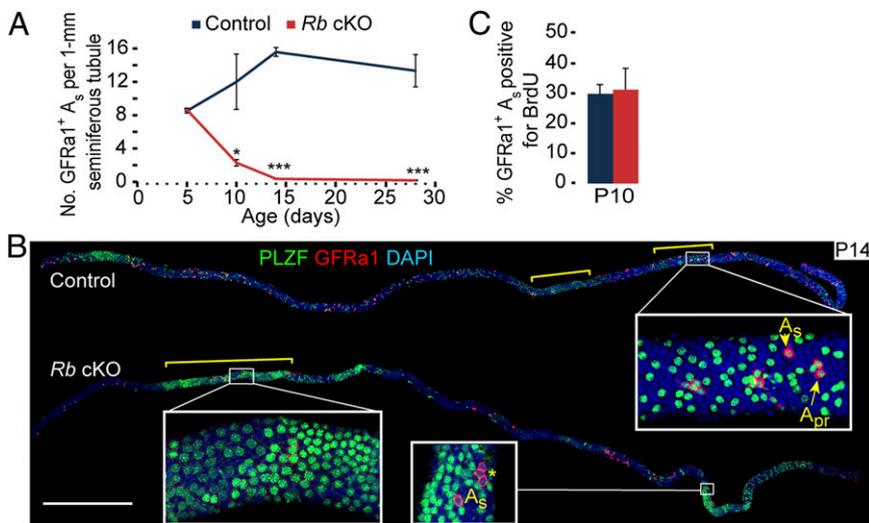


Fig. 4. *Rb* cKO seminiferous tubules show both loss of GFRa1⁺ A_s spermatogonia and increased numbers of progenitor spermatogonia. (A) Density of GFRa1⁺ A_s spermatogonia in seminiferous tubules of control and *Rb* cKO testes ($n \geq 3$ for all points, except $n = 2$ for P5). * $P < 0.05$; *** $P < 0.001$. (B) Representative image of whole-mount seminiferous tubule staining for PLZF (green) and GFRa1 (red) expressing cells at P14. Nuclei counterstained with DAPI (blue). Confocal images were taken along the length of the tubule and stitched together. Yellow brackets indicate representative areas with denser PLZF⁺ spermatogonial clusters, roughly representing stages II–VII. GFRa1⁺ A_s spermatogonia are rarely seen in the *Rb* cKO tubule. One example of GFRa1⁺ A_s spermatogonium is next to a chain of three cells (asterisk). (Insets) Higher magnification of boxed areas. (Scale bar, 1 mm.) (C) BrdU-labeling index of GFRa1⁺ A_s spermatogonia in control and *Rb* cKO testes ($n = 3$ and 6, respectively; $P > 0.05$); BrdU injected 4 h before mice being killed. Error bars indicate SD. *P* values from two-tailed Student *t* test.

