

DISCOVERING MECHANISMS DURING SPERMATOGONIAL DIFFERENTIATION
THAT PREPARE MALE GERM CELLS FOR MEIOSIS

BY

TAYLOR A. JOHNSON, M.S., M.S.

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DIRECTOR OF DISSERTATION: CHRISTOPHER B. GEYER, PH.D.

DEPARTMENT OF ANATOMY & CELL BIOLOGY

Spermatogenesis, the male germ cell maturation process, allows men to become and remain fertile for decades. Male fertility relies on fate decisions of different types of spermatogonia – spermatogonial stem cells maintain the germline long-term, undifferentiated progenitor spermatogonia divide and await the differentiation signal (retinoic acid, RA), and differentiating spermatogonia proceed forward into meiosis as spermatocytes to ultimately become sperm. Spermatogonial differentiation and meiotic initiation are indispensable transitions in spermatogenesis that remain poorly understood. The three studies within this dissertation, using a combination of *in vivo* and *in vitro* approaches, detail spermatogonial requirements in these two transitions. The first study (chapter two) scrutinizes and redefines a presumed dogma – the requirement of RA for the spermatogonia-to-spermatocyte (mitotic-to-meiotic) transition. The second study (chapter three) deciphers the differential responsiveness of spermatogonia to RA in the adult testis. The third study (chapter four) introduces a novel RNA binding protein required for the commitment to and completion of spermatogonial differentiation. The collective findings from

these three studies both enrich our understanding of spermatogonial biology and propose avenues for treatments that can enhance or discontinue spermatogonial differentiation.

**Discovering mechanisms during spermatogonial differentiation that prepare
male germ cells for meiosis**

A Dissertation

Presented to the faculty of the Department of Anatomy & Cell Biology
Brody School of Medicine at East Carolina University

In partial fulfillment of the requirements for the degree
Doctor of Philosophy in Biomedical Sciences

By

Taylor A. Johnson, M.S., M.S.

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By

Taylor A. Johnson, M.S., M.S.

APPROVED BY:

DIRECTOR OF DISSERTATION: _____
Christopher B. Geyer, Ph.D.

COMMITTEE MEMBER: _____
Jeffrey B. Eells, Ph.D.

COMMITTEE MEMBER: _____
Jessica M. Ellis, Ph.D.

COMMITTEE MEMBER: _____
Karen Schindler, Ph.D.

COMMITTEE MEMBER: _____
Ann Sperry, Ph.D.

INTERIM CHAIR OF THE DEPARTMENT OF ANATOMY AND CELL BIOLOGY:

Espen E Spangenburg, Ph.D.

INTERIM DEAN OF THE GRADUATE SCHOOL:

Kathleen Cox, Ph.D.

DEDICATION

To my brother Evan Johnson, my parents Erika and Thomas Johnson; we did it.

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Thank you to my brother Evan, my mother Erika, and my father Thomas; your unconditional love, support, sacrifices, and guidance along the way cannot be measured. We've laughed, stressed, vented, and celebrated together, and who would've thought that the Johnson boys would both become doctors in life. We've come from humble beginnings, and at the end of the day, my success is our success.

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LIST OF SYMBOLS/ABBREVIATIONS

A_{Aligned}	Type A aligned undifferentiated progenitor spermatogonia
A_{Diff}	Type A differentiating spermatogonia
AMH	Anti- Müllerian hormone
ANOVA	Analysis of variance
A_{Paired}	Type A paired undifferentiated progenitor spermatogonia
A_{Single}	Type A single undifferentiated progenitor spermatogonia
A_{Undiff}	Type A undifferentiated progenitor spermatogonia
BMP	Bone morphogenic protein
BSA	Bovine serum albumin
BTB	Blood Testis Barrier
CCR-NOT	Carbon catabolite repression – negative on TATA-less
CDS	Coding sequence
CITS	Crosslink-induced truncation sites
CRE	Cre recombinase
CSF	Colony stimulating factor 1
CXCR4	Chemokine (c-x-c motif) receptor 4' (CXCR4)
CYP26	Cytochrome P450, family 26
CYP26A1	Cytochrome P450, family 26, subfamily a, polypeptide 1
CYP26B1	Cytochrome P450, family 26, subfamily b, polypeptide 1
CYP26C1	Cytochrome P450, family 26, subfamily c, polypeptide 1
DAPI	4',6-diamidino-2-phenylindole

DAVID	Database for Annotation, Visualization and Integrated Discovery
DAZL	Deleted in azoospermia-like
DDX4	DEAD box polypeptide 4
DEG	Differentially expressed gene
DMC1	DNA meiotic recombinase 1
DMEM	Dulbecco's modified Eagle medium
DMRT1	Doublesex and mab-3 related transcription factor 1
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DRAQ5	1,5-bis((2-(dimethylamino)ethyl)amino)-4,8-dihydroxyanthracene-9,10-dione.
E	Embryonic day
eCLIP-Seq	Enhanced crosslinking coupled with immunoprecipitation and RNA-Seq
EDTA	Ethylenediaminetetraacetic acid
eGFP	Enhanced green fluorescent protein
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
FMRP	Fragile X mental retardation protein
FSC	Forward scatter
FSH	Follicle-stimulating hormone
γ H2AX	Gamma H2A histone protein 4
GATA4	GATA binding protein 4
GDNF	Glial cell line derived neurotrophic factor

GFRA1	Glial cell line derived neurotrophic factor family receptor alpha 1
GO	Gene ontology
H1F6	H1.6 linker histone, cluster member
HBSS	Hanks' balanced salt solution
HEB	Hypotonic extraction buffer
HEPES	N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid
HPG	Hypothalamic-pituitary-gonadal
ICB	Intracellular bridges
ICSI	Intracytoplasmic sperm injection
ID4	Inhibitor of DNA binding 4
IIF	Indirect immunofluorescence
IME1	Inducer of meiosis
IP	Intraperitoneal injection
KIT	proto-oncogene receptor tyrosine kinase
KITL	KIT Ligand
KO	Knockout
LH	Luteinizing hormone
LIN28A	LIN-28 homolog A
LRAT	Lecithin-retinol acyltransferase
M6A	N6-methyladenosine
MEIOC	Meiosis specific with coiled-coil domain
MIS	Meiosis-inducing substance
MPS	Meiosis-preventing substance

mRNA	Messenger RNA
mTORC1	Mammalian target of rapamycin complex 1
NANOS2	Nanos C2HC-type zinc finger 2
NGN3	Neurogenin 3
OCT	Optical cutting temperature
P	Postnatal day
PAS	Periodic acid Schiff's stain
PBS	Phosphate buffered saline solution
PFA	Paraformaldehyde
PGC	Primordial germ cell
PIWI	P-element induced wimpy testis
PIWIL	PIWI like RNA-mediated gene silencing
PMSF	Phenylmethylsulfonyl fluoride
PRDM	PR domain zing finger protein
PTM	Peritubular myoid cell
qRT-PCR	Quantitative reverse transcription polymerase chain reaction
RA	Retinoic acid
RALDH	Retinaldehyde dehydrogenase
RAR	Retinoic acid receptor
RARA	Retinoic acid receptor, alpha
RARB	Retinoic acid receptor, beta
RARE	Retinoic acid response element
RARG	Retinoic acid receptor, gamma

RBM46	RNA-binding motif protein 46
RBP	RNA-binding protein
ReBP4	Retinol binding protein 4
RDH10	Retinol dehydrogenase 10 (all-trans)
RE	Retinyl ester
REC8	Meiotic recombination protein
RET	Ret proto-oncogene
RNA	Ribonucleic acid
RNA-Seq	RNA-sequencing
ROR2	Receptor tyrosine kinase-like orphan receptor 2
RRM	RNA recognition motif
RPL19	Ribosomal protein L19
RXR	Retinoid X receptor
RXRA	Retinoid X receptor alpha
RXRB	Retinoid X receptor beta
RXRG	Retinoid X receptor gamma
SE	Super enhancers
SOHLH1	Spermatogenesis and oogenesis specific basic helix-loop-helix1
SOHLH2	Spermatogenesis and oogenesis specific basic helix-loop-helix2
SOX	SRY-box transcription factor
SRY	Sex determining region of Y
SSC	Spermatogonial stem cell
STRA8	Stimulated by retinoic acid gene 8

SYCP3	Synaptonemal complex protein 3
TEX14	Testis expressed gene 14
TH	Thyroid hormone
TNAP	Tissue nonspecific alkaline phosphatase
TRA98	Germ cell-specific antigen
TUT	Tumorous testis
UCHL1	Ubiquitin carboxy-terminal hydrolase L1
UTR	Untranslated region
VAD	Vitamin A deficiency
WIN 18,446	N,N'-1,8-Octanediylbis (2,2-dichloroacetamide)
WT	Wild-type
YBX2	Y-box-binding protein 2
YTHDC2	YTH domain containing 2
ZBTB16	Zinc finger and BTB domain containing 16

CHAPTER I: INTRODUCTION

A. Germline origin and testis development

The foundation of the male germline and gonad starts in the early stages of development. During week five of human fetal development, the indifferent gonad begins to form. Two mesoderm-derived tissues, mesothelium and mesenchyme (embryonic connective tissue), give rise to the external and internal portions (respectively) of the future gonad. Meanwhile, the umbilical vesicle houses the future germ line, which are termed primordial germ cells (PGCs). PGCs must undergo specification and lineage restriction within the umbilical vesicle before migrating into the developing gonad.

In the mid-1950's, Alexander Chiquoine first identified PGCs within the proximal epiblast through their expression of 'tissue non-specific alkaline phosphatase' (TNAP)¹. TNAP⁺ PGCs migrate from the base of the allantois (a portion of the umbilical vesicle) to the gonadal ridge, a region formed from mesothelium²⁻⁴. Since these discoveries, the family of 'PR domain zinc finger proteins' (PRDMs) have been implicated in PGC specification. For example, in PRDM1 knockout (KO) embryos, PGCs could form, but the number of PGCs was significantly lower. *Prdm1* KO PGCs were also unable to proliferate, migrate, or form a functional germ line⁵. PRDM14 is also required for PGC specification through both epigenetic reprogramming and the re-expression of pluripotency genes^{6,7}. Pluripotency genes are expressed until the PGCs migrate into the gonadal ridge, where the cells proceed as unipotent progenitors^{8,9}. In addition to PRDMs, the family of 'bone morphogenic proteins' (BMP), specifically BMP2, BMP4, and BMP8b, are required for

both repressing the expression of somatic cell genes and activating the expression of PGC specific genes^{10–12}.

Following specification, the PGCs migrate from the allantois towards the hindgut of the developing fetus, and then laterally to the site of the developing gonad^{3,13–15}. Successful PGC migration relies on the expression of several genes. For example, PGCs lacking ‘receptor tyrosine kinase-like orphan receptor 2’ (ROR2) readily underwent apoptosis and demonstrated significantly impaired polarization towards the gonadal ridge¹⁶. The absence of ‘chemokine (c-x-c motif) receptor 4’ (CXCR4) in PGCs presented with a similar phenotype – PGCs were unable to colonize the developing gonad and migrate into the gonadal ridge¹⁷. ‘Receptor tyrosine kinase and its ligand’ (KIT/KITL) are required for both PGC function and migration following specification^{18,19}. KIT and KITL are both necessary for PGC colony expansion and motility initiation^{18,20}. Testes of *Kit* KO mice contained significantly fewer PGCs at embryonic (E) ages (e.g. E10, E11) within the allantois, hindgut, mesenchyme and gonadal ridges compared to wild type mice (WT)^{19,20}. Once PGCs have migrated into the gonadal ridge, proliferation of the mesothelium will stimulate the formation of gonadal cords – the site of future spermatogenesis.

B. Sex determination and prenatal testis development

Early in development, PGCs are considered bipotential, in that they have not yet committed to either the male or female fate. In mammals, sex determination occurs following expression of the ‘sex-determining region of Y’ (*Sry*) gene located on the Y chromosome^{21–24}. SRY is expressed by somatic cells (future Sertoli cells) in the nascent testis and activates expression of the downstream effector ‘SRY-box transcription factor’ 9 (SOX9)^{21,25,26}. As a transcription factor, SOX9 activates other developmental genes, including the gene for ‘anti-Müllerian hormone’

(AMH)^{26–28}. AMH is a hormone that represses the development of the Mullerian duct system which, in females, forms the future uterus, uterine tubes, and upper vagina^{29–31}. The requirement of SOX9 expression in the development of male reproductive structures was validated in male mice that lacked *Sox9* (loss-of-function approach) and in female mice that overexpressed *Sox9* (gain-of-function approach)^{32–34}. In mammals with these disrupted signaling cues, gonads containing ovarian and testicular tissue can form (termed the ovotestis). Clinically, these situations are rare, and the etiology is poorly understood^{35,36}.

Shortly following sex determination (week 7-8 in humans), the maturing Sertoli cells surround the male germ cells within the gonadal cords to form the seminiferous cords (future seminiferous tubules) and rete testis³⁷. PGCs are now referred to as prospermatogonia or gonocytes^{38–41}. Later in development, somatic cells termed peritubular myoid cells (PTMs) migrate to the exterior of the cords and form the basal lamina in conjunction with the Sertoli cells^{40–42}. As these events unfold, somatic cells within the mesenchyme receive signals from Sertoli cells to differentiate into Leydig cells^{43,44}. Leydig cells secrete the steroid hormone testosterone, which is essential for activating three key events: 1) development of the male duct system (e.g., epididymis, vas deferens); 2) development of the external genitalia; and 3) descent of the testis into the scrotum^{45–47}.

The proliferation activity of male germ cells changes as the testis develops. Using the mouse testis as a model, prospermatogonia were shown to proliferate until E14-16^{48–51}. Prospermatogonia arrest in the G0/G1 phase of the cell cycle and remain near the center of the seminiferous cord until after birth⁵¹. Shortly after birth, mouse prospermatogonia resume proliferation and migrate to the periphery of the testis cords.

The time that it takes germ cells to enter meiosis differ between males and females. In males, prospermatogonia are protected from entry into meiosis until after birth⁵¹. Meiosis initiates ~8 days after birth in male mice and during puberty (~12-14 years of age) in boys. In sharp contrast, female germ cells (termed oogonia after sex determination) enter meiosis in the fetal ovary in response to exposure to retinoic acid (RA; to be discussed in detail shortly)^{52,53}. After responding to RA, oogonia will proceed through mitosis, then into meiosis I as oocytes before arresting in diplotene. These oocytes remain arrested in meiosis I in association with somatic granulosa cells (analogous to Sertoli cells) as primordial follicles and will only complete meiosis I if they are selected following puberty.

Once the germline is specified for the male fate and the supporting cells form the tubule architecture, the next step is for the germline to be established.

C. Postnatal establishment of the male germline

Shortly after birth in mice (postnatal (P)1-2), prospermatogonia re-enter the meiotic cell cycle and relocate to the periphery of the testis cords; those that fail these two tasks will undergo apoptosis^{54,55}. Germ cells are flanked by Sertoli cells within the cord and PTMs outside the cord^{40-42,51}. Upon resuming mitosis, prospermatogonia proliferate⁵¹ and by P3-4, prospermatogonia transition – seemingly simultaneously – into all three types of spermatogonia - spermatogonial stem cells (SSCs), undifferentiated progenitor spermatogonia (A_{Undiff}) and differentiating (A_{Diff}) spermatogonia. The factors that direct adoption of these three spermatogonial fates is currently unknown⁵⁶. The formation of spermatogonia begins the first round, or wave, of spermatogenesis⁵⁷. The first A_{Diff} spermatogonia will enter meiosis as preleptotene spermatocytes (~ P8), complete meiosis and form haploid round spermatids (~P21) and exit the testis as sperm (~ P35 in mice)⁵⁸⁻

⁶⁰. Whereas first wave spermatogenesis begins shortly after birth in mice, the process begins upon entering puberty in boys. Germ cells within the human testis progress similarly to those in mice, although the process takes longer to complete (~70 days in humans compared to ~35 days in mice)^{61,62}.

Tubule histology and germ cell morphology have been historically used to follow germ cell progression. More recently, scientists have identified multiple protein markers of germ cell fate at different points in spermatogenesis. SSCs and early A_{Undiffs} express ‘Glial cell line derived neurotrophic factor family receptor alpha 1’ (GFRA1). GFRA1 heterodimerizes with ‘ret proto-oncogene’ (RET) to bind the ligand ‘glial cell line derived neurotrophic factor’ (GDNF) and facilitate SSC self-renewal and maintenance of the SSC population^{54,58,63}. The levels of GDNF in the testis are critical – loss of GDNF causes progressive cell loss, while its overexpression leads to a surplus of A_{Undiffs}⁶⁴. Another spermatogonial fate marker, ‘zinc finger and BTB domain-containing protein 16’ (ZBTB16, also known as PLZF) is expressed in all A_{Undiff} spermatogonia prior to entering differentiation^{65,66}. KIT is a cell surface receptor that is required for fertility and expressed in spermatogonia undergoing differentiation^{67–69}. ‘Synaptonemal complex protein 3’ (SYCP3) is localized to the synaptonemal complexes of spermatocytes, and the expression pattern of SYCP3 changes as the spermatocytes progress through the different phases of meiosis⁷⁰. Collectively, by using specific antibodies to detect these markers (and others), researchers have tracked germ cell fate and progression to better understand requirements for different transitions and events during spermatogenesis^{71–76}.

D. Organization of cells in the adult testis

Prospermatogonia in the neonatal testis become the spermatogonia that differentiate during the first wave of spermatogenesis^{56,77}. In subsequent waves of spermatogenesis (termed steady-state spermatogenesis) in adult testes, the source of germ cells is provided solely from the SSCs⁷⁷. Figure 1.1 demonstrates the progression of germ cells, from SSC to spermatozoa. Briefly, SSCs, making up less than 0.1% of the total germ cell population in the mouse testis, are largely singular cells (A_{Single}) that are distributed throughout the testis. Daughter cells of an SSC division that forgo the self-renewal program and initiate proliferation⁵⁶ will divide into pairs (A_{Paired}) then chains (A_{Aligned}) of 4, 8, 16, or 32 A_{Undiffs} , connected by intracellular bridges (ICBs)^{78–80}. These ICBs are $\sim 1\mu\text{m}$ in diameter and are formed by the action of ‘testis expressed gene 14’ (TEX14) which prevents abscission of cells during cytokinesis^{81–83}. These chains of A_{Undiffs} are destined to respond to RA and become type A_1 differentiating spermatogonia. As A_{Diffs} , they undergo five divisions ($A_1 - A_2 - A_3 - A_4 - \text{Intermediate} - B$) before dividing a sixth time and entering meiosis as preleptotene spermatocytes^{78,79} (Fig. 1.2). These primary spermatocytes will continue through the lengthy meiotic program, from preleptonema, leptonema, zygonema, pachynema, to diplonema. At the conclusion of meiosis I, the newly formed secondary spermatocytes quickly complete meiosis II to form four haploid round spermatids⁸⁴. These round spermatids must then complete the specialized morphogenetic program of spermiogenesis, during which they elongate and condense their nuclei while developing a flagellum to form sperm⁸⁴ (Fig. 1.1). The sperm will eventually leave the testis, transit the epididymis where they gain motility, and exit through the remainder of the male reproductive duct system and out the penis⁸⁵.

In the neonatal testis, Sertoli cells physically span the developing seminiferous epithelium, and their nuclei reside near the basement membrane of the seminiferous cord. During the first two-three weeks of postnatal development in the mouse, as germ cell populations expand, Sertoli cells

extend from the base of the cords and contribute to the formation of lumina^{86,87}. Seminiferous cords, with this new lumina, are now called seminiferous tubules. Sertoli cells proliferate until approximately P15, then terminally differentiate in response to thyroid hormone (TH) signaling⁸⁸. Dysregulation of TH production can either increase proliferation (if TH production decreases) or decrease Sertoli cell proliferation and cause premature mitotic arrest (if TH production increases)⁸⁹.

Sertoli cells have several roles in the testis, particularly in the organization and function of germ cells within the tubule. Sertoli cells form the blood-testis barrier (BTB), a compilation of tight junction complexes that divide the seminiferous tubule into two compartments – the adluminal (housing meiotic spermatocytes and postmeiotic spermatids) and basal (housing SSCs, A_{Undiffs} and A_{Diffs}). This barrier restricts passage of charged molecules, antibodies, and other agents into the adluminal compartment^{90–92}. Each Sertoli cell can support ~20-30 germ cells within the testis⁹³ by providing key nutrients such as iron, pyruvate, lactate, and RA for use in germ cell progression^{94–97}. In addition to these nutrients, Sertoli cells can synthesize GDNF upon receiving stimulation from follicle stimulating hormone (FSH) for use in SSC regulation⁸⁹. Sertoli cells also play a central role in the creation and maintenance of an immunoregulatory environment within the seminiferous tubule^{94,98}.

Additional cell types within the adult testis also contribute to daily spermatogenic function. For example, PTMs are multifunctional smooth muscle cells that physically wrap around the Sertoli cells and germ cells to encase the tubules⁹⁹. Innervation from the autonomic nervous system stimulate peristalsis-like actions of the PTMs, helping to facilitate the exodus of immotile testicular sperm into the epididymides¹⁰⁰. Like Sertoli cells, PTMs can secrete proteins that affect germ cell behavior, including RA catabolic enzymes as well as GDNF^{101–103}. External to the PTMs is the

interstitial compartment, which houses the neurovascular network, along with macrophages and Leydig cells. Particularly of note, Leydig cells secrete testosterone as well as ‘colony stimulating factor 1’ (CSF1), which promotes the proliferation of GFRA1+ spermatogonia¹⁰⁴.

