# Retinoblastoma-E2F Transcription Factor Interplay Is Essential for Testicular Development and Male Fertility

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The retinoblastoma (RB) protein family members (pRB, p107 and p130) are key regulators of cell cycle progression, but also play crucial roles in apoptosis, and stem cell self-renewal and differentiation. RB proteins exert their effects through binding to E2F transcription factors, which are essential developmental and physiological regulators of tissue and organ homeostasis. According to the canonical view, phosphorylation of RB results in release of E2Fs and induction of genes needed for progress of the cell cycle. However, there are eight members in the E2F transcription factor family with both activator (E2F1-3a) and repressor (E2F3b–E2F8) roles, highlighting the functional diversity of RB-E2F pathway. In this review article we summarize the data showing that RB-E2F interaction is a key cell-autonomous mechanism responsible for establishment and maintenance of lifelong male fertility. We also review the expression pattern of RB proteins and E2F transcription factors in the testis and male germ cells. The available evidence supports that RB and E2F family members are widely and dynamically expressed in the testis, and they are known to have versatile roles during spermatogenesis. Knowledge of the function and significance of RB-E2F interplay for testicular development and spermatogenesis comes primarily from gene knock-out (KO) studies. Several studies conducted in Sertoli cell-specific *pRB*-KO mice have demonstrated that pRB-mediated inhibition of E2F3 is essential for Sertoli cell functional maturation and cell cycle exit, highlighting that RB-E2F interaction in Sertoli cells is paramount to male fertility. Similarly, ablation of either pRB or E2F1 in the germline results in progressive testicular atrophy due to germline stem cell (GSC) depletion, emphasizing the importance of proper RB-E2F interplay for germline maintenance and lifelong sperm production. In summary, while balanced RB-E2F interplay is essential for cell-autonomous maintenance of GSCs and, the pRB-E2F3 system in Sertoli cells is critical for providing GSC niche thus laying the basis for spermatogenesis.

**Keywords: retinoblastoma protein, spermatogenesis, testis, E2F transcription factor, germ cell, Sertoli cell**

# RB-E2F PATHWAY

Strict regulation of the cell cycle is critical during testicular development and steady-state spermatogenesis. The mechanisms that define whether a cell stays in the $G_1$ state or transits to S phase or $G_0$ are ultimately responsible for normal development of any tissue and its function under homeostasis. The $G_1$/S transition is controlled by the interaction between retinoblastoma (RB) tumor suppressor proteins and E2F transcription factors. In $G_1$ RB family proteins form complexes with E2Fs and various chromatin modifiers to repress E2F activity on the promoters of the genes that are needed to enter the S phase. The function of RB is thus growth-inhibitory and misregulation of RB-E2F interplay is one of the hallmarks of cancer (1). The RB-dependent repression on E2F-driven transcription is relieved upon phosphorylation of RB by cyclin-dependent kinases (CDKs) resulting in cellular growth, DNA synthesis and advancement of the cell cycle (**Figure 1**). It is considered that many different stimuli that affect cell fate decisions are channeled through CDKs to control the phosphorylation status of RB to ultimately control the progress or arrest of the cell cycle at $G_1$ (2).
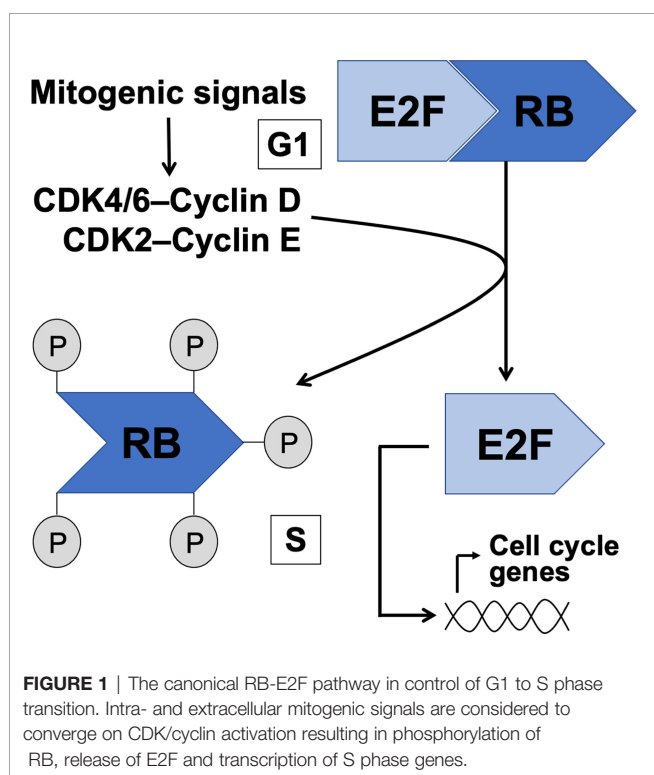
Efficient entry in the S phase depends on numerous phosphorylation events on RB resulting in the dissociation of the RB-E2F complex and activation of E2F transcription factors. While numerous different kinases have a capacity to phosphorylate RB (3), the role of CDK4/6 and CDK2 in G1/S transition is probably the most critical (4). The transcriptional targets of E2F include *cyclin E, Cdk1, DNA polymerase-alpha* and *E2f*s themselves (5). While the molecular control of CDK-RB-

E2F pathway has been much studied and relatively well understood, the versatility in RB and E2F protein families brings a layer of complexity to the big picture.

The retinoblastoma protein family consists of three proteins (pRB, p107 and p130; also known as RB1, Retinoblastoma-like 1 and Retinoblastoma-like 2 (RBL1-2), respectively) that share significant homology, yet only *pRb* is found frequently mutated in cancer (6). While some degree of functional redundancy in the RB protein family is apparent - especially between p107 and p130, as observed in mouse knock-out studies - the data support an indispensable role for pRB in development and oncogenesis [reviewed in (7)]. Yet, in a physiological context evaluation of such aspects is often irrelevant due to cell-type dependent expression patterns, as we will later see also in the testis. Thus far eight different *E2f* genes (*E2f1-8*) are known, giving rise to nine distinct E2F proteins. E2F1, E2F2 and E2F3A are generally considered as transcriptional activators, whereas E2F3B and E2F4-8 have been assigned transcriptional repressor functions. However, at least in some developmental contexts E2Fs display functional plasticity and may interconvert between repressor and activator functions (8–12). While activator E2Fs are generally considered to function in cell proliferation, repressor E2Fs are involved in cell cycle exit and differentiation (13). However, such clear-cut functional dichotomy in a physiological context barely exists, and the downstream effects of E2F activation are context-dependent. A notable feature in the E2F protein family is functional redundancy, and in many cases two or more *E2f* genes need to be ablated in order to achieve a phenotypic change (14).

RB family proteins are so called pocket proteins that interact widely with different proteins (>300 proteins have been identified as possible binding partners), not just with E2Fs (15, 16). Within the E2F family they show preferences in terms of interaction partners, and while E2F1-3 predominantly associate with pRB, E2F4-5 preferentially interact with p107 or p130 (7). Conversely, E2F6 is an RB-independent transcriptional repressor and instead forms a complex with Polycomb group proteins (17), and E2F7/8-driven transcriptional repression is also independent from CDK-mediated phosphorylation of RBs (18). Notably, the function of RBs goes beyond gatekeeping $G_1$/S transition and they play critical roles in quiescent, senescent and differentiating cells by maintaining $G_0$/$G_1$. Among E2Fs the interaction of E2F1 with pRB is considered unique and even hyperphosphorylation of pRB does not fully preclude this interaction (19, 20). Conspicuously, E2F1 is also the most extensively studied of the E2F family members and future investigations will be required to elucidate the nuances in tissue-, cell- and context-dependent action of different E2Fs.

In addition to its canonical role in control of the cell cycle and proliferation, the RB-E2F pathway has also been implicated in regulation of heterochromatin and chromosome stability, and apoptosis (2), all of which are very relevant to spermatogenesis. A number of studies have shown that increased E2F activity due to RB loss-of-function has an adverse effect on chromosome stability and causes aneuploidy (2, 21). One of the underlying mechanism may be deregulation of the pericentric heterochromatin, an



**FIGURE 1** | The canonical RB-E2F pathway in control of G1 to S phase transition. Intra- and extracellular mitogenic signals are considered to converge on CDK/cyclin activation resulting in phosphorylation of RB, release of E2F and transcription of S phase genes.
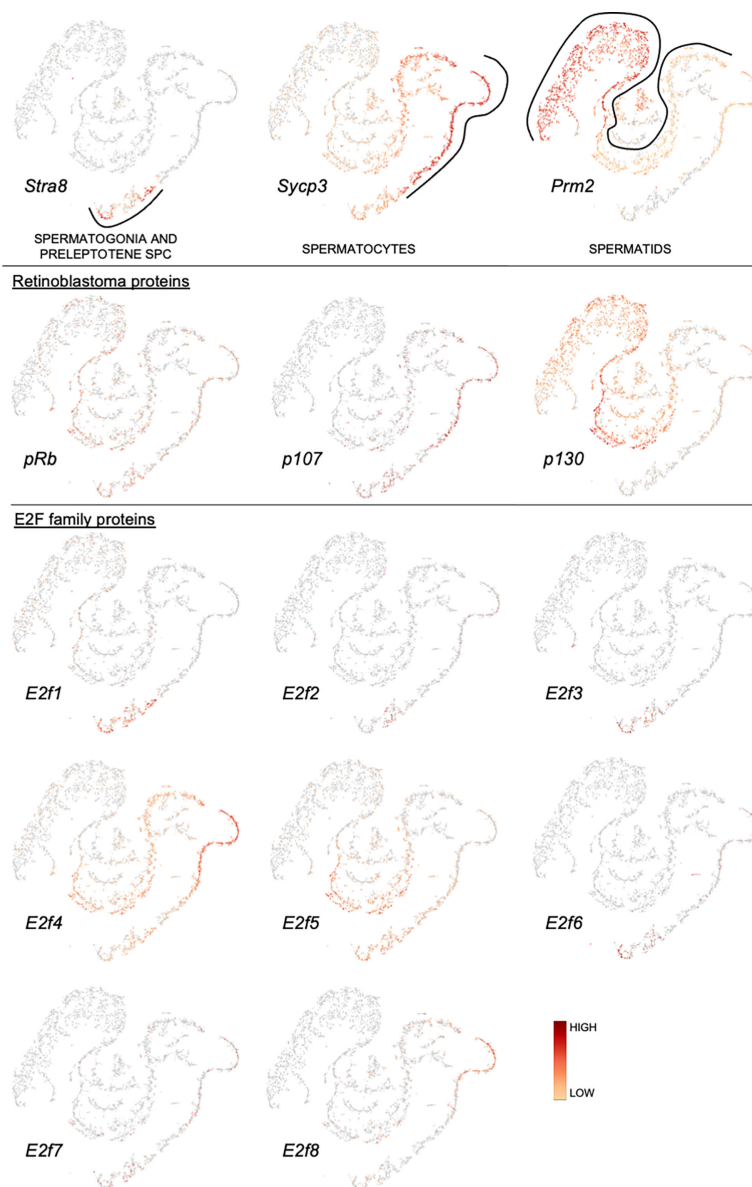
**FIGURE 2** | Expression of retinoblastoma protein family and E2F transcription factor family mRNAs along a pseudotime trajectory of adult mouse spermatogenic cells. The data is extracted from Hermann et al. 2018 (26) using Loupe Cell Browser v6.0.0 from 10x Genomics. *Stra8*, *Sycp3* and *Prm2* are included to denote spermatogonia/preleptotene spermatocytes, spermatocytes and spermatids, respectively.

essential chromosomal region for proper segregation during mitosis and meiosis (22, 23). Considering the role of activator E2Fs in cell cycle progression, it is somewhat counterintuitive that increased E2F activity is also able to induce apoptosis *via* DNA damage signaling pathways. According to a model proposed by Dick & Rubin (2013) this involves extensive post-transcriptional modifications in both RB and E2F1 allowing expression of pro-apoptotic genes, but repression of E2F-dependent cell cycle genes (2). All these different functional aspects of RB-E2F pathway make it a key regulator of testicular development and physiology, as will be highlighted in this article.