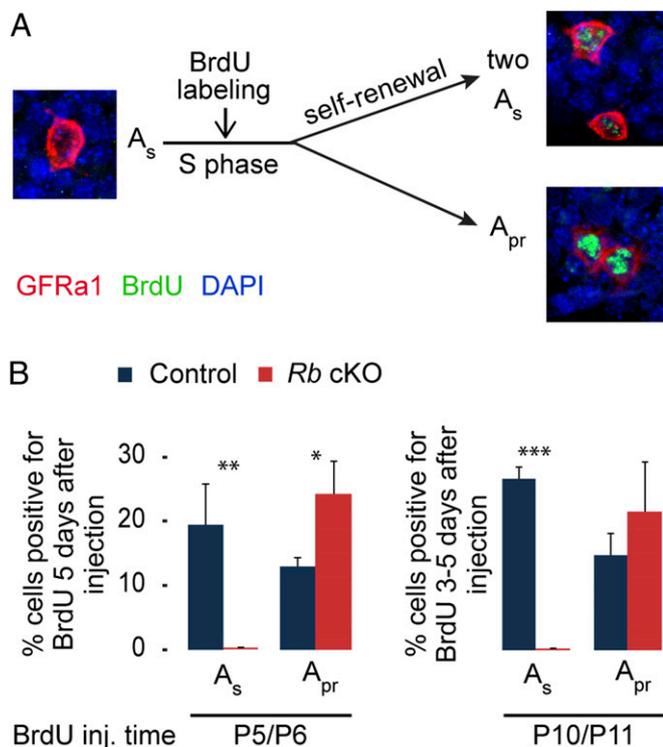


Fig. 5. *Rb* deficiency blocks self-renewal of GFRA1⁺ A_s spermatogonia. (A) Outline of experiment to identify newly generated GFRA1⁺ A_s spermatogonia through BrdU tracing. BrdU marks progeny of cells that were in S phase at time of labeling. Nuclei counterstained with DAPI (blue). (B) Mice at P5/P6 or P10/P11 were injected with a single pulse of BrdU. Five days after injection (3–5 d in the P10/P11 experiment), testes were collected for whole-mount seminiferous tubule preparation. Shown are percentages of GFRA1⁺ A_s and A_{pr} spermatogonia that retained BrdU label at time of testis recovery (mean ± SD, *n* = 3 for each group). **P* < 0.05; ***P* < 0.01; ****P* < 0.001 (two-tailed Student *t* test).

between P5 and P28. We found that during this time frame, nearly all PLZF⁺ A_s cells expressed GFRA1 in control testes and that this expression pattern was faithfully represented in *Rb* cKO testes.

Next, we compared the density of GFRA1⁺ A_s spermatogonia between control and *Rb* cKO testes at P5, when the SSC pool is being established in the mouse testis (35). We found that at this time point, the density of GFRA1⁺ A_s spermatogonia in *Rb* cKO testes was similar to that in controls (Fig. 4A). However, thereafter, the density of GFRA1⁺ A_s spermatogonia declined dramatically in *Rb* cKO testes (Fig. 4A). Consistently, we observed that lengthy stretches of seminiferous tubules contained no GFRA1⁺ A_s spermatogonia in *Rb* cKO testes at P10–P28, whereas GFRA1⁺ A_s spermatogonia were distributed evenly throughout the length of seminiferous tubules in controls (Fig. 4B; example at P14). Taken together, these observations establish that the population of GFRA1⁺ A_s spermatogonia is rapidly depleted in *Rb* cKO testes, confirming the phenotype of premature SSC exhaustion.

Given that RB is a master regulator of cell-cycle progression, we then examined whether the rapid loss of GFRA1⁺ A_s spermatogonia in *Rb* cKO testes was due to altered proliferative activity. We conducted a BrdU incorporation study to test this possibility. P10 mice were injected with BrdU and killed 4 h later, after which we assayed BrdU incorporation in GFRA1⁺ A_s spermatogonia in whole mounts of seminiferous tubules. We found that GFRA1⁺ A_s spermatogonia incorporated BrdU in *Rb* cKO testes at a similar rate as controls (Fig. 4C), demonstrating

that *Rb* deficiency does not affect the proliferative activity of GFRA1⁺ A_s spermatogonia.

To determine whether increased cell death contributed to the progressive loss of GFRA1⁺ A_s spermatogonia in *Rb* cKO testes, we assayed cell apoptosis by staining whole-mount tubules for GFRA1 and active caspase-3. We found no GFRA1⁺ A_s spermatogonia that were positive for active caspase-3 in either control or *Rb* cKO testes at P10 (>45 GFRA1⁺ A_s spermatogonia examined for each genotype). These results suggest that the loss of GFRA1⁺ A_s spermatogonia in *Rb* cKO testes is not due to increased apoptosis.

