

**Title:** Retinoid signaling controls spermatogonial differentiation by regulating expression of replication-dependent core histone genes

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### **Summary statement**

Up-regulation of replication-dependent core histone genes by retinoic acid signaling contributes to spermatogonial differentiation.

### **Abstract**

Retinoic acid (RA) signaling is critical for spermatogonial differentiation, which is a key step for spermatogenesis. We explored the mechanisms underlying spermatogonial differentiation by targeting expression of a dominant-negative mutant of RA receptor  $\alpha$  (RAR $\alpha$ ) specifically to the germ cells of transgenic mice to subvert the activity of endogenous receptors. Here we show that (i) inhibition of retinoid signaling in germ cells completely blocked spermatogonial differentiation identical to vitamin A-deficient (VAD) mice; (ii) the blockage of spermatogonial differentiation by impaired retinoid signaling resulted from an arrest of entry of the undifferentiated spermatogonia into S phase; and (iii) retinoid signaling regulated spermatogonial differentiation through controlling expression of its direct target genes including replication-dependent core histone genes. Altogether, our results demonstrate that the action of retinoid signaling on spermatogonial differentiation in vivo is direct through spermatogonia self, and provide the first evidence that this is mediated by regulation of expression of replication-dependent core histone genes.

## Introduction

Spermatogenesis is a highly organized and complex process that allows for the continuous production of millions of haploid spermatozoa throughout adult male life and for transferring the intact genome and appropriate epigenome from generation to generation (Clermont, 1972, Oatley and Brinster, 2008). The transition of undifferentiated spermatogonia into A<sub>1</sub> spermatogonia (termed spermatogonial differentiation) is an initial and irreversible step of spermatogenesis (de Rooij, 2001). The undifferentiated spermatogonia can be subdivided into A<sub>single</sub> (A<sub>s</sub>), A<sub>paired</sub> (A<sub>p</sub>) and A<sub>aligned</sub> (A<sub>al</sub>) spermatogonia and contain spermatogonial stem cells (SSCs) and progenitor spermatogonia. This cohort of undifferentiated spermatogonia express self-renewal and proliferation associated genes such as *Pou5f1* (Pesce et al., 1998), *Lin28a* (Tong et al., 2011, Zheng et al., 2009), *Mir-21* (Niu et al., 2011), *Mir-17~92* (Tong et al., 2012), *Foxo1* (Goertz et al., 2011), *Nanos2* (Sada et al., 2009, Zhou et al., 2015), *Neurog 3* (*Ngn3*) (Nakagawa et al., 2007), *Sox3* (Laronda and Jameson, 2011), *Taf4b* (Falender et al., 2005), and *Zbtb16* (*Plzf*) (Buaas et al., 2004, Costoya et al., 2004) to maintain the capacity for self-renewal and proliferation. During spermatogonial differentiation, the undifferentiated spermatogonia downregulate those self-renewal associated genes and upregulate genes associated with differentiation such as *Sohlh1* (Ballow et al., 2006), *Sohlh2* (Hao et al., 2008), *Stra8* (Endo et al., 2015, Zhou et al., 2008), *Kit* (Schrans-Stassen et al., 1999), *Ccnd2* (Beumer et al., 2000), and *Sall4a* (Hobbs et al., 2012, Gely-Pernot et al., 2015). Despite this fact, the molecular mechanisms that govern spermatogonial differentiation remain incomplete.

Retinoic acid (RA), an active derivative of vitamin A, is essential for spermatogonial differentiation because (i) the transition of the undifferentiated spermatogonia into A<sub>1</sub> spermatogonia is blocked in vitamin A deficient (VAD) rodents, and (ii) RA administration to VAD animals reinitiates spermatogonial differentiation (Clagett-Dame and Knutson, 2011, Griswold et al., 1989, Huang and Hembree, 1979, Morales and Griswold, 1987, Wilson et al., 1953, Wolbach and Howe, 1925, Wolgemuth and Chung, 2007, van Pelt and de Rooij, 1990). There are 12 stages of cycle of seminiferous epithelium (hereafter referred to as epithelial stages I-XII) in the mouse (Clermont, 1972, Hogarth and Griswold, 2010, Oakberg, 1956). Although the undifferentiated spermatogonia in epithelial stages II-VIII are competent for spermatogonial differentiation in the adult mouse testis,

spermatogonial differentiation occurs only in epithelial stages VII/VIII as the RA level reaches its peak (de Rooij, 2001, Endo et al., 2015, Hasegawa and Saga, 2012, Hogarth et al., 2015, Hogarth and Griswold, 2010). Moreover, RA treatment could induce precocious differentiation of the undifferentiated spermatogonia in epithelial stages II-VII into A1 spermatogonia (Hogarth et al., 2015, Endo et al., 2015). However, the mechanisms underlying RA-induced spermatogonial differentiation remain largely unknown.

The action of RA on expression of target genes is mediated through two families of nuclear hormone receptors, the RA receptors (RARs) and the retinoid X receptors (RXRs), each with three subtypes:  $\alpha$ ,  $\beta$ , and  $\gamma$ , which are encoded by distinct genes (Chambon, 1996). RAR/RXR usually function as RAR/RXR heterodimers, which bind to RA-response elements (RAREs) in regulatory regions of the target genes (Bastien and Rochette-Egly, 2004). RAREs are typically composed of two direct repeats of a core hexameric motif, PuG(G/T)TCA separated by a 5 bp spacer sequence (referred to as DR5) (Bastien and Rochette-Egly, 2004). Several subtypes of RARs and RXRs are expressed in both Sertoli cells and germ cells including spermatogonia and exert redundant functions (Vernet et al., 2006b, Gaemers et al., 1998). Ikami et al. showed that ectopic expression of *Rarg* could induce the differentiation of RARG-negative undifferentiated spermatogonia by RA (Ikami et al., 2015). Global inactivation of individual *Rar* such as *Rara* results in male sterility and aberrant spermatogenesis (Lufkin et al., 1993). Several lines of compound mutants lacking multiple RARs or RXRs have been studied in testis, suggesting that retinoid signaling plays a critical role in spermatogonial differentiation (Gely-Pernot et al., 2012, Gely-Pernot et al., 2015). However, the RA target genes implicated in spermatogonial differentiation need to be identified.

To address these questions in the current study, we used conditional dominant-negative mouse models to block retinoid signaling specifically in germ cells. We demonstrate that impaired retinoid signaling in germ cells resulted in a complete blockage of spermatogonial differentiation. One of the major biological functions of RA is to inhibit cell proliferation (Bohnsack and Hirschi, 2004, Clagett-Dame and Knutson, 2011); however, RA is capable of stimulating cell proliferation in some type of cells such as neural crest-derived mesenchyme in the forebrain (Schneider et al., 2001) and neonatal germ cells (Busada et al., 2014). We report here that RA-induced entry into S phase of the undifferentiated spermatogonia could be critical for

spermatogonial differentiation. We further show that retinoid signaling could directly control expression of replication-dependent core histone genes that is essential for entry into S phase during spermatogonial differentiation. These findings thus provide novel insights into the molecular mechanisms by which retinoid signaling could regulate expression of replication-dependent core histone genes, thereby control spermatogonial differentiation *in vivo*.