# DYNAMIC EXPRESSION OF RBS AND E2FS IN RODENT TESTIS

## mRNA Expression in Mouse Spermatogenic Cells

Application of single-cell RNA-sequencing technology (scRNA-seq) for the analysis of gene expression during spermatogenesis has provided a powerful tool to better understand its molecular regulation and identify the genes/pathways involved in it in various organisms (24–32). We took advantage of a previously published adult mouse testis scRNA-seq dataset (26) and Loupe
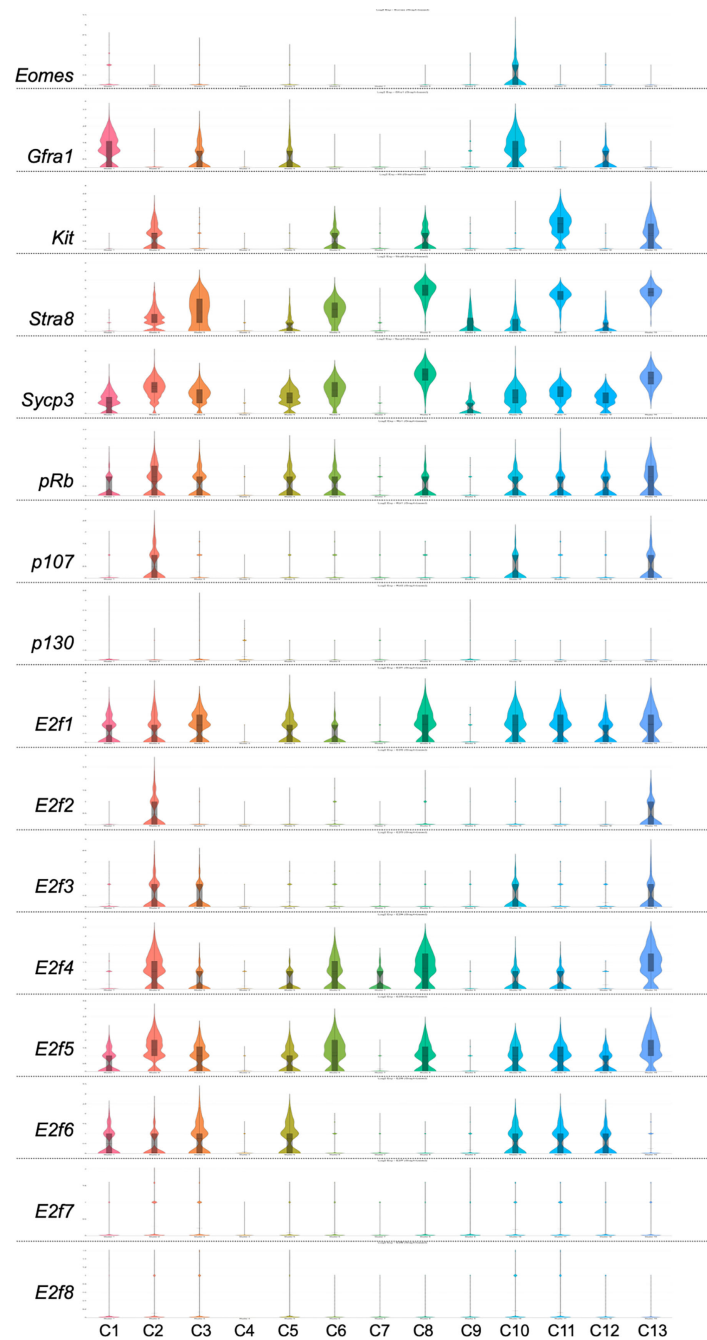
**FIGURE 3** | Violin plots displaying the expression of *Eomes, Gfra1, Kit, Stra8, Sycp3, pRb, p107, p130* and *E2f1-E2f8* in spermatogonial cells of an adult mouse. The data is extracted from Hermann et al. 2018 (26) using Loupe Cell Browser v6.0.0 from 10x Genomics. Spermatogonia are divided into clusters 1-13 (C1-13).

Cell Browser v6.0.0 from 10x Genomics to visualize the distribution of RB and E2F family mRNAs along the entire spermatogenic trajectory (**Figure 2**), or specifically in spermatogonia (**Figure 3**) and round spermatids (**Figure 4**). We chose the dataset of Hermann et al. (2018) because it is the first comprehensive scRNA-seq analysis of adult mouse spermatogenic cells distinguishing 11 cell types (26) and enabling feasible analysis

of their gene expression signature. For this analysis spermatogonia were further divided into clusters (1-13; **Figure 3**), with clusters 1, 3, 5 and 10 representing undifferentiated spermatogonia as defined by expression of *Gfra1* (33, 34) and *Eomes* (35, 36), and clusters 2, 3, 6, 8, 11 and 13 representing differentiating spermatogonia with characteristic expression for *Kit* (37, 38), *Stra8* (39, 40) and *Sycp3* (41, 42). Round spermatids were divided into early, mid and late
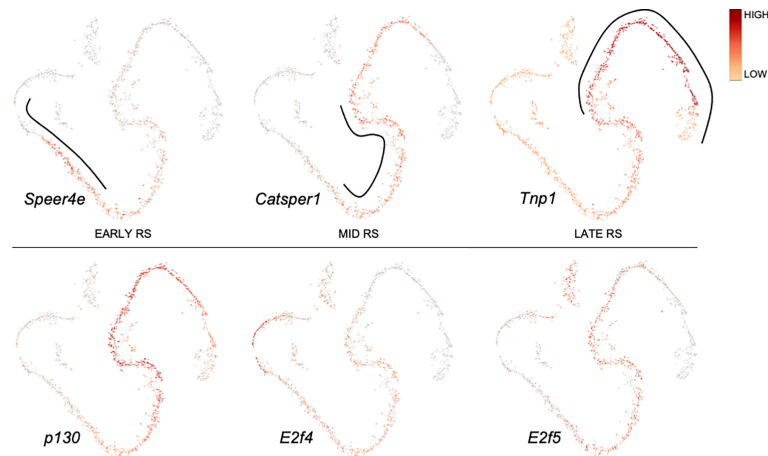
**FIGURE 4** | Expression of *p130*, *E2f4* and *E2f5* along a pseudotime trajectory of sorted mouse round spermatids. The data is extracted from Hermann et al. 2018 (26) using Loupe Cell Browser v6.0.0 from 10x Genomics. Expression for *Speer4e, Catsper1* and *Tnp1* are included to demarcate early, mid and late round spermatids, respectively.

steps (**Figure 4**) based on expression of *Speer4e, Catsper1* and *Tnp1* (26). Besides the entire spermatogenic trajectory (**Figure 2**), we decided to focus on GSCs/spermatogonia (**Figure 3**) because they are the foundation of spermatogenesis, and round spermatids (**Figure 4**) because *p130*, *E2f4* and *E2f5* display intriguing expression patterns in these cells. It should be noted that the data presented in **Figures 2–4** are from a previously published dataset (26) and their reliability have not been validated with independent approaches.

## pRB (Rb1)
*pRB* is expressed rather uniformly throughout spermatogenic differentiation (**Figure 2**), and also in most cell clusters within the spermatogonial compartment (**Figure 3**).

## p107 (Rbl1)
*p107* is expressed in spermatocytes, and to a lesser extent in early spermatids (**Figure 2**) and spermatogonia (**Figure 3**).

## p130 (Rbl2)
*p130* is expressed in spermatids (**Figure 2**) with the highest levels in mid-to-late round spermatids (**Figure 4**).

## E2f1
*E2f1* is expressed in spermatogonia and early spermatocytes (**Figure 2**), which is also supported by RNA *in situ* results (43). *E2f1* expression is rather uniform in all spermatogonial clusters (**Figure 3**) from GSCs (*Eomes/Gfra1*+; clusters 1 and 10) to differentiating spermatogonia (*Kit/Sycp3/Stra8*+; clusters 2, 3, 6, 8, 11 and 13).

## E2f2
There are very few cells expressing *E2f2* along the spermatogenic trajectory (**Figure 2**).

## E2f3
*E2f3* is expressed in a limited fashion in spermatogonia and preleptotene spermatocytes (**Figure 2**).

## E2f4
*E2f4* is expressed in spermatogonia, spermatocytes and round spermatids (**Figure 2**). In spermatogonia (**Figure 3**) the highest *E2f4* levels are seen in clusters 2, 6, 8 and 13 (*Kit/Sycp3/Stra8*+) corresponding to differentiating spermatogonia. In round spermatids *E2f4* levels are highest in early and mid steps (**Figure 4**).

## E2f5
*E2f5* closely follows the expression pattern of *E2f4* but is clearly less expressed in spermatocytes and early round spermatids (**Figure 2**). Within the round spermatid population the highest levels are seen in mid to late steps (**Figure 4**). Expression in spermatogonial clusters is rather uniform but with highest levels in differentiating spermatogonia (**Figure 3**).

## E2f6
*E2f6* expression is restricted to spermatogonia and only sporadically detected elsewhere (**Figure 2**). Within the spermatogonial compartment *E2f6* is enriched in clusters that are also positive for *Gfra1* (1, 3, 5 and 10) and thus potential GSCs (**Figure 3**).

## E2f7-8
Spermatogenic expression for *E2f7* and *E2f8* is low. Late spermatocytes and/or early round spermatids show limited *E2f8* expression (**Figure 2**).

## Protein Expression in Rodent Testis
The protein expression of all RB and most E2F family members have been studied in a handful of articles. The expression pattern for each protein is summarized in **Figure 5**.

**FIGURE 5** | Expression of RB and E2F family proteins in spermatogenic cells and Sertoli cells. Mitotic/premeiotic germ cells are divided into type A (A), type Intermediate (Int) and type B (B) spermatogonia. Meiotic germ cells are presented as preleptotene (Pl), leptotene (L), zygotene (Z), early and late pachytene (P) and diplotene (D) spermatocytes, and secondary spermatocytes, m2m. Post-meiotic germ cells are round spermatids (RS) and elongating spermatids (STD).

## pRB (RB1)

In the mouse testis Nalam et al. (2009) reported the staining of pRB in Sertoli cells and spermatogonia (44), while Rotgers et al. (2014) observed the same but extended the staining to preleptotene spermatocytes (45). In the rat testis Yan et al. (2001) confirmed pRB staining in Sertoli cells and spermatogonia but also showed that the highest levels of Sertoli cell pRB in seminiferous epithelial stages VII-VIII (46). The protein expression of pRB in spermatogenic cells is to some extent in conflict with mRNA level findings (**Figure 2**), and suggests that *pRB* mRNA is not translated to protein in post-mitotic male germ cells.