No Newly Generated GFRA1⁺ A_s Spermatogonia. Upon mitotic division, an A_s spermatogonium either self-renews by forming two new A_s spermatogonia or takes a step toward differentiation by forming an A_{pr} two-cell chain (Fig. 5A). Having found that *Rb*-deficient GFRA1⁺ A_s spermatogonia proliferate at a rate comparable with controls (Fig. 4C), we wondered whether they fail to self-renew, forming A_{pr} spermatogonia only, leading to their depletion. To test this hypothesis, we performed a BrdU-tracing experiment to trace the mitotic progeny of A_s spermatogonia in both control and *Rb* cKO testes. In this experiment, mice were given a single injection of BrdU at P5/P6 and killed 5 d later. If *Rb*-deficient GFRA1⁺ A_s spermatogonia failed to self-renew, we would expect to detect only BrdU⁺ GFRA1⁺ A_{pr} spermatogonia, but no BrdU⁺ GFRA1⁺ A_s spermatogonia, in *Rb* cKO mice at the time of testis recovery.

In control testes, we readily detected both BrdU⁺ GFRA1⁺ A_s and BrdU⁺ GFRA1⁺ A_{pr} spermatogonia in seminiferous tubules, as expected given that wild-type A_s spermatogonia undergo both self-renewal and differentiation (Fig. 5B). In *Rb* cKO testes, by contrast, we found BrdU⁺ GFRA1⁺ A_{pr} spermatogonia but no BrdU⁺ GFRA1⁺ A_s spermatogonia (Fig. 5B), suggesting that *Rb*-deficient GFRA1⁺ A_s spermatogonia were not capable of self-renewal. Similarly, we observed the absence of BrdU⁺ GFRA1⁺ A_s spermatogonia in *Rb* cKO testes when mice were injected with BrdU at P10/P11 and killed 3–5 d later (Fig. 5B). Accordingly, we surmise that upon cell division, *Rb*-deficient GFRA1⁺ A_s spermatogonia exclusively form A_{pr} spermatogonia and do not self-renew to form new GFRA1⁺ A_s spermatogonia; each GFRA1⁺ A_s cell can therefore initiate only one round of spermatogenesis.

Extended Proliferation of Progenitor Spermatogonia. The histological analysis revealed that progenitor (transit-amplifying) spermatogonia might overproliferate in *Rb* cKO testes (Fig. 3 and Fig. S4). To better understand this phenomenon, we closely

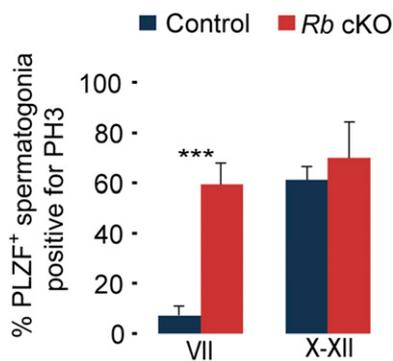


Fig. 6. *Rb* deficiency increases mitotic activity of PLZF⁺ spermatogonia during the mitotically inactive phase. PH3-labeling index of PLZF⁺ spermatogonia at indicated seminiferous tubule stages in sections of control and *Rb* cKO testes at P10 (mean ± SD, *n* = 4 for each group). ****P* < 0.001 (two-tailed Student *t* test).

examined whole-mount seminiferous tubules at P14. In controls, the density of A_{spa} spermatogonia (particularly A_{al} progenitor spermatogonia) rises and falls periodically along the length of the seminiferous tubules, peaking in epithelial stages II–VII (Fig. 4B, yellow brackets) and reaching its lowest point in stages VIII–IX (30, 32, 33). This periodic variation occurs largely because active proliferation of A_{spa} spermatogonia is limited to stages IX to ~III, coupled with the fact that they transition to become differentiating spermatogonia in stages VII–VIII. In the *Rb* cKO testes, we observed stretches of seminiferous tubules that did not contain any A_{spa} spermatogonia because of premature SSC exhaustion. However, in the areas that still retained A_{spa} spermatogonia, the chains of these cells were larger and more densely packed than in controls (Fig. 4B, yellow brackets). These overly accumulated cells were nearly all A_{al} progenitor spermatogonia (GFRA1⁻ PLZF⁺ chains as well as GFRA1⁺ PLZF⁺ chains of four or more cells). These results confirm that, in *Rb* cKO testes, progenitor spermatogonia undergo a marked expansion—albeit transiently—because of the eventual exhaustion of the SSCs from which the progenitor spermatogonia derive.