## RESULTS

**Inactivation of Retinoid Signaling in Spermatogonia Impaired Spermatogenesis.** To directly determine whether retinoid signaling in germ cells controls spermatogenesis, we employed a conditional dominant-negative mutant of RAR $\alpha$ 403 (dnRAR) transgene strategy (Rosselot et al., 2010). RAR $\alpha$ 403, which is a truncated form of human RAR $\alpha$ , retains the ability to dimerize and bind the RARE but lose its transcriptional activation function (Damm et al., 1993). Previous studies have demonstrated that RAR $\alpha$ 403 can completely block wild-type RARs/RXRs function in a dose-dependent manner (Damm et al., 1993). A dnRAR transgene was inserted into the ROSA26R locus and was preceded by a floxed STOP sequence that is excised in cells expressing the CRE, activating the expression of dnRAR (Fig. S1A) (Rosselot et al., 2010). We conditionally expressed dnRAR in a subset of spermatogonia using a Stra8-Cre transgenic line with Cre expression in germ cells starting at ~3dpp (Sadate-Ngatchou et al., 2008). Throughout this study, we referred to hereafter two different genotypes of mice as: germ cell mutant (dnRAR<sup>flox/flox</sup>, Stra8-Cre), heterozygous germ cell mutant (dnRAR<sup>flox/+</sup>, Stra8-Cre).

To test whether the expression of dnRAR can impair retinoid signaling, we used a RARE/*lacZ* reporter line containing a RARE driven *lacZ* transgene, which allows the distribution of retinoid signaling to be visualized by 5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactoside (X-gal) staining (Rossant et al., 1991). No *lacZ* activity was detected in testes from the mice expressing both alleles of the dnRAR transgene (dnRAR<sup>flox/flox</sup>, Stra8-Cre, RARE/*lacZ*), whereas strong *lacZ* staining was seen in the control testes (Fig. S1B). However, in those animals expressing a single allele of dnRAR (dnRAR<sup>flox/+</sup>, Stra8-Cre, RARE/*lacZ*) testes, *lacZ* activity was reduced but still detectable (Fig. S1B), suggesting that the dnRAR in this line can block endogenous retinoid signaling in a dose-dependent manner as previously shown (Rosselot et al., 2010). Histologically, the seminiferous epithelium in control testes contained *lacZ*-positive spermatogonia and spermatocytes (Fig. S1C). Significantly, no *lacZ*-positive cells can be found in testes from the mice expressing both alleles of the dnRAR transgene (Fig. S1C), but more than 40% of seminiferous tubes contained *lacZ*-positive spermatogonia in the testes from the mice expressing a single allele of the dnRAR (Fig. S1C).

Germ cell mutant males were sterile but they exhibited normal copulating behavior. Germ cell mutant testes were much smaller than control littermate testes

[Fig. 1A, B, at age of 2 wk, control =  $0.231 \pm 0.016$  (mean testis weight/body weight  $\times 100 \pm$  s.d.), germ cell mutants =  $0.132 \pm 0.006$ ;  $P < 0.001$ ,  $n = 12$ ; At age of 12 wk, control =  $0.368 \pm 0.014$ , germ cell mutants =  $0.079 \pm 0.012$ ;  $P < 0.001$ ,  $n = 8$ ]. Histological examinations of adult germ cell mutants showed severe defects in spermatogenesis (Fig. 1E, F). In contrast to control seminiferous tubules that contained the full complement of germ cells (Fig. 1C, D), adult germ cell mutant seminiferous tubules showed a reduced diameter and contained only morphologically normal Sertoli cells and undifferentiated spermatogonia cells (Fig. 1E, F). Moreover, compared with control testes, seminiferous epithelium were also devoid of differentiated germ cells, containing only Sertoli cells and undifferentiated spermatogonia at the basal membrane in 2- or 3-week-old germ cell mutants (Fig. S2A-D).

**Germ Cell Mutants Exhibits Complete Blockage of Spermatogonial Differentiation.** To further characterize the remaining spermatogonia in germ cell mutants, we employed immunostaining with antibodies to spermatogonial markers. By immunostaining for STRA8, a marker for differentiated spermatogonia, STRA8 positive germ cells were not observed in adult germ cell mutant testes (Fig. 2B), whereas control testes showed many seminiferous tubules with STRA8 positive germ cells (Fig. 2A), demonstrating that the undifferentiated spermatogonia in adult germ cell mutants fail to differentiate. Consistent with this, KIT positive germ cells were rarely seen in germ cell mutant testes in contrast to control testes that contained many seminiferous tubules with KIT positive germ cells (Fig. S3A, B). We next examine the undifferentiated spermatogonial markers, LIN28 and PLZF, in both control and germ cell mutant testes. The LIN28-expressing (Fig. 2C, D) or PLZF-expressing (Fig. S3C, D) undifferentiated spermatogonia in germ cell mutants were similar to that in controls. Coimmunostaining for SOX9, a marker for Sertoli cell, showed normal Sertoli cell development in adult germ cell mutant testes (Fig. 2C, D and Fig. S3C, D). These results revealed complete blockage of spermatogonial differentiation in adult germ cell mutants. Furthermore, immunostaining with spermatogonial markers showed that seminiferous tubules were depletion of STRA8-expressing differentiated spermatogonia (Fig. S2E-H) and had normal LIN28-expressing undifferentiated spermatogonia (Fig. S2I-L) at 2- or 3-week-old, indicating that inactivation of retinoid signaling in spermatogonia also causes impaired

spermatogonial differentiation during the first wave of spermatogenesis. Taken together, the observed defects in germ cell mutant testes are identical to the abnormalities present in VAD animals. We further found that the defects in spermatogonial differentiation first occurred by the 4.5-day-old (Fig. 2E-G). We did not observe significant difference in apoptosis of PLZF-expressing spermatogonia between control and germ cell mutant testes using a TUNEL assay (Fig. S4A-C). Thus, we conclude that inactivation of retinoid signaling in germ cells causes complete blockage of spermatogonial differentiation in both the first wave of spermatogenesis and adult spermatogenesis.

**Inactivation of Retinoid Signaling Blocks Entry into S Phase in the Undifferentiated Spermatogonia** . The above data indicated that impaired retinoid signaling in germ cells causes the blockage at the differentiation of undifferentiated spermatogonia into A<sub>1</sub> spermatogonia. To pinpoint the spermatogonial differentiation defects incurred by the impaired retinoid signaling in germ cells, we administered a short-term (4h) 5-ethynyl-2'-deoxyuridine (EdU) pulse to control and germ cell mutant mice. We found that the ratio (EdU<sup>+</sup>PLZF<sup>+</sup>/ PLZF<sup>+</sup>) of both EdU positive and PLZF positive cells (EdU<sup>+</sup>PLZF<sup>+</sup>) to PLZF positive cells in germ cell mutant testes was significantly lower than that of controls (Fig. 3A-C), indicating that germ cell mutants had more undifferentiated spermatogonia in the G<sub>0</sub>/G<sub>1</sub> phase of the cell cycle. Only a subset of undifferentiated spermatogonia, which are arrested in the G<sub>0</sub>/G<sub>1</sub> phase of the cycle, are competent for spermatogonial differentiation (Kluin and de Rooij, 1981, Endo et al., 2015). We thus speculated that inaction of retinoid signaling could result in G<sub>1</sub>/S phase transition arrest of the undifferentiated spermatogonia, accounting for impaired spermatogonial differentiation observed in germ cell mutant testes.