Altogether, the testis houses somatic cells and several generations of germ cells. The interplay between germ cells and somatic cells ensures long-term viability of the germline and consistent sperm production.

E. RA synthesis and signaling in the testis

Developmental biologists have known for almost 100 years that male fertility requires vitamin A¹⁰⁵. Vitamin A is an essential micronutrient, meaning the body needs it to perform daily functions, but cannot produce it¹⁰⁶. Therefore, it must be consumed from the diet. The vitamin A family includes retinol, retinal, retinyl esters (REs) and beta-carotene (Fig. 1.3). The two major forms found in the human diet are preformed vitamin A (retinol, retinal, REs, RA) from animal products and provitamin A carotenoids (e.g., beta-carotene) from plant-derived foods¹⁰⁷. Upon internalization, retinoid derivatives either circulate within the blood for distribution to target organs or are transported to the liver for repackaging and/or storage. The synthesis of RA primarily relies on a two-step enzymatic process within the host tissue. First, retinol is converted into retinal (also known as retinaldehyde) by ‘retinol dehydrogenases’ (RDH, e.g., RDH10)^{108,109}. Thereafter, retinal is converted into RA by one of several ‘retinal dehydrogenases’ (RALDH, e.g., RALDH1, 2, 3)^{110,111} (Fig. 1.3). These enzymes have differential expression in tissues, with RALDH1 and RALDH2 being expressed in the testes¹¹¹. Due to the short half-life in tissues (<90 minutes in mammals), RA must be rapidly used within the resident tissue before it is degraded^{112–114}.

Several studies have clearly shown the requirement for vitamin A in maintaining male fertility. Testes from mice and rats fed vitamin A deficient (VAD) diets contain tubules lacking differentiating spermatogonia, meiotic spermatocytes, postmeiotic spermatids, or sperm^{105,115–117}. This VAD condition is reversible – when vitamin A is reincorporated into the diet, spermatogenesis is not only restored, but germ cells proceed through the process together in synchrony, which is markedly different than in unaltered adult mammals^{105,115–117}.

There are two primary sources of RA within the testis – Sertoli cells and late-stage spermatocytes (e.g., in pachytene). Evidence for this comes from mouse studies where enzymes responsible for converting vitamin A family members, enzymes responsible for synthesizing RA, or specific cells within the testis were deleted^{109,118,119}. Evidence for the Sertoli cell source of RA comes from the deletion of RDH10 in juvenile mice; deletion of *Rdh10* in Sertoli cells resulted in the degeneration of seminiferous tubules, and the deletion of *Rdh10* in Sertoli cells and germ cells prevented spermatogonia differentiation¹⁰⁹. When RA was administered to these juvenile mice, spermatogenesis could resume¹⁰⁹. In a separate study, conditional deletion of RALDHs from Sertoli cells resulted in the accumulation during the first wave of A_{Undiffs} that were unable to differentiate¹¹⁸. These studies have positioned Sertoli cells as one required source of RA, particularly during the first wave of spermatogenesis. Pachytene spermatocytes also express RALDH2 enzymes, and thus have been posited to contribute to RA synthesis in the adult testis^{111,118–121}. Depletion of spermatocytes via VAD or treatment with hydroxyurea (killing cells in S phase) significantly decreased RALDH2 mRNA levels and lowered testicular RA levels^{111,119}. Hydroxyurea treatments also impaired spermatid elongation and spermiation, which was rescued after RA treatment¹¹⁹. Collectively, pachytene spermatocytes have been proposed as an additional source of RA, which may support distinct processes (e.g., spermatid elongation and spermiation)

compared to Sertoli cell-derived RA (e.g., spermatogonia differentiation and entry into meiosis)¹¹⁹. Macrophages positioned within the periphery of the seminiferous epithelia (near borders between PTMs) also contain RALDH enzymes, and depletion of macrophages has shown to hinder, but not prevent, spermatogonial differentiation¹²². At this time however, the volume of RA produced by macrophages and the potential involvement of macrophage-derived RA in facilitating different transitions in spermatogenesis is unknown¹²².

F. Vitamin A transport, signaling and regulation in the testis

Once taken in from the diet, vitamin A is transported through circulation through binding to lipoproteins (e.g., beta-carotene) or ‘retinol binding protein 4’ (ReBP4) (e.g., retinol). Mice lacking ReBP4 have significantly lower levels of retinol and retinyl esters compared to WT mice¹²³. Although no histological abnormalities were noted in ReBP4 KO mice fed a normal diet, several abnormalities were evidenced once they were put on VAD; 1) presence of large vacuoles; 2) tubules devoid of germ cell generations; 3) sloughing of immature germ cells into the lumen; and 4) failure of spermatids to undergo spermiation¹²³.

Vitamin A has two fates once it reaches its target organ – to be converted into a bioactive derivative (e.g. RA) or be repackaged and stored as retinyl esters for future use. If “designated” for storage, vitamin A requires the enzyme ‘Lecithin-retinol acyltransferase’ (LRAT) for conversion into an RE. Although *Lrat* KO mice fed a normal diet were viable and fertile with histologically normal testes, they were more susceptible to developing VAD compared to WT mice¹²⁴. Additionally, meiotic spermatocytes in *Lrat* KO mice were present when maintained on a normal diet, but notably absent on a VAD diet¹²⁴.

RA is a lipophilic molecule and thus can readily enter tissues and cells. In spermatogonia, RA can bind to a ‘retinoic acid receptor’ (RAR) to regulate transcriptional changes¹²⁵. RAR can then heterodimerize with a ‘retinoid X receptor’ (RXR) (Fig. 1.3). Each of the three RARs and RXRs come in forms of alpha, beta, and gamma (RARA, RARB, and RARG; RXRA, RXRB, RXRG). This heterodimeric complex relocates to the nucleus where it binds to ‘retinoic acid response elements’ (RAREs) to regulate gene expression. RARB is undetectable at both mRNA and protein levels in the testis. Accordingly, *Rarb* KO mice were fertile with normal testis histology and spermatogenesis¹²⁶. RARA is readily detectable in Sertoli cells but is expressed at low levels in germ cells¹²⁷. Adult whole-body *Rara* KO mice had testes with disrupted seminiferous epithelia nearly devoid of germ cells, and *Rara* KO mice died within a few months¹²⁸. Tamoxifen was injected into *Tg(Sox9-cre)/Rara-lox* mice at P15 to generate Sertoli cell-specific *Rara* KO mice, which had abnormal spermatogenesis and spermiogenesis, increased vacuolization and substantial germ cell apoptosis once mice reached adulthood, a phenotype mimicking to *Rara*-whole body KO males¹²⁹. Both whole body- and Sertoli cell deletion of *Rara* resulted in infertility^{128,130–132}. The third RAR, RARG, is detectable only in late A_{Undiffs} and early A_{Diffs} in the mouse testis^{74,127}. Testes of *Rarg* KO mice had a progressive loss of germ cells over time due to impaired spermatogonia differentiation (and a concomitant buildup of undifferentiated progenitors)¹²⁷. Conversely, the recombinant expression of RARG in SSCs and early progenitors (using a transgene in which the *Gfra1* promoter was linked to the *Rarg* coding sequence) drove progenitors into differentiation precociously¹³³. Interestingly, when RARG was deleted, germ cells could proceed through both the first wave and the subsequent steady-state spermatogenesis. This suggests RARA may play a compensatory role¹²⁷.

In comparison to RARs, loss of RXR function has less detrimental effects on spermatogenesis. Of the three RXRs, only loss of RXRB caused impaired spermatogenesis; Sertoli cell specific conditional as well as conventional *Rxrb* KO mice demonstrated age-related spermatogenic impairments (like RARG KO studies)^{132,134}. *Rxrb* KO mice had accumulated cholesterol esters in Sertoli cells, lower sperm counts, and impaired sperm release from the seminiferous epithelia^{134,135}. Experiments using mice containing a dominant-negative version of RARA that could dimerize with RXRs were infertile, demonstrating the importance of RAR/RXR signaling for spermatogonia differentiation¹³⁶. More specifically, germ cells from this dominant negative line could not enter differentiation, like VAD mice¹³⁶.

The levels of RA within tissues are the result of the balance in synthesis and breakdown, or catabolism. There are three cytochrome P450 enzymes (CYP26A1, B1, and C1) with established roles in RA catabolism throughout the body. *Cyp26c1* is undetectable in the testis, and global *Cyp26c1* KO mice were viable and fertile¹³⁷. In comparison, the enzymatic activity of CYP26B1 is particularly critical for early male germ cell development and for preventing prospermatogonia exposure to RA in the developing testis⁵¹⁻⁵³. After birth, CYP26B1 is most readily detectable in PTMs¹⁰¹, suggesting a role in insulating cord from RA produced by cells in neighboring cords and cells within the interstitium¹²⁰. In testes of *Cyp26b1* KO mice, Sertoli cells precociously entered meiosis, revealing a role for CYP26B1 in protecting fetal prospermatogonia from RA exposure¹³⁸. However, deletion of CYP26B1 in both Sertoli cells and germs cells disrupted spermatogenesis and led to subfertility¹³⁹. Whereas CYP26B1 has been positioned as the primary RA catabolic enzyme, CYP26A1 has a seemingly minor role in spermatogenesis. *Cyp26a1* is an RA responsive gene, and as such its expression increases in multiple tissues after RA treatment^{74,140,141}. Knockout mice of *Cyp26a1* in germ cells only, Sertoli cells only, and both

cell types had tubule histology indistinguishable from WT testes¹³⁹. Deletion of *Cyp26a1* and *Cyp26b1* in germ cells or Sertoli cells increased vacuolization and delayed spermiation, but sperm was still produced, and mice remained fertile¹³⁹. There was also no compounding effect noted on male reproduction when both *Cyp26a1* and *Cyp26b1* were deleted, compared to *Cyp26b1* knockout mice¹³⁹. Altogether, the regulation of RA is critical for ensuring proper spermatogenesis.

G. RA utilization by spermatogonia during spermatogenesis

Germ cells undergo several transitions during spermatogenesis to produce sperm. Tubule sections with the highest RA levels contain these key transitions – spermatogonial differentiation, meiotic initiation, spermatid elongation, and spermiation¹¹⁹. For the purposes of the research described in this dissertation, **we will focus on the first two transitions: 1) initiation of differentiation, as $A_{Undiffs}$ convert into A_{Diff} spermatogonia; and 2) initiation of meiosis, as A_{Diffs} convert into spermatocytes.**

In mammals, germ cells from each sex enter meiosis on different temporal timelines. In males, germ cells do not enter meiosis until after birth, while in females germ cells enter meiosis during fetal development. This raises a conundrum – is meiotic initiation an intrinsic, pre-programmed property of germ cells, or a specialized response to an external stimulus (or stimuli)? The concepts of a ‘meiosis-inducing substance’ (MIS) and a ‘meiosis-preventing substance’ (MPS) were first proposed in the 1970’s in experiments using cultures enriched with male and female germ cells¹⁴². When male and female germ cells were cultured together, the male germ cells also entered meiosis, which supported the existence of a diffusible MIS¹⁴². Further research conducted two decades later cemented the concept of a MIS. When fetal spermatogonia (~E12.5 in mice) were removed from the gonadal ridge and cultured with either female genital ridge cells

or lung cells (chosen due to their different environment and embryonic origin), the male germ cells entered meiosis¹⁴³. When female germ cells were cultured with male gonadal ridge cells however, no meiosis was seen¹⁴³. These results validated the existence of both an MIS and MPS. The identities of both the MIS and MPS were later revealed in studies using both the fetal ovary and the fetal testis^{52,53}. In the fetal ovary, meiosis was induced by RA derived from the adjacent meiosis. In comparison, in the fetal testis, RA was metabolized by CYP26B1, thus preventing germ cells from entering meiosis until after birth^{52,53}. Therefore, RA was labelled the long sought-after MIS, and CYP26B1 the corresponding MPS.

‘Stimulated by retinoic acid 8’ (*Stra8*) is an RA-response gene informally referred to as the *meiotic gatekeeper* in literature. *Stra8* is detectable at both the mRNA and protein level in differentiating spermatogonia and in spermatocytes^{144–146}. Both *Stra8* mRNA and protein levels increase in response to RA, leading researchers to posit STRA8 is responsible for directing germ cells into meiosis¹⁴⁵. Interestingly, *Stra8* KO studies have shown conflicting results that do not support the requirement of STRA8 for meiotic initiation^{147,148}. Testes from *Stra8* KO mice on a mixed C57BL/6 x 129/Sv background contained germ cells that could replicate their DNA during preleptonema at the beginning of meiosis, express meiotic genes, and progress into early pachynema days later¹⁴⁷. These authors concluded that STRA8 was dispensable for spermatogonia differentiation and meiotic initiation¹⁴⁷. A separate group published a similar study in the same year using *Stra8* KO mice on a congenic C57BL/6 background. In those mice, germ cells did not progress as far, arresting during preleptonema¹⁴⁸. Thus, STRA8 is required for progression through meiosis on both backgrounds, but there are strain-specific differences in the timing of the appearance of the phenotype. It remains unclear whether STRA8, and more importantly RA, is required for meiotic initiation. **The current dogma – which is based more on presumption than**

evidence – is that RA is required for meiotic initiation, however this has never been experimentally tested.

We and others have convincingly shown RA is required to initiate spermatogonial differentiation^{67,118,133}. However, how spermatogonia respond (or not) to RA was poorly understood. To define RA responsiveness in the foundational pool of spermatogonia, neonatal mouse pups were treated with exogenous RA and spermatogonia were categorized based on their response to RA⁷⁴ (Fig. 1.4). We discovered the following: 1) SSCs cannot respond to RA (“RA-insensitive”), and thus are protected from differentiation; 2) A_{Undiffs} are “RA-responsive” and are poised to respond to RA but have not yet done so; and 3) A_{Diffs} have already responded to RA (“RA-responded”)⁷⁴. To extend these findings to spermatogonia undergoing steady-state spermatogenesis in the adult testis, one must consider that the adult testis contains the full complement of germ cells, multiple sources of RA within the tubule, and a distinct architecture of the seminiferous epithelium which is subdivided into basal and adluminal compartments. Despite these differences between the neonatal and adult testis, the concepts pertaining to RA signaling in spermatogonia are likely quite similar – the different subtypes of spermatogonia reside near one another and appear to be exposed to RA together, but they must respond to RA differentially. This differential response of spermatogonia to RA presents as ignoring/not responding to RA or responding and differentiating with the commitment to complete the rest of spermatogenesis. **To-date however, the molecular mechanisms by which spermatogonial differentiation is directed in response to RA levels during adult steady-state spermatogenesis remain undefined.**

H. Molecular mediators of spermatogonial differentiation

Several genes required for differentiation have detectable levels of mRNAs in undifferentiated spermatogonia, but only become effectively transcribed into protein in response to RA at differentiation. Examples include *Kit* and ‘spermatogenesis and oogenesis specific basic helix-loop-helix’ factors (*Sohlh1* and *Sohlh2*)^{67,71}. In response to RA, mRNAs were recruited into heavy polysome complexes, resulting in increased protein levels for KIT, SOHLH1 and SOHLH2 without corresponding increases in mRNA abundance^{67,71}, suggesting RA exerts a significant role in stimulating protein synthesis. Genome-wide transcriptome analysis using bulk and single-cell RNA-sequencing (RNA-seq) revealed relatively few genes had differential mRNA levels at initiation of spermatogonial differentiation^{149,150}. The requirement for some of these genes has been verified using *in vivo* KO models. For example, in mice with whole-body deletions of the germline specific *Sohlh1* and *Sohlh2* genes, spermatogonia were unable to differentiate^{151,152}. In mice with a single mutation in the *Kit* gene, (resulting in impaired activity of KIT), homozygous mice were completely sterile, with tubules containing only Sertoli cells and SSCs¹⁵³. Deletion of another SOX gene (*Sox3*) presented with a similar phenotype – impaired spermatogonial differentiation, although there was some recovery of the germline as they aged^{154,155}.

Several meiotic genes are transcriptionally upregulated during differentiation (e.g. ‘meiotic recombination protein’ (REC8), ‘synaptonemal complex protein 3’ (SYCP3), ‘deleted in azoospermia-like’ (DAZL))^{156–158}. These genes are not required for spermatogonial differentiation, but rather for spermatocyte progression through meiosis. Thus, the activation of these genes in spermatogonia may occur in preparation for subsequent meiosis. This also suggests post-transcriptional regulation plays an active – and likely critical – role in regulating protein synthesis in spermatogonia.

Key post-transcriptional control over mRNA fate is often exerted by RNA-binding proteins (RBPs), which regulate mRNA storage, decay, or the efficiency of translation. Arguably the best-studied RBP in the male germline is ‘nanos C2HC-type zinc finger 2’ (NANOS2), which binds and suppresses expression of differentiation- and meiosis-associated mRNAs such as *Stra8*, thus preventing differentiation and meiotic initiation^{159–161}. NANOS2, expressed in prospermatogonia and SSCs, binds specific cohorts of mRNA with P-bodies to initiate their decay in cooperation with the ‘carbon catabolite repression – negative on TATA-less’ (CCR-NOT) deadenylase complex^{159–161}. The requirement for *Nanos2* is clear, as its loss results in complete male germline extinction prior to birth¹⁶⁰. The *Nanos2* gene is remarkably conserved, as CRISPR-Cas9 removal of *Nanos2* in cattle, goats, pigs, and mice also resulted in male sterility¹⁶². Two members of the “P-element induced wimpy testis” (PIWI) family, “PIWI like RNA-mediated gene silencing 2” (PIWIL2) and “PIWI like RNA-mediated gene silencing 4” (PIWIL4), are critical for protein synthesis and the regulation of DNA methylation of transposons^{163–165}. “DEAD-box helicase 4” (DDX4) is also active in methylating DNA and the piRNA processing pathway¹⁶⁶. DAZL is required for germ cells to respond to RA, and absence of *Dazl* depletes the germline^{156,167}. Deletion of the “Y-box-binding protein 2” gene (*Ybx2*, also referred to as *Msy2*) leads to aberrant post-transcriptional control in spermatocytes, with reduced numbers of post-meiotic mRNAs, as well as sterility in both male and female mice^{168–170}. **Collectively, RBPs are critical for the regulation of gene expression throughout spermatogenesis, resulting in the preservation and maintenance of long-term fertility. Identifying a novel RBP, particularly one essential for spermatogonial differentiation, would provide novel insights into the molecular mechanisms that govern this key transition in spermatogenesis.**

I. Potential avenues for repressing and enhancing male fertility

Findings from the work detailed here are applicable to both sides of the fertility pendulum – identifying novel therapeutic targets for male contraceptive drugs that safely and reversibly inhibit spermatogenesis and developing new strategies to improve spermatogenesis in men struggling with subfertility or infertility.

The development of effective male contraception treatments would provide men a new option to assist in family planning. Having more options would be expected to reduce both population growth and the number of abortions. Recent population studies have shown that men are more willing than in years past to use contraceptives^{171,172}. Excluding changes in lifestyle habits (e.g., abstinence, rhythm method), men have two reliable, available contraceptive options – condoms and vasectomies. Condoms are an economic, non-surgical option, which provide a physical barrier to prevent sperm from entering the female reproductive system. Condoms are also quite effective, with a user failure rate of 2% when used perfectly (consistent and correct use) and a failure rate of 13% with typical use (including inconsistent/incorrect use)^{173,174}. Vasectomy, the surgical ligation of the ductus deferens, has been used since the 1890s as a non-reversible male contraceptive therapy/treatment¹⁷⁵. The success rate (causing sterility) is 99.7%, when measured ~12 weeks after the procedure¹⁷⁶, and the likelihood of complications post-procedure is low¹⁷⁷. Vasectomies can also be reversed (termed vasovasostomy) to restore fertility, however the success rate is contingent on two key factors – overall patient health and the amount of time between the vasectomy and the vasovasostomy. For example, if the reversal was performed less than three years after the vasectomy, fertility restoration rates approach ~75%. However, this success rate is ~30% if the reversal was done 15 years after the vasectomy¹⁷⁸.

Alternative contraceptive approaches have been explored over the past few decades. One approach is to target the body's hypothalamic-pituitary-gonadal (HPG) axis; exogenous testosterone, in combination with endogenous progesterone, could theoretically be used to induce negative feedback responses and suppress levels of the gonadotrophins luteinizing hormone (LH) and FSH^{179,180}. Although successful in isolation, developing testosterone-based targeting strategies have proved challenging. Recent studies have shown androgen-based contraceptives result in severe mood changes and depression in men; one study even reported a suicide^{181,182}. Therefore, any future therapies must consider the overall well-being of the patients before proceeding. Several non-hormonal treatments (e.g., Gossypol, Adjudin) have also been proposed, but all these approaches to-date have shown significant noteworthy side effects¹⁸³. Triptonide, a natural compound derived from the Chinese herb *Tripterygium Wilfordii Hook F*, was recently shown to induce sperm head-bending and disrupt motility¹⁸⁴. Daily treatment over several weeks caused male infertility in rodents and monkeys, and cessation of Triptonide treatment led to the resumption of spermatogenesis¹⁸⁴. No evident toxicities were documented in these subjects in critical organs (e.g., liver, kidney), although its efficacy and toxicity in humans are unknown.