## p107 (RBL1)

Rotgers et al. (2014 & 2019) reported p107 staining solely in pachytene spermatocytes in adult WT mouse (45), which is in agreement with single-cell RNA-sequencing data (**Figure 2**). In the rat testis p107 was localized to spermatogonia and spermatocytes (46).

## p130 (RBL2)

In the mouse testis Sertoli cells were found positive for p130 (45, 47). Interestingly, the authors did not mention a weak staining for p130 in spermatocytes and round spermatids that would also be supported by enrichment of *p130* mRNA in these germ cells (**Figure 2**). In the rat testis p130 protein expression is seen in Sertoli cells, Leydig cells and peritubular myoid cells, and excluded from differentiating germ cells (46).

## E2F1

The expression of E2F1 in the mouse testis has been reported only in one study. El-Darwish et al. (2006) showed the expression in Intermediate to type B spermatogonia and in leptotene to early pachytene spermatocytes (48). The highest levels of E2F1 were observed in stages IX-XI, *i.e* leptotene and zygotene spermatocytes. These findings align with mRNA expression analysis, although leave the protein expression in type A

spermatogonia elusive. Based on functional analyses (discussed later in the text) it is probable that E2F1 is expressed at the protein level already in type A spermatogonia. In the rat testis the E2F1 staining differs slightly with type A spermatogonia already expressing it, and the expression extending to mid-to-late pachytene spermatocytes (48).

## E2F2

El-Darwish et al. (2006) also studied the localization of E2F2 in rodent testis. In the rat testis E2F2 is most highly expressed in pachytene and diplotene spermatocytes of stages VII-XIII, but also in secondary spermatocytes (48). In the mouse the results show abundant E2F2 staining in nearly all mouse testicular cell types, excluding condensing spermatids. Compared to the very restricted pattern of expression in the rat and the scanty mRNA expression in mouse (**Figure 2**) the results concerning the staining in the mouse would require further investigation and validation.

## E2F3

The data concerning E2F3 expression in the mouse testis is contradictory. While the expression in Sertoli cells is supported by all available data (45, 47, 48), germ cell expression remains somewhat elusive. While El-Darwish et al. (2006) and Rotgers et al. (2014) reported E2F3 staining in spermatogonia and preleptotene spermatocytes (45, 48), which is compatible with mRNA expression (**Figure 2**), Rotgers et al. (2019) did not confirm spermatogonial staining with E2F3a-specific or pan-E2F3 antibodies in the adult mouse testis (47). Isoform unspecific E2F3 antibody, however, did stain spermatogonia in the juvenile testis. In the adult rat testis E2F3 expression was seen in Sertoli cells and spermatogonia (48).

## E2F4

Strong E2F4 staining is seen in the nuclei of testicular somatic cells: Sertoli cells, Leydig cells and peritubular myoid cells both in

mouse and rat (48). The authors speculate though, that only the Sertoli cell staining is specific because parallel staining of *E2f4* KO mouse testis sections show that only Sertoli staining was absent. Interestingly, there is a granular staining for E2F4 from spermatocytes to elongated spermatids both in mouse and rat.

## E2F5

E2F5 is expressed from type Intermediate spermatogonia to mid-Pachytene spermatocytes in the rat testis (48). To our knowledge E2F5 localization in the mouse testis has not been studied.

As far as we know there are no studies where the localization of E2F6, E2F7 or E2F8 in rodent testis would have been studied.

# FUNCTIONAL CONSEQUENCES OF RB-E2F PATHWAY LOSS-OF-FUNCTION IN THE TESTIS

## pRB Is Essential for Establishment and Maintenance of GSCs

The fact that *pRB*-null mice are embryonically lethal (49–51) has complicated the study of pRB's tissue-specific functions. In the testicular context, however, this limitation has been circumvented by creation of cell type-specific conditional KO mice (44, 45, 52–54) and *ex vivo* culture (55). Of note, *p107*- and *p130*-deficient mice are viable, healthy and fertile (56, 57). We will first focus on the effects of germ cell-specific ablation of pRB and then summarize the role of pRB in Sertoli cells. The available literature indicates that pRB is indispensable for formation and maintenance of germline stem cells (GSCs). Lifelong ability of sperm production depends on GSCs, whose cell fate decisions need to be tightly balanced in order to maintain high and continuous production of sperm throughout the reproductive life span. In theory, GSCs must undergo both self-renewal divisions to sustain the GSC pool and differentiation divisions to give rise to transit-amplifying progenitor spermatogonia that are destined to complete spermatogenesis. Mechanistically it is not entirely clear to what extent these cell fate decisions are cell-autonomous and how/when the molecular cues in the GSC niche microenvironment are incorporated into the regulation of these events. The stem cell capacity in the mouse testis is thought to reside in a subset of undifferentiated spermatogonia ($A_{undiff}$), although in regenerative conditions $A_{undiff}$ display functional plasticity suggesting that the number of potential stem cells markedly exceeds that of steady-state stem cells (58, 59).

Given its role as the gatekeeper of G1/S transition, or quiescence vs. proliferation, pRB is understandably a critical regulator of GSCs. The evidence for this comes from various mouse models where *pRB* has been conditionally deleted from germ cells at different ages: prenatally from primordial germ cells (*pRB*-KO$^{Blimp1}$) (53) or ED15.5-17 gonocytes (*pRB*-KO$^{Ddx4}$) (52, 54), and postnatally from progenitor-$A_{undiff}$ (*pRB*-KO$^{Ngn3}$) (54) and progenitor/differentiating spermatogonia (*pRB*-KO$^{Stra8}$) (54). Contrary to its role in somatic stem cells where inactivation of RB family proteins often results in stem cell expansion, increased apoptosis, altered cell fate/differentiation

defects, and initiation of cancer (60), pRB-deficient male GSCs have been reported to lose their capacity to self-renew, possibly explaining why no testicular tumors were observed (52, 54). Notably, the differentiation capacity of *pRB*-null germ cells was not affected but young adult males (until 2-3 months of age) were able to sire offspring. However, due to gradual depletion of GSCs they became infertile with age (52, 54).

Despite the fact that pRB is expressed very early in the germ lineage, its absence does not seem to have any effects on fetal germ cells, termed gonocytes (or prospermatogonia), before ED14.5 (53, 55) which is the time when WT gonocytes start to enter mitotic quiescence (61). Recently, Du et al. (2021) discovered that at ED16.5, when practically all control gonocytes had stopped proliferating, the majority of pRB-KO$^{Blimp1}$ were still engaged in the cell cycle resulting in two-fold higher number of germ cells at the same time point. Subsequently, there is a massive wave of apoptosis in pRB-KO$^{Blimp1}$ testes that ablates the germline by the time of onset of the first round of spermatogenesis (PND3.5-6.5) (53). Interestingly, in this time window the classical effects of pRB-deficiency are also recapitulated in the germline: mitotic overexpansion followed by increased apoptosis. It is not exactly clear what induces programmed cell death in practically all pRB-KO$^{Blimp1}$ germ cells perinatally, but there are a number of factors that might contribute to it, including a failure of pRB-KO$^{Blimp1}$ gonocytes to inhibit the onset of meiosis, a demarcating feature of all fetal male germ cells (53). It has been well documented that the gonocyte population experiences a wave of apoptosis between ED13.5 and ED17.5 (62, 63), which is considered to eradicate genetically or epigenetically defective male germ cells that are developmentally incompetent (64). Considering that the cellular functions of pRB are not limited to cell cycle regulation, it is likely that the reason for germline ablation in pRB-KO$^{Blimp1}$ mice is due to failure of pRB-deficient gonocytes to pass this developmental quality control check-point.

Perinatal testicular development in *pRB*-KO$^{Ddx4}$ mice (*pRB* deleted in ED15.5-17 gonocytes) is apparently normal although the number of spermatogonia in the pre/peripubertal testis is higher than in control mice (52). Despite a previously established role for pRB in control of gonocyte cell cycle exit before birth (55), this is not due to extended proliferation of gonocytes and *pRB*-KO$^{Ddx4}$ mice are born with a normal number of germ cells (52), suggesting that conditional deletion of *pRB* at the time of gonocyte quiescence, does not disrupt this state. Notably, and in contrast to pRB-KO$^{Blimp1}$ mice, the GSC pool is formed in pRB-KO$^{Ddx4}$ mice because GFRa1-positive (GDNF family receptor alpha 1) cells are observed at the basement membrane of the seminiferous epithelium (52). These GSCs, however, show very limited, if any, self-renewal capacity (52, 54). Although it has not been studied whether the GSC niche forms properly in pRB-KO$^{Ddx4}$ mice, it is conceivable that inability to self-renew is likely GSC-intrinsic rather than due to extrinsic factors, such as lack of GDNF (glial cell line-derived neurotrophic factor) (36, 65). Hu et al. (2013) also report that pRB-KO$^{Ddx4}$ GSC exit from self-renewal is followed by expansion of progenitor-$A_{undiff}$ population (52). This is supported by increased density of

spermatogonia in peripubertal mice but suffers some limitations because the authors use PLZF (promyelocytic leukemia zinc finger) as a marker of $A_{undiff}$, while PLZF is also expressed in differentiating spermatogonia (66, 67). Nonetheless, these data support that pRB regulates the timely and stage-dependent cell cycle entry and exit in spermatogonia.

If pRB is ablated in progenitor-$A_{undiff}$ or differentiating spermatogonia, as in $pRB$-KO$^{Ngn3}$ and $pRB$-KO$^{Stra8}$ mice, there are no effects on male fertility, but the testes of respective mice are often cystic and have areas that are devoid of spermatogenic cells, and sometimes, as Yang et al. (2013) report, house cells with neoplastic features (54). These results suggest that the consequences of pRB-deficiency in differentiation-committed and differentiating germ cells and all subsequent spermatogenic cell types are significant but possibly compensated by other RB family members, as also suggested by **Figure 2**, given the continuation of qualitatively normal spermatogenesis in adult $pRB$-KO$^{Ngn3}$ and $pRB$-KO$^{Stra8}$ mice.

To summarize, the spermatogenic phenotype that germ cell-specific $pRB$ deletion inflicts highly depends on the developmental stage where the deletion has been induced. While $pRB$-KO$^{Blimp1}$ mice are sterile, pRB-KO$^{Ddx4}$ mice undergo 1-2 rounds of spermatogenesis, and both $pRB$-KO$^{Ngn3}$ and $pRB$-KO$^{Stra8}$ mice display qualitatively normal spermatogenesis and are fertile (52–54). These data demonstrate that pRB is a critical regulator of formation and maintenance of GSCs but dispensable for spermatogenic differentiation of their progeny.

## RB-E2F3 in Sertoli Cell Maturation

In addition to germ cells, the developmental and functional consequences of pRB ablation have also been studied in Sertoli cells (44, 45). Sertoli cells are the only somatic cell type inside the seminiferous tubules and they are paramount to testicular development, function and spermatogenesis (68). It is considered that the number of Sertoli cells ultimately defines sperm production capacity because each Sertoli cell is able to support a finite number of spermatogenic cells (69). Therefore, any factors that impinge on Sertoli cell proliferation or apoptosis are a potential threat to male fertility. Sertoli cells are specified in the mouse XY gonad following the expression *Sry* (sex-determining region Y) and its most significant downstream target gene *Sox9* (SRY-box 9) at ED10.5-11 (61). Sertoli cells then coordinate the differentiation of all other testicular cell types, including the germ cells. Sertoli cells undergo maturation in pubertal testis involving polarization, formation of the blood-testis barrier, a profound change in the transcriptome/proteome and exit from the cell cycle. Notably, Sertoli cells of an adult mouse do not proliferate but are in a seemingly terminally-differentiated state. However, under specific *in vitro* circumstances they are able to resume the cell cycle (70, 71). As discussed in detail below, similar to many other somatic cells, but not germ cells, Sertoli cells fail to enter a functionally mature mitotically-quiescent state if lacking pRB (44, 45, 52, 60).