To test this hypothesis, we examined cell cycle progression of the undifferentiated spermatogonia (THY1<sup>+</sup> spermatogonia) in control and germ cell mutants by fluorescence-activated cell sorting (FACS) analysis. We found that, compared with control testes, impaired retinoid signaling in spermatogonia inhibited cell cycle progression, by significantly increasing the G<sub>1</sub> population of the undifferentiated spermatogonia (Fig. 3D,E), suggesting that the undifferentiated spermatogonia in the germ cell mutants underwent an arrest of entry into S phase. To confirm that impaired spermatogonial differentiation results from an arrest of entry of the undifferentiated spermatogonia into S phase, we then injected WIN18,466 to



chemically inhibit RA synthesis and block spermatogonial differentiation in the control mice. As predicted, WIN18,466 lead to a substantial accumulation of the undifferentiated spermatogonia in G1 phase while spermatogonial differentiation is blocked (Fig 3F). Because injected RA induced differentiation of the undifferentiated spermatogonia into A<sub>1</sub> spermatogonia, we predicted that RA could rescue the G1/S transition arrest by WIN18,466 treatment. We first found that, after RA injection, WIN18,466 resulted in the accumulation of cells in the G1 phase was significantly reduced (Fig. S5A,B). We then showed that STRA8-positive spermatogonia incorporated EdU in RA-treated mice as previously reported, whereas both mice without RA treatment and germ cell mutant mice with RA administration did not contain STRA8-positive spermatogonia in the testes (Fig. 3G-I; Fig. S5C-E), indicating newly differentiating spermatogonia entry into mitotic S phase through RA induction.

Collectively, these findings provide strong evidence that impaired spermatogonial differentiation in the germ cell mutant testes results from an arrest of entry into S phase in the undifferentiated spermatogonia.

**Retinoid Signaling Controls Spermatogonial Differentiation Through Expression of the Target Genes Including Replication-dependent Core Histone Genes.** To investigate alterations in gene expression that result from impaired retinoid signaling, we conducted RNA-seq to profile the transcriptome of germ cell mutant and control THY1<sup>+</sup> spermatogonia. Gene ontology (GO) analysis of the genes at the top of the ranked genes indicated enrichment in genes associated with roles in reproduction, transcription, and spermatogenesis (Fig. 4A). In total, we identified 1633 and 742 transcripts (Reads Per Kilobase of transcript per Million mapped reads (RPKM) > 1) that were significantly (p-value < 0.05, > 1.5-fold difference) down- and up-regulated, respectively, in the germ cell mutants compared with the controls (Table S1). It is of note that a dramatic upregulation of a specific subset of transcripts encoding proteins previously reported to be expressed by the undifferentiated spermatogonia in germ cell mutants relative to controls (Fig. 4B). In contrast, expression of genes known to be involved in spermatogonial differentiation was significantly downregulated in germ cell mutants compared to controls (Fig. 4B). This finding suggested that the expression program of spermatogonia in germ cell mutants was switched to the undifferentiated spermatogonia program.

The above data showed an arrest of entry of the undifferentiated spermatogonia into S phase in germ cell mutants. Interestingly, we found that the majority of transcripts of replication-dependent core histone genes, histone cluster 1 (*Hist1*) (Osley, 1991, Kurat et al., 2014, Marzluff et al., 2002) were downregulated in germ cell mutants (Fig. 4C; Table S1). In mammals, the genes for the five histones H1, H2A, H2B, H3 and H4 are clustered in two loci, *Hist1* and *Hist2* (Osley, 1991, Kurat et al., 2014, Marzluff et al., 2002). Transcription of the *Hist1* and *Hist2* cluster genes is initiated at the G1/S transition and downregulated soon after completion of genome duplication in the S phase (Kurat et al., 2014, Osley, 1991). The downregulation of individual *Hist1* genes was further validated by qRT-PCR on RNA from isolated germ cell mutant and control THY1<sup>+</sup> spermatogonia. Seven out of eight *Hist1* genes examined showed significant decreases in expression in germ cell mutants compared to controls (Fig. 4D). Downregulation of the *Hist1* cluster genes is consistent with G1/S transition arrest of the undifferentiated spermatogonia in germ cell mutants. To further investigate whether RA controls the transcription of *Hist1* genes, we examined expression of the individual *Hist1* genes in THY1<sup>+</sup> spermatogonia from mice with WIN18,466 treatment alone and WIN18,466 treated mice with RA exposure. As shown in Fig. 4E, RA treatment resulted in a significant increase in *Hist1* mRNA levels. Taken together, we conclude that retinoid signaling could play an in vivo role in the regulation of the expression of replication-dependent core histone genes located in the *Hist1* cluster.

In addition, we found that expression of *E2F* and *Ccnd2*, which are crucial for regulation of cell cycle, was significantly suppressed in the undifferentiated spermatogonia of germ cell mutants, whereas, in VAD mice, RA administration stimulated *Ccnd2* expression in spermatogonia (Fig. S6), as previously reported.

**Retinoid Signaling Regulates Expression of Replication-dependent Core Histone Genes.** The mouse *Hist1* cluster is located on chromosome 13 and the histone genes in the *Hist1* cluster are arranged in 3 subclusters (Marzluff et al., 2002) (Fig. 5A). Given that most of *Hist1* gene transcripts are coordinately regulated in THY1<sup>+</sup> spermatogonia by retinoid signaling, we assume that expression of *Hist1* genes is directly controlled by retinoid signaling.

To test this hypothesis, we found a putative RARE upstream of the histone-encoding genes of the *Hist1* cluster. The RARE is located between -671 and -655

relative to the transcription start site of *h2bl*, the first *Hist1* gene (Fig. 5A). Notably, chromatin immunoprecipitation assays (ChIPs) using a RARG antibody performed on mouse testis at day 5 revealed that RARG is present at the RARE region upstream of the *Hist1* cluster similar to the *Stra8* promoter (Fig. 5B). Compared with controls, there was a significant decrease in acetylated H4 (H4Ac) levels at the regulatory regions of both *Hist1* and *Stra8* in the germ cell mutants (Fig. 5C). In addition, RA administration to WIN18,466 treated mice induces a striking upregulation in H4Ac at the regulatory regions of both *Hist1* and *Stra8* (Fig. 5D). To further examine whether the -671/-655 sequences is a functional RARE, we inserted ~1.5kb upstream regulatory region of *Hist1* cluster containing this putative RARE into the luciferase reporter vector and transfected the reporter into RARs/RXRs-expressing cells, P19 cells (Kruyt et al., 1991, Schoorlemmer et al., 1995). As shown in Fig. 5e, luciferase activity was significantly induced by RA treatment compared to vehicle-treated controls; however, RA-induced luciferase activity was significantly inhibited by cotransfected with dnRAR vector. Furthermore, a mutation of the RARE in the reporter significantly disrupted the RA-induced luciferase activity (Fig. 5E). Collectively, these results reveal that a functional RARE is present upstream of the *Hist1* cluster and that the *Hist1* cluster genes could be direct targets for retinoid signaling.

## DISCUSSION

**Retinoid Signaling Directly Controls Spermatogonial Differentiation.** Retinoid signaling is central to spermatogonial differentiation. We and others have demonstrated that testicular RA mainly originates from Sertoli cells especially in puberty, whereas it remains elusive that action of RA on spermatogonial differentiation is through germ cells or Sertoli cells or both Sertoli and germ cells, as its receptors, RARs and RXRs are expressed in both Sertoli cells and the undifferentiated spermatogonia (Gely-Pernot et al., 2012, Tong et al., 2013, Vernet et al., 2006b, Gaemers et al., 1998, DeFalco et al., 2015). For instance, genetic ablation of *Rarg* in germ cells only resulted in mild spermatogonial differentiation defects in younger than one-year-old mutants, indicating either the retinoid signaling in Sertoli cells was involved in spermatogonial differentiation, or other subtypes of RARs in spermatogonia compensate for loss of RARG function, as it is the case for many other developmental process (Gely-Pernot et al., 2012, Mark et al., 2009). Thus, to better understand whether the action of retinoid signaling on spermatogonial differentiation can be direct on the germ cells, we have to completely inactivate retinoid signaling specifically in germ cells. In this study, we used dnRAR<sup>fl/fl</sup> transgenic mice with a germ cell-specific expressing Cre line to impair retinoid signaling in germ cells. We find that, if there was no retinoid signaling in germ cells, spermatogonial differentiation was completely blocked during the first wave of spermatogenesis and in adult spermatogenesis. Therefore, our findings presented in this study provide the strong functional evidence that retinoid signaling directly controls spermatogonial differentiation through spermatogonia self.