Several studies have also targeted RA signaling in the design of contraceptives. Multiple compounds, functioning as RAR-antagonists, have shown to be both successful and reversible in rodents, but clinical trials have not yet begun with these antagonists¹⁸⁵. Pharmacologic inhibition of RALDHs can also block spermatogonial differentiation, leading to the accumulation of A_{Undiffs} in the testis^{186–188}. RALDH is inhibited by the compound N,N'-1,8-Octanediylbis (2,2-dichloroacetamide) (WIN 18,446; also referred to as BDAD). In the 1960's, men treated with WIN 18,446 showed significant reductions in sperm production¹⁸⁹. Although the drug was well tolerated overall, an unexpected side-effect occurred - men who consumed alcohol while undergoing

treatment developed “disulfiram reactions”, which include excessive sweating, vomiting, nausea, and impaired cardiovascular function¹⁸⁹. Considering >60% of men in the United States drink alcohol¹⁹⁰, and global alcohol consumption has steadily increased over the past few decades (+70% since 1990)¹⁹¹, a contraceptive designed to specifically target RA synthesis with these alcohol-related side effects would be unlikely to be adopted and accepted.

Results from these studies are also applicable to enhance fertility in infertile or subfertile men. Approximately one in seven couples struggle with infertility worldwide, and men contribute to ~50% of all cases¹⁹². Infertility is also a significant issue in countries where diets contain limited nutritional value, thus increasing the likelihood of male subfertility or infertility later in life^{193,194}. Men with metabolic and cardiovascular diseases such as diabetes and obesity have reduced fecundity/fertility, with lower sperm counts and testosterone levels^{195–197}. Therefore, targeting specific transitions (particularly at spermatogonial differentiation) to enhance spermatogenesis, even if temporary, could greatly help couples struggling with infertility.

J. Rationale for study and Specific Aims

The overarching goal of this research is to decipher requirements directing spermatogonial differentiation prior to entering meiosis. Here, I report my progress on accomplishing and completing the following goals: 1) critically examine the requirement of RA for the transition into meiosis (and define the point of meiotic initiation or entry); 2) evaluate the effects of RA signaling on the development of spermatogonial subpopulations during steady-state spermatogenesis; and 3) determine the role of a recently-identified but highly evolutionarily conserved RBP (RNA-binding motif protein 46; RBM46) in spermatogonial development and progression.

Aim 1: Determine the requirement of RA signaling for the initiation, progression, and completion of meiosis in the mammalian testis. The rationale for goal #1 originates from the presumption that RA functions in the male as the MIS. We hypothesized that **RA is required for initiation of spermatogonial differentiation, but not for the initiation, progression, and completion of meiosis.** To directly test this *in vivo*, we used a steady-state synchronization model. In this model, we treated the testis with WIN 18,446 to block endogenous RA signaling and consequentially fill the testis with A_{Undiffs}. Thereafter, the administration of a singular RA pulse initiates differentiation in all spermatogonia (except the SSCs), and these newly differentiating spermatogonia move through spermatogenesis in synchrony. This model was adapted to one where WIN 18,446 was administered continuously throughout development. Thus, after receiving the pulse of RA, germ cells proceeded through an ‘RA deficient’ environment devoid of any endogenous RA. This was compared to ‘RA sufficient’ testes, where WIN 18,446 treatments were discontinued after receiving the RA pulse. These models, in conjunction with *in vitro* cultures, allowed for careful evaluation of the requirement of RA post-differentiation.

Aim 2: Decipher the responsiveness of spermatogonia populations to RA and their fate post-RA exposure in the adult testis. The rationale for goal #2 is to extend our previous findings of differential RA responses from the first wave spermatogenesis in the neonatal testis⁷⁴ into steady state spermatogenesis in the adult testis. There is a notable gap in our understanding of how spermatogonia that respond to RA modulate their fate and progress in spermatogenesis. We hypothesized that **spermatogonia maintain this differential response in the adult, and the capacity to precociously advance in response to RA is limited to specific spermatogonia populations.** To test this, I utilized *in vivo* mouse models, both adult wild type and those

undergoing synchronization, to assess spermatogonial response to RA and potential precocious progression into differentiation and meiosis.

Aim 3: Elucidate the requirement for and role of Rbm46 in mammalian spermatogenesis. The rationale for goal #3 stems from the lack of molecular mechanisms that govern spermatogonial differentiation in response to RA. This is a critical gap in knowledge given the importance of regulating gene expression changes during spermatogenesis. In performing a functional screen, we identified a germline specific RBP, RBM46, with an undefined function in spermatogenesis. Thus, we hypothesized that **RBM46 plays a critical role in post-transcriptional control during germ cell development, differentiation, and progression in the mammalian testis.** We employed multiple mouse models, including *Rbm46* KO and *Rbm46*-FLAG tagged transgenics, to define its requirement and role in male germ cell progression.

K. Chapter 1 Figures

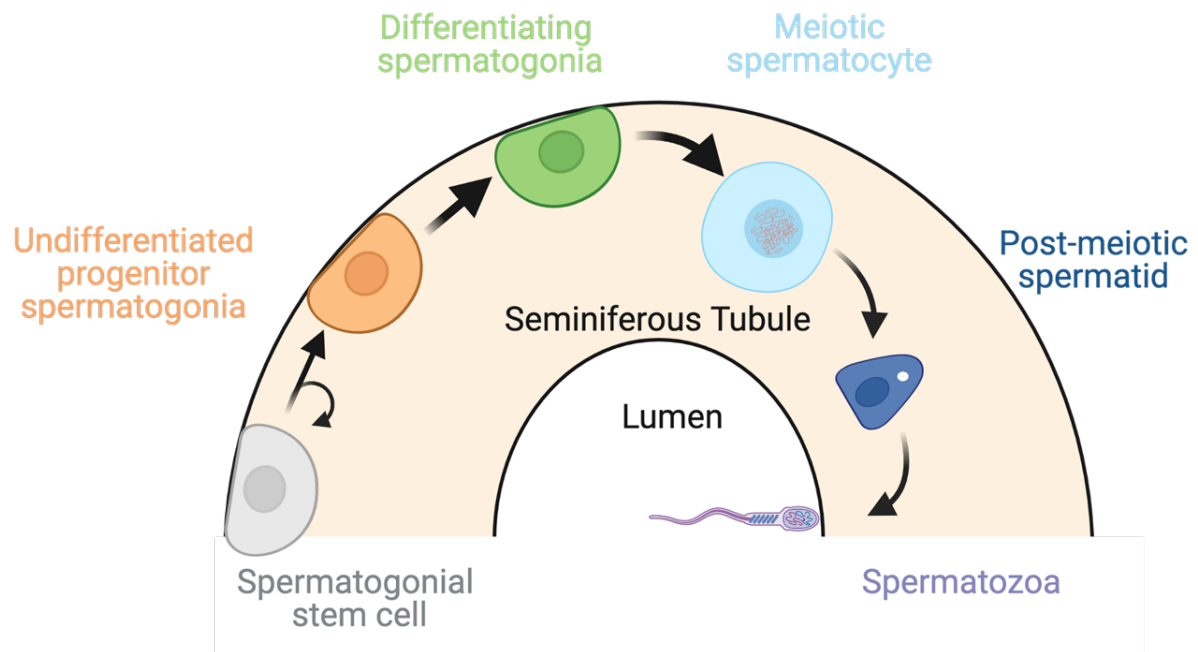


Fig. 1.1 Progression of germ cells through spermatogenesis.

Schematic of germ cells and their transitions in spermatogenesis. Spermatogonial stem cells self-renew or forgo renewal to become mitotically active undifferentiated progenitor spermatogonia. Progenitors will continue to divide until signaled to enter differentiation. Differentiating spermatogonia will proceed through the fixed differentiation program and complete differentiation before entering meiosis as spermatocytes. Spermatocyte will proceed through and complete meiosis before entering spermiogenesis as a post-meiotic spermatid. Spermatids will undergo a specialized process known as spermiogenesis (not depicted here) that results in the formation of spermatozoa. Created using BioRender.com.

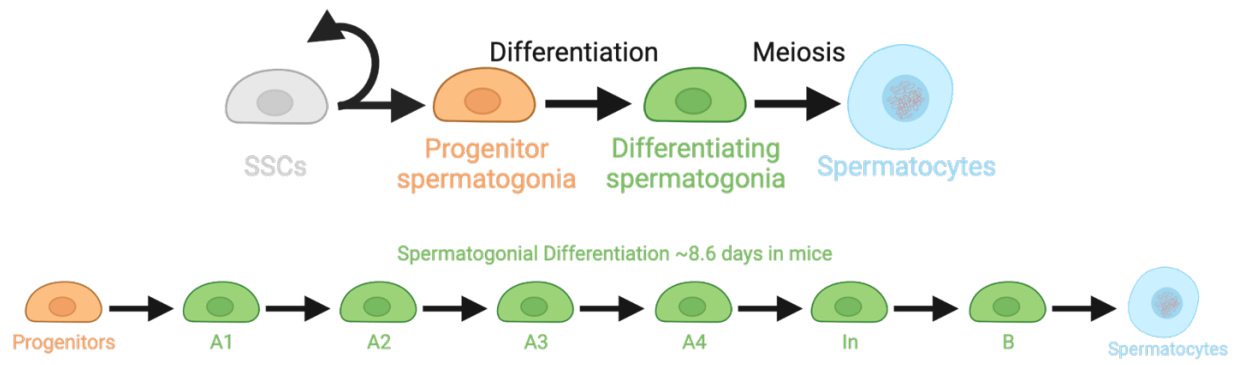


Fig. 1.2 Sub-types of differentiating spermatogonia in the mouse testis.

Schematics of spermatogonia progression into meiosis as spermatocytes. Once germ cells forgo self-renewal, the progenitors will divide before differentiating. As differentiating spermatogonia, germ cells undergo five additional divisions – these germ cells are classified as type A1, A2, A3, A4, Intermediate (In) and B differentiating spermatogonia. This differentiation program takes approximately 8.6 days in mice, and germ cells must progress through this fixed program before dividing a sixth time and entering meiosis as pre-leptotene spermatocytes. Created using BioRender.com.

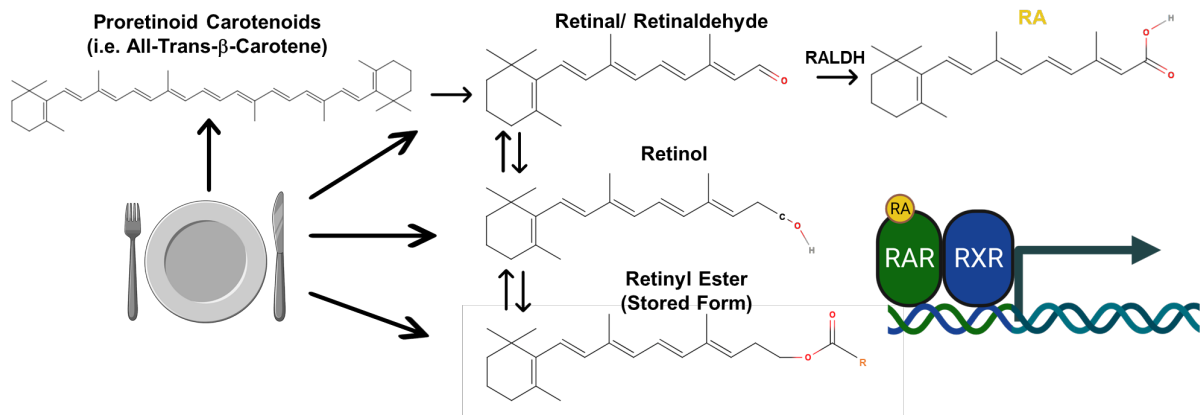


Fig. 1.3 Summary of RA synthesis.

Retinoic acid (RA) is derived from different members of the Vitamin A family internalized through the diet – this includes proretinoid carotenoids, retinal/retinaldehyde, retinol, and retinyl esters. These members can be reversibly converted for 1) storage (e.g., retinyl ester) and future use, 2) distribution throughout the body, or 3) synthesis of RA. Retinaldehyde dehydrogenases (RALDH) irreversibly convert retinal into RA. RA is then internalized into the nucleus and can bind to an RA receptor (RAR), which then heterodimerizes with a retinoid x receptor (RXR) before binding to RA response elements (RAREs) in DNA to manipulate transcription. Created using BioRender.com.

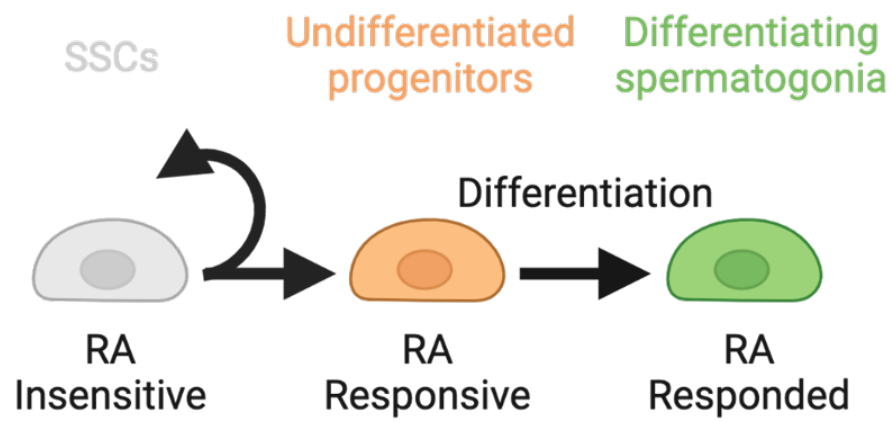


Fig. 1.4 Concept of differential responsiveness to RA in the neonatal testis.

Spermatogonia can be categorized based on the intrinsic ability to respond to RA. The spermatogonial stem cells (SSCs) are RA-insensitive, and thus cannot respond to RA. Undifferentiated progenitor spermatogonia are RA responsive and await the RA signal to enter differentiation. Finally, the differentiating spermatogonia responded to the RA. Created using BioRender.com.

CHAPTER II: MAMMALIAN MALE GERM CELLS INITIATE MEIOSIS IN THE ABSENCE OF RETINOIC ACID

This chapter is modified from a manuscript in final preparations for Proceedings of the National Academy of Sciences (PNAS).

A. Summary

Retinoic acid (RA) is the proposed mammalian ‘meiosis inducing substance’. However, evidence for this role comes from studies in the fetal ovary, where germ cell differentiation and meiotic initiation are temporally inseparable. In the postnatal testis, these events are separated by >1 week. Exploiting this difference, we discovered that although RA is required for spermatogonial differentiation, it is dispensable for the subsequent initiation, progression, and completion of meiosis. Indeed, the meiotic transcriptome program was largely unaffected by the absence of RA, but instead, spermiogenesis gene expression and spermatid morphogenesis were disrupted. Together, these data support an RA-independent model for male meiotic initiation.

B. Introduction

Meiosis is essential for sexual reproduction, and its specialized processes have been intensely studied in lower eukaryotes and in mammals. In mammals, two landmark studies using fetal mice identified retinoic acid (RA) as the long sought-after ‘meiosis-inducing factor’ (MIS)^{52,53}. However, the relevance of these observations to male meiotic initiation is questionable because in the postnatal mouse testis, a pulse of RA is essential for differentiation of mitotic spermatogonia^{115,198}, which precedes meiotic initiation by 8.6 days¹⁹⁹. Therefore, the conclusion

that a second pulse of RA is the sole molecular trigger for meiotic initiation is not supported by direct experimentation. Currently, the only evidence that RA directs male meiotic initiation is correlative activation of meiosis genes such as '*stimulated by retinoic acid 8*' (*Stra8*) and '*meiotic recombination protein 8*' (*Rec8*)^{145,156}, which are themselves not required for meiotic initiation, but rather for meiotic progression^{147,148,200}. Indeed, in *Stra8* KO mice on an inbred C57BL/6 background, preleptotene spermatocytes completed meiotic DNA replication but failed to load the meiotic cohesin REC8¹⁴⁸, while on a mixed genetic background preleptotene spermatocytes were formed, completed meiotic S-phase^{148,200}, and even reached pachynema¹⁴⁷. Therefore, the requirement for RA in meiotic initiation and progression needs to be critically examined using additional strategies.

Here, we used both in vitro and in vivo models paired with loss-of- and gain-of-function approaches (by preventing endogenous RA synthesis and administering exogenous RA) to examine the requirement for RA as the instructive signal for male meiotic initiation. We discovered that, in the absence of RA, male germ cells initiated, progressed through, and completed meiosis as haploid spermatids. The initiation of meiosis in RA-deficient male germ cells was accompanied by rather modest changes in gene expression, and the activation of meiosis-related genes was largely unaffected. However, there were significant changes in expression of genes encoding factors involved in and required for the morphogenetic changes during the later developmental program of spermiogenesis that creates sperm. An examination of this process revealed spermatids in RA-deficient testes failed to properly elongate and, consequently, no sperm were produced. In conclusion, our data support an RA-independent model of meiotic initiation in the mammalian testis.

C. Materials and methods

C.1 Synchronizing spermatogenesis and blocking retinoic acid (RA) synthesis *in vivo*

All procedures using mice adhered to guidelines outlined in the National Research Council Guide for the Care and Use of Laboratory Animals and were approved by the Animal Care and Use Committee at East Carolina University (approval #A3469-01). Uchl1-eGfp mice were obtained from The Jackson Laboratory (stock 22476²⁰¹). Originally on a C57BL/6 genetic background, these were outcrossed with CD-1 mice (Charles River Laboratories, Stock#022) to increase litter size and reduce dam cannibalism^{202–204}. Both mouse lines were used for these studies, and the day of birth designated as postnatal (P)0. Mice were humanely euthanized prior to P7 by decapitation, and after P7 by asphyxiation in CO₂ followed by cervical dislocation.

Spermatogenesis was synchronized similarly to previous reports^{186,187,205,206}. N^oN-1,8-Octanediylbis 2,2-dichloroacetamide (WIN18,446) (Cayman Chemical, 14018) was resuspended in vehicle (dimethyl sulfoxide (DMSO), final concentration 100 µg/g of body weight) was given orally for ten consecutive days (P1-P11) to block spermatogonial differentiation. Next, on P11, mice received one subcutaneous injection of 10 µl exogenous RA (10 µg/µl) in DMSO to initiate spermatogonial differentiation. Following RA injection, mice were treated in one of two regimens: 1) discontinuation of WIN18,446 treatment, enabling resumption of endogenous RA synthesis (hereafter referred to as ‘RA-sufficient’ synchronization); 2) continuation of WIN18,446 treatment to block endogenous RA synthesis (hereafter referred to as ‘RA-deficient’ synchronization). During RA-deficient synchronization, WIN 18,446 was administered orally from P11-14 and via intraperitoneal injection from P15 onward. Mice were humanely euthanized on specific subsequent days.

C.2 Fluorescence-activated cell sorting (FACS)

‘Enhanced green fluorescent protein’ (EGFP)+ germ cells from ‘Ubiquitin carboxy-terminal hydrolase L1’ (Uchl1)-eGfp mice were sorted on a Becton Dickinson AriaFusion cell sorter. A 100 mW 488 nm laser was used for excitation of the EGFP signal, and a 530/30 bandpass filter was used for detection of the emitted fluorescence. Dead cells were removed using forward and side scatter gating as well as fluorescence gating on cells excluding 4’6-diamidino-2-phenylindole (DAPI) (Thermo Fisher D1306). Single DNA-containing cells were identified using 1,5-bis((2-(di-methylamino)ethyl)amino)-4, 8-dihydroxyanthracene-9,10-dione (DRAQ5) fluorescent probe (Thermo Fisher 62254). Doublets were removed using forward scatter (FSC)-height vs FSC-area plots. An 85 µm nozzle was used for sorting, and the flow rate was controlled to 5,000-9,000 events/sec. Cells were recovered in sorting buffer (15% fetal bovine serum (FBS) + 10 mM ethylenediaminetetraacetic acid (EDTA) + 10 mM N-2-hydroxyethylpiperazine-N-sulfonic acid (HEPES) in 1X phosphate buffered saline (PBS)) in 5 ml polypropylene tubes (Corning LS, 352063) pre-coated with 10% bovine serum albumin (BSA). Cell purity was assessed by reanalyzing a small aliquot of the sorted cells by immunostaining.

C.3 *In vitro* cell culture

Single cell suspensions were prepared as described previously²⁰⁷. DNA content analysis was performed as described previously²⁰⁸, using “Basic Protocol 1” with minor adjustments. Testes were placed in Hanks' Balanced Salt Solution (HBSS, Thermo Fisher Scientific, 14175095), detunicated, and transferred to a solution containing 4.5 ml of 0.25% trypsin (Thermo Fisher Scientific, 15050065) and 0.5 ml DNase 1 (7 mg/ml, Sigma-Aldrich, DN25) in HBSS and

incubated at 37°C for 3 min. An additional 1 ml DNase 1 in HBSS was added, and the mixture was pipetted to further break up the testes, followed by an additional one min incubation at 37°C. One ml of FBS was added to deactivate the trypsin, and the mixture was triturated to break apart any remaining clumps of intact testis. The cell mixture was filtered through a 40 µm sieve and centrifuged at 600 x g for 7 min. The resulting cell pellets were resuspended in serum-free Dulbecco's modified eagle medium (DMEM)/F12 with Glutamax (Thermo Fisher Scientific 10565018) containing 1% Penicillin-Streptomycin (ThermoFisher Scientific 15070063). Media were made fresh for each experiment and sterile filtered through a 0.2 µm filter. Cultures were maintained at 34°C in 5% CO₂.