The consequences of Sertoli cell-specific ablation of pRB have been explored in three studies, all of which relied on Cre expression under *Anti-Müllerian hormone* promoter (*pRB*-KO$^{Amh}$) resulting in pRB loss of function from ED14.5 (44, 45,

47). The studies show congruent results: pRB-deficiency leads to rapid testicular atrophy and male infertility. However, while the gene expression is misregulated at least as early as in PND10 testis, testicular development and onset of spermatogenesis in juvenile mice appear unaffected in $pRB$-KO$^{Amh}$ mice, and until 6-weeks of age there are no obvious changes in testicular histology (44, 45). Subsequently, the changes in the phenotype are fast and the male mice become infertile by early adulthood. The testicular phenotype of $pRB$-KO$^{Amh}$ mice also includes Sertoli cell vacuolization, sloughing of Sertoli cells and immature germ cells from the seminiferous epithelium, increased germ cell apoptosis and Leydig cell hyperplasia (44, 45, 47). Interestingly, this might present an evolutionarily conserved function of RB proteins given a comparable phenotype in *Drosophila* following somatic deletion of *Rbf* (a pRB homolog) (72, 73).
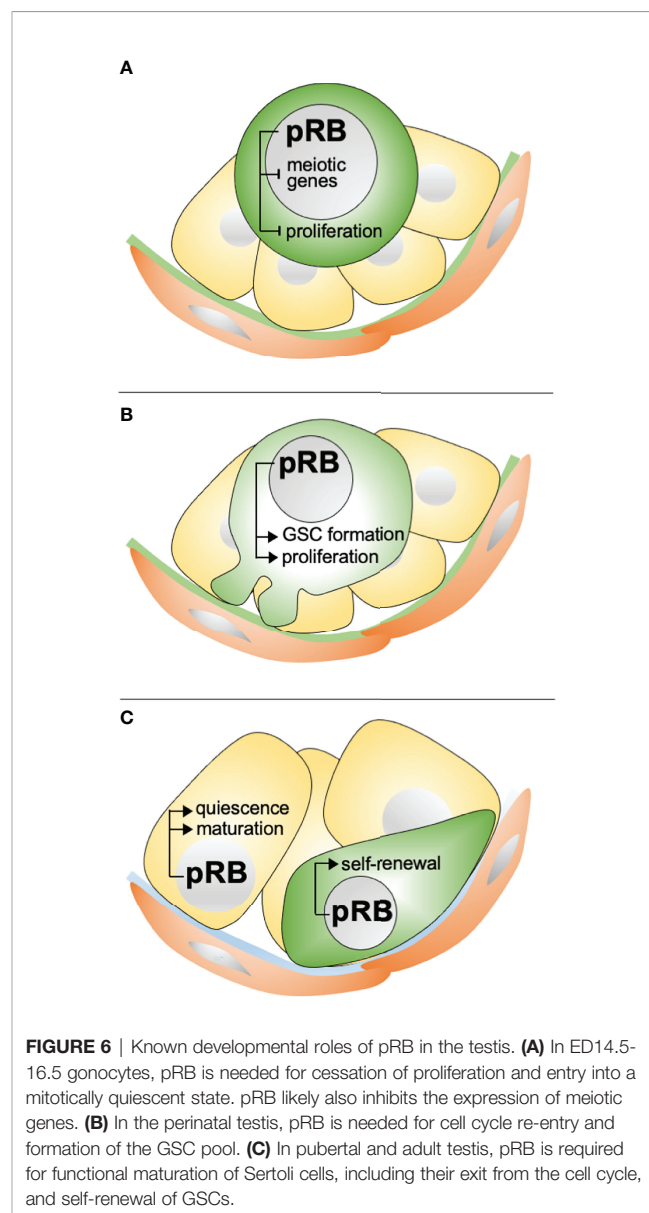
**FIGURE 6 |** Known developmental roles of pRB in the testis. **(A)** In ED14.5-16.5 gonocytes, pRB is needed for cessation of proliferation and entry into a mitotically quiescent state. pRB likely also inhibits the expression of meiotic genes. **(B)** In the perinatal testis, pRB is needed for cell cycle re-entry and formation of the GSC pool. **(C)** In pubertal and adult testis, pRB is required for functional maturation of Sertoli cells, including their exit from the cell cycle, and self-renewal of GSCs.

There are a few remarkable changes in pRB-ablated Sertoli cell physiology that have a common nominator: inability to mature functionally. First of all, while WT Sertoli cells exit the cell cycle at around PND15, $pRB$-KO$^{Amh}$ Sertoli cells continue to proliferate at a constant rate at least until PND30 (45). This is followed by increased apoptosis probably due to activation of p53 pathway, thus precluding tumor formation (44). While the likely reason for spermatogenic failure in these mice is complex and multifactorial [loss of BTB (blood-testis barrier) integrity, apoptosis of pRB-deficient Sertoli cells to nurture and maintain spermatogenic cells etc.] it has not been carefully studied at which step spermatogenesis fails and whether functional GSCs were sustained in adult $pRB$-KO$^{Amh}$ mice. Considering the critical cell-autonomous role of pRB in GSCs, as described above, this is an interesting question and would deserve further investigation. Given the timeline of male infertility in these mice [2-3 months (44) and 3-6 months (45)] it is conceivable that GSCs are able to undergo at least some rounds of self-renewal in these conditions. This is also supported by qPCR data showing reduced but measurable levels of GSC-associated transcripts: $Gfra1$ and $Lin28$ (47). However, $Pou5f1$ ($Oct4$) expression is lost in $pRB$-KO$^{Amh}$ testis suggesting a loss of progenitor-A$_{undiff}$, where $Pou5f1$ expression is enriched (36).

Interestingly, according to Rotgers et al. (2014) the BTB first organizes normally in 6-week old $pRB$-KO$^{Amh}$ mice but then disintegrates (45). However, the data from Nalam et al. (2009) indicate that the BTB is not functional even at this early time point and allows the leakage of a tracer from the interstitium to the adluminal compartment of seminiferous tubules, an immune-privileged site in normal testis (44). These findings suggest that pRB is not developmentally needed for formation of the BTB but is indispensable for its integrity and maintenance, which is another sign of Sertoli cell dedifferentiation and immaturity in $pRB$-KO$^{Amh}$ mice. Interestingly, Sertoli cells deficient for pRB display seminiferous epithelial cyclic activity, as judged by fluctuation of androgen receptor (AR) staining intensity between seminiferous tubule cross-sections (47). AR is one of the proteins that are known to be expressed in Sertoli cells in a seminiferous epithelial stage-dependent manner with the highest levels in early-to-mid stages and lowest levels in late stages (74, 75).

In WT testis pRB interacts with E2F3, the only activator E2F that is expressed in Sertoli cells (45). $E2f3$ gene encodes two isoforms, $E2f3a$ and $E2f3b$, both of which are expressed in the mouse testis. Interestingly, while Sertoli cells also express p130, Rotgers et al. (2014) do not confirm an interaction between E2F3 and p130 in WT testis. In $pRB$-KO$^{Amh}$ testis, however, E2F3 is found to form a complex with both p130 and p107, whose expression is induced in pRB-deficient Sertoli cells (45). This is in agreement with previous findings showing that when pRB is absent p107 and p130 also bind to activator E2Fs (E2F1-3), not just the repressors (E2F4-5) (76). While these findings imply that there might be a compensatory mechanism, the severity of the phenotype indicates that such a mechanism does not have any functional relevance. Already in their original paper Rotgers et al. (2014) provide data showing that impairment of spermatogenesis

due to pRB-deficiency is attenuated by simultaneous silencing of $E2f3$ in vivo (45). These results were corroborated by a follow-up study where $pRB$-$E2f3$ compound-KO$^{Amh}$ mice were created (47). These mice display full spermatogenesis but interestingly the litter size is markedly reduced when compared to control. This might be due to disturbance of non-canonical, i.e. non-E2F-mediated, pRB functions in compound-KO$^{Amh}$ mice, including chromatin organization and nuclear architecture which are pronouncedly relevant in the spermatogenic context (77, 78). While spermatogenesis is qualitatively normal in $pRB$-KO$^{Amh}$/$E2f3$-haploinsufficient mice, they display age-dependent testicular atrophy, and continuously cycling Sertoli cells, suggesting that $E2f3$ gene dosage is critical for spermatogenesis. Notably, adult Sertoli cells of $pRB$-$E2f3$ compound-KO$^{Amh}$ mice are non-proliferative and seem to be functionally comparable to their WT counterparts (47). In summary, these data show that spermatogenic failure, prolonged proliferation, increased apoptosis and dedifferentiation of Sertoli cells are mediated by adverse and non-restricted action of E2F3 in Sertoli cells of $pRB$-KO$^{Amh}$ mice, and highlights the role of pRB as a key regulator in life-long maintenance of the non-renewable Sertoli cell population.

Considering the spermatogenic rescue experiments by simultaneous deletion (47) or shRNA-mediated in vivo knock-down of $E2f3$ (45) on a $pRB$-KO$^{Amh}$ background, it is surprising that $E2f3$-KO$^{Amh}$ mice, where $E2f3$ has been deleted from fetal Sertoli cells, are fertile and show no signs of impaired spermatogenesis (47). The authors hypothesize that E2F4 might fully compensate for lack of E2F3 in these mice. While this might be possible there are, however, at least two obvious pitfalls in this interpretation. Firstly, E2F4 is considered a transcriptional repressor, whereas E2F3a isoform is classically considered a transcriptional activator. Secondly, E2F4 primarily associates with p107 and p130, not with pRB. Thus, further investigations are warranted to address this question. Results from these studies further support the concept that Sertoli cells are not terminally-differentiated but in a state of continuous cell cycle repression (79), and show that pRB is responsible for maintaining this non-proliferative state. These conclusions, however, suffer some limitations because pRB is ablated already in fetal Sertoli cells. An inducible $pRB$-deletion in adult Sertoli cells would be required to further elucidate this question. The developmental roles of pRB in the testis are summarized in **Figure 6**.

## pRB Is Not Involved in Testicular Tumorigenesis

To our knowledge, there are only very limited data available supporting a relationship between pRB-deficiency and increased tumorigenesis in the testis. Targeted deletion of $pRB$ from prenatal germ cells results in germline ablation and infertility (44, 53, 54), and focal GCNIS-like (germ cell neoplasia in situ) like cells are only observed when $pRB$ is deleted from progenitors and/or differentiating spermatogonia, arguably due to inability to commit to the spermatogenic lineage (54), an interpretation that might have some merit given that progenitor-A$_{undiff}$ are enriched

for pluripotency-associated transcripts (36). Ablation of pRB in Sertoli cells leads to continued engagement in the cell cycle but also increased apoptosis thus precluding tumor formation (44). Interestingly, Nalam et al. (2010) also studied the compound effects of *pRB* ablation on an inhibin-α-knockout (*Inha* KO) background (80). *Inha* KO mice are known to develop gonadotropin-dependent gonadal stromal tumors, which originate from Sertoli cells (81). Unexpectedly, the double-KO mice did not display exacerbation of the tumorigenic phenotype but Sertoli cell dysfunction took place earlier in these mice when compared to *pRB*-KO$^{Amh}$ mice (80). In summary, there are no strong indications that pRB would have tumor suppressor properties in the mouse testis.