Gely-Pernot et al have recently analyzed mice simultaneously lacking all RARs or all RXRs specifically in undifferentiated spermatogonia and found that testicular defects of both mutants are not identical to that of VAD mice (Gely-Pernot et al., 2015). The phenotype we see in our study is more severe than that reported by Gely-Pernot et al. They reported that excision of genes for all 3 *Rxrs* resulted in an age dependent testicular degeneration but spermatogonial differentiation proceeded normally and some cells entered meiosis. In contrast to our results they showed that ablation of all *Rxrs* or all *Rars* in spermatogonia did not alter the first wave of spermatogenesis. This difference may be due to the fact that the dnRAR used in our studies could bind up all available *Rxrs* and *Rars*. Because the dnRAR would sequester all *Rxrs* it is possible that some of the defects we seen in our mutant

testes could be due to the lack of action of other RXR binding partners such as vitamin D receptor (VDR), thyroid receptor (TR), or the PPAR receptor. However, we feel this result is unlikely because the knockout of the *Vdr* or *Ppar* still results in the completion of spermatogenesis although with a reduced sperm count (Blomberg Jensen et al., 2013, Yao et al., 2015). In addition, male mice lacking individual *TR* or both *TR $\alpha$ 1* and *TR $\beta$ 1* are still fertile and inactivation of *TR $\alpha$ 1* actually results in a larger testis with enhanced Sertoli and germ cell numbers (Cooke, 1991, Gao et al., 2014, Gothe et al., 1999, Holsberger et al., 2005). Therefore, the observations from Gely-Pernot et al and the fact in this report that germ cell mutants exhibits complete defects in spermatogonial differentiation identical to VAD mice reveal that RARs could act both with and without an RXR in germ cells, or that RXRs could act both with and without an RAR in germ cells. Indeed, it has been demonstrated that RAR functions in Sertoli cells independently of RXRs (Vernet et al., 2006a). Thus, the transgenic model using in this study should be powerful for exploring the molecular mechanisms of retinoid signaling in spermatogonial differentiation, meiotic initiation and spermiogenesis.

**RA-induced Entry of the Undifferentiated Spermatogonia into S Phase Are Crucial to Spermatogonial Differentiation.** RA, a potent regulator of cell growth, exerts pleiotropic effects in regulating cellular proliferation and differentiation, depending on the cell types present during embryogenesis and in adult tissues (Bohnsack and Hirschi, 2004, Chambon, 1996, Clagett-Dame and Knutson, 2011). We here provide functional evidence that, in the testis, retinoid signaling may control entry of the undifferentiated spermatogonia into S phase, and then promote spermatogonial differentiation. First, impaired retinoid signaling in germ cells causes the arrest of the undifferentiated spermatogonia into S phase, accounting for the blockage of spermatogonial differentiation: (i) a significant accumulation of the undifferentiated spermatogonia in the G0/G1 phase occurred in germ cell mutants while the differentiation of the undifferentiated spermatogonia into A1 spermatogonia is blocked; (ii) inhibition of RA synthesis by WIN18,466 resulted in a blockade of entry into S phase in the undifferentiated spermatogonia as previously suggested by Van Pelt et al. (van Pelt et al., 1995, van Pelt and de Rooij, 1990); (iii) expression of a burst of replication-dependent core histone genes, whose expression is induced right before and during S phase (Kurat et al., 2014), was downregulated in the undifferentiated spermatogonia of germ cell mutants; and (iv) expression of *Ccnd2*

and *E2F*, key regulators for the progression from G1 to S phase (Bohnsack and Hirschi, 2004), was reduced in the germ cell mutants. Second, during reinitiation of spermatogonial differentiation in WIN18,466-treated mice upon RA administration, early differentiating spermatogonia (STRA8-positive spermatogonia) enter into the S phase. Consistent with this, transcription of *Hist1* cluster genes and *Ccnd2* is upregulated in spermatogonia during RA-induced spermatogonial differentiation. Finally, this hypothesis is also supported by previous published data. For instance, during RA-induced precocious spermatogonial differentiation in vivo, the undifferentiated spermatogonia in epithelial stages II-VI, which are arrested in G0/G1 phase of the cell cycle, are released from the G1 block and subsequently enter into mitotic S phase (Endo et al., 2015, Kluin and de Rooij, 1981). Furthermore, undifferentiated spermatogonia in the epithelial stage VII-VIII have been shown to enter mitotic S phase during spermatogonial differentiation (Endo et al., 2015).

**Retinoid Signaling Can Regulate Expression of Replication-dependent Core Histone Genes in the Undifferentiated Spermatogonia.** The regulation of cell cycle progression is tightly controlled within cells. At the G1/S phase transition, the cell duplicates the genome and that process requires the initiation of DNA replication and the coupling activation of core histone gene expression, whereas, outside of S phase, core histone synthesis is suppressed to avoid overproduction (Osley, 1991). Expression of the core histone genes that encode proteins for packaging the newly synthesized genome is exquisitely controlled at transcriptional and posttranscriptional levels within cells (Osley, 1991, Eliassen et al., 1998). Our studies showed that impaired RA signaling in germ cells inhibited the transcription of replication-dependent core histone genes in the undifferentiated spermatogonia. In contrast, RA treatment upregulated expression of core histone gene expression in spermatogonia. Collectively, these data indicate that RA-dependent signaling in spermatogonia controls replication-dependent core histone gene expression. Because the expression of many histone genes such as *Hist1* cluster genes is coordinately activated upon entry into S phase, it is possible that a common pathway (e.g. transcriptional factors) contributes to their simultaneous regulation. Using bioinformatic analyses, ChIP assays and luciferase reporter experiments, we identified a functional RARE present in the upstream regulatory region of *Hist1* cluster. Thus, we demonstrate that retinoid signaling directly controls the transcription of *Hist1* cluster genes in spermatogonia.

This, together with a wealth of evidence that CCND2 plays critical roles in the mitotic G1/S transition, leads us to conclude that retinoid signaling directly functions on the undifferentiated spermatogonia to upregulate the expression of *Ccnd2* for initiating DNA synthesis and to activate the transcription of replication-dependent histone genes for packaging the new genome. This results in the induction of the entry of the undifferentiated spermatogonia into S phase (Fig. 6). It will be of interest in the future to study whether retinoid signaling could promote the premeiotic G1/S transition.

Recent studies reveal that several RA target genes have also been implicated in spermatogonial differentiation. Endo et al demonstrated that RA target gene, *Stra8*, could promote spermatogonial differentiation (Endo et al., 2015). Gely-Pernot et al indicated that *Sall4a*, a RA target gene, could control spermatogonial differentiation through upregulation of *Kit* expression (Gely-Pernot et al., 2015). Busada et al showed that RA could also utilize non-genomic pathways via PI3K/AKT/mTOR signaling to stimulate translation of mRNA for *Kit* during spermatogonial differentiation (Busada et al., 2014). Based on these results, we postulate that retinoid signaling may induce the entry of the undifferentiated spermatogonia into S phase through upregulation of expression of replication-dependent histone genes and *Ccnd2*, and subsequently promote the differentiation of undifferentiated spermatogonia into A<sub>1</sub> spermatogonia with RA-induced expression of *Stra8* (Endo et al., 2015, Zhou et al., 2008), *Sall4* (Gely-Pernot et al., 2015), and *Kit* (Schrans-Stassen et al., 1999, Busada et al., 2014) (Fig. 6).