C.4 Meiotic Chromosome Spreads

Meiotic chromosome spreads were prepared as previously described²⁰⁹. Testes were dissected, detunicated, and incubated in ice-cold Hypotonic Extraction Buffer (HEB, 30 mM Tris HCl, 50 mM sucrose, 17 mM trisodium citrate dihydrate, 5 mM Ethylenediaminetetraacetic acid (EDTA), 0.5 mM dithiothreitol (DTT), and 0.2 mM phenylmethylsulfonyl fluoride (PMSF), pH 8.2) for 30-60 min based on individual testis weights. Testes were removed from HEB, separated into 3 mm chunks using forceps, and transferred to a 23 µl drop of 100 mM sucrose (pH 8.2). Tissue was then minced using a scalpel until a cloudy solution formed, and remaining pieces were removed with forceps and discarded. Cell suspensions were prepared by adding another 23 µl of 100 mM sucrose to the cell suspension and mixing by trituration. Slides were placed in a Coplin jar containing a fixative solution of 1% paraformaldehyde (PFA) and 0.15% Triton X-100 in 1X PBS (pH 9.2), removed after 1 hr at room temperature and tilted to allow a drop to form in the corner of the slide. Twenty µl of the cell suspension was mixed with the drop by trituration, and

the slide tilted to allow even coating by the cell suspension. Slides were dried for 2 hr in a humid chamber, then allowed to air dry completely (~30 min after removal of the humid chamber lid). Slides were then washed with 0.4% Photo-Flo 200 (Eastman Kodak Company, 146-4502) in 1X PBS twice for 5 min each, then with 0.4% Photo-Flo 200 in H₂O once for 5 min. Slides were air-dried and stored at -80°C prior to thawing for immunostaining meiotic spreads, using conventional methods.

C.5 Histology and Indirect Immunofluorescence (IIF)

For histological analyses, whole testes were immersion-fixed in Bouin's solution and Periodic Acid Schiff (PAS) staining was done using standard methods, and immunostaining was performed on PFA-fixed cryosections as previously described¹⁴⁶. For histological analyses, whole testes were immersion-fixed in Bouin's solution for 24 hr at 4°C, washed overnight in 1X PBS, dehydrated through an ethanol series, processed using standard methods, and embedded in paraffin. Sections (5 µm) were cut and stained with Periodic Acid Schiff using standard methods, and images were taken on a Zeiss Axio Observer A1 microscope outfitted with a Zeiss AxioCam 503 color digital camera and Zen software (Carl Zeiss Microscopy, LLC). For IIF, testes were immersion-fixed for 24 hr at 4°C in fresh 4% PFA, washed overnight in 1X PBS, and then incubated in 30% sucrose at 4°C for 24 hr. Testes were then frozen in optical cutting temperature (OCT) solution, and 5 µm cryosections cut for immunostaining. Sections were blocked in 3% BSA solution for 30 min before incubation with primary antibodies at room temperature for 1 hr. Primary antibodies used were the following: anti-‘germ cell-specific antigen’ (TRA98, 1:1000, ab82527, Abcam, rat monoclonal), anti-‘glial cell derived neurotrophic factor family receptor alpha 1’ (GFRA1, 1:800, AF560, R&D Systems, goat polyclonal), anti-‘proto-oncogene receptor

tyrosine kinase' (KIT, 1:1000, AF1356, R&D Systems, goat polyclonal) anti-'zinc finger and BTB domain containing 16' (ZBTB16/PLZF, 1:400, AF2944, R&D Systems, goat polyclonal), anti-'H1.6 linker histone, cluster member' (H1F6, 1:500²¹⁰), anti-'doublesex and mab-3 related transcription factor 1' (DMRT1, 1:1000²¹¹), anti-STRA8 (1:3000, ab49602), anti-'gamma H2A histone family member x' (γ H2AX, 1:400, ab11174). Primary antibody was omitted in negative controls. Following stringency washes, sections were incubated in secondary antibodies (1:500, Alexa Fluor donkey anti-rabbit-488, Alexa Fluor donkey anti-goat-488, Alexa Fluor donkey anti-goat-555, Alexa Fluor donkey anti-rat-555, ThermoFisher Scientific). Fluorescently conjugated anti-'synaptonemal complex protein 3' (SYCP3; SYCP3-488, 1:200, abcam ab205846), lectin (lectin-488, 1:500, Thermo Fisher L21409 and lectin-594, 1:500, Thermo Fisher L32459) were used. Coverslips were mounted with Fluoroshield mounting medium (enQuire Bioreagents, NC1666520), and images obtained using an Olympus Fluoview FV1000 confocal laser-scanning microscope (Olympus America).

C.6 DNA Content Measurement for DNA Ploidy

Testis single cell suspensions from P23 and P30 RA-sufficient and RA-deficient CD-1 males were fixed in ice-cold 70% ethanol at the concentration of 1×10^6 cells/ml and stored at -20°C until the day of analysis. On the day of analysis, ethanol-suspended cells were washed with PBS and resuspended in 20 μ g/ml PI/Triton X-100 staining solution with 5 μ g/ml RNase A and kept in the dark at room temperature for 30 min. The DNA content analysis was performed using Cytex Aurora flow cytometer equipped with 50 nW 561 laser. For emission, YG4 band pass (661/18) was used. Flow rate was set to low, and data were analyzed using a linear scale. Testicular

single cell suspensions from adult wild-type (WT) CD-1 testes were used for calibration and as a positive control.

C.7 qRT-PCR

Mice with synchronized spermatogenesis were euthanized at P17 for isolation of RA-sufficient type B spermatogonia and at P19 for isolation of RA-sufficient or RA-deficient preleptotene spermatocytes. FACS was used to isolate $2-3 \times 10^6$ intermediate/type B spermatogonia and $2-3 \times 10^6$ preleptotene spermatocytes (3-4 males, each litter). Total RNA was isolated from three independent preparations of cells using the GeneJet RNA purification kit (Thermo Scientific) and used for quantitative reverse transcription-polymerase chain reaction (qRT-PCR) and RNA sequencing (RNA-seq). For qRT-PCR analysis, 0.5 μ g total RNA was reverse transcribed to generate cDNA using the iScript cDNA Synthesis Kit (Bio-Rad) according to manufacturer's instructions. qPCR was performed using the SsoFast EvaGreen Supermix (Bio-Rad) on the Bio-Rad CFX-384 or CFX-96 real-time PCR System, and beta-actin was used for normalization.

C.8 RNA-Seq

Fluorescence-activated cell sorting (FACS) of isolated Uchl1-EGFP⁺ germ cells were used for total RNA isolation from two or three independent preparations of cells using the GeneJet RNA purification kit (Thermo Scientific). Subsequently, cDNA libraries were produced from total RNA with the Illumina TruSeq Stranded mRNA kit according to manufacturer recommendations. Libraries were sequenced with an Illumina NovaSeq6000 instrument with single-end (75 bp) chemistry to ~30M/sample depth. All datasets (raw and analyzed) produced for these studies have

been deposited into a publicly available database at NIH GEO (GSE194376). To scrutinize GEO accession GSE194376, reviewers should go to <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE194376> and enter reviewer token udqvmikurhcvzir into the box. Adapter of RNA-seq reads were trimmed with cutadapt(v1.9) and reads with quality less than 20 were removed. STAR(v2.5.2b) was used to map the reads to reference mm9 with --outFilterMismatchNoverLmax 0.04. Reads were then annotated with featureCounts(v1.5.1) to gene level. DESeq2 was used to determine differentially expressed genes (DEGs) based on Log2Fold-Change > 1 and an adjusted p-value < 0.05. Four separate pairwise DEG comparisons were performed, A_{Aligned} undifferentiated spermatogonia (A_{Undiff}) vs A1 differentiating spermatogonia, A1 vs A3 differentiating spermatogonia, A3 vs intermediate/type B differentiating spermatogonia, and In/B vs preleptotene spermatocytes. Differentially expressed genes (DEGs) were used to produce heatmaps with the package ‘pheatmap’ (v1.0.12) in R (v4.1.0) with genes clustered on complete linkage based on the Euclidean distance. Gene clusters in each heatmap were identified by k-means and genes in individual smaller clusters were further combined to generate gene lists representing the major clusters in each heatmap. Gene ontology (GO) enrichment analysis was performed with ShinyGo v0.75 with default parameters and background normalization²¹² and AmiGO2 Biological pathways V2017.5²¹³.

C.9 Statistics

Statistical differences between experimental groups were determined using One-Way analysis of variation (ANOVA) and Student’s t-test, with significance levels set at P<0.05. Error bars show one standard deviation.

D. Results

D.1 Synchronizing spermatogenesis to examine gene expression during spermatogonia differentiation and meiotic initiation.

Currently, little is known about the requisite program of differentiation that spermatogonia must complete prior to meiotic initiation. To study this, we adapted an *in vivo* model of synchronized steady state spermatogenesis^{186,205}. As shown in the timeline (Fig. 2.1A, synchronization was accomplished by dosing mice with the potent and highly selective RA synthesis inhibitor WIN 18,446 beginning at postnatal day P1, prior to endogenous RA signaling and when testes contain only prospermatogonia, the precursors of spermatogonia^{214,215}. Daily WIN 18,446 treatment through P10 blocked normal spermatogonia differentiation, and thus testes at P11 were filled with STRA8-/KIT- undifferentiated stem and progenitor spermatogonia (Fig. 2.1B). At P11, a single injection of exogenous RA was given to induce all spermatogonia to differentiate (except spermatogonial stem cells (SSCs) which are RA-insensitive⁷⁴). RA is extremely labile, with a half-life in mice <0.5 hr^{113,114}, and thus is not expected to persist in the testis past P12. Twenty-four hrs later in these 'RA-sufficient' testes, nearly all germ cells were STRA8+/KIT+ type A₁ differentiating spermatogonia (Fig. 2.1C) that proceeded through the remaining stages (A₂₋₄, In, B) of differentiation (Fig. 2.1D-E) and entered meiosis on P19 as STRA8+ preleptotene spermatocytes (Fig. 2.1F). These spermatocytes completed meiosis to form round spermatids by P30 (Fig. 2.S1A-C). Elongating spermatids appeared by P37 (Fig. 2.S1F), and by P43 condensing spermatids were clustered around lumina in preparation for spermiation (Fig. 2.S1G). Sperm were readily detectable in the epididymis by P50 (Fig. 2.S1H).

Using this model system, we next identified gene expression changes at the transcriptome level: 1) prior to differentiation; 2) at the onset, midpoint, and end of differentiation; and 3) at

meiotic initiation. To isolate each of these distinct cell types for bulk RNA-seq, we utilized transgenic mice expressing EGFP downstream of the promoter for *Uchl1*. Originally created to label motor neurons, testes of these mice were also EGFP⁺ (Fig. 2.1G-G'), with high levels in spermatogonia and low levels in preleptotene spermatocytes entering meiosis (Fig. 2.S2A-B²⁰⁶). This consistent difference in fluorescence intensity enabled the first isolation, from testes with synchronized spermatogenesis, of highly enriched (>95% pure) populations of millions of EGFP^{bright} premeiotic spermatogonia and EGFP^{dim} preleptotene spermatocytes entering meiosis (Fig. 2.S2A-B²⁰⁶). EGFP^{bright} type intermediate (In)/B differentiating spermatogonia and EGFP^{dim} preleptotene spermatocytes from testes of *Uchl1*-EGFP mice with synchronized spermatogenesis were used for bulk RNA-seq to measure changes in mRNA abundance during spermatogonial differentiation and entry into meiosis. Four pairwise comparisons were made between temporally adjacent cell types, including undifferentiated spermatogonia (A_{Undiff}) vs A₁ differentiating spermatogonia, A₁ vs A₃ differentiating spermatogonia, A₃ vs In/B differentiating spermatogonia, and In/B vs preleptotene spermatocytes to identify differentially expressed genes (log₂ fold-change > 1, and p_{Adj} ≤ 0.01. We found 1,859 DEGs between A_{Undiff}-A₁ spermatogonia (1,082 higher in A_{Undiff}, 777 higher in A₁), which were grouped into six clusters (Fig. 2.1H). Messages encoding prototypical markers of stem and progenitor spermatogonia (e.g., *Id4*, *Nanos2*, *Tcl1*, *Upp1* and *Zbtb16*) declined in differentiating spermatogonia, while those encoding markers of differentiating spermatogonia (e.g., *Kit*, *Sohlh2*, and *Stra8*) increased (Fig. 2.1H-I). Notably, genes in cluster 4, which had elevated mRNA levels at the onset of differentiation, were significantly enriched for cell proliferation biological processes (e.g., regulation of cytokinesis, positive regulation of cell cycle process, regulation of cell division; (Fig. 2.1J). A relatively small number of DEGs were identified between types A₁ and A₃ differentiating spermatogonia (383 higher in A₁, 171 higher in

A₃), which clustered into six groups based on expression pattern (Fig. 2.1K). Surprisingly, GO analysis identified no significant enrichment of biological processes among genes in any of the six A₁ vs A₃ clusters, suggesting no significant biological changes are underway at the midpoint of spermatogonial differentiation. As spermatogonial differentiation progressed to the mid A₃ and late In/B stages, however, we observed more changes in mRNA levels (2,461 DEGs, 1,407 higher in A₃, 1,054 higher in In/B) that grouped into eight DEG clusters (Fig. 2.2K). Pathway analysis of these clusters demonstrated mRNAs from genes involved in developmental programs were reduced in the A₃-to-In/B transition (Fig. 2.2L), while those involved in meiosis were increased at the In/B spermatogonia stage (Fig. 2.2M). These data confirm activation of genes constituting the meiotic program during spermatogonial differentiation prior to meiotic entry. Just prior to meiotic entry, we observed the highest number of mRNA abundance changes between In/B spermatogonia and preleptonema spermatocytes (3,349 DEGs, 2,133 higher in In/B, 1,216 higher in PL) which grouped into six DEG clusters (Fig. 2.2N). Biological pathways corresponding to translational control and a variety of biosynthetic pathways were overrepresented among genes whose mRNA levels decreased upon meiotic initiation, while there was increased mRNA abundance for genes involved in spermiogenesis and sperm function (Fig. 2.2O).

D.2 RA-deficient spermatogonia both enter and complete meiosis *in vivo*.

The transcriptomic data revealed: 1) activation of the gene expression program for many meiotic genes occurred during spermatogonial differentiation well in advance of meiotic initiation; 2) surprisingly, the levels of many meiotic mRNAs were not significantly altered during preleptonema, when the second round of RA signaling presumably occurs^{119,216}. Since the requirement for RA in male meiotic initiation has never been experimentally tested, we modified

the synchronization protocol (Fig. 2.1A) into a loss-of-function approach to generate ‘RA-deficient’ testes by continuously blocking RA synthesis with WIN 18,446 after inducing differentiation at P11 (Fig. 2.2A). Loss of RA signaling in vivo at the time of meiotic initiation was confirmed in these testes using multiple approaches. RA-induced mRNAs (e.g., *Stra8*, *Rec8*^{144,145,156}) were not upregulated in preleptotene spermatocytes from WIN 18,446-treated mice, while those for the RA-independent meiotic strand exchange factor *Dmc1* were significantly elevated (Fig. 2.S3A). In addition, STRA8 protein was undetectable, while SYCP3 was elevated in both RA-deficient and -sufficient testes (Fig. 2.S3B). In RA-deficient testes, morphologically normal STRA8- preleptotene spermatocytes were formed at P19 (Fig. 2.2B-C), on the same day as in control ‘RA-sufficient’ testes (Fig. 2.1F). The expression of meiotic genes such as ‘DNA meiotic recombinase 1’ (*Dmc1*) and *Sycp3* and formation of preleptotene spermatocytes in RA-deficient testes revealed the onset of meiotic initiation was not reliant upon the signal provided by RA.

We next assessed meiotic progression in RA-deficient testes and found morphologically normal DMRT1-/H1F6+ zygotene/pachytene spermatocytes appeared by P23 (Fig. 2.2D-F), and round spermatids appeared on P30 (Fig. 2G-J), as in RA-sufficient testes (Fig. 2.S1A-E). In stark contrast to control RA-sufficient testes that contained the next generation of KIT+ differentiating spermatogonia (Fig. 2.S4), RA-deficient testes at P30 contained only two discrete populations of germ cells – lectin+ round spermatids and basally-located undifferentiated ZBTB16+/KIT- spermatogonia (Fig. 2.2I-J); spermatocytes from any potential subsequent waves of differentiation were never observed. These results provide further evidence that continuous WIN 18,446 treatment effectively blocked RA synthesis and thus further spermatogonial differentiation in vivo.

We next determined whether spermatogenic cells that apparently entered and completed meiosis in RA-deficient testes in vivo faithfully completed landmark cytological processes of meiosis. We first examined changes in DNA content using flow cytometry – during preleptonema, 2N/2C spermatocytes replicated their DNA but then failed to divide, becoming 2N/4C. At the end of meiosis I, 2N/4C diplotene spermatocytes divide twice, successively forming two 2N/2C secondary spermatocytes and four 1N/1C round spermatids. As expected, RA-sufficient testes from P23 mice contained 2C and 4C cells (corresponding to a population of somatic cells/spermatogonia and zygotene/pachytene spermatocytes, respectively), while those from P30 mice contained 1C, 2C, and 4C cells (round spermatids, somatic cells+spermatogonia, and spermatocytes from subsequent waves of differentiation, respectively) (Figs. 2.3A, 2.S5). Testes from RA-deficient mice at P23 also contained 2C and 4C cells (Figs. 2.3B, 2.S5), revealing zygotene/pachytene spermatocytes indeed replicated their DNA. By P30, testes from RA-deficient mice contained 1C and 2C cells, revealing the initial synchronized cohort of germ cells completed meiosis as haploid (1C) round spermatids (Figs. 2.3B, 2.S5) in the absence of RA. Importantly, in contrast to RA-sufficient testes, P30 RA-deficient testes did not contain 4C spermatocytes, further confirming inhibition of RA signaling in these mice (Figs. 2.3B, 2.S5). We next assessed whether spermatocytes in RA-deficient testes successfully completed key events during leptotema, zygonema, and pachynema of the first meiotic prophase. Immunostaining for established markers of chromosome synapsis (SYCP3) and double strand break formation (γ H2AX) revealed spermatocytes in RA-deficient testes (Fig. 2.3F-H) resembled those produced in RA-sufficient testes (Fig. 2.3C-E). Likewise, meiotic chromosome spreads from RA-deficient testes from P19-23 demonstrated normal-appearing SYCP3⁺ synaptonemal complexes after P20, and γ H2AX assumed its characteristic localization to the XY, or sex body by P21 (Fig. 2.3I-K).

The current model for male germ cell meiosis postulates that initiation is triggered by rising RA levels, which occur in the normal testis at stages VII-VIII of the seminiferous epithelium^{216,217}. To test this model, we employed a gain-of-function approach by injecting RA into mice with synchronized spermatogenesis whose testes contained KIT⁺ spermatogonia both at the middle and at late stages of differentiation (Fig. 2.4A-B). We predicted that, if these differentiating spermatogonia were both responsive to and awaiting the signal provided by RA, then they should activate STRA8 and precociously enter meiosis. Differentiating spermatogonia at P14 (type A3) and P16 (type In) were indeed RA-responsive, as evidenced by activation of STRA8 in response to exogenous RA (100 µg), as expected (Fig. 2.4C vs 2.4F, I). By P19, testes from mice given vehicle or the second pulse of RA (at P14 or P16, respectively) all contained STRA8⁺ preleptotene spermatocytes, revealing the timing of meiotic initiation was not advanced, and thus RA was not a limiting factor for the initiation of meiosis in spermatogonia at mid (type A₃, Fig. 2.4G-H) and late (type In/B, Fig. 2.4J-K) stages of differentiation.

D.3 RA is dispensable for spermatogonia differentiation and meiotic initiation *in vitro*.

We next sought to verify these *in vivo* results by examining meiotic initiation and progression *in vitro*, where RA levels are more easily manipulated. Testis single cell suspensions, in which tubule architecture was disrupted (Fig. 2.5A), were isolated five days following *in vivo* RA-induced differentiation, when differentiating (type In) spermatogonia were the most advanced germ cell type (Fig. 2.5B). Cultures were maintained in serum-free media lacking retinoids and harvested on successive days for immunostaining with established fate markers of spermatogonia (DMRT1⁺/TRA98⁺) and meiosis progression (DMRT1⁻/SYCP3⁺). At P16 (day 0 of culture), 99.3% of TRA98⁺ germ cells in single cell suspensions were spermatogonia (Figs. 2.5C, D-D');

note: somatic Sertoli cells are TRA98-/DMRT1+²¹¹). By P19 (3 days in culture), only 23.6% were spermatogonia, and 75.2% had become preleptotene spermatocytes, as expected (Figs. 2.5C, E-E'). On P20 (4 days in culture), 45.5% were in preleptonema, and 40.1% were in leptonema (Figs. 2.5C, F-F'). On P21 (5 days in culture), 21.8% were in leptonema and 51.6% were in zygonema (Figs. 2.5C, G-G'). On P22 (6 days in culture), 79.0% were in zygonema/pachynema (Figs. 2.5C, H-H').