## E2F1 Has a Multifaceted Role in the Testis

Similar to other tissues and cellular contexts, E2F1's role in the testis has deserved most attention within the E2F family. E2F1 transcription factor is a critical regulator of the cell cycle, and a direct target of pRB. However, like all activator E2Fs, E2F1 in particular displays intriguing functional dichotomy and is able to induce both proliferation and apoptosis (13). This is apparent also in the testis. E2F1 has been associated with progressive degeneration of the seminiferous epithelium (43, 82–84), GSC maintenance (43, 84), apoptosis of germ cells (43, 85) and Sertoli cells (44), germ cell neoplasia *in situ* (86), testicular tumors (82), testicular descent in human (87, 88) and mouse (84), and human male infertility (87, 88).

Our understanding of E2F1's testicular function comes primarily from mouse work and use of *E2f1$^{tm1Meg}$* mouse strain (89) (referred to *E2f1*-null hereafter). *E2f1*-null mice display characteristics of progressive testicular atrophy that is manifested in a background strain-dependent manner implying that genetic background of the mice has an impact on the time course and severity of the phenotype (43, 82–84, 89). Interestingly, on C57Bl/6 background the first signs of spermatogenic impairment can be observed as early as 20 days of age when the number of meiotic cells is reduced in *E2f1*-null mice when compared to control (43). E2F1 is an early regulator of the spermatogonial compartment and likely promotes spermatogonial apoptosis during the first wave of spermatogenesis (43). Somewhat counterintuitively, loss of E2F1 does not result in accumulation of spermatogonia during steady-state spermatogenesis but their gradual depletion and occurrence of tubules with Sertoli-cell-only phenotype (43, 82, 84). While the underlying mechanisms are not fully understood it is likely that GSCs in *E2f1*-null mice are lost *via* loss of self-renewal capacity and escape to differentiation. The phenotype thus shares remarkable but limited resemblance to pRB-KO$^{Ddx4}$ mice (52). While *E2f1*-null males remain fertile for at least 5-6 months (43, 82, 84), pRB-KO$^{Ddx4}$ mice become infertile by 2-3 months (52, 54). This is a significant difference because it shows that GSCs are able to self-renew in *E2f1*-null but not in pRB-KO$^{Ddx4}$ mice.

While the exact explanation for the discrepancy in time course is yet to be discovered, there are a couple of likely explanations: compensation by other E2F activators and non-E2F-mediated effects of pRB (discussed above). Notably, apart

from *E2f1* no other *E2f*s were differentially expressed in *E2f1*-null testis casting doubts for compensation at the transcriptional level (43), yet leaving the door open for post-transcriptional mechanisms. Interestingly, when human E2F1 is overexpressed in the mouse testis, increased germ cell apoptosis is observed, accompanied with accumulation of undifferentiated spermatogonia (85), thus parallelling GDNF or NANOS2 overexpression phenotype (65, 90). Together these data demonstrate that E2F1 is a critical regulator of GSC maintenance and germ cell apoptosis. Although the definitive data are missing, as how E2F1 might be involved in GSC fate decisions, the literature provides some clues about the potential mechanisms. Transcriptomic changes in *E2f1*-null testis have been studied at 20 days of age by Rotgers et al. (2015) (43), and 3 and 7 months of age by Jorgez et al. (2021) (84). However, choice of the time point and method of study (microarray vs. qPCR) makes the results of these studies hard to compare. While the histological differences in a 20-day-old *E2f1*-null and WT mouse testis are relatively small making comparison between the genotypes feasible at this time point, the transcriptome at this age is different from adult testis with full spermatogenesis which complicates the comparison between the time points. Then again, the cellularity already in a 3-month-old and particularly in a 7-month-old *E2f1*-null testis is dramatically different from WT testis thereby complicating the analysis. For these reasons we will look at the findings separately.

Rotgers et al. (2015) identify a couple of E2F1 candidate target genes in a 20-day old mouse testis that, based on available studies, might be relevant for the development of the phenotype: *Cnot1 (CCR4-NOT Transcription Complex Subunit 1)* and *Chd1* (Chromodomain Helicase DNA Binding Protein 1) both of which are downregulated in *E2f1*-null testes (43). CNOT1 is particularly interesting because it directly interacts with NANOS2, a key intrinsic regulator of the male germline (90, 91). NANOS2 is involved in both degradation and sequestration of specific mRNA molecules in ribonucleoprotein complexes, and its loss results in GSC depletion and rapid germline ablation (59, 90–93). CNOT1 mediates NANOS2 interaction with CCR4-NOT, a major cytoplasmic deadenylase that primes mRNAs for degradation (91, 93). Importantly, CCR4-NOT–CNOT1–NANOS2 interaction is critical for NANOS2 function and maintenance of the male germline (91), a plausible mechanism also explaining GSC depletion in *E2f1*-null mice. Another interesting E2F1-candidate gene discovered by the microarray analysis is *Chd1*. While its role in spermatogenesis has not been studied, two of its family members CHD4 and CHD5 are critical for sperm production, albeit at the opposite ends of this complex process. CHD4 has been shown to be highly expressed in GSCs where its involved in their maintenance and self-renewal (94), whereas CDH5 is required for spermiogenesis and especially for chromatin condensation in elongating spermatids (95, 96).

Jorgez et al. (2021) studied the expression of select 66 mRNAs by qPCR and subsequent proteomic analyses (84). They found changes in the expression of cyclin genes and other E2F1 cell cycle targets. Interestingly, Wnt signaling pathway genes were differentially expressed between the genotypes, and all studied

Wnt ligands were found generally upregulated, especially WNT4. Wnt signaling has been thought to prime GSCs for differentiation and promote exit from the self-renewing state (97–101). Boyer et al. (2012) reported that treatment of cultured GSCs with WNT4 reduces their stemness both *in vitro* and after transplantation (100). These data would support a model where GSCs in *E2f1*-null testis are subjected to differentiation-priming environment at the expense of self-renewal, thus resulting in their depletion over time. To explore this possibility Jorgez et al. (2021) generated a compound *E2f1*-null/*Wnt4*-null mouse line, where *Wnt4* was conditionally deleted from *Stra8*-expressing germ cells, that is progenitor-A$_{undiff}$/differentiating spermatogonia. Remarkably, the spermatogenic capacity is qualitatively restored in these mice and they display nearly normal fertility parameters, demonstrating that many of the adverse effects of E2F1-deficiency can be overcome by simultaneous removal of WNT4. While the above findings provide a mechanism for GSC loss in *E2f1*-null mice, further studies are needed to address how the RB-E2F pathway is integrated in the control of GSC fate in WT mice, and how is it mechanistically linked with GDNF signaling (36, 65, 102) and mTOR (mammalian target of rapamycin) pathway (67, 103, 104) to balance self-renewal and differentiation of GSCs.

A typical feature of spermatogenic impairment when E2F1 is either deleted or over-expressed is spermatocyte apoptosis (43, 82, 85). While this leads to subfertility, *E2f1*-null male mice are able to sire viable offspring because some spermatocytes manage to avoid cell death. The reason for *E2f1*-null spermatocyte apoptosis is not known but there is data to support that DNA damage is not the underlying cause (43). Further studies are needed to unveil what induces spermatocyte apoptosis if E2F1 is either absent or overexpressed. Considering the expression pattern of E2F1 (highly expressed in premeiotic cells and early spermatocytes; **Figures 2, 5**) and its role as a transcriptional activator, it is conceivable that the expression of E2F1-target genes needs to be delicately balanced for successful meiosis. It is likely that spermatocyte apoptosis is induced in a cell-intrinsic manner, also considering the fact that despite being expressed in peritubular cells and in the testicular interstitium, E2F1-deficiency was not found to affect testicular somatic cells or gonadotropin or androgen levels (43, 84). However, albeit not

known to express E2F1, the Sertoli cells of *E2f1*-null mice display some transcriptomic changes when compared to controls. Surprisingly, while some of the changes may be explained by a change in cellularity in *E2f1*-null vs. control mice testis (43), misregulation of genes involved in tight and adherens junctions were shown to have functional consequences and the blood-testis barrier of 3-month-old *E2f1*-null mice was found leaky (84).

**Table 1** provides a summary of the spermatogenic phenotypes of different KO mouse strains where one or two genes encoding proteins of RB-E2F pathway has been deleted.

## Repressor E2Fs E2F4 and E2F5 Are Needed for the Development of Male Reproductive Tract

Compared to activator E2Fs, the functions of repressor E2Fs have been less investigated. While both *E2f4* and *E2f5*-deficient mice are viable, they have a shortened lifespan (105–107). *E2f4*-mutants display developmental defects in multiple tissues and have a high early postnatal lethality due to susceptibility to infections (106, 107). *E2f4*-deficient male mice were found subfertile/infertile although the histology of male reproductive organs was reported normal (106, 107), and the underlying basis for this observation therefore remains elusive. There is a lack of knowledge concerning fertility of *E2f5*-mutant mice. However, *e2f5* mutation in the zebrafish results in male infertility due to a spermatogenic arrest in prophase I, and subsequent apoptosis of spermatocytes (108). Rotgers et al. (2014, 2019) report a clear decrease in *E2f4* and *E2f5* expression in *pRB*-KO$^{Amh}$ mouse testis which is, however, likely due to lack of meiotic and postmeiotic germ cells (cf. **Figure 2**). Interestingly, E2F4 and E2F5 have been shown to display redundant roles in controlling the development of the male reproductive tract. *E2f4*-deficiency within the efferent ducts on a *E2f5* heterozygous background leads to a loss of multiciliated cells from the efferent ducts, dilation of the seminiferous tubules and the rete testis, and infertility.

The other repressor E2Fs have been even less studied in the testis. *E2f6*-deficient mice are born at an expected Mendelian frequency, are viable and fertile, and grow and develop normally (109). Pohlers et al. (2005) have reported that E2F6 is needed to suppress the expression of germline genes in somatic tissues (109).

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**TABLE 1 |** Summary of spermatogenic phenotypes in different mouse strains with a deletion in gene(s) encoding RB-E2F pathway proteins.

| Mouse strain | Time of deletion | Lineage where deleted | Normal establishment of the GSC pool | Qualitatively/Quantitatively normal 1st round of spermatogenesis | Qualitatively/Quantitatively normal spermatogenesis in adulthood | Ref |
|---|---|---|---|---|---|---|
| *pRB*-KO$^{Blimp1}$ | ED6.5 | germline | No | No/No | No/No | (53) |
| *pRB*-KO$^{Ddx4}$ | ED15.5-17 | germline | n.a. | Yes/No | No/No | (52, 54) |
| *pRB*-KO$^{Ngn3}$ | postnatal | germline | Yes | Yes/n.a | Yes/No | (54) |
| *pRB*-KO$^{Stra8}$ | postnatal | germline | Yes | Yes/n.a | Yes/No | (54) |
| *pRB*-KO$^{Amh}$ | ED14.5 | Sertoli | Yes | Yes/Yes | No/No | (44, 45) |
| *E2f3*-KO$^{Amh}$ | ED14.5 | Sertoli | Yes | Yes/Yes | Yes/Yes | (47) |
| *pRB*-KO$^{Amh}$/*E2f3*-KO$^{Amh}$ | ED14.5 | Sertoli | Yes | Yes/n.a | Yes/No | (47) |
| *pRB*-KO$^{Amh}$/*E2f3*$^{+/-}$ | ED14.5 | Sertoli | Yes | Yes/n.a | Yes/No | (47) |
| *E2f1*$^{tm1Meg}$ | zygotic | universal | Yes | Yes/No | No/No | (43, 84) |
| *E2f1*$^{tm1Meg}$/*Wnt4*-KO$^{Stra8}$ | zygotic/ postnatal | universal/ germline | Yes | Yes/n.a | Yes/No | (84) |

*n.a, not analyzed.*

However, the significance or functions of E2F6 in the germline cells are not known but mRNA level analysis (**Figure 2**) suggests they might be subtle. No fertility problems have been reported in mice deficient for *E2f7* or *E2f8*, whereas the double-null mice are early embryonically lethal precluding any analysis on germline effects (110).