## **MATERIALS and METHODS**

**Mice.** The dnRAR<sup>flox/flox</sup> were a generous gift from the Mendelsohn laboratory (Rosselot et al., 2010). (Lecureuil et al., 2002)The *Stra8*-Cre line was generated in the Braun laboratory (Sadate-Ngatchou et al., 2008). RARE/*lacZ* reporter line was created in the Rossant laboratory (Rossant et al., 1991). All animal experiments were approved by the Animal Care and Use Committee at Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences.

**Histological and Immunohistochemical Analyses.** Testes were fixed in Bouins solution or 4% paraformaldehyde (PFA), embedded in paraffin and sectioned. Sections were deparaffinized, rehydrated, and stained with hematoxylin and eosin. For immunohistochemical studies, slides were boiled in 10mM sodium citrate buffer, pH 6.0, for 15min, brought to room temperature, washed in PBS with 0.1% Triton X-100, and then incubated for 60min at room temperature with blocking buffer (10% donkey serum, 1% BSA, 0.1% Triton X-100 in PBS). The sections were then incubated with a 1:50 dilution of rat anti-GCNA IgM (kindly provided by Dr. G. Enders, University of Kansas, Kansas City, KS) and rabbit anti-STRA8 IgG or rabbit anti-PLZF (Santa Cruz Biotechnology) or rabbit anti-SOX9 IgG (Millipore) or 1:50 dilution of rat anti-KIT (Millipore) overnight at 4°C and rabbit anti-STRA8 IgG or rabbit anti-PAlexa Fluor 488 and 594 conjugated donkey secondary antibody (Jackson ImmunoResearch Laboratories) at 1:500 dilution were added. After 60 min at room temperature, the sections were washed in PBS, rinsed quickly in pure ethanol, mounted in Prolong Gold Antifade medium with DAPI (Molecular Probes), and then analyzed by a fluorescence microscope (Olympus).

**Isolation of Mouse THY1<sup>+</sup> Spermatogonia and Flow Cytometry.** THY1<sup>+</sup> spermatogonia were isolated using magnetic activated cell sorting (MACS) with magnetic microbeads conjugated to anti-THY1<sup>+</sup> (Miltenyi Biotech) as described previously (Tong et al., 2012). To determine cell cycle progression, THY1<sup>+</sup> spermatogonia were fixed in 75% ethanol at 4°C overnight, washed with PBS, and incubated with 25µg/ml RNase A (Sigma) and 50µg/ml propidium iodide (Sigma) in PBS at 37°C for 30 min, followed by FACS analysis on a BD LSRII SORP (BD Biosciences).

**RNA-Seq Analyses.** Total RNA was extracted from THY1<sup>+</sup> spermatogonia isolated from control and germ cell mutant mice using Trizol reagent (Invitrogen). Libraries of



cDNA were constructed by the Omics core of CAS-MPG Partner Institute for Computational Biology at Shanghai Institutes for Biological Sciences using the TrueSeq Stranded Total RNA Library Prep Kit (Illumina) following manufacturerKitinstruction. Libraries were sequenced using single reads (100nt) on Illumina HiSeq 2000 (Illumina). Sequencing reads were mapped to the ENSEMBL mouse reference genome (GENCODE vM6/GRCm38) using TopHat with standard setting. Confidently aligned reads for each sample were analyzed with the Cuffdiff2 program to determine differential expression between control and germ cell mutant samples. Genes with a fold change  $\geq 1.5$  were selected as differential genes.

**Quantitative RT-PCR Assays.** Total RNA was extracted using Trizol reagent, and treated with DNaseI (Ambion). Total RNA was reverse transcribed using an iScript cDNA Synthesis Kit (Bio-Rad). Quantitative PCR was performed with Fast SYBR Green PCR mastermix (Applied Biosystems) on the Applied Biosystems 7500 Fast system (Applied Biosystems). Relative expression of genes was analyzed by the comparative  $C_T$  method with use of ribosomal protein S2 (*Rps2*) as a normalized control. RT-PCR primer sequences were listed in Table S2.

**ChIP Assay.** As previously described (Tong et al., 2011), mouse spermatogonia were cross-linked with 1% formaldehyde/PBS for 10 min at room temperature and quenched by adding glycine to a final concentration of 0.25M for 5 min. Cells were collected and washed twice with cold PBS containing 1x protease inhibitor cocktail (Roche). Cell pellets were lysed in 1ml of lysis buffer (1% SDS, 50mM Tris-HCl pH8.0, 10mM EDTA and 1x protease inhibitor cocktail) for 10min on ice, and sonicated on ice to obtain a chromatin size of 200 to 500bp. After pre-clearing with protein A/G agarose beads (Upstate), an equivalent amount of sheared chromatin was immunoprecipitated with antibody overnight at 4°C followed by an incubation with protein A/G for 1h. Antibodies used in ChIP assays were anti-RARG (Santa Cruz), anti-acetylated histone H4 (H4Ac) (Upstate), and IgG control (Santa Cruz). Protein-DNA complexes were eluted in fresh 1% SDS/0.1M NaHCO<sub>3</sub>. Crosslinking was reversed and protein was removed. DNA was recovered and purified for PCR using specific primers listed in Table S3.

**Luciferase Assay.** The pGL3-Hist1-luciferase reporter vector was constructed by replacing the cytomegalovirus (CMV) promoter sequence of the pGL3-luciferase reporter plasmid with -1.5kb upstream of *Hist1* cluster containing a putative RARE

region (-671/-655: AGGCCAAGGGGAAGTGA). The mutations were introduced into the RARE region of pGL3-Hist1-Luciferase construct to generate the pGL3-mHist1-luciferase reporter. The pGL3-Rarb-luciferase was generated by replacing the CMV promoter of the pGL3-luciferase plasmid with *Rarb* promoter sequence, which contains classic RARE. The pGL3-Hist1-luciferase, pGL3-mHist1-luciferase, or pGL3-Rarb-luciferase was cotransfected with a *Renilla* vector into P19 cells using Lipofectamine 2000 transfection reagent according to the manufacturer's instruction (Invitrogen). At 24h posttransfection, the cells were treated with or without  $10^{-6}$ mol/L RA for another 24h before harvesting cells for dual luciferase assay according to the manufacturer's protocol (Promega). The relative luciferase activity was normalized to Renilla luciferase activity.

**WIN18,466, RA Administration and EdU Labeling.** For WIN18,466 treatment (Hogarth et al., 2013), 2-day-old animals were pipette-fed with WIN18,466 (100 $\mu$ g/g body weight ) suspended in 1% gum tragacanth for 7 consecutive days. For RA injection experiments, mice were received an i.p. injection of all-trans-RA (Sigma) (400 $\mu$ g/injection) in 10 $\mu$ l of DMSO for 24h.

For EdU labeling, as described previously (Tong et al., 2013), mice were i.p. injected with EdU (Invitrogen)(50 $\mu$ g/g body weight) in PBS. The mice were euthanized 4h later and testes were fixed in 4% PFA/PBS solution, embedded in paraffin and sectioned. The sections were immunostained with PLZF or STRA8 antibody first and the EdU incorporation was then detected by Click-It EdU Alexa Fluor 594 Imaging Kit according to the manufacture's protocol (Invitrogen).