D.4 The meiotic gene expression program is largely unaltered in RA-deficient testes.

A role has been proposed for RA in regulating STRA8-mediated gene expression changes critical for meiotic initiation^{218,219}. However, STRA8 is not strictly required for meiotic initiation^{147,200}, and our data presented here reveal, after a single transient dose of RA to induce differentiation, male germ cells initiated and completed meiosis ~8 days later in the absence of RA. We therefore examined the requirement for RA in regulating normal gene expression changes during meiotic initiation. RNA-seq was employed to identify the transcriptomes of In/B spermatogonia (EGFP^{bright}) and meiotic preleptotene spermatocytes (EGFP^{dim}) from *Uchl1*-EGFP mice with synchronized spermatogenesis in the presence or absence of RA. As expected, there were dramatic changes (largely increases) in abundance of numerous key meiotic mRNAs as RA-sufficient differentiating spermatogonia initiated meiosis as preleptotene spermatocytes (Fig. 2.6A). However, in preleptotene spermatocytes from RA-deficient vs RA-sufficient testes, these mRNAs exhibited minimal differences in their abundance (Fig. 2.6A).

We next assessed other genes whose expression was also unchanged in the absence of RA. The abundance of 2,497 mRNAs was similar when comparing differentiating type In/B spermatogonia and preleptotene spermatocytes from either RA-sufficient or RA-deficient testes

(Fig. 2.6B). In agreement with our targeted analysis (Fig. 6B), genes in ‘common cluster 5’, which had significantly elevated mRNA levels in both preleptotene spermatocyte groups ($\pm$ RA), exhibited significant overrepresentation of genes associated with meiosis GO terms (Fig. 2.6C). These data reinforce our observations, based on cellular morphology and expression of established markers of germ cell identity, that male germ cells similarly initiated meiosis in both RA-sufficient and -deficient testes.

D.5 Male germ cells fail to complete spermiogenesis and produce sperm in RA-deficient testes.

We identified the 4,768 genes with significantly differential mRNA abundance in RA-sufficient preleptotene spermatocytes vs differentiating In/B spermatogonia that were significantly changed in RA-deficient preleptotene spermatocytes. Interestingly, genes in ‘RA-sufficient cluster 5’, which were induced in RA-sufficient but not RA-deficient preleptotene spermatocytes, contained a significant overrepresentation of spermiogenesis genes, including those involved in cilia function, axoneme assembly, flagellar motility, spermatid development, and gamete development (Fig. 2.6D-E).

Due to these significant yet rather unexpected changes in expression of spermiogenesis genes, we assessed whether spermiogenesis was successfully completed in RA-deficient testes, using the well-described length of each phase of spermatogenesis and spermiogenesis^{199,220–222} as a temporal guide. As expected, P39 RA-sufficient testes contained numerous condensing spermatids (Fig. 2.6F) and at P50, RA-sufficient epididymides were filled with sperm (Fig. 2.6G). In contrast, P39 RA-deficient testes contained spermatogonia and condensing spermatids with highly misshapen heads (Fig. 2.6H) and, consequently, RA-deficient P50 epididymides were

empty, with no apparent sperm (Fig. 2.6I). In conclusion, the pulse of RA at meiotic initiation is required for normal completion of spermiogenesis and the formation of testicular sperm.

E. Discussion

Taken together, results presented here reveal the signal provided by RA is not a requisite checkpoint for meiotic initiation in the testis as previously proposed^{52,53,223–225}. Instead, RA's primary role in the male in terms of meiosis is to initiate the lengthy preceding program of spermatogonia differentiation. It is during this essential differentiation program that gene expression changes occur that are sufficient for initiation, progression through, and completion of meiosis. In the absence of RA following spermatogonial differentiation, the morphogenesis of haploid spermatids was disrupted, resulting in the lack of testicular sperm and consequent empty epididymides.

It has been known for nearly 100 years that vitamin A, and thus RA, are essential for normal spermatogenesis and male fertility^{105,226}. Until recently, loss-of-function approaches to deplete testicular RA were done by feeding rodents a vitamin A-deficient (VAD) diet. VAD testes contained only spermatogonia and, in some studies, limited numbers of preleptotene spermatocytes^{116,226–228}. Animals with VAD re-fed vitamin A or injected with either retinol or RA recovered full spermatogenesis^{115,207,225,226,229}. This resumption of spermatogenesis occurred in a synchronized fashion²²⁵ and led to restoration of fertility. However, VAD often caused incomplete RA deficiency – in one study, 93% (14/15) of VAD rats had testes containing numerous meiotic spermatocytes¹¹⁵. In more recent experimental approaches, researchers depleted RA using the potent and selective RA synthesis inhibitor WIN 18,446 (used in this study)^{112,230}. Careful analyses of WIN 18,446-treated animals revealed an initial and essential requirement for RA at

spermatogonial differentiation (formation of type A1 spermatogonia)¹⁸⁶. Therefore, since differentiation requires RA, its subsequent roles in processes such as meiosis, spermiogenesis, and spermiation in males have thus far remained unexamined. Our results reveal in the absence of RA, the meiotic gene expression program was largely unaltered and male germ cells entered and completed meiosis, forming haploid spermatids unable to complete spermiogenesis.

The fetal gonad has long been employed as a model system to study germ cell entry into meiosis I, which occurs in the ovary but not the testis^{143,231,232}. In 2006, two studies reported that RA provides the ‘meiosis inducing substance’ (MIS) in the ovary, and that its action is prevented in the testis by the catabolic enzyme ‘cytochrome P450, family 26, subfamily b, polypeptide 1’ (CYP26B1), which serves as the ‘meiosis preventing substance’ (MPS)^{52,53}. In lower organisms such as yeast, meiosis initiates downstream of a carbon substrate switch – from glucose to acetate – in response to the aptly named ‘inducer of meiosis’ (IME1^{233,234}). This master regulator of meiotic initiation is recruited to meiotic gene promoters to activate their transcription. In mammals, an analogous role has been proposed for STRA8, which is activated in type A1 differentiating spermatogonia and again in preleptonema spermatocytes by RA^{53,144,145,148}. However, a conserved role is unlikely for three key reasons. First, STRA8 does not resemble the pioneering transcription factor IME1, as STRA8 lacks binding preference for meiosis-specific gene promoters, and Stra8 KO spermatocytes had relatively few gene-specific changes²¹⁸. Second, although Stra8 KO germ cells in congenic C57BL/6 mice arrest during preleptonema^{148,200}, those in a mixed genetic background progress through meiosis for up to another week, progressing to zygonema and even pachynema¹⁴⁷. These disparate phenotypes suggest existence of genetic modifier(s) of STRA8 in the C57BL/6 background. Since STRA8 is evolutionarily conserved in all mammals, it is difficult to conceive that it might serve as the meiotic gatekeeper only in one specific background of mice.

Third, we showed here that male germ cells initiated, progressed through, and completed meiosis without RA (and thus detectable STRA8) during preleptonema. However, in our treatment paradigm, male germ cells clearly expressed STRA8, albeit transiently, eight days prior to meiosis, with at the initiation of differentiation. This likely explains how the phenotype of germ cells in RA-deficient testes differs so dramatically from that of Stra8 KO germ cells, which arrest in both backgrounds by pachynema.

In rodent testes, four critical cell transitions – spermatogonial differentiation, meiotic initiation, spermatid elongation, and spermiation – are spatially adjacent and occur within seminiferous epithelium stages VII-IX^{199,220,235}. There is some evidence in the literature for each of these transitions' dependency on the rise in RA levels, which occurs at stages VII-VIII^{119,216}. However, these events are not similarly colocalized in testes from other species, including humans²³⁶. Due to this, it is difficult to imagine how discrete changes in RA levels could play key conserved roles in their regulation in those species.

In the fetal ovary, RA is essential for meiosis. However, the differentiation of oogonia and subsequent meiotic entry of preleptotene oocytes occur at ~E13-E14 and thus these events have not been temporally isolated. Thus, it is possible that RA serves a similar role in the ovary as in the testis, to drive commitment to a differentiation program that culminates in meiosis. The female germline is not a stem cell-based system like the testis, which relies on the constant proliferation of spermatogonia to generate millions of gametes daily. This may in part explain why there is such a difference in length of the differentiation program in mouse testes (8.6 days) versus the ovary (~1 day).

The preparation, or commitment of differentiating spermatogonia to meiosis is supported by transcriptome analyses, which revealed a subset of mRNAs encoding proteins with established

roles in meiosis were upregulated during differentiation. This reveals that, days prior to meiotic initiation, preparations are already underway to generate the gene products necessary for the meiotic program. This meshes with recently published work from the Namekawa laboratory, who identified regions of DNA containing clusters of transcriptional enhancers (termed super-enhancers, or SEs) that were specific to meiotic genes. Interestingly – and with direct relevance to the results presented here – these meiotic SEs were enriched in spermatogonia with H3K4me3 and H3K27ac²³⁷. These histone modifications are characteristic of genes poised for transcription, and these results reveal preparation for meiosis occurs well in advance (~8.6 days) of STRA8 activation by RA during preleptonema. The observed initiation and progression into meiosis without RA *in vitro*, like *in vivo*, supports the existence of an intrinsic clock mechanism that preserves a set number of divisions and a rigid timeline for meiosis following RA-induced differentiation.

F. Acknowledgements

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to support studies was provided by G.H., B.P.H., and C.B.G. C.B.G. designed experiments, supervised the overall project, and wrote the manuscript with input from all authors.

Table 2.1 Antibodies

<i>Antigen</i>	<i>Host</i>	<i>Source</i>	<i>Dilution</i>	<i>Catalog #</i>
DMRT1	Rabbit	Gift from <i>Heckert</i>	1:1000	Lei et al 2007
γ H2AX	Rabbit	Abcam	1:400	AB11174
GFRA1	Goat	R&D Systems	1:800	AF560
H1F6	Guinea Pig	Gift from <i>Handel</i>	1:500	Inselman et al 2003
KIT	Goat	R & D Systems	1:1000	AF1356
Lectin-488	Peanut	Thermo Fisher Scientific	1:1000	L21409
Lectin-594	Peanut	Thermo Fisher Scientific	1:1000	L32459
RARG	Rabbit	Cell Signaling	1:500	8965T
STRA8	Rabbit	Abcam	1:3000	Ab49602
SYCP3-488	Mouse	Abcam	1:200	Ab205846
TRA98	Rat	Abcam	1:1000	Ab82527
ZBTB16/PLZF	Goat	R & D Systems	1:1000	AF2944

G. Chapter 2 Figures

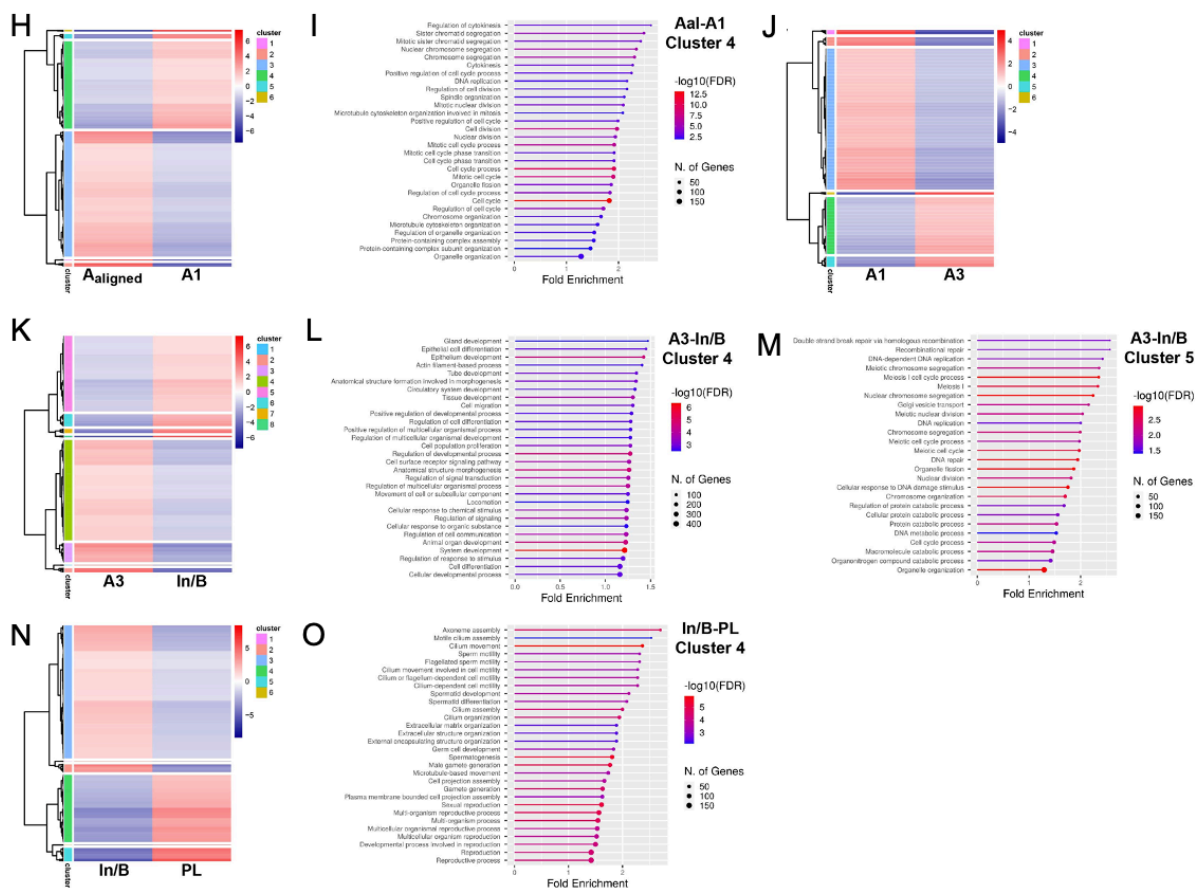


Fig. 2.1 Adapting a model of synchronized spermatogenesis to study spermatogonial differentiation and meiotic initiation *in vivo*.

(A) Synchronization timeline for treatments to generate RA-sufficient mice. (B-F) Testis sections from mice with RA-sufficient spermatogenesis are shown, with ages indicated on each image. Sections were immunostained for the indicated germ cell fate markers (colors indicated on each image). Tubules are encircled, in immunostained sections, by white dashed lines. (G-G') Testes from P>60 Uchl1-eGfp transgenic mice are shown, illuminated by white (G) or fluorescent (G') light. (H-O) Heatmaps showing log2Fold-change values of mRNA abundance difference between the noted cell populations (listed below). Fold-change is according to the color scale and unsupervised gene clusters are shown to the left. (I, L, M, O) Lollipop plot indicates the Gene Ontology Enrichment Analysis of genes from the noted clusters. Scale bar (in B) = 40 μ m.

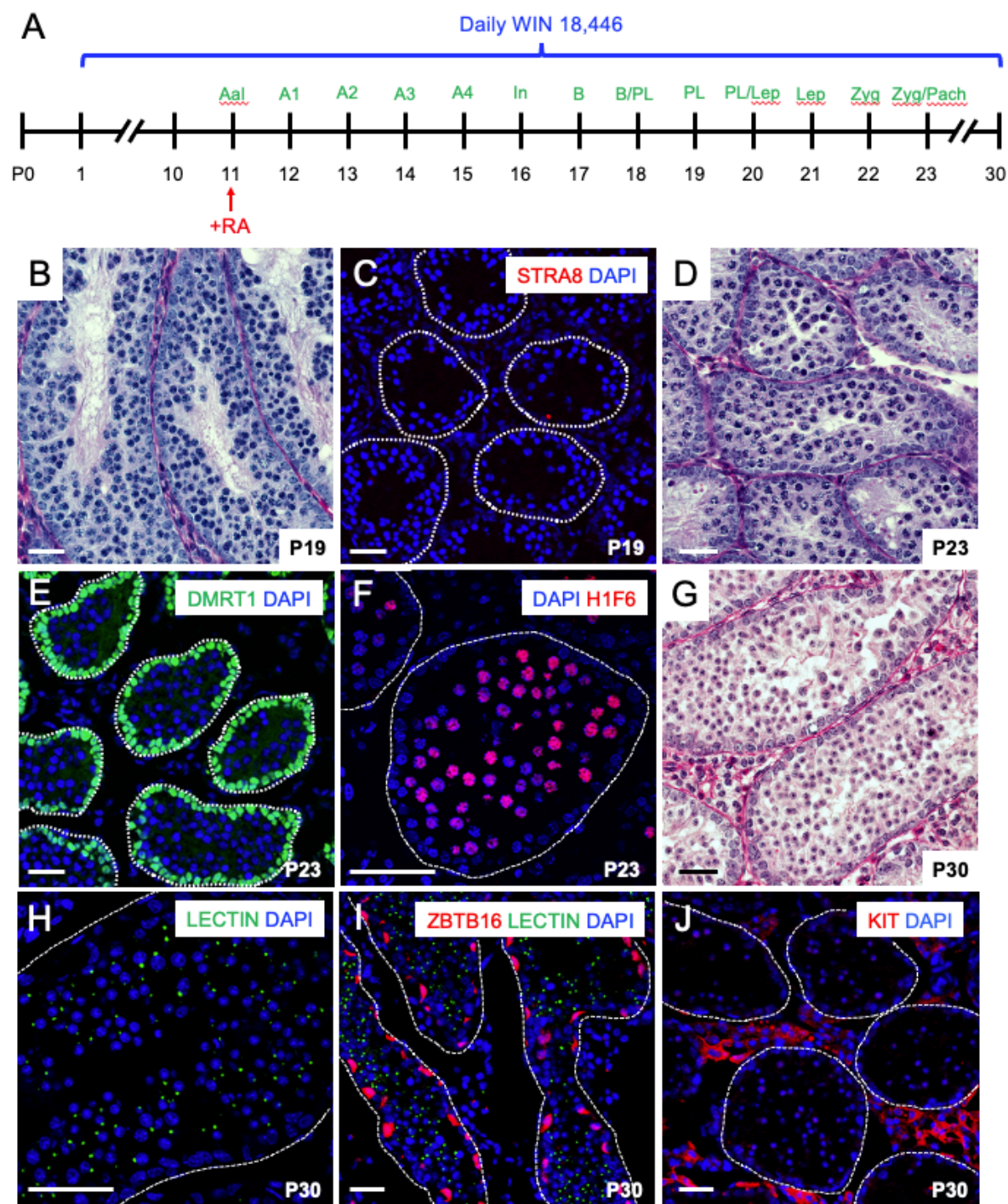


Fig. 2.2 Male germ cells initiate and complete meiosis in the absence of RA *in vivo*.

(A) Synchronization timeline for single RA injection at P11 (to induce differentiation) and continuous WIN 18,446 treatments to generate RA-deficient testes. (B-J) Testis sections from mice with RA-deficient spermatogenesis are shown, with ages indicated on each image. Sections in B, D, and G were stained with PAS. In C, E-F, and H-J, sections were immunostained for the indicated germ cell fate markers (colors indicated on each image), or with lectin to mark spermatid acrosomes (H, I), and nuclei were counterstained with DAPI (blue). Tubules are encircled by white dashed lines. Scale bars = 40 μ m.