## CONCLUDING REMARKS

Considering its role as the gatekeeper of cell cycle, it is no wonder that the RB-E2F pathway is critically important for development and function of the testis. Both the germline and the somatic Sertoli cells depend on proper regulation of its activity during formation of the testis and under steady-state spermatogenesis. Continuous production of sperm relies on lifelong maintenance of GSCs and functionally mature Sertoli cells, two fundamental outcomes of normal function of RB-E2F pathway, highlighting its importance for male fertility. Despite the fact that the functional consequences of pRB, E2F1 and E2F3-deficiency in the testis have been relatively well characterized, further studies are warranted to elucidate how RB-E2F is integrated into the regulation of the germline at the mechanistic level plus shed light on the diversity of RB-E2F signaling beyond pRB and E2F1 and E2F3. Notably,

despite relatively high level of mRNA expression in germ cells, the spermatogenic functions of p107, p130, E2F4 and E2F5 are virtually undefined. Examining their roles would probably require creation of compound inducible KO mouse models to circumvent functional redundancy within both the RB and E2F families, and for targeted analyses of spermatogenic functions. Their temporally restricted patterns of expression along the spermatogenic trajectory are intriguing and worthy of deeper investigation.

## AUTHOR CONTRIBUTIONS

J-AM and JT: Conception of the article, writing and editing. All authors contributed to the article and approved the submitted version.

## ACKNOWLEDGMENTS

## REFERENCES

1. Burkhart DL, Sage J. Cellular Mechanisms of Tumour Suppression by the Retinoblastoma Gene. *Nat Rev Cancer* (2008) 8:671–82. doi: 10.1038/nrc2399

2. Dick FA, Rubin SM. Molecular Mechanisms Underlying RB Protein Function. *Nat Rev Mol Cell Biol* (2013) 14:297–306. doi: 10.1038/nrm3567

3. Munro S, Carr SM, La Thangue NB. Diversity Within the pRb Pathway: Is There a Code of Conduct? *Oncogene* (2012) 31:4343–52. doi: 10.1038/onc.2011.603

4. Rubin SM, Sage J, Skotheim JM. Integrating Old and New Paradigms of G1/S Control. *Mol Cell* (2020) 80:183–92. doi: 10.1016/j.molcel.2020.08.020

5. DeGregori J, Kowalik T, Nevins JR. Cellular Targets for Activation by the E2F1 Transcription Factor Include DNA Synthesis- and G1/S-Regulatory Genes. *Mol Cell Biol* (1995) 15:4215–24. doi: 10.1128/MCB.15.8.4215

6. Weinberg RA. The Retinoblastoma Gene and Gene Product. *Cancer Surv* (1992) 12:43–57.

7. Stevens C, La Thangue NB. E2F and Cell Cycle Control: A Double-Edged Sword. *Arch Biochem Biophys* (2003) 412:157–69. doi: 10.1016/S0003-9861(03)00054-7

8. Weijts BGMW, Bakker WJ, Cornelissen PWA, Liang K-H, Schaftenaar FH, Westendorp B, et al. E2F7 and E2F8 Promote Angiogenesis Through Transcriptional Activation of VEGFA in Cooperation With HIF1. *EMBO J* (2012) 31:3871–84. doi: 10.1038/emboj.2012.231

9. Chong J-L, Wenzel PL, Sáenz-Robles MT, Nair V, Ferrey A, Hagan JP, et al. E2f1-3 Switch From Activators in Progenitor Cells to Repressors in Differentiating Cells. *Nature* (2009) 462:930–4. doi: 10.1038/nature08677

10. Trikha P, Sharma N, Opavsky R, Reyes A, Pena C, Ostrowski MC, et al. E2f1-3 are Critical for Myeloid Development. *J Biol Chem* (2011) 286:4783–95. doi: 10.1074/jbc.M110.182733

11. Wenzel PL, Chong J-L, Sáenz-Robles MT, Ferrey A, Hagan JP, Gomez YM, et al. Cell Proliferation in the Absence of E2F1-3. *Dev Biol* (2011) 351:35–45. doi: 10.1016/j.ydbio.2010.12.025

12. Lee B-K, Bhinge AA, Iyer VR. Wide-Ranging Functions of E2F4 in Transcriptional Activation and Repression Revealed by Genome-Wide Analysis. *Nucleic Acids Res* (2011) 39:3558–73. doi: 10.1093/nar/gkq1313

13. Polager S, Ginsberg D. P53 and E2f: Partners in Life and Death. *Nat Rev Cancer* (2009) 9:738–48. doi: 10.1038/nrc2718

14. DeGregori J, Johnson DG. Distinct and Overlapping Roles for E2F Family Members in Transcription, Proliferation and Apoptosis. *Curr Mol Med* (2006) 6:739–48. doi: 10.2174/1566524010606070739

15. Cobrinik D. Pocket Proteins and Cell Cycle Control. *Oncogene* (2005) 24:2796–809. doi: 10.1038/sj.onc.1208619

16. Kitajima S, Takahashi C. Intersection of Retinoblastoma Tumor Suppressor Function, Stem Cells, Metabolism, and Inflammation. *Cancer Sci* (2017) 108:1726–31. doi: 10.1111/cas.13312

17. Attwooll C, Oddi S, Cartwright P, Prosperini E, Agger K, Steensgaard P, et al. A Novel Repressive E2F6 Complex Containing the Polycomb Group Protein, EPC1, That Interacts With EZH2 in a Proliferation-Specific Manner. *J Biol Chem* (2005) 280:1199–208. doi: 10.1074/jbc.M412509200

18. Ouseph MM, Li J, Chen H-Z, Pécot T, Wenzel P, Thompson JC, et al. Atypical E2F Repressors and Activators Coordinate Placental Development. *Dev Cell* (2012) 22:849–62. doi: 10.1016/j.devcel.2012.01.013

19. Dick FA, Dyson N. pRB Contains an E2F1-Specific Binding Domain That Allows E2F1-Induced Apoptosis to be Regulated Separately From Other E2F Activities. *Mol Cell* (2003) 12:639–49. doi: 10.1016/S1097-2765(03)00344-7

20. Cecchini MJ, Dick FA. The Biochemical Basis of CDK Phosphorylation-Independent Regulation of E2F1 by the Retinoblastoma Protein. *Biochem J* (2011) 434:297–308. doi: 10.1042/BJ20101210

21. van Harn T, Foijer F, van Vugt M, Banerjee R, Yang F, Oostra A, et al. Loss of Rb Proteins Causes Genomic Instability in the Absence of Mitogenic Signaling. *Genes Dev* (2010) 24:1377–88. doi: 10.1101/gad.580710

22. Yadav RP, Mäkelä J-A, Hyssälä H, Cisneros-Montalvo S, Kotaja N. DICER Regulates the Expression of Major Satellite Repeat Transcripts and Meiotic Chromosome Segregation During Spermatogenesis. *Nucleic Acids Res* (2020) 48:7135–53. doi: 10.1093/nar/gkaa460

23. Gonzalo S, García-Cao M, Fraga MF, Schotta G, Peters AHFM, Cotter SE, et al. Role of the RB1 Family in Stabilizing Histone Methylation at Constitutive Heterochromatin. *Nat Cell Biol* (2005) 7:420–8. doi: 10.1038/ncb1235

24. Guo J, Grow EJ, Mlcochova H, Maher GJ, Lindskog C, Nie X, et al. The Adult Human Testis Transcriptional Cell Atlas. *Cell Res* (2018) 28:1141–57. doi: 10.1038/s41422-018-0099-2

25. Green CD, Ma Q, Manske GL, Shami AN, Zheng X, Marini S, et al. A Comprehensive Roadmap of Murine Spermatogenesis Defined by Single-Cell RNA-Seq. *Dev Cell* (2018) 46:651–67.e10. doi: 10.1016/j.devcel.2018.07.025

26. Hermann BP, Cheng K, Singh A, Roa-De La Cruz L, Mutoji KN, Chen IC, et al. The Mammalian Spermatogenesis Single-Cell Transcriptome, From Spermatogonial Stem Cells to Spermatids. *Cell Rep* (2018) 25:1650–67.e8. doi: 10.1016/j.celrep.2018.10.026

27. Tan K, Song H-W, Thompson M, Munyoki S, Sukhwani M, Hsieh T-C, et al. Staged Developmental Mapping and X Chromosome Transcriptional Dynamics During Mouse Spermatogenesis. *Nat Commun* (2019) 10:1251. doi: 10.1038/s41467-019-09182-1

28. Shami AN, Zheng X, Munyoki SK, Ma Q, Manske GL, Green CD, et al. Single-Cell RNA Sequencing of Human, Macaque, and Mouse Testes Uncovers Conserved and Divergent Features of Mammalian Spermatogenesis. *Dev Cell* (2020) 54:529–47.e12. doi: 10.1016/j.devcel.2020.05.010

29. Yu X-W, Li T-T, Du X-M, Shen Q-Y, Zhang M-F, Wei Y-D, et al. Single-Cell RNA Sequencing Reveals Atlas of Dairy Goat Testis Cells. *Zoological Res* (2021) 42:401–5. doi: 10.24272/j.issn.2095-8137.2020.373

30. Qian P, Kang J, Liu D, Xie G. Single Cell Transcriptome Sequencing of Zebrafish Testis Revealed Novel Spermatogenesis Marker Genes and Stronger Leydig-Germ Cell Paracrine Interactions. *Front Genet* (2022) 13:851719. doi: 10.3389/fgene.2022.851719

31. Guo J, Nie X, Giebler M, Mlcochova H, Wang Y, Grow EJ, et al. The Dynamic Transcriptional Cell Atlas of Testis Development During Human Puberty. *Cell Stem Cell* (2020) 26:262–76. doi: 10.1016/j.stem.2019.12.005

32. Guo J, Sosa E, Chitiashvili T, Nie X, Rojas EJ, Oliver E, et al. Single-Cell Analysis of the Developing Human Testis Reveals Somatic Niche Cell Specification and Fetal Germline Stem Cell Establishment. *Cell Stem Cell* (2021) 28:764–78.e4. doi: 10.1016/j.stem.2020.12.004

33. Hara K, Nakagawa T, Enomoto H, Suzuki M, Yamamoto M, Simons BD, et al. Mouse Spermatogenic Stem Cells Continually Interconvert Between Equipotent Singly Isolated and Syncytial States. *Cell Stem Cell* (2014) 14:658–72. doi: 10.1016/j.stem.2014.01.019