**X-gal Staining.** *dnRAR<sup>fl/fl</sup>*, *Stra8-Cre<sup>+</sup>* (female) mice were crossed with RARE/*lacZ* reporter line harboring RARE-*Hspa1b-lacZ* alleles to obtain *dnRAR<sup>fl/+</sup>*, *Stra8-Cre<sup>+</sup>*, RARE/*lacZ*, and *dnRAR<sup>fl/+</sup>*, RARE/*lacZ* mice. The *dnRAR<sup>fl/+</sup>*, *Stra8-Cre<sup>+</sup>*, RARE/*lacZ* (female), and *dnRAR<sup>fl/+</sup>*, RARE/*lacZ* mice were used to produce *dnRAR<sup>fl/fl</sup>*, *Stra8-Cre<sup>+</sup>*, RARE/*lacZ*, *dnRAR<sup>fl/+</sup>*, *Stra8-Cre<sup>+</sup>*, RARE/*lacZ* and *dnRAR<sup>fl/fl</sup>*, RARE/*lacZ* mice. Testes, epididymis, and kidneys from animals bearing RARE-*Hspa1b-lacZ* alleles were fixed in 4% paraformaldehyde (PFA) in PBS for 2h at room temperature, washed, stained in bromo-chloro-indolyl-galactopyranoside (X-gal) at 37°C overnight, washed and then photographed. The stained testes were then processed, embedded in paraffin, and sectioned. Sections were counterstained with fast red.

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## **Competing Financial Interests Statement**

The authors declare no competing financial interests.

## **Author Contributions**

Y.C. carried out most experiments and data analysis; L.M. and G.W. performed RNA-seq data analysis; C.H., M.G. and M.H.T. analyzed data and wrote the manuscript. All the authors were involved in the discussion on the manuscript.

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## Figures

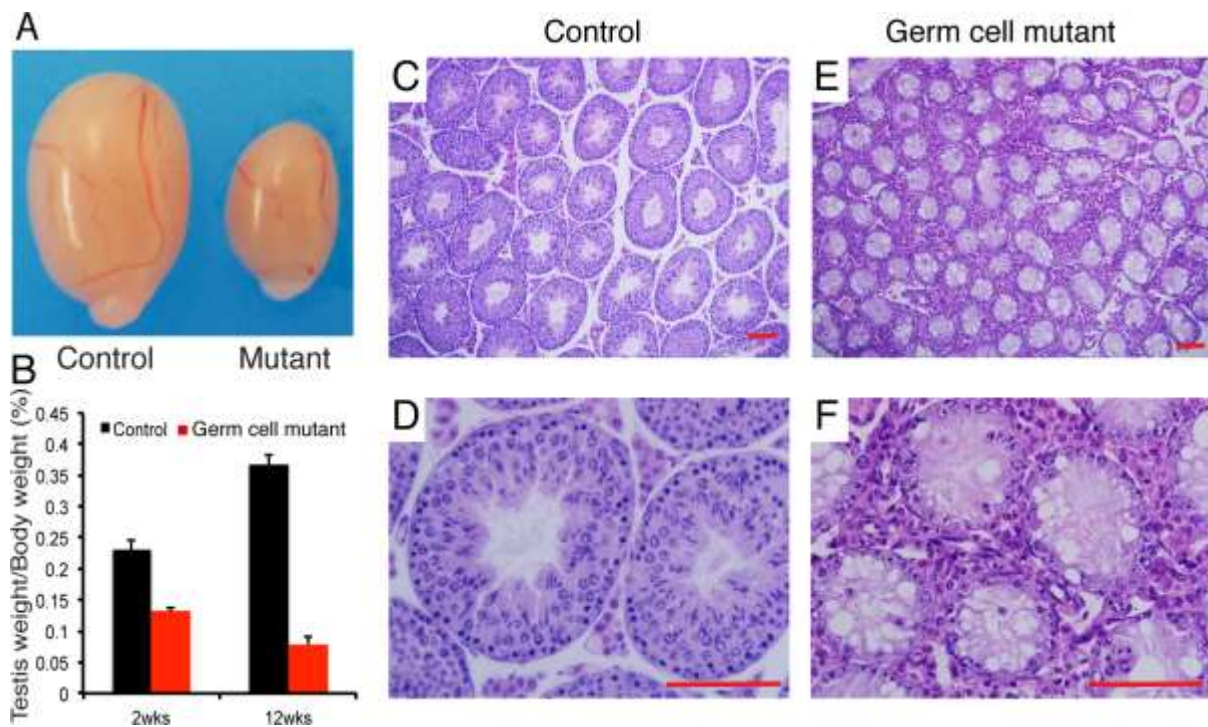


Figure 1. Impaired spermatogenesis in germ cell mutant testes. (A) Gross morphology of representative testes from a 8-week-old control and age-matched germ cell mutants. (B) Comparisons of testis weight from 2 or 12-wk-old control and mutants ( $n = 8-12$  for each genotype per data point). (C-F) Hematoxylin and eosin staining of control (C and D) and germ cell mutant (e and f) testes at 8-week-old. Scale bar, 20 $\mu$ m.

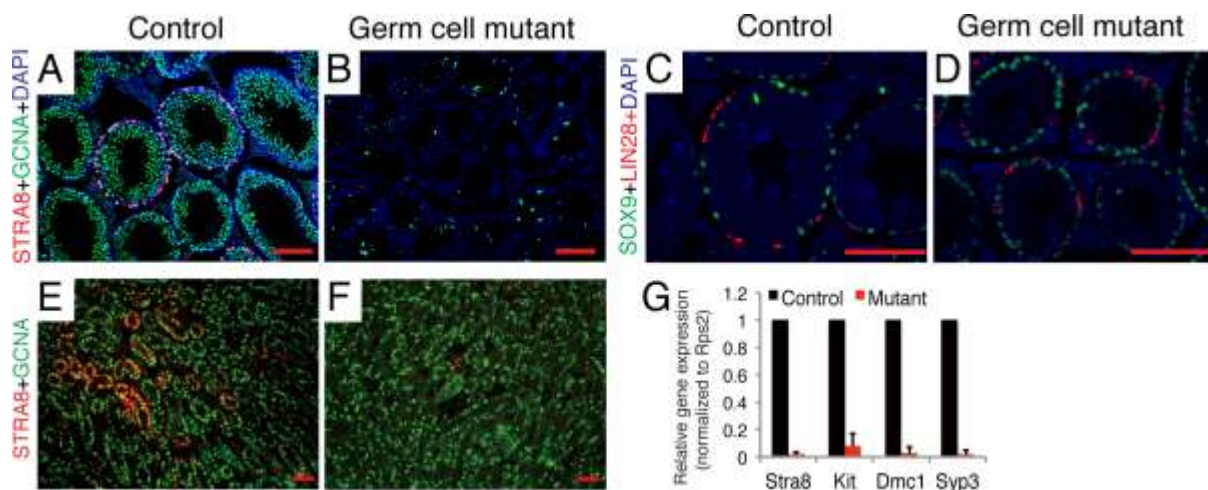


Figure 2. Complete blockage of spermatogonial differentiation in germ cell mutants. (A and B) Immunohistochemical staining for STRA8 (red) in sections of 8-week-old control (A) and germ cell mutant (B) testes. Costaining for a germ cell marker, GCNA (green). (C and D) Immunohistochemical staining for LIN28 (red) and SOX9 (green) in sections of 8-week-old control (C) and germ cell mutant (D) testes. (E and F) Immunohistochemical staining for STRA8 (red) in sections of 4.5-day-old control (E) and germ cell mutant (F) testes. Costaining for a germ cell marker, GCNA (green). *Scale bar*, 10 $\mu$ m. (G) RT-qPCR analysis of mRNA levels of marker for spermatogonial differentiation in control and germ cell mutant testes at 4.5-day-old. Data are expressed as mean  $\pm$  s.d. fold changes compares with controls, normalized to *Rps2*. n=3-4, \*P<0.01; Student t test.