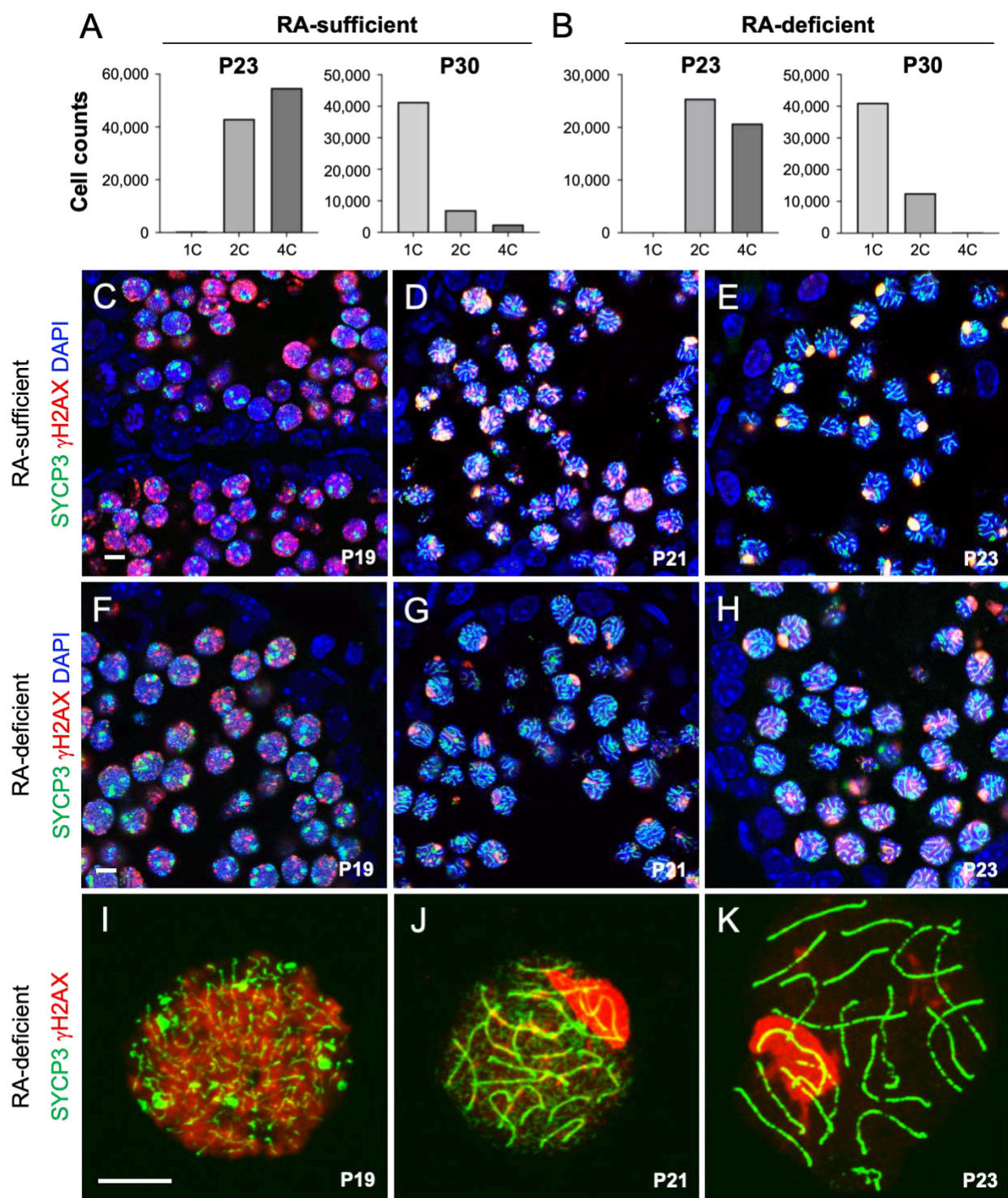


Fig. 2.3 Landmark cytological events of meiosis are faithfully completed in the absence of RA in vivo.

(**A-B**) DNA content was assessed by flow cytometry in RA-sufficient (**A**) and RA-deficient (**B**) testes from mice at P23 and P30. At P23, testes from both groups contained 2C and abundant 4C cells, indicating spermatocytes had indeed replicated their DNA in the absence of RA. At P30, testes from both groups contained abundant 1C and 2C cells, indicating haploid spermatids were indeed formed in the absence of RA. In addition, RA-sufficient P30 testes contained the next generation of 4C spermatocytes, while those from RA-deficient mice did not. Testes containing spermatocytes from RA-sufficient (**C-E**) and RA-deficient (**F-H**) mice at the ages indicated on each image exhibited similarly normal gH2AX (red) and SYCP3 (green) localization patterns, and DAPI labeled nuclei (in blue). (**I-K**) Meiotic chromosome spreads from RA-deficient testes had normal immunostaining patterns for gH2AX (red) and SYCP3 (green) with ages indicated on each image. Scale bars = 10 μ m.

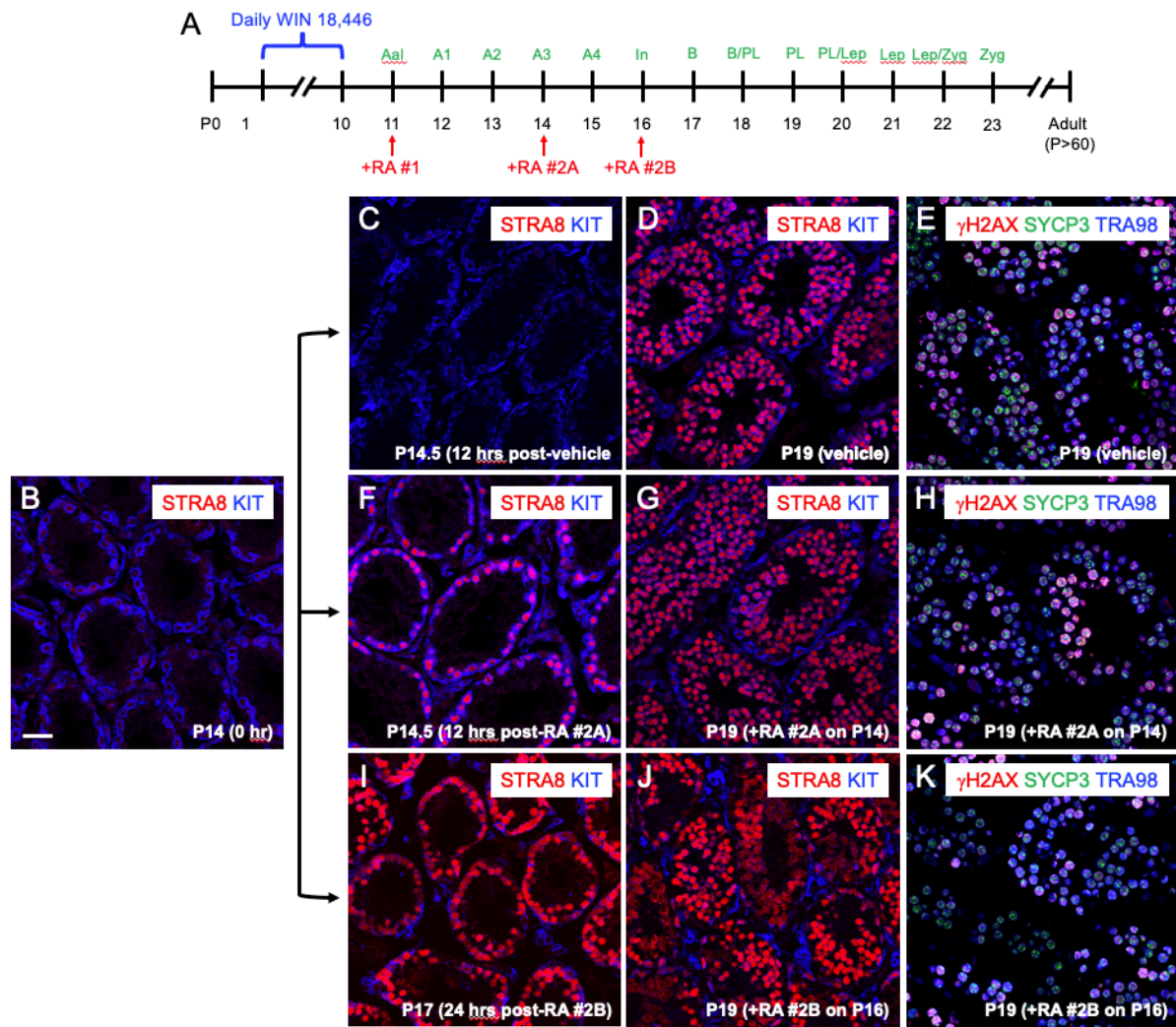


Fig. 2.4 Precocious RA does not advance the timing of meiotic initiation.

(A) Spermatogenesis was synchronized on this timeline, and exogenous RA was given to all mice on P11 to induce spermatogonia differentiation (+RA #1). Then, groups of mice received either vehicle or a second dose of RA at P14 (+RA #2A, A3 spermatogonia) or P16 (+RA #2B, In spermatogonia). (B-K) Immunostaining for protein markers (color coding provided on each image) on testis sections from mice that received only RA #1 (B-E), RA #1 plus RA #2A (F-H), or RA #1 plus RA #2B (I-K). Scale bar = 25 μ m.

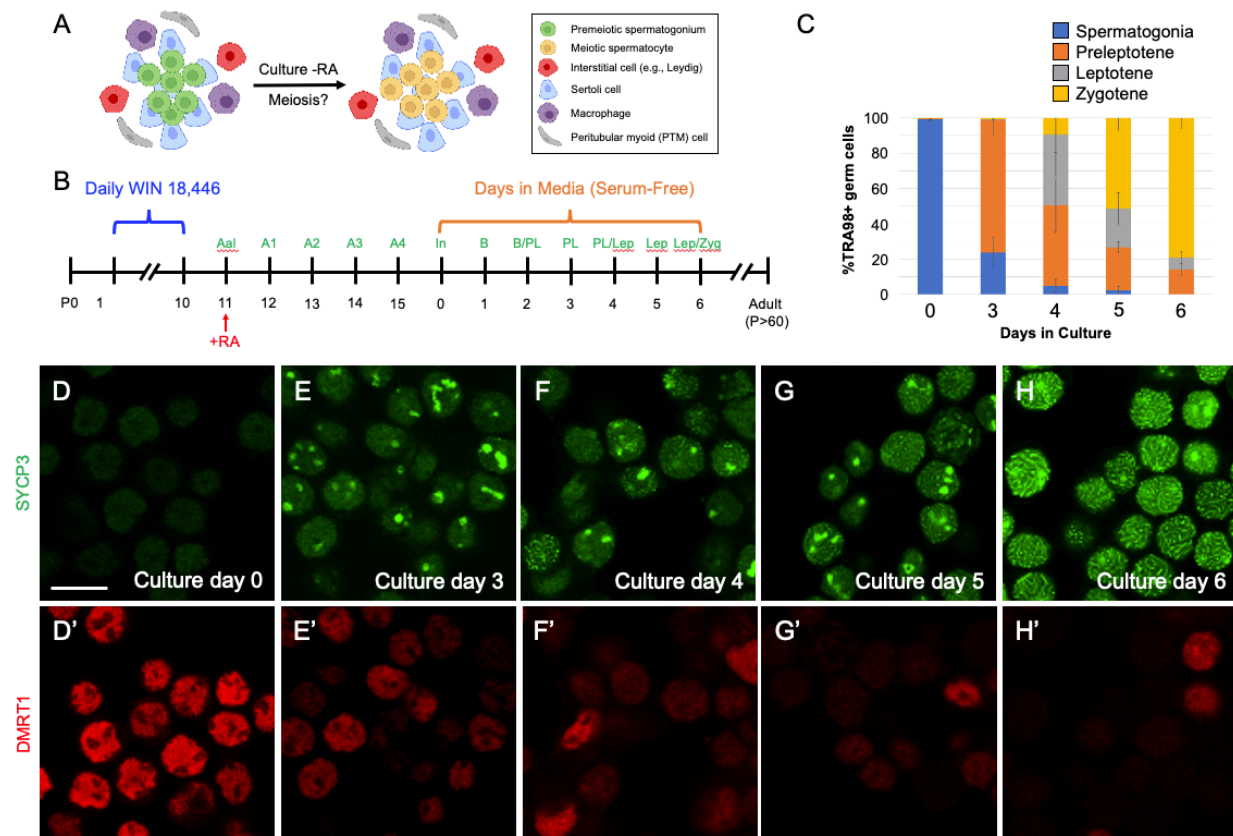


Fig. 2.5 Spermatogonia complete differentiation and initiate meiosis in retinoid-free media *in vitro*.

(A-B) Mice with synchronized spermatogenesis were given a single injection of RA on P11 and euthanized on P15. Testes were collected for single cell testis suspensions and maintained in serum-free media for 6 days. Cultures were harvested 12 hr after plating (day 0) and then on subsequent days. **(C)** Quantitation of the identity of TRA98⁺ germ cells based on characteristic localization patterns of SYCP3 and DMRT1. **(D-H')** Immunostaining was done on fixed cells using antibodies against SYCP3 (green) and DMRT1 (red). Day of culture is indicated on each image. Scale bar = 25 μ m.

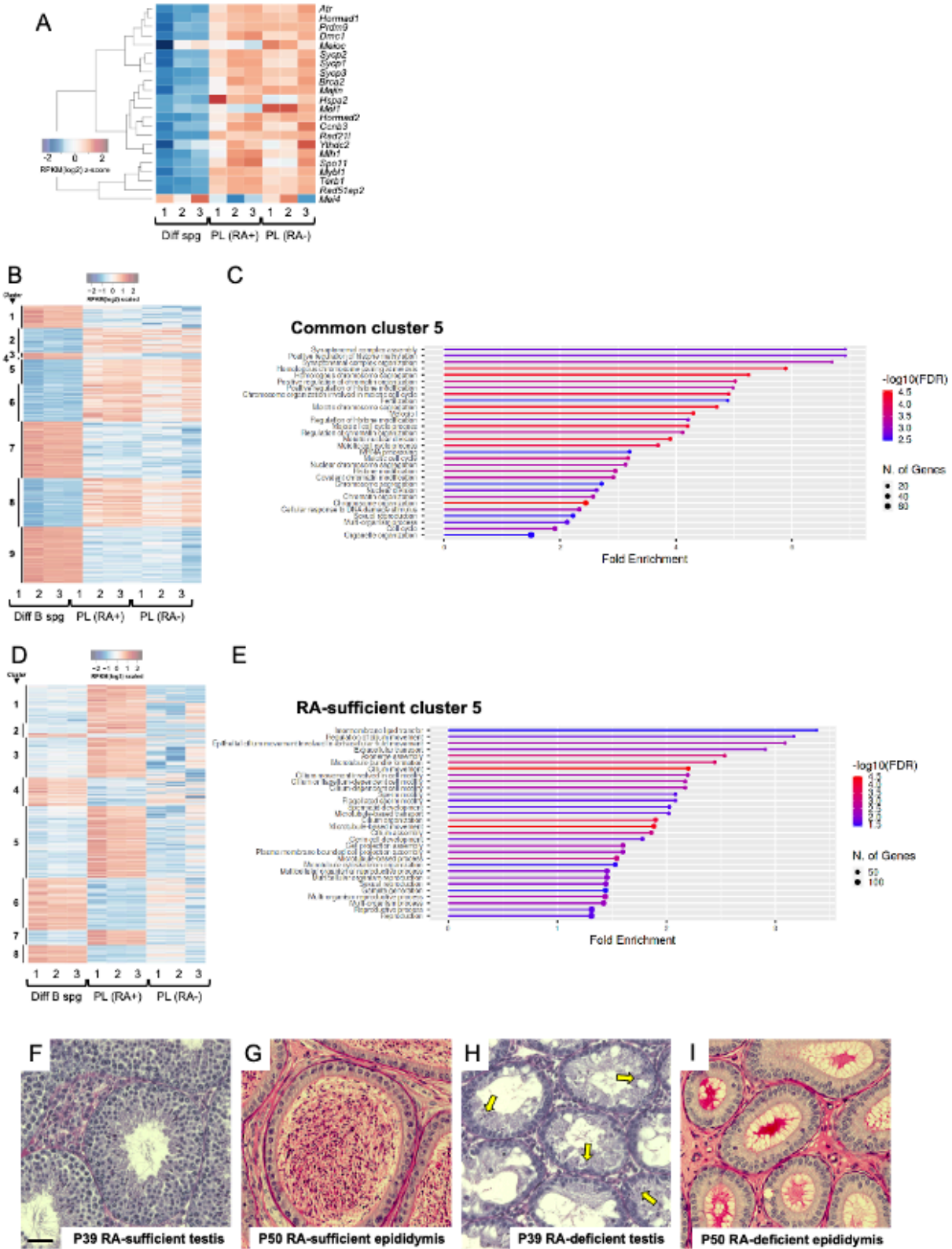


Fig. 2.6 Gene expression changes in RA-deficient germ cells reveal a requirement for the second pulse of RA in completion of spermiogenesis.

(A) Heatmap showing log-normalized mRNA abundance for a panel of meiotic genes in the noted samples (Diff spg = type B spermatogonia, P17; PL = preleptotene spermatocytes, P19, RA+ = RA-sufficient, RA- = RA-deficient) according to the scale. (B) Heatmap showing scaled mRNA abundance of significantly differentially expressed genes between differentiating B spermatogonia and preleptotene spermatocytes from both RA-sufficient and RA-deficient testes. Abundance is according to the color scale and unsupervised gene clusters are shown to the left. (C) Lollipop plot indicates the Gene Ontology Enrichment Analysis of genes from cluster 5 in A. (D) Heatmap showing scaled mRNA abundance of significantly differentially expressed genes between differentiating B spermatogonia and preleptotene spermatocytes from both RA-sufficient and RA-deficient testes. Abundance is according to the color scale and unsupervised gene clusters are shown to the left. (E) Lollipop plot indicates the Gene Ontology Enrichment Analysis of genes from cluster 5 in C. (F-I) PAS-stained sections from RA-sufficient (F-G) or RA-deficient (H-I) testis and epididymis, with ages indicated on each image. Yellow arrows (in H) indicate remaining undifferentiated spermatogonia. Scale bar (in F) = 50 μ m.

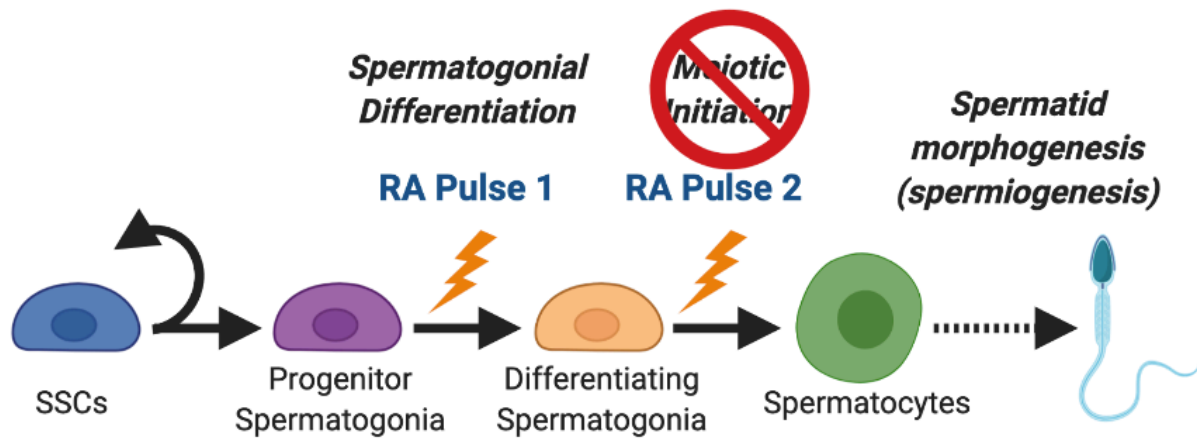


Fig. 2.S0 Abstract synopsis figure.

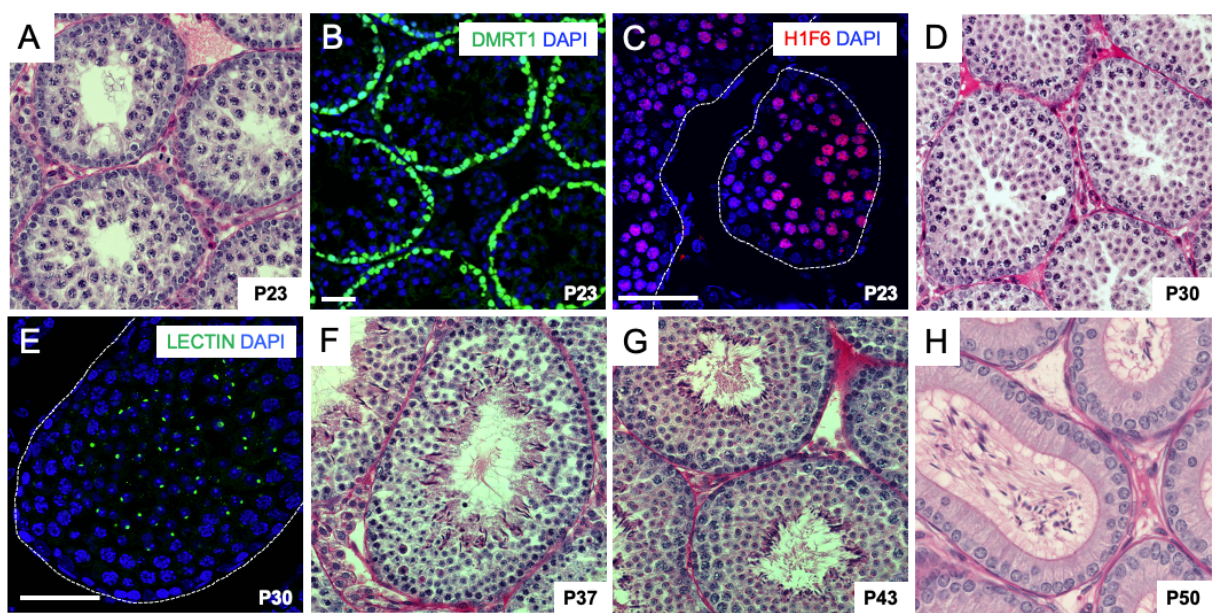


Fig. 2.S1 Timeline for the completion of synchronized spermatogenesis in RA-sufficient mice.

Sections from Bouin's-fixed testes (**A, D, F, G**) and epididymis (**H**) were stained with PAS. (**B, C, E**) Immunostaining was done on cryosections, with markers (and corresponding colors) indicated on each image. Nuclei were counterstained with DAPI (blue). Scale bars = 25 μm .