To investigate the mechanism underlying this expansion of progenitor spermatogonia in *Rb* cKO tubules, we examined the proliferation status of A_{spa} (PLZF⁺) spermatogonia using the mitotic marker phospho-(Ser10)-histone H3 (PH3). We studied these spermatogonia in tubule cross-sections at stages VII and X–XII, representing, respectively, the mitotically inactive and active phases. We found no significant difference in the PH3 labeling index in PLZF⁺ spermatogonia between control and *Rb* cKO testes during the mitotically active phase (stages X–XII). However, during the mitotically inactive phase (stage VII), when only ~7% of control PLZF⁺ spermatogonia were positive for PH3, ~59% were positive in *Rb* cKO testes (Fig. 6). Taken together with the findings that GFRA1⁺ A_{s} spermatogonia proliferate at a normal rate and are progressively lost in *Rb* cKO testes (Fig. 4), these results indicate that *Rb*-deficient progenitor spermatogonia (non-SSC A_{spa} spermatogonia) fail to arrest at a time when control spermatogonia are mitotically quiescent, leading to their increased accumulation.

In wild-type seminiferous tubules, excess spermatogonia are subject to density control regulation, undergoing cell death at the differentiating spermatogonial stage (36, 37). We expected that the overproduced progenitor spermatogonia in *Rb* cKO testes would be culled when they progress to become differentiating spermatogonia. To test this prediction, we counted apoptotic cells in control and *Rb* cKO testis sections at P10, at which time spermatogonia (and, in some tubules, early spermatocytes) comprise the majority of testicular germ cells. Indeed, we observed more apoptotic cells in *Rb* cKO than in control tubules (Fig. S5). As a consequence of this density-control regulation, the increased number of progenitor spermatogonia at P10 and P14 (Fig. 3D) did not lead to any significant increase in the number of preleptotene spermatocytes at the same times and up to P28 in *Rb* cKO testes (Fig. S3). We concluded that spermatogonial density control is unaffected in *Rb* cKO tubules, and thus that *Rb* is dispensable for this process.

Although *Rb* deficiency resulted in extended proliferation of progenitor spermatogonia, our histological analysis indicated that these cells subsequently differentiated into spermatozoa in a stage-dependent manner similar to that observed in controls. Given the known functional redundancy among the *Rb* family genes (5, 38), functional compensation from other *Rb* family genes may contribute to the absence of a significant differentiation phenotype in spermatogenesis of *Rb* cKO mice.

Regulation of self-renewal and differentiation of stem cells depends on both intrinsic and extrinsic factors (23, 25, 26, 39). One possible explanation for the diversion of SSC fate from self-renewal to differentiation in *Rb* cKO testes is that RB is involved

in the intracellular signaling essential for SSC entry into the self-renewal pathway. Alternatively, *Rb*-deficient germ cells may fail to acquire self-renewal capacity in response to cues from stem-cell niches in the postnatal testis. Because a definitive marker for self-renewing SSCs is lacking at present, the question of whether the GFRA1⁺ A_{s} spermatogonia seen in young *Rb* cKO testes had properly transitioned to the SSC state awaits further investigation.

Interestingly, a phenotype similar to that reported here in *Rb* cKO mice has been observed in *Id4*-null mice. *Id4*-null SSCs were shown to lose their self-renewal capacity, albeit much more gradually than in *Rb* cKO mice, without affecting their differentiation to form functional sperm (40). It will be important to determine whether RB and ID4 interact directly in regulating SSC self-renewal, as has been shown for RB and ID2 in other systems (41, 42). Other factors known to regulate mouse SSC maintenance intrinsically include NANOS2, PLZF, GFRA1, RET, and ETV5. NANOS2 functions in SSCs to maintain their undifferentiated state (22, 27). PLZF, GFRA1, RET, and ETV5 regulate proliferation of cells within the A_{spa} population, and, as assayed by transplantation, the absence of any one of these factors eliminates or reduces the number of SSCs (22, 43–46). It will be of interest to determine whether RB governs SSC self-renewal through interaction with these known factors or others yet to be identified.