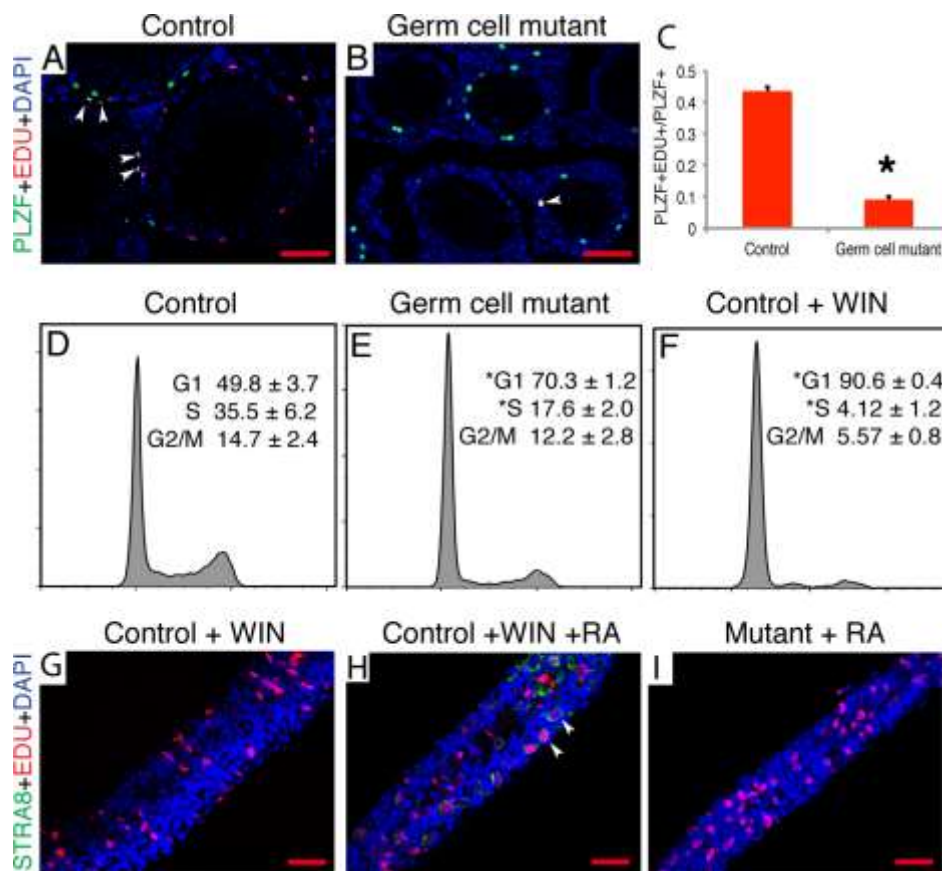


Figure 3. Retinoid signaling regulated G1/S phase transition of the undifferentiated spermatogonia. (A and B) Immunostaining for PLZF (green) and EdU (red) in sections of 4-week-old control (A) and germ cell mutant testes (B). Arrowheads indicate both PLZF and EdU positive spermatogonia (orange). (C) Quantification of proliferative spermatogonia in control and germ cell mutant testes at 4 wk of age. The number of both EdU and PLZF positive cells was scored per number of PLZF positive cells. All seminiferous tubules at each section were counted (n=3-4). Error bars represent standard deviations. \* $P < 0.05$  (Student *t*-test). (D-F) FACS analysis showing the effect of retinoid signaling on cell cycle progression in spermatogonia. The data (*inset*) represent means  $\pm$  s.d. of four independent analyses and shows one of the representative FACS. \* $P < 0.01$  (Student *t*-test). (G-I) Whole-mount immunostaining of seminiferous tubules for STRA8 (green) and EdU (red) in WIN18,466-treated controls (G), RA-injected WIN18,466-treated controls (H), and RA-injected germ cell mutants (I). Arrowheads indicate both STRA8 and EdU positive representative spermatogonia. Scale bar, 20 $\mu$ m.

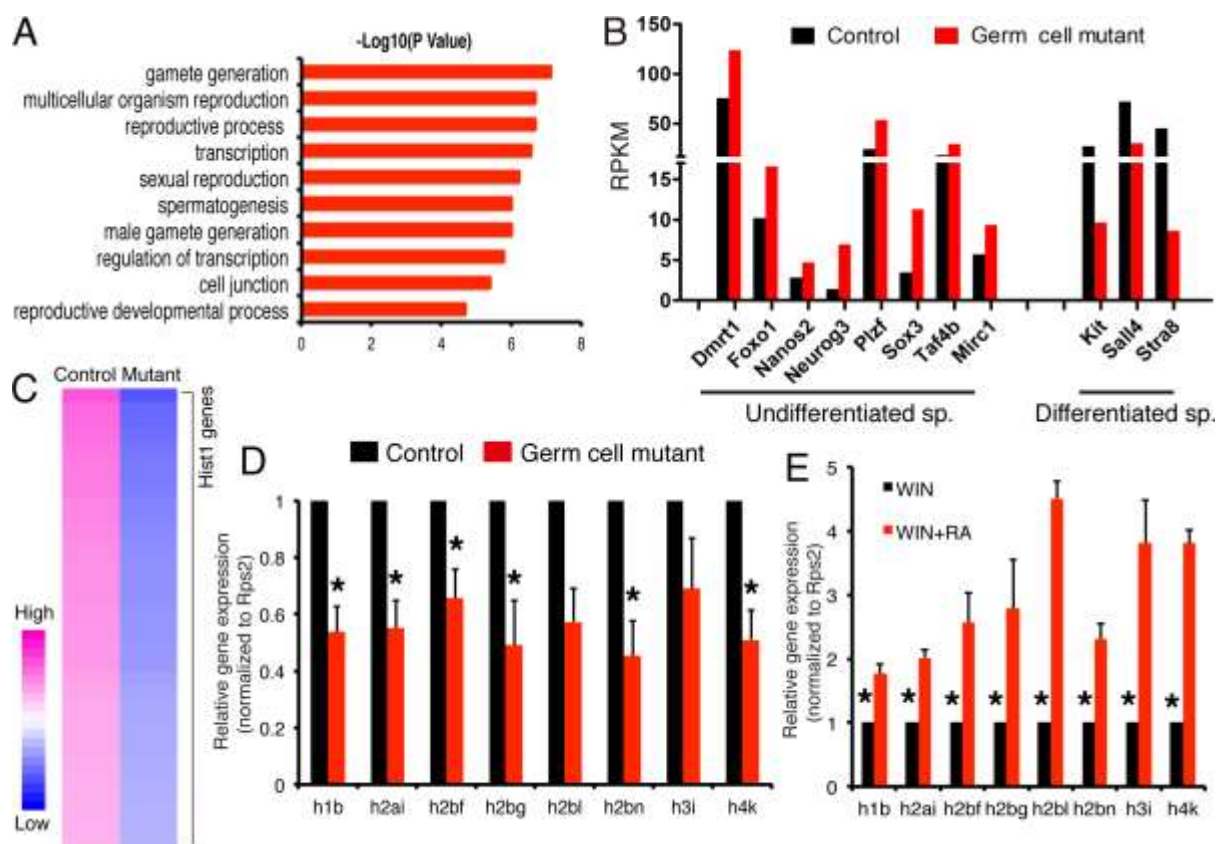


Figure 4. Alterations of the mRNA transcriptome in germ cell mutant spermatogonia. (A) Gene ontology (GO) term enrichment analyses of retinoid signaling regulated genes. The top 10 most enriched biological processes based on their p-values are shown. (B) Differential expression of genes reported previously to be expressed by undifferentiated or differentiated spermatogonia between controls and germ cell mutants. (C) A heat map showing expression of 36 *Hist1* cluster genes in control and germ cell mutant spermatogonia. (D) RT-qPCR analysis of mRNA levels of *Hist1* cluster genes in control and germ cell mutant spermatogonia. (E) RT-qPCR analysis of mRNA levels of *Hist1* cluster genes in WIN18,466-treated and RA-injected WIN18,466-treated mouse spermatogonia. Data (D and E) are expressed as fold differences compared with controls (D) or (WIN18,466-treated), respectively, normalized *Rps2* (mean  $\pm$  s.d.,  $n=3$ ;  $*P<0.05$ , Student *t*-test).