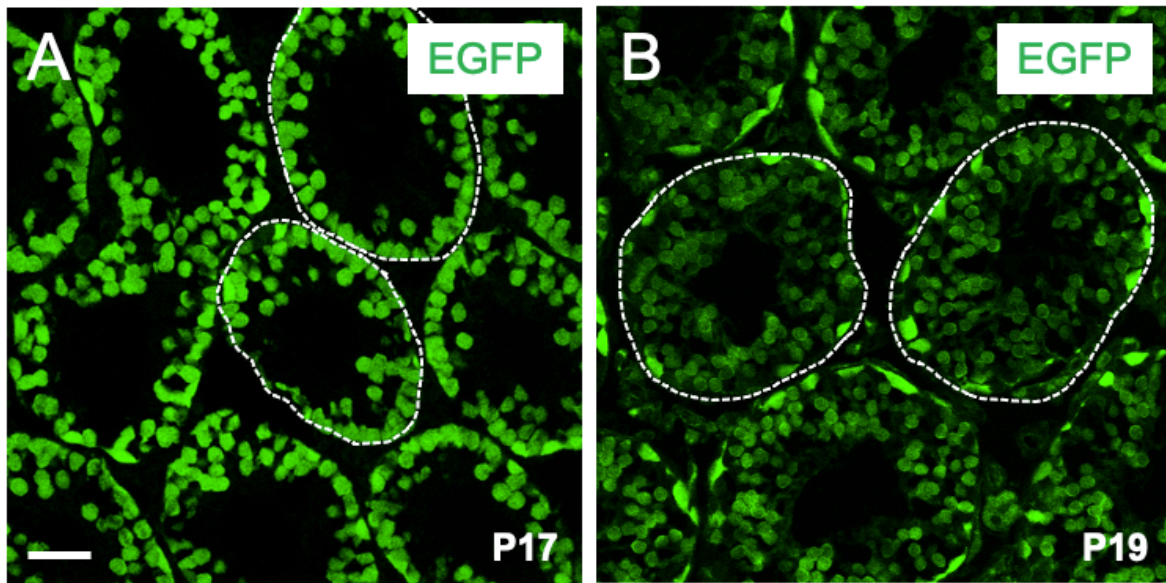


Fig. 2.S2 Spermatogonia are UCHL1^{bright}, and preleptotene spermatocytes are UCHL1^{dim}.

(A-B) Representative testis cryosections are shown from *Uchl1-eGfp* mice aged P17 and P19, respectively. Scale bar (in **A**) = 25 μ m.

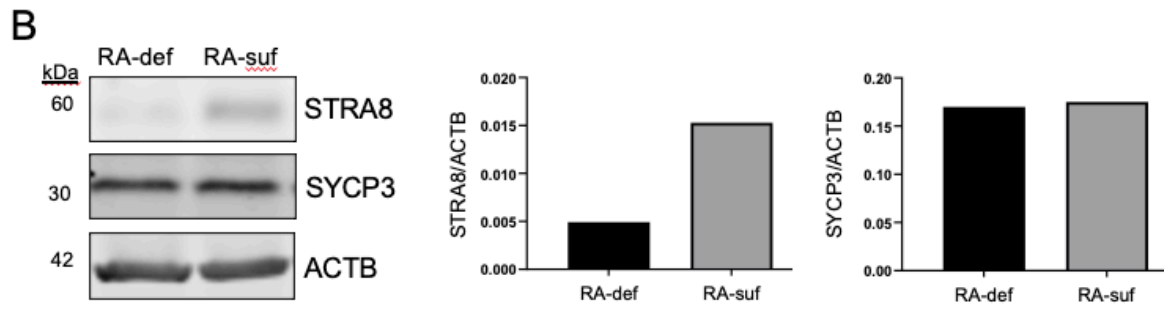
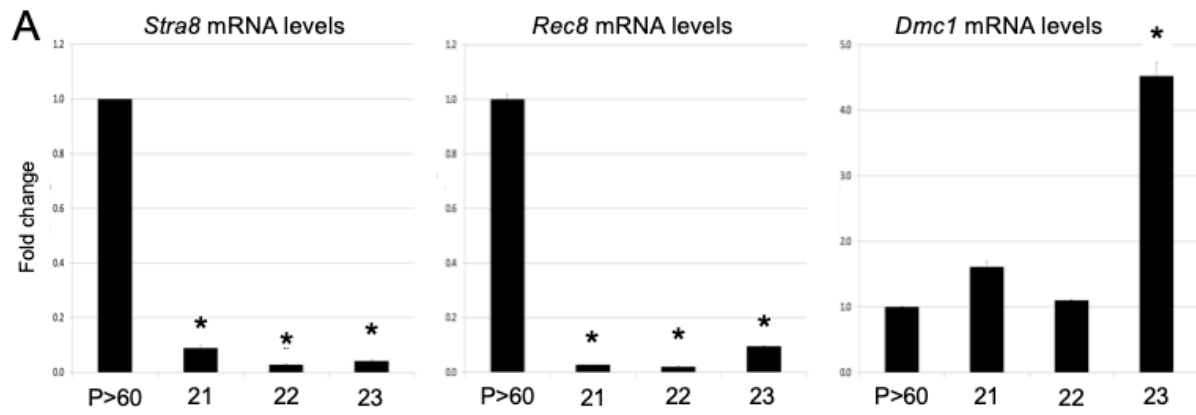


Fig. 2.S3 Confirming testis RA deficiency.

(A) RNA was isolated from RA-deficient testes at the ages indicated and compared to WT adult (P>60) testes using qRT-PCR to quantify Stra8, Rec8, and Dmc1 mRNA levels. (B) Immunoblotting was done using whole testis lysates from P19 RA-sufficient and -deficient testes to detect STRA8 and SYCP3, and ACTB was used as a loading control. Densitometry results are shown to the right of the blots.

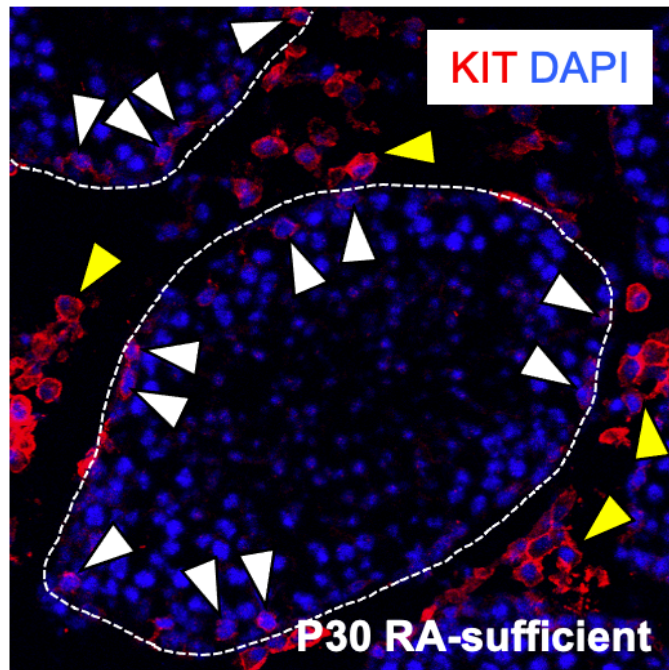
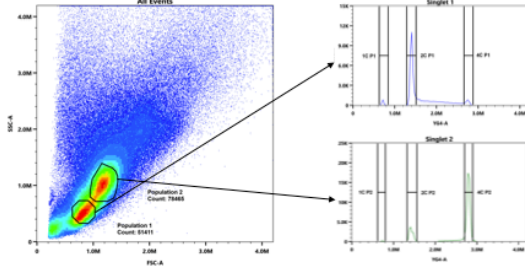


Fig. 2.S4 Confirming subsequent generations of differentiating spermatogonia in P30 testes.

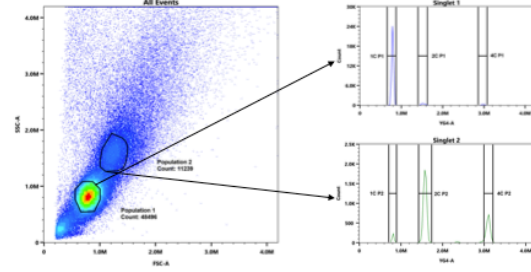
P30 mouse testis sections stained with KIT (red) and DAPI (blue) to detect differentiating spermatogonia (indicated by white arrowheads). Dashed white line represents the periphery of the seminiferous tubule. Yellow arrowheads indicate the presence of KIT⁺ interstitial cells.

P23 RA-sufficient



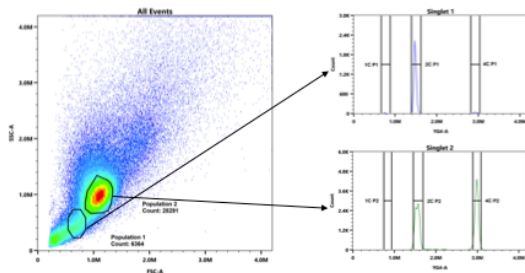
Population 1			Population 2		
DNA Content	Median Value	% CV	DNA Content	Median Value	% CV
1C	735.88	2.92	1C	754.36	2.25
2C	1431.36	2.25	2C	1443.10	3.0
4C	2777.09	1.89	4C	2833.24	1.33

P30 RA-sufficient



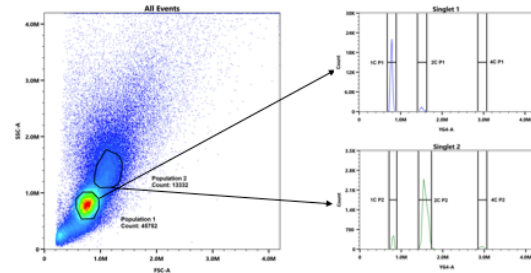
Population 1			Population 2		
DNA Content	Median Value	% CV	DNA Content	Median Value	% CV
1C	812.07	2.50	1C	837.84	3.00
2C	1552.66	2.57	2C	1608.10	2.41
4C	3015.40	1.45	4C	3138.24	1.41

P23 RA-deficient



Population 1			Population 2		
DNA Content	Median Value	% CV	DNA Content	Median Value	% CV
1C	766.88	6.94	1C	851.59	5.58
2C	1509.54	1.84	2C	1576.03	3.06
4C	2958.46	1.34	4C	3019.24	1.30

P30 RA-deficient



Population 1			Population 2		
DNA Content	Median Value	% CV	DNA Content	Median Value	% CV
1C	803.85	2.45	1C	826.92	2.53
2C	1535.43	2.32	2C	1601.64	3.47
4C	2976.66	1.58	4C	2979.65	1.55

Fig. 2.S5 Ploidy determination by flow cytometry.

Single cell suspension from P23 and P30 mouse testes with RA-sufficient vs -deficient synchronized spermatogenesis stained with PI. MFI values indicate DNA doubling between 1C and 2C, and 2C and 4C.

EVALUATING SPERMATOGENIAL RESPONSIVENESS TO RA IN THE ADULT TESTIS

Retinoic acid (RA) has been touted as the master regulator for germ cell progression in spermatogenesis. In fact, a long-standing dogma is the requirement of RA. Using a battery of assays, RA was deemed non-essential for meiosis – a staggering and surprising conclusion. Tangential to this study was a separate RA-centric project focused the regulation of RA for the progression of spermatogonia in the adult testis. The inspiration for this second project arose from two simple concepts observed in the neonatal testis – spermatogonia respond differently to RA and the testis employs multiple mechanisms to regulate RA signaling. By understanding this dichotomy between RA signaling and spermatogonia progression, germ cell fate can be leveraged for the design of treatments that impede or expedite germ cell progression. The two concepts from the neonatal testis were explored in the adult testis, but other findings and observations dovetailed with the RA-meiosis project – particularly the progression of germ cells after differentiation. Whereas studies in chapter two exclusively used synchronized pups and emphasized the absence of RA, studies in chapter three were conducted using synchronized pups and adult mice and emphasized an abundance of RA. The findings from chapter three proffer spermatogonial differentiation as the key transition to focus on for the future fertility treatments.

CHAPTER III: DIFFERENTIAL RESPONSIVENESS OF SPERMATOGONIA TO RETINOIC ACID DICTATES PRECOCIOUS DIFFERENTIATION BUT NOT MEIOTIC ENTRY DURING STEADY-STATE SPERMATOGENESIS

This chapter is modified from a manuscript under review in Biology of Reproduction (BOR).

A. Summary

The foundation of mammalian spermatogenesis is provided by the pool of spermatogonia that contain spermatogonial stem cells (SSCs) and transit-amplifying progenitors that differentiate in response to retinoic acid (RA). This initial response to RA maintains normal ratios of undifferentiated progenitor (A_{Undiff}) and differentiating spermatogonia (A_{Diff}) and ensures a committed pool of spermatogonia to enter meiosis. It was recently shown these populations are maintained in part by their differential responsiveness to RA. Here, we expand on those findings to define the extent to which RA responsiveness during steady-state spermatogenesis in the adult testis regulates spermatogonial fate. Our results reveal both A_{Undiff} and A_{Diff} spermatogonia throughout the testis can respond to exogenous RA, but their fates were quite different – A_{Undiff} s precociously differentiated and proceeded into meiosis on a normal timeline, while A_{Diff} s did not hasten their entry into meiosis. This reveals spermatogonia responding to RA must complete the 8.6-day differentiation program prior to their entry into meiosis. Exposure to exogenous RA enriched testes with preleptotene and pachytene spermatocytes one and two seminiferous cycles later, supporting recent clinical studies reporting increased sperm production and enhanced fertility in subfertile men on long-term RA analog treatment. Collectively, our results reveal a well-buffered system exists within mammalian testes to regulate spermatogonial RA exposure, that

exposed spermatogonia must still complete a normal-length differentiation program, and that daily RA treatments increased numbers of advanced germ cells by directing undifferentiated progenitor spermatogonia to continuously differentiate.

B. Introduction

A key requirement for successful germ cell progression through spermatogenesis is provided by the essential micronutrient vitamin A. Vitamin A deficiency (VAD) in mammals results in reduced seminiferous tubule size, disrupted spermatogenesis, and impaired sperm production^{105,226}; these effects are reversible, as spermatogenesis resumes following the reintroduction of vitamin A into the diet²²⁵. Dietary vitamin A is converted into a retinyl ester (RE) for long-term storage or into retinal/retinaldehyde, which is further oxidized into the morphogen RA. RA synthesis is achieved by retinaldehyde dehydrogenases (RALDHs), which are active in distinct testicular cells. In the developing testis, Sertoli cells provide the main source of RA, and this role is taken over by spermatocytes in adult testes^{109,119}. Macrophages located just outside the seminiferous tubules, some of which are positioned at junctions between peritubular myoid cells, also contain RALDH enzymes and thus may contribute to testicular RA synthesis¹²². Once synthesized, the dogma states that RA binds to a RA receptor (RARA, RARB, RARG) which then heterodimerizes with a RXR (RXRA, RXRB, RXRG) to activate or repress transcription at RA response element (RARE)-containing promoters of target genes²³⁸. RA signaling has been shown, both in somatic tissues as well as in spermatogonia, to also act through nongenomic means, for example by activating ‘mammalian target of rapamycin complex 1’ (mTORC1) to enhance cap-dependent translation^{71–73}.

In the mammalian testis, divisions of SSCs produce daughter cells that either remain as

SSCs (self-renewal) or proliferate as A_{Undiff} that will eventually differentiate in response to RA (Fig. S1)⁷⁹. The requirement for RA in differentiation is unequivocal, and thus represents a pivotal spermatogonial cell fate decision, which is the commitment to initiate meiosis^{239,240}. Defining the regulatory logic underlying how spermatogonia manage this fate decision is fundamental to our understanding of how proper ratios of SSCs, A_{Undiff} , and A_{Diff} spermatogonia are maintained to ensure life-long male fertility.

In human small-scale clinical trials, researchers assessed the ability of exogenous RA to enhance fertility of subfertile or infertile men with low sperm counts. When these men were given the RA analog Isotretinoin^{241,242}, they exhibited significantly improved sperm counts, and in some cases the ability to father children, either through intercourse or by intracytoplasmic sperm injection (ICSI)^{241,242}. Despite these exciting preliminary findings, the mechanisms by which RA enhanced sperm production and the long-term outcomes of continuous RA treatment on the male germline remain unknown.

In a recent report from our laboratory⁷⁴, the foundational spermatogonial populations in the neonatal testis were categorized both *in vitro* and *in vivo* based on their responsiveness to RA. SSCs could not respond to RA (RA-insensitive), undifferentiated progenitors could respond to RA but had not yet done so, and differentiating spermatogonia had already responded to RA. Based on our results in the developing testis, we proposed these responses were based on a lack of expression of a cognate RAR (likely, RARG) in SSCs, and ‘cytochrome p450, family 26’ (CYP26)-mediated RA catabolism in A_{Undiff} ⁷⁴. Whether these mechanisms function similarly during steady-state spermatogenesis in the juvenile and adult testis remains unknown. Broadly speaking, adult spermatogonial subtypes are predicted to manage their response to RA based on two non-mutually exclusive scenarios: 1) differential distribution of RA gradients within the testis;

2) cell type-specific differential expression of cognate RARs.

In the first, non-cell autonomous scenario, a differential gradient of the lipophilic morphogen RA is formed based on the balanced action of enzymes directing its synthesis (e.g., RALDHs) or catabolism (e.g., CYP26s)^{109,216,243–245}. This concept is supported by reports measuring RA levels, which differ along the mouse seminiferous tubules^{119,216,246}. Specifically, RA levels are highest in tubule segments containing seminiferous epithelial stages VII-IX in the mouse^{186,240}. Perhaps coincidentally, these stages in mice contain germ cells undergoing key RA-related transitions such as spermatogonial differentiation, meiotic initiation, spermatid elongation, and spermiation¹¹⁹. This suggests RA levels themselves are regulatory and predict RA levels would be limiting in stages I-VI and X-XII. An issue complicating extension of this model to other species – notably primates and humans – is that these key cell transitions do not similarly align in this spatial manner in those species^{236,247,248}. Thus, it is difficult to conceive how these critical events are co-regulated by an RA gradient in primates and humans.

In the second, cell autonomous scenario, differential reception of the RA signal is thought to occur through binding one or more of the three RARs²⁴⁹, which are expressed in cell- and tissue-specific patterns. In the testis, RARB is undetectable, and male knockout mice were fertile²⁵⁰. RARA is expressed, albeit at different levels, in both spermatogonia and Sertoli cells, and is not essential for spermatogonial differentiation in global, spermatogonia- or Sertoli cell-specific knockout (KO) mouse models^{128,131,132}. RARG is the most likely candidate for managing the spermatogonial RA response, since it is readily detectable in nuclei of RA-responsive A_{Undiffs}¹²⁰. In addition, when recombinant RARG was expressed precociously in SSCs and very early A_{Undiffs} under control of the '*glial cell line derived neurotrophic factor family receptor alpha 1*' (*Gfra1*) promoter, those cells differentiated early in response to RA¹³³. Confounding this issue, however,

is that it was shown in mouse KO models RARG was not essential for spermatogonial differentiation¹²⁰. Therefore, at this point it can be best summarized that RARG is sufficient but not necessary for spermatogonial differentiation, and redundant mechanisms must exist to mediate spermatogonial response to RA.

Here, we examined how spermatogonial responses to RA direct differentiation and meiotic initiation during steady-state spermatogenesis. Our results reveal the adult mammalian testis contains a well-buffered system that employs multiple modalities to manage the spermatogonial response to RA during steady-state spermatogenesis. In addition, we found that while exogenous RA did not hasten entry into meiosis, as recently reported²¹⁷, it did advance $A_{Undiffs}$ into the 8.6 day differentiation program, leading to increased numbers of spermatocytes two weeks later. Ultimately, this buffering system ensures the preservation of long-term spermatogenesis, but could be temporarily overridden to increase sperm output to enhance fertility.

C. Materials and Methods

C.1 Animal Care and Treatments

All animal procedures adhered to the National Research Council Guide for the Care and Use of Laboratory Animals guidelines and were approved by the East Carolina University Animal Care and Use Committee (A3469-01). Mouse strains used for this study were outbred CD-1 (Charles River Laboratories, stock #022) and inbred C57Bl/6N (Charles River Laboratories, stock #027).

Adult mice (~6-10 weeks of age) were subjected to at least one of the following treatments:

- 1) 100 μ l of sterile RA (R2625 Sigma Aldrich; 10 μ g/ μ l via intraperitoneal (IP) injection; 2) 5 μ g/g body weight of the CYP26 inhibitor talarozole (Cat #HY-14531; Medchem Express; 1 μ g/ μ l

via oral administration) prepared in sterile vehicle ('dimethyl sulfoxide' (DMSO)); 3) equivalent volume of sterile DMSO via IP injection or oral administration as vehicle-only control. Mice were age-matched prior to receiving treatments.

Synchronized spermatogenesis was performed similar to previous reports as a model for steady-state spermatogenesis^{186,205,206}. Briefly, N,N'-1,8-Octanediylbis 2,2-dichloroacetamide (WIN 18,446; Cayman Chemical, #14018) was resuspended in DMSO (final concentration = 100 µg/g of body weight) and given orally to CD-1 mice for ten consecutive days (P1-P11) to block spermatogonial differentiation. On P11, mice received one subcutaneous injection of 10 µl exogenous RA (10 µg/µl) to stimulate differentiation. WIN 18,446 treatments may continue post-differentiation if mice are subjected to an "RA-deficient" synchronization schedule. Mice received either one injection of RA at P11 or two injections at either P11 + P14 or P11 + P16. Mice were humanely euthanized via CO₂ asphyxiation followed by cervical dislocation.