In summary, our data demonstrate that *Rb* plays an unexpected role in maintaining the germ-line stem cell pool in the mouse testis by regulating SSC self-renewal. Although *Rb* deficiency results in overexpansion of many somatic stem-cell populations, it leads to rapid exhaustion of SSCs. In addition to its role in SSC self-renewal, we also find that *Rb* plays a separate role, as a cell-cycle regulator, in progenitor spermatogonia. *Rb* is required for these cells to become quiescent during the mitotically inactive phase (stages III–VIII) in the seminiferous tubules. Despite these defects, however, *Rb* deficiency does not impair the ability of SSCs to differentiate into mature spermatozoa, which is in contrast to RB's general roles in fate decision and differentiation of somatic stem cells. *Rb*'s regulatory roles in spermatogenesis—including its essential function in male germ-line stem cell renewal—are remarkably different from its known roles in somatic lineages.

Materials and Methods

Mice. Mice carrying the *Rb* conditional allele (28) were a gift from Tyler Jacks and Jacqueline Lees (Massachusetts Institute of Technology). *Mvh*^{Cre-mOrange/+} mice were generated by gene targeting in C57BL/6 ES cells (Fig. S1). The targeting vector replaced a portion of exon 1 and intron 1 of the *Mvh* locus with the *Cre-mOrange* gene. The *mOrange* plasmid (47) used to generate the targeting vector was a gift from Roger Tsien (University of California, San Diego, CA). *Rb*-conditional mutants and littermate controls were obtained by crossing *Rb*^{fl/fl} and *Rb*^{fl/+}; *Mvh*^{Cre-mOrange/+} mice. All experiments involving mice were approved by the Committee on Animal Care at the Massachusetts Institute of Technology.

Histological Analysis and Seminiferous Tubule Staging. Testes were fixed overnight in Bouin's solution, embedded in paraffin, and sectioned. Slides were then dewaxed, rehydrated, and stained with periodic acid-Schiff-hematoxylin. Stage-identifying criteria were described previously (24). Spermatogonia and preleptotene spermatocytes were counted in stage VII tubule cross-sections and normalized to the number of Sertoli cells (a supporting somatic lineage) to correct for variation in tubule size.

Immunohistochemistry. Immunohistochemical staining of testis sections was carried out as described previously (48). Primary antibodies against active caspase-3 (ab13847, Abcam), BrdU (OBT0030, Accurate Chemical and Scientific), GFRA1 (AF560, R&D Systems), histone H3 (phospho S10, ab5176, Abcam), MVH (AF2030, R&D Systems), PLZF (OP128, EMD Biosciences), and RB (sc-7905, Santa Cruz Biotechnology) were used in the study.

Whole-Mount Seminiferous Tubule Staining. Mouse testes were dissected to remove the tunica albuginea, and seminiferous tubules were untangled using forceps. Whole-mount immunohistochemical staining was carried out as described previously (49). Stained tubules were spread on glass slides and imaged. Primary antibodies used are listed in the previous paragraph.

For BrdU incorporation experiments, mice were injected intraperitoneally with 100 mg/kg body weight of BrdU 4 h before sacrifice. For BrdU-tracing experiments, BrdU was given 3–5 d before mice were killed. Testes were then removed and processed for whole-mount immunohistochemical staining, following the procedure described previously, except that the tubules were denatured with 3.5 N HCl for 2 min before blocking. The percentages of GFRa1⁺ A_s and A_{pr} spermatogonia positive for BrdU label were determined using an LSM710 confocal microscope (Zeiss). In each animal, we counted all

GFRa1⁺ A_s and A_{pr} cells positive or negative for BrdU in at least two seminiferous tubules, each at least 10 mm in length.

To determine the density of GFRa1⁺ A_s spermatogonia, we counted all GFRa1⁺ A_s cells in at least two seminiferous tubules, each at least 10 mm in length, in each animal. The density was obtained by dividing the total number of GFRa1⁺ A_s spermatogonia by the length of the tubule.

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