34. Nakagawa T, Sharma M, Nabeshima Y, Braun RE, Yoshida S. Functional Hierarchy and Reversibility Within the Murine Spermatogenic Stem Cell Compartment. *Science* (2010) 328:62–7. doi: 10.1126/science.1182868

35. Sharma M, Srivastava A, Fairfield HE, Bergstrom D, Flynn WF, Braun RE. Identification of EOMES-Expressing Spermatogonial Stem Cells and Their Regulation by PLZF. *Elife* (2019) 8:e43352. doi: 10.7554/eLife.43352

36. La HM, Mäkelä J-A, Chan A-L, Rossello FJ, Nefzger CM, Legrand JMD, et al. Identification of Dynamic Undifferentiated Cell States Within the Male Germline. *Nat Commun* (2018) 9:2819-018-04827-z. doi: 10.1038/s41467-018-04827-z

37. Schrans-Stassen BH, van de Kant HJ, de Rooij DG, van Pelt AM. Differential Expression of C-Kit in Mouse Undifferentiated and Differentiating Type A Spermatogonia. *Endocrinology* (1999) 140:5894–900. doi: 10.1210/endo.140.12.7172

38. Pellegrini M, Filipponi D, Gori M, Barrios F, Lolicato F, Grimaldi P, et al. ATRA and KL Promote Differentiation Toward the Meiotic Program of Male Germ Cells. *Cell Cycle* (2008) 7:3878–88. doi: 10.4161/cc.7.24.7262

39. Zhou Q, Li Y, Nie R, Friel P, Mitchell D, Evanoff RM, et al. Expression of Stimulated by Retinoic Acid Gene 8 (Stra8) and Maturation of Murine Gonocytes and Spermatogonia Induced by Retinoic Acid. *Vitro Biol Reprod* (2008) 78:537–45. doi: 10.1095/biolreprod.107.064337

40. Ikami K, Tokue M, Sugimoto R, Noda C, Kobayashi S, Hara K, et al. Hierarchical Differentiation Competence in Response to Retinoic Acid Ensures Stem Cell Maintenance During Mouse Spermatogenesis. *Development* (2015) 142:1582–92. doi: 10.1242/dev.118695

41. Yuan L, JG L, Zhao J, Brundell E, Daneholt B, Höög C. The Murine SCP3 Gene is Required for Synaptonemal Complex Assembly, Chromosome Synapsis, and Male Fertility. *Mol Cell* (2000) 5:73–83. doi: 10.1016/S1097-2765(00)80404-9

42. Park M, Lee Y, Jang H, Lee O-H, Park S-W, Kim J-H, et al. SOHLH2 is Essential for Synaptonemal Complex Formation During Spermatogenesis in Early Postnatal Mouse Testes. *Sci Rep* (2016) 6:20980. doi: 10.1038/srep20980

43. Rotgers E, Nurmio M, Pietila E, Cisneros-Montalvo S, Toppari J. E2F1 Controls Germ Cell Apoptosis During the First Wave of Spermatogenesis. *Andrology* (2015) 3:1000–14. doi: 10.1111/andr.12090

44. Nalam RL, Andreu-Vieyra C, Braun RE, Akiyama H, Matzuk MM. Retinoblastoma Protein Plays Multiple Essential Roles in the Terminal Differentiation of Sertoli Cells. *Mol Endocrinol* (2009) 23:1900–13. doi: 10.1210/me.2009-0184

45. Rotgers E, Rivero-Muller A, Nurmio M, Parvinen M, Guillou F, Huhtaniemi I, et al. Retinoblastoma Protein (RB) Interacts With E2F3 to Control Terminal Differentiation of Sertoli Cells. *Cell Death Dis* (2014) 5:e1274. doi: 10.1038/cddis.2014.232

46. Yan W, Kero J, Suominen J, Toppari J. Differential Expression and Regulation of the Retinoblastoma Family of Proteins During Testicular Development and Spermatogenesis: Roles in the Control of Germ Cell Proliferation, Differentiation and Apoptosis. *Oncogene* (2001) 20:1343–56. doi: 10.1038/sj.onc.1204254

47. Rotgers E, Cisneros-Montalvo S, Nurmio M, Toppari J. Retinoblastoma Protein Represses E2F3 to Maintain Sertoli Cell Quiescence in Mouse Testis. *J Cell Sci* (2019) 132:jcs229849. doi: 10.1242/jcs.229849

48. El-Darwish KS, Parvinen M, Toppari J. Differential Expression of Members of the E2F Family of Transcription Factors in Rodent Testes. *Reprod Biol Endocrinol* (2006) 4:63. doi: 10.1186/1477-7827-4-63

49. Lee EY, Chang CY, Hu N, Wang YC, CC L, Herrup K, et al. Mice Deficient for Rb are Nonviable and Show Defects in Neurogenesis and Haematopoiesis. *Nature* (1992) 359:288–94. doi: 10.1038/359288a0

50. Clarke AR, Maandag ER, van Roon M, van der Lugt NM, van der Valk M, Hooper ML, et al. Requirement for a Functional Rb-1 Gene in Murine Development. *Nature* (1992) 359:328–30. doi: 10.1038/359328a0

51. Jacks T, Fazeli A, Schmitt EM, Bronson RT, Goodell MA, Weinberg RA. Effects of an Rb Mutation in the Mouse. *Nature* (1992) 359:295–300. doi: 10.1038/359295a0

52. Hu YC, de Rooij DG, Page DC. Tumor Suppressor Gene Rb is Required for Self-Renewal of Spermatogonial Stem Cells in Mice. *Proc Natl Acad Sci U. S. A.* (2013) 110:12685–90. doi: 10.1073/pnas.1311548110

53. Du G, Oatley MJ, Law NC, Robbins C, Wu X, Oatley JM. Proper Timing of a Quiescence Period in Precursor Prospermatogonia is Required for Stem Cell Pool Establishment in the Male Germline. *Development* (2021) 148:dev194571. doi: 10.1242/dev.194571

54. Yang Q-E, Gwost I, Oatley MJ, Oatley JM. Retinoblastoma Protein (RB1) Controls Fate Determination in Stem Cells and Progenitors of the Mouse Male Germline. *Biol Reprod* (2013) 89:113. doi: 10.1095/biolreprod.113.113159

55. Spiller CM, Wilhelm D, Koopman P. Retinoblastoma 1 Protein Modulates XY Germ Cell Entry Into G1/G0 Arrest During Fetal Development in Mice. *Biol Reprod* (2010) 82:433–43. doi: 10.1095/biolreprod.109.078691

56. Cobrinik D, MH L, Hannon G, Mulligan G, Bronson RT, Dyson N, et al. Shared Role of the pRB-Related P130 and P107 Proteins in Limb Development. *Genes Dev* (1996) 10:1633–44. doi: 10.1101/gad.10.13.1633

57. Lee MH, Williams BO, Mulligan G, Mukai S, Bronson RT, Dyson N, et al. Targeted Disruption of P107: Functional Overlap Between P107 and Rb. *Genes Dev* (1996) 10:1621–32. doi: 10.1101/gad.10.13.1621

58. de Rooij DG. The Nature and Dynamics of Spermatogonial Stem Cells. *Development* (2017) 144:3022–30. doi: 10.1242/dev.146571

59. Makela J-A, Hobbs RM. Molecular Regulation of Spermatogonial Stem Cell Renewal and Differentiation. *Reproduction* (2019) 158:R169–87. doi: 10.1530/REP-18-0476

60. Sage J. The Retinoblastoma Tumor Suppressor and Stem Cell Biology. *Genes Dev* (2012) 26:1409–20. doi: 10.1101/gad.193730.112

61. Mäkelä JA, Koskenniemi JJ, Virtanen HE, Toppari J. Testis Development. *Endocr Rev* (2019) 40:857–905. doi: 10.1210/er.2018-00140

62. Coucouvanis EC, Sherwood SW, Carswell-Crumpton C, Spack EG, Jones PP. Evidence That the Mechanism of Prenatal Germ Cell Death in the Mouse is Apoptosis. *Exp Cell Res* (1993) 209:238–47. doi: 10.1006/excr.1993.1307

63. Wang RA, Nakane PK, Koji T. Autonomous Cell Death of Mouse Male Germ Cells During Fetal and Postnatal Period. *Biol Reprod* (1998) 58:1250–6. doi: 10.1095/biolreprod58.5.1250

64. Nguyen DH, Soygur B, Peng S-P, Malki S, Hu G, Laird DJ. Apoptosis in the Fetal Testis Eliminates Developmentally Defective Germ Cell Clones. *Nat Cell Biol* (2020) 22:1423–35. doi: 10.1038/s41556-020-00603-8

65. Meng XJ, Lindahl M, Hyvonen ME, Parvinen M, de Rooij DG, MW H, et al. Regulation of Cell Fate Decision of Undifferentiated Spermatogonia by GDNF. *Sci (80-. ).* (2000) 287:1489–93. doi: 10.1126/science.287.5457.1489

66. La HM, Makela JA, Chan AL, Rossello FJ, Nefzger CM, Legrand JMD, et al. Germline Stem Cell Activity Is Sustained by SALL4-Dependent Silencing of Distinct Tumor Suppressor Genes. *Stem Cell Rep* (2017) 9:956–71. doi: 10.1016/j.stemcr.2017.08.001

67. Chan AL, La HM, Legrand JMD, Makela JA, Eichenlaub M, De Seram M, et al. Distinct Germline Progenitor Subsets Defined Through Tsc2-Mtorc1 Signaling. *EMBO Rep* (2015) 16:467–80. doi: 10.15252/embr.201439379

68. Mäkelä JA, Toppari J. Endocrinology of the Testis and Male Reproduction. In: M Simoni, I Huhtaniemi, editors. *Endocrinology of the Testis and Male Reproduction.* Cham:Springer International Publishing (2017). p. 1–39. doi: 10.1007/978-3-319-29456-8_13-1

69. Rebourcet D, Darbey A, Monteiro A, Soffientini U, Tsai YT, Handel I, et al. Sertoli Cell Number Defines and Predicts Germ and Leydig Cell Population Sizes in The Adult Mouse Testis. *Endocrinology* (2017) 158:2955–69. doi: 10.1210/en.2017-00196

70. Ahmed EA, Barten-van Rijbroek AD, Kal HB, Sadri-Ardekani H, Mizrak SC, van Pelt AM, et al. Proliferative Activity *In Vitro* and DNA Repair Indicate That Adult Mouse and Human Sertoli Cells are Not Terminally Differentiated, Quiescent Cells. *Biol Reprod* (2009) 80:1084–91. doi: 10.1095/biolreprod.108.071662

71. Mäkelä JA, Toppari J, Rivero-Muller A, Ventela S. Reconstruction of Mouse Testicular Cellular Microenvironments in Long-Term Seminiferous Tubule Culture. *PloS One* (2014) 9:e90088. doi: 10.1371/journal.pone.0090088

72. Greenspan LJ, Matunis EL. Retinoblastoma Intrinsically Regulates Niche Cell Quiescence, Identity, and Niche Number in the Adult Drosophila Testis. *Cell Rep* (2018) 24:3466–3476.e8. doi: 10.1016/j.celrep.2018.08.083