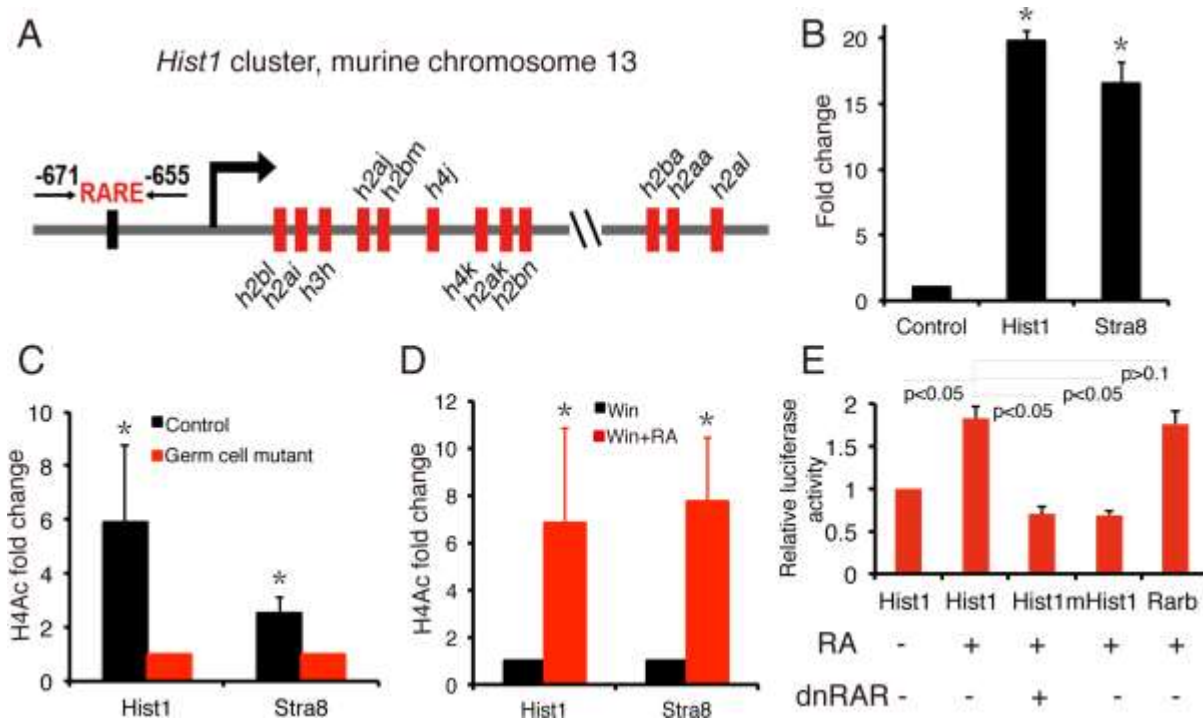


Figure 5. Functional RARE occurs upstream of the *Hist1* cluster. (A) The histone genes in the mouse *Hist1* and the position of putative RARE are shown. (B) Native ChIP with RARG or IgG antibodies followed by qPCR with primers encompassing RARE upstream of *Hist1*, *Stra8* (positive controls), or the control site (-2kb upstream of *Hist1* RARE) revealing the presence of RARG at the upstream RARE of *Hist1* and *Stra8*. Mean fold enrichment of three independent experiments at the *Hist1* and *Stra8* site is relative to the amount of DNA at the control site. Error bars represent the s.d.. \* $P < 0.001$ . (C) The level of acetylated histone H4 (H4Ac), a mark for open and actively transcribed chromatin, at RARE upstream of both *Hist1* and *Stra8* is significantly higher in control spermatogonia than in germ cell mutant spermatogonia. (mean  $\pm$  s.d.,  $n=3$ ; \* $P < 0.01$ , Student *t*-test). (D) The H4Ac level at RARE upstream of both *Hist1* and *Stra8* is significantly increased in RA-injected WIN18,466 treated mouse spermatogonia compared with WIN18,466 treated mouse spermatogonia. (mean  $\pm$  s.d.,  $n=3$ ; \* $P < 0.01$ , Student *t*-test). (E) Quantitative evaluation of the putative RARE at upstream of *Hist1* cluster. Relative firefly luciferase activity was normalized to Renilla for individual conditions. Data shown in graph represent the mean  $\pm$  s.d. fold change from control media (without RA treatment) of three

independent experiments. \* $P < 0.01$ . HIST1: a luciferase reporter containing the putative RARE at upstream of *Hist1* cluster; mHIST1: a luciferase reporter containing mutant putative RARE as described in *Materials and Methods*; Rarb: a luciferase reporter containing classic RARE at the Rarb gene promoter.

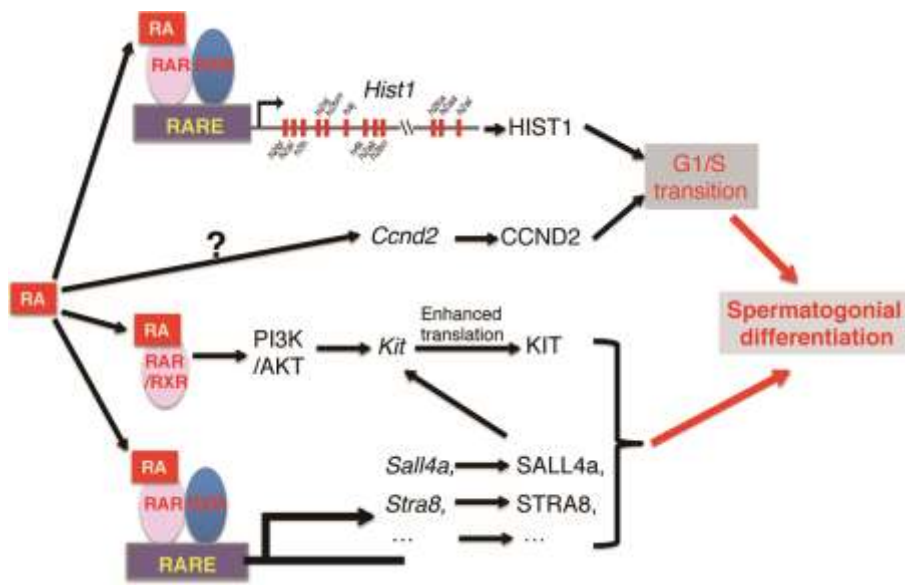


Figure 6. A proposed model for retinoid signaling-induced spermatogonial differentiation. RA signaling induces spermatogonial differentiation through (1) activating expression of genes (e.g. *Hist1*, *Ccnd2*) required for G1/S phase transition and (2) stimulating other gene expression such as *Stra8*, *Sall4a*, *Kit* in both genomic and non-genomic pathways.