73. Dominado N, La Marca JE, Siddall NA, Heaney J, Tran M, Cai Y, et al. Rbf Regulates Drosophila Spermatogenesis *via* Control of Somatic Stem and Progenitor Cell Fate in the Larval Testis. *Stem Cell Rep* (2016) 7:1152–63. doi: 10.1016/j.stemcr.2016.11.007

74. Bremner WJ, Millar MR, Sharpe RM, Saunders PT. Immunohistochemical Localization of Androgen Receptors in the Rat Testis: Evidence for Stage-Dependent Expression and Regulation by Androgens. *Endocrinology* (1994) 135:1227–34. doi: 10.1210/endo.135.3.8070367

75. Faisal I, Cisneros-Montalvo S, Hamer G, Tuominen MM, Laurila P-P, Tumiati M, et al. Transcription Factor USF1 Is Required for Maintenance of Germline Stem Cells in Male Mice. *Endocrinology* (2019) 160:1119–36. doi: 10.1210/en.2018-01088

76. Chen H-Z, Tsai S-Y, Leone G. Emerging Roles of E2Fs in Cancer: An Exit From Cell Cycle Control. *Nat Rev Cancer* (2009) 9:785–97. doi: 10.1038/nrc2696

77. Dick FA, Goodrich DW, Sage J, Dyson NJ. Non-Canonical Functions of the RB Protein in Cancer. *Nat Rev Cancer* (2018) 18:442–51. doi: 10.1038/s41568-018-0008-5

78. Krishnan B, Yasuhara T, Rumde P, Stanzione M, Lu C, Lee H, et al. Active RB Causes Visible Changes in Nuclear Organization. *J Cell Biol* (2022) 221:e202102144. doi: 10.1083/jcb.202102144

79. Tarulli GA, Stanton PG, Meachem SJ. Is the Adult Sertoli Cell Terminally Differentiated? *Biol Reprod* (2012) 87:1–11,13. doi: 10.1095/biolreprod.111.095091

80. Nalam RL, Andreu-Vieyra C, Matzuk MM. Absence of Inhibin Alpha and Retinoblastoma Protein Leads to Early Sertoli Cell Dysfunction. *PloS One* (2010) 5:e11797. doi: 10.1371/journal.pone.0011797

81. Matzuk MM, Finegold MJ, Su JG, Hsueh AJ, Bradley A. Alpha-Inhibin is a Tumour-Suppressor Gene With Gonadal Specificity in Mice. *Nature* (1992) 360:313–9. doi: 10.1038/360313a0

82. Yamasaki L, Jacks T, Bronson R, Goillot E, Harlow E, Dyson NJ. Tumor Induction and Tissue Atrophy in Mice Lacking E2F-1. *Cell* (1996) 85:537–48. doi: 10.1016/S0092-8674(00)81254-4

83. Hoja M-R, Liu J-G, Mohammadieh M, Kvist U, Yuan L. E2F1 Deficiency Impairs Murine Spermatogenesis and Augments Testicular Degeneration In SCP3-Nullizygous Mice. *Cell Death Differentiation* (2004) 11:354–6. doi: 10.1038/sj.cdd.4401362

84. Jorgez CJ, Seth A, Wilken N, Bournat JC, Chen CH, Lamb DJ. E2F1 Regulates Testicular Descent and Controls Spermatogenesis by Influencing WNT4 Signaling. *Development* (2021) 148:dev191189. doi: 10.1242/dev.191189

85. Agger K, Santoni-Rugiu E, Holmberg C, Karlström O, Helin K. Conditional E2F1 Activation in Transgenic Mice Causes Testicular Atrophy and Dysplasia Mimicking Human CIS. *Oncogene* (2005) 24:780–9. doi: 10.1038/sj.onc.1208248

86. Novotny GW, Sonne SB, Nielsen JE, Jonstrup SP, Hansen MA, Skakkebaek NE, et al. Translational Repression of E2F1 mRNA in Carcinoma *in Situ* and Normal Testis Correlates With Expression of the miR-17-92 Cluster. *Cell Death Differentiation* (2007) 14:879–82. doi: 10.1038/sj.cdd.4402090

87. Jorgez CJ, Wilken N, Addai JB, Newberg J, Vangapandu HV, Pastuszak AW, et al. Genomic and Genetic Variation in E2F Transcription Factor-1 in Men With Nonobstructive Azoospermia. *Fertil Steril* (2015) 103:44–52.e1. doi: 10.1016/j.fertnstert.2014.09.021

88. Rocca MS, Di Nisio A, Sabovic I, Ghezzi M, Foresta C, Ferlin A, et al. E2F1 Copy Number Variations Contribute to Spermatogenic Impairment and Cryptorchidism by Increasing Susceptibility to Heat Stress. *Andrology* (2019) 7:251–6. doi: 10.1111/andr.12583

89. Field SJ, FY T, Kuo F, Zubiaga AM, Kaelin WGJ, Livingston DM, et al. E2F-1 Functions in Mice to Promote Apoptosis and Suppress Proliferation. *Cell* (1996) 85:549–61. doi: 10.1016/S0092-8674(00)81255-6

90. Sada A, Suzuki A, Suzuki H, Saga Y. The RNA-Binding Protein NANOS2 is Required to Maintain Murine Spermatogonial Stem Cells. *Science* (2009) 325:1394–8. doi: 10.1126/science.1172645

91. Suzuki A, Saba R, Miyoshi K, Morita Y, Saga Y. Interaction Between NANOS2 and the CCR4-NOT Deadenylation Complex is Essential for Male Germ Cell Development in Mouse. *PloS One* (2012) 7:e33558. doi: 10.1371/journal.pone.0033558

92. Zhou Z, Shirakawa T, Ohbo K, Sada A, Wu Q, Hasegawa K, et al. RNA Binding Protein Nanos2 Organizes Post-Transcriptional Buffering System to Retain Primitive State of Mouse Spermatogonial Stem Cells. *Dev Cell* (2015) 34:96–107. doi: 10.1016/j.devcel.2015.05.014

93. Codino A, Turowski T, van de Lagemaat LN, Ivanova I, Tavosanis A, Much C, et al. NANOS2 is a Sequence-Specific mRNA-Binding Protein That Promotes Transcript Degradation in Spermatogonial Stem Cells. *iScience* (2021) 24:102762. doi: 10.1016/j.isci.2021.102762

94. Cafe SL, Skerrett-Byrne DA, De Oliveira CS, Nixon B, Oatley MJ, Oatley JM, et al. A Regulatory Role for CHD4 in Maintenance of the Spermatogonial Stem Cell Pool. *Stem Cell Rep* (2021) 16:1555–67. doi: 10.1016/j.stemcr.2021.04.003

95. Zhuang T, Hess RA, Kolla V, Higashi M, Raabe TD, Brodeur GM, et al. CHD5 is Required for Spermiogenesis and Chromatin Condensation. *Mech Dev* (2014) 131:35–46. doi: 10.1016/j.mod.2013.10.005

96. Li W, Wu J, Kim S-Y, Zhao M, Hearn SA, Zhang MQ, et al. Chd5 Orchestrates Chromatin Remodelling During Sperm Development. *Nat Commun* (2014) 5:3812. doi: 10.1038/ncomms4812

97. Yeh JR, Zhang X, Nagano MC. Indirect Effects of Wnt3a/beta-Catenin Signalling Support Mouse Spermatogonial Stem Cells *In Vitro*. *PloS One* (2012) 7:e40002. doi: 10.1371/journal.pone.0040002

98. Takase HM, Nusse R. Paracrine Wnt/beta-Catenin Signaling Mediates Proliferation of Undifferentiated Spermatogonia in the Adult Mouse Testis. *Proc Natl Acad Sci USA* (2016) 113:E1489–97. doi: 10.1073/pnas.1601461113

99. Tokue M, Ikami K, Mizuno S, Takagi C, Miyagi A, Takada R, et al. SHISA6 Confers Resistance to Differentiation-Promoting Wnt/beta-Catenin Signaling in Mouse Spermatogenic Stem Cells. *Stem Cell Rep* (2017) 8:561–75 doi: 10.1016/j.stemcr.2017.01.006

100. Boyer A, Yeh JR, Zhang X, Paquet M, Gaudin A, Nagano MC, et al. CTNNB1 Signaling in Sertoli Cells Downregulates Spermatogonial Stem Cell Activity *via* WNT4. *PloS One* (2012) 7:e29764. doi: 10.1371/journal.pone.0029764

101. Chassot AA, Le Rolle M, Jourden M, Taketo MM, Ghyselinck NB, Chaboissier MC. Constitutive WNT/CTNNB1 Activation Triggers Spermatogonial Stem Cell Proliferation and Germ Cell Depletion. *Dev Biol* (2017) 426:17–27. doi: 10.1016/j.ydbio.2017.04.010

102. Kitadate Y, Jorg DJ, Tokue M, Maruyama A, Ichikawa R, Tsuchiya S, et al. Competition for Mitogens Regulates Spermatogenic Stem Cell Homeostasis in an Open Niche. *Cell Stem Cell* (2019) 24:79–92.e6. doi: 10.1016/j.stem.2018.11.013

103. Hobbs RM, Seandel M, Falciatori I, Rafii S, Pandolfi PP. Plzf Regulates Germline Progenitor Self-Renewal by Opposing Mtorc1. *Cell* (2010) 142:468–79. doi: 10.1016/j.cell.2010.06.041

104. Busada JT, Niedenberger BA, Velte EK, Keiper BD, Geyer CB. Mammalian Target of Rapamycin Complex 1 (Mtorc1) Is Required for Mouse Spermatogonial Differentiation. *Vivo Dev Biol* (2015) 407:90–102. doi: 10.1016/j.ydbio.2015.08.004

105. Lindeman GJ, Dagnino L, Gaubatz S, Xu Y, Bronson RT, Warren HB, et al. A Specific, Nonproliferative Role for E2F-5 in Choroid Plexus Function Revealed by Gene Targeting. *Genes Dev* (1998) 12:1092–8. doi: 10.1101/gad.12.8.1092

106. Humbert PO, Rogers C, Ganiatsas S, Landsberg RL, Trimarchi JM, Dandapani S, et al. E2F4 is Essential for Normal Erythrocyte Maturation and Neonatal Viability. *Mol Cell* (2000) 6:281–91. doi: 10.1016/S1097-2765 (00)00029-0

107. Rempel RE, Saenz-Robles MT, Storms R, Morham S, Ishida S, Engel A, et al. Loss of E2F4 Activity Leads to Abnormal Development of Multiple Cellular Lineages. *Mol Cell* (2000) 6:293–306. doi: 10.1016/S1097-2765(00)00030-7

108. Xie H, Kang Y, Wang S, Zheng P, Chen Z, Roy S, et al. E2f5 is a Versatile Transcriptional Activator Required for Spermatogenesis and Multiciliated Cell Differentiation in Zebrafish. *PloS Genet* (2020) 16:e1008655. doi: 10.1371/journal.pgen.1008655

109. Pohlers M, Truss M, Frede U, Scholz A, Strehle M, Kuban R-J, et al. A Role for E2F6 in the Restriction of Male-Germ-Cell-Specific Gene Expression. *Curr Biol* (2005) 15:1051–7. doi: 10.1016/j.cub.2005.04.060

110. Li J, Ran C, Li E, Gordon F, Comstock G, Siddiqui H, et al. Synergistic Function of E2F7 and E2F8 Is Essential for Cell Survival and Embryonic Development. *Dev Cell* (2008) 14:62–75. doi: 10.1016/j.devcel.2007. 10.017