C.2 Tissue preparation and histological staining

Testes were fixed in either Bouin's solution or 4% paraformaldehyde (PFA) for 24 hrs at 4°C and subsequently washed in multiple changes of 1X PBS overnight. Bouin's-fixed samples were dehydrated through an ethanol series and processed for paraffin embedding using standard methods. Five µm tissue sections were subjected to periodic acid Schiff's (PAS) staining using conventional methods. Coverslips were mounted with Permount, and images taken using an Axio Observer A1 microscope equipped with an AxioCam 503 color digital camera (Carl Zeiss Microscopy).

C.3 Indirect immunofluorescence (IIF)

Immunostaining was performed as before^{72–74,206,251}. Briefly, PFA-fixed testes were washed in 1X phosphate buffered saline (PBS) overnight, soaked in 30% sucrose for 24 hrs, and frozen in optical cutting temperature (OCT) compound (Tissue Tek) prior to sectioning. Five μ m sections were incubated in blocking solution (1X PBS + 3% bovine serum albumin (BSA) + 0.1% Triton X-100) for at least 30 mins at room temperature. Thereafter, primary antibodies (Table 3.1) were diluted with blocking solution and incubated on tissue sections for 1 hr at room temperature. One section per slide was incubated in blocking solution without primary antibody as a negative control. After 15 mins in washing solution (1X PBS + 0.1% Triton X-100), sections were incubated in blocking solution with corresponding fluorescent secondary antibody (1:500) for 1 hr at room temperature. Slides were washed, covered in 4'6-diamidino-2-phenylindole (DAPI) with mounting medium (EnQuire BioReagents) or 1:1 1X PBS/Glycerol solution, and coverslipped. All incubation and wash steps were performed in humid opaque containers. Imaging was done using either the Celldiscover7 (Carl Zeiss Microscopy) or Fluoview FV1000 laser scanning confocal microscope (Olympus America).

C.4 Testicular single cell suspensions

Adult mouse testes were used to make single cell suspensions, as previous described⁷⁴. Briefly, testes were detunicated and transferred to a solution containing 4.5 ml of 0.25% trypsin (Gibco) and 0.5 ml DNase 1 (7 mg/ml, Sigma-Aldrich) and incubated in a 37°C water bath for 3 min. An additional 1 ml DNase 1 was added and the mixture was pipetted up and down to further break up the testes, followed by a 1 min incubation in a 37°C water bath. One ml of fetal bovine serum (FBS, ATCC) was added to deactivate the trypsin, and the mixture was triturated to break

apart any remaining clumps of intact testes. The cell mixture was filtered through a 40 μm sieve and centrifuged at 500 x g for 7 min. The resulting cell pellet was suspended in Dulbecco's modified eagle medium (DMEM; Gibco) containing 1X penicillin-streptomycin (Thermo-Fisher Scientific). Cells were plated at a density of 1.4×10^6 cells/well into a 12-well plate containing glass coverslips and allowed to adhere for 2 hrs at 37°C with 5% CO₂ prior to treatment. Cells were treated with either DMSO or talarozole (25 nM) for 2 hrs, followed by an addition of vehicle or RA (0.001, 0.01, and 1.0 μM) for 8 hrs. These concentrations were determined based on published IC(50) values shown to achieve effective inhibition of the CYP enzymes at the minimum dosage²⁴³. These cells were cultured for a total of 12 hrs, and experiments were repeated in triplicate.

C.5 qRT-PCR

Total RNA was isolated from tissues using the miRNeasy Mini Kit (Qiagen) following the manufacturer's protocol and quantified using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific). Real-time quantitative polymerase chain reaction (qRT-PCR) was performed using the iTaq Universal SYBR Green One-Step Kit (Bio-Rad) using a StepOnePlus real-time PCR thermocycler (Applied Biosystems)²⁰⁶. Message abundance was normalized to the average C_t values of the housekeeping gene '*ribosomal protein L19*' (*Rpl19*) and expressed as $2^{-\Delta C_t}$. Primer sets are listed in Table 3.2.

C.6 Statistics and data analyses

Student's t-test, one-way analysis of variance (ANOVA), and Tukey's test were utilized to determine statistical significance at $p < 0.05$. For *in vivo* assays, at least 3 mice were used per age

per treatment. For adult *in vivo* experiments, the average number of tubule cross-sections quantified per testis exceeded 100. For single cell suspension experiments, trials were performed thrice. QuPath, Microsoft Excel, and GraphPad Prism were used to quantify data. GraphPad Prism and ImageJ were both used for formatting figures and data.

D. Results

D.1 Exogenous RA causes widespread spermatogonial response across the testis.

The extent of germ cell response to RA during adult steady state spermatogenesis was first determined by examining testes of mice treated with vehicle or 10 $\mu\text{g}/\mu\text{l}$ RA. CD-1 mice were euthanized 12, 24, and 48 hrs after treatment, and expression of ‘stimulated by RA gene 8’ (STRA8) protein was used as an established proxy for RA-responsive germ cells within the testis^{71,74}. Immunostaining revealed significantly increased numbers of STRA8+ spermatogonia in RA-treated testes 12 hrs after treatment, with ~80% of tubules containing STRA8+ spermatogonia (~3.5 fold increase over vehicle-treated controls (Fig. 3.1A, D, G)). Twenty-four hrs post-RA, numbers of STRA8+ spermatogonia were significantly lower than at 12 hrs post-RA, but greater than in vehicle-treated testes (Fig. 3.1B, E, G). STRA8 was undetectable by 48 hrs later, as both the numbers of STRA8+ spermatogonia and the percentage of tubules with STRA8+ spermatogonia in RA-treated testes were not different from vehicle-treated controls (Fig. 3.1C, F, G). To ensure these results were not strain-dependent, treatments were repeated in adult mice on a C57Bl/6N background. Immunostaining revealed a similar STRA8 expression pattern in both vehicle- and RA-treated testes within the 48 hr acute response window (Fig. 3.S2) to those evidenced in CD-1 mice (Fig. 3.1). Collectively, these findings reveal spermatogonia throughout adult testes were indeed RA-responsive, supporting the concept of differential localized RA

concentrations along the length of the seminiferous tubules.

The precise timing of mouse spermatogenesis enables subdivision of the adult seminiferous epithelia into 12 specific stages (I-XII), with each tubule section containing an expected profile of specific germ cell types²⁵². Mouse seminiferous tubule stages VII-IX were previously shown to have higher levels of RA than other stages²¹⁶, and it is during stage IX that differentiation occurs to form type A₁ spermatogonia^{199,221}. Using established staging criteria²⁵², fluorescent lectin labeling to visualize spermatid acrosomes, and immunostaining for STRA8, the stages containing RA-responsive germ cells were identified. All tubule stage groupings 12 hrs after RA treatment contained STRA8+ germ cells (Fig. 3.1H, I). These RA-responsive germ cells in RA-treated testes (versus vehicle-treated controls) were predominantly in stages I-VI (Fig. 3.1J).

Within the testis, RARG is restricted to spermatogonia and is thus predicted to be a primary determinant in WT testes for RA responsiveness^{74,133}. Therefore, to identify potential RA-responsive spermatogonia, RARG was immunolocalized in adult testes. Surprisingly, the numbers of RARG+ spermatogonia increased ~2.5 fold compared to vehicle-treated controls 12 hrs post-RA treatment (Fig. 3.2A-E). This increase in RARG+ spermatogonia was retained at 24 hrs post-RA, and higher than vehicle-treated samples, but significantly less than at 12 hrs post-RA treatment (Fig. 3.2B, C, E). Like the transient increase in STRA8+ germ cells, numbers of RARG+ spermatogonia decreased 48 hrs post-RA treatment, comparable to vehicle-treated testes (Fig. 3.2D, E). To determine the locations of RARG+ germ cells, staging of tubules was performed by immunostaining for RARG, along with lectin and DAPI. RARG+ spermatogonia were predominantly housed within stages VI-IX of vehicle-treated testes, in agreement with prior reports of RARG expression in late A_{Undiffs} and A_{Diffs} (type A₁)^{120,127}. In comparison, RARG+ spermatogonia were present 12 hrs after RA treatment throughout the testes in all tubule stage

groupings (Fig. 2F-J).

Since stages I-VI contain SSCs (predominantly type A_s) as well as A_{Undiffs} (types A_{pr}, and A_{al}) and A_{Diff} (types A₄ – B) spermatogonia^{220,221,252,253}, the specific subtypes were identified that responded to RA. First, we verified adult SSCs were RA insensitive, as in the developing testis⁷⁴, by co-immunostaining for STRA8 and GFRA1, the most restricted fate marker for SSCs and early progenitors⁶³. Indeed, we did not observe GFRA1+/STRA8+ spermatogonia 12 hours post-RA (Fig. 3.S3A). These results were corroborated by staining of testes 12 hours post-RA with RARG and GFRA1 – indeed GFRA1+ germ cells were predominantly RARG- (Fig. 3.S3B) as expected. Next, the numbers of RA-responsive A_{Undiffs} progenitors were identified by co-immunostaining for both STRA8 and ‘zinc finger and BTB domain containing 16’ ZBTB16 (also termed PLZF), an established marker of A_{Undiffs}²⁵⁴. Twelve hrs post-RA, there was a ~3.5-fold increase in STRA8+/ZBTB16+ A_{Undiffs} (Fig. 3.3A-E), and ~80% of tubules contained these cells. By twenty-four hrs post-RA, numbers of STRA8+/ZBTB16+ spermatogonia were comparable to vehicle-treated controls (Fig. 3.3C, E). Staging these tubules 12 hrs after RA revealed these RA-responsive A_{Undiffs} were present in all tubule stages throughout the testis (Fig. 3.3F-J). To quantify adult RA-responsive A_{Diffs}, co-immunostaining was performed using markers for STRA8 along with ‘kit proto-oncogene receptor tyrosine kinase’ (KIT), a bona fide marker expressed at high levels in A_{Diffs} and a lesser extent in preleptotene spermatocytes^{255,256}. Twelve hrs post-RA, there were significant increases in numbers of STRA8+/KIT+ germ cells (Fig. 3.4A-E). By 24 hrs post-RA, numbers were higher than vehicle-treated controls, but significantly lower than 12 hr RA-treated testes. By 48 hrs post-RA, numbers of STRA8+/KIT+ germ cells were comparable between RA- and vehicle-treated testes (Fig. 3.4A-E). Staging these tubules 12 hrs after RA revealed RA-responsive KIT+ germ cells (likely A_{Diffs}) in all tubule stages (Fig. 3.4F-J). Collectively, these

results reveal both A_{Undiffs} and A_{Diffs} throughout the adult testis have the capacity to respond to RA.

D.2 CYP26-mediated catabolism partially mediates the male germ cell RA response in the adult testis.

ZBTB16⁺ germ cells were shown to be RA-responsive in all tubule stages 12 hrs post-RA treatment (Fig. 3.3) but are somehow protected from differentiating in the unaltered WT testis until stages VII-IX. We previously showed that, in the developing testis, CYP26-based catabolism protected A_{Undiffs} from precociously differentiating in response to RA⁷⁴. Here, we examined the extent to which CYP26-mediated catabolism also modulated responsiveness of adult undifferentiated progenitor spermatogonia. To assess this, adult mice were treated with vehicle-alone, vehicle + RA, or the specific and potent CYP26 inhibitor talarozole²⁵⁷. Immunostaining of testes of mice 12 hrs after treatment revealed talarozole treatment increased numbers of STRA8⁺/ZBTB16⁺ A_{Undiffs} ~2-fold as compared to vehicle-treated controls (Fig. 3.5A-D). Interestingly, talarozole treatment increased numbers of STRA8⁺ A_{Undiffs} (as well as the percentage of STRA8⁺/ZBTB16⁺ tubules), but not to the same extent as RA-treated testes (Fig. 3.5A-E). To validate these *in vivo* results, adult testes were cultured as single cell suspensions and treated with vehicle-alone or talarozole with increasing RA concentrations (0.001-1 μ M). qRT-PCR revealed *Stra8* mRNA levels were significantly higher in talarozole + RA treated samples compared to those treated with vehicle + RA (Fig. 3.5F). Collectively, these results reveal RA catabolism is involved, to a degree, in regulating RA levels and thus germ cell RA response during adult steady-state spermatogenesis.

D.3 A single pulse of RA caused undifferentiated progenitor spermatogonia to precociously differentiate but they still enter ~ 8 days later, on the expected timeline.

We next examined whether the response to RA by the $A_{Undiffs}$ was transient or if they became committed to differentiate and ultimately enter meiosis. To distinguish between these possibilities, adult mice were injected with vehicle-alone or RA (=day 1) and euthanized eight days later (=day 9) (Fig. 3.6A). This timeline was selected because the length of one cycle of the seminiferous epithelium (I-XII) in mice is 8.6 days¹⁹⁹, and eight days after responding to exogenous RA, spermatogonia committed to differentiation would be expected to be entering meiosis as preleptotene spermatocytes¹⁹⁹. Co-immunostaining was done to identify preleptotene spermatocytes based on strong nuclear STRA8 signal and punctate staining for the spermatocyte marker ‘synaptonemal complex protein 3’ (SYCP3)⁷⁰. Compared to vehicle-treated controls, testes of RA-treated mice had ~30% more preleptotene spermatocytes, which were present in more tubules (~1.8-fold increase) (Fig. 3.6B-E). These results suggest $A_{Undiffs}$ that responded to exogenous RA committed to and completed differentiation and entered meiosis on an expected timeline.

D.4 RA exposure does not hasten differentiating spermatogonia to precociously enter meiosis.

During steady-state spermatogenesis, the RA-responsive spermatogonial pool contains both $A_{Undiffs}$ and A_{Diffs} , which had already responded to RA. It was previously reported that A_{Diffs} , in mice that received a second bolus of exogenous RA, would exit the differentiation program and enter meiosis²¹⁷. To test whether A_{Diffs} at the midpoint or near the end of differentiation were indeed awaiting a second pulse of RA for meiosis, an *in vivo* model of synchronized adult steady-state spermatogenesis was used (Fig. 3.S4).

Spermatogenesis was synchronized by treating mice from P1-P10 with WIN 18,446, a potent and selective inhibitor of RA synthesis (Fig. 3.S4)²⁰⁶. On P11, a single bolus of RA was injected to initiate differentiation. Groups of mice with synchronized spermatogenesis were then subjected to one of the following treatments: 1) RA once on P11, to induce differentiation and formation of type A₁ spermatogonia at the beginning of the differentiation program; 2) RA on P11 and P14, when testes contained type A₃ spermatogonia near the midpoint of the differentiation program; 3) RA on P11 and P16, when testes contained type Intermediate (In) spermatogonia near the end of the differentiation program²⁰⁶. To ensure the synchronized model properly mirrored events in steady-state spermatogenesis, co-immunostaining for STRA8 and KIT was done to detect RA-responsive spermatogonia. As in the adult testes (Fig. 3.4), differentiating spermatogonia were STRA8+/KIT+ 24 hrs post-RA (Fig. 3.S5A, B). To verify STRA8 was induced specifically in response to the initial pulse of exogenous RA, experiments were repeated but mice were continuously treated with WIN 18,446 after P11 to generate mice with RA-deficient testes (Fig. S5A, C). In these mice, differentiating spermatogonia were also STRA8+ 24 hrs post-RA treatment. The histology of P19 testes (with preleptotene spermatocytes as the most abundant germ cell type)²⁰⁶ that were treated with a single pulse of RA on P11 was compared to those that received second pulses at P14 or P16. If the second pulse of RA had hastened meiosis, then most germ cells in those testes should be advanced beyond preleptonema. Surprisingly, there were no apparent differences in the germ cell complement in testes from mice receiving only the single RA injection with those receiving a second, revealing no evidence for precocious meiotic entry (Figs. 3.7A, B; 3.S6). To verify these results, spermatocyte identity was assessed by co-immunostaining for STRA8 (an established marker of preleptotene spermatocytes^{147,217}) and SYCP3, which exhibits a characteristic pattern during each stage of meiosis. In this steady-state synchronization

model with a single pulse of RA at P11, testes were enriched with spermatocytes in preleptonema at P19, leptonema/zygonema at P21, and early pachynema at P23²⁰⁶. If a second pulse of RA at P14 or P16 indeed advanced entry into meiosis, then spermatocytes in leptonema, zygonema, and/or pachynema would predominate at P19. However, testes from mice receiving the second pulse of RA at either P14 or P16 did not contain significantly more spermatocytes beyond preleptonema than controls receiving a single pulse at P11 (Fig. 3.7C). To verify that this lack of advancement of differentiated spermatogonia into meiosis phenotype was not unique to the steady-state synchronization model, adult mice were treated with vehicle or RA and sacrificed 24, 48, and 72 hours later and histologically examined for accumulation of preleptotene or leptotene spermatocytes, which would indicate a truncation of differentiation and hastened entry into meiosis. In testes of RA-treated mice, there were no differences in the complement of spermatocytes as compared to vehicle-treated controls (Fig. 3.S7). Collectively, these findings reveal a single exogenous pulse of RA neither shortened the differentiation timeline nor hastened meiotic entry or progression.

D.5 Continuous RA administration enriches production of advanced spermatogenic cells.

We next examined whether multiple pulses of RA were required for spermatogonia to precociously exit differentiation and enter meiosis. To test this, groups of adult mice were injected daily with vehicle-alone or RA for eight consecutive days (D1-D8) and euthanized on D9 or D17 (Fig. 3.8A). On this treatment schedule, germ cells are predicted to complete one seminiferous cycle by D9 and two cycles by D17. Co-immunostaining was done to detect both SYCP3 and ‘H1.6 linker histone, cluster member’ (H1F6, also termed H1T), the latter of which is detectable in spermatocytes from mid-pachynema onward and in round and elongating spermatids^{258,259}. At

D9, there were no significant differences in numbers of H1F6+ spermatocytes in testes from mice receiving vehicle-alone or daily pulses of exogenous RA (Fig. 3.8A-C). At D17, after two seminiferous epithelial cycles, significantly more H1F6+ spermatocytes (16% increase) were observed in testes from mice receiving eight daily doses of RA (Fig. 3.8A-C). This reveals daily RA exposure provided continual stimulation of undifferentiated progenitor spermatogonia to differentiate, thus forming a larger-than-normal cohort of germ cells proceeding together through spermatogenesis.

A concern with repeated dosages of RA is the long-term effect on the germline, with the potential that transiently enriched numbers of A_{Diffs} would occur at the expense of the subsequent germ cell cohort or the self-renewing SSC pool. Thus, we wanted to ensure the germline was preserved in the presence of RA injections for eight days. To assess this, numbers of ZBTB16+ A_{Undiffs} were quantified in D17 testes after daily injection of vehicle-alone or RA. Since SSCs are RA-insensitive (Fig. 3.S3 and⁷⁴), we predicted they would not differentiate in response to these daily RA pulses and thus continue to generate A_{Undiffs}. Indeed, numbers of A_{Undiffs} were similar between vehicle- and RA-treated testes at D17 (Fig. 3.8D-F). Altogether, these results reveal consistent RA exposure can enrich a generation of germ cells without adversely affecting SSCs and provide further evidence that the RA insensitivity is a key feature of these cells.

E. Discussion

Here, we expand on our prior results in the developing testis⁷⁴ to examine the relationship between differential responses to RA and spermatogonial development during steady-state spermatogenesis. A_{Undiffs} outside seminiferous epithelium stages VII-VIII precociously responding to exogenous RA both committed to and completed differentiation before entering meiosis ~8 days

later as preleptotene spermatocytes. CYP26-mediated RA catabolism played a role, albeit not a primary one as in the neonatal testis, in limiting exposure of A_{Undiffs} to RA. A_{Diffs} were also RA-responsive, but their response did not shorten the 8.6 day differentiation timeline, and it did not hasten their entry into or progression through meiosis. Daily exposure for an entire 8 day seminiferous epithelial cycle increased the numbers of advanced germ cells, presumably by directing A_{Undiffs} to continuously commit to differentiate and enter meiosis, and this treatment did not appear to adversely affect SSCs. Results here provide an explanation for recent findings from small scale clinical trials, in which repeated treatment with retinoids transiently increased sperm output and enhanced male fertility^{241,242}.

Our studies revealed exogenous RA drives widespread germ cell response throughout the testis. We chose to focus on the acute response to RA and detailed the degree of response over 48 hours. This response was indeed transient, with STRA8 signal diminishing over time, only germ cells in specific tubule stages (VII-VIII) expressing STRA8 thereafter. Due to this acute window, we noted the presence of RA responsive A_{Undiffs} and RA responsive A_{Diffs} in all seminiferous tubule stage groupings. Our findings contrast with a recent report of STRA8⁺ A_{Undiffs} post-RA injection only in stages II-VI and not in later stages²¹⁷. We account for this discrepancy based on time of analyses – multiple timepoints post-treatment vs 24 hrs post-treatment. Also noteworthy, we observed this transient expression of STRA8 in mice of multiple genetic backgrounds: CD-1 and C57Bl6N as well as on a CD-1xC57Bl/6J mixed background (data not shown).

STRA8 expression is commonly used in the field as a proxy for RA signaling^{144,147,148,217,219,260}. Although the specific function of this putative transcription factor is unknown, STRA8 is unique to germ cells and to vertebrates, and has been associated with pluripotency-associated genes and meiotic initiation^{145,260}. Intriguingly, STRA8 is expressed in

both A_{Diff}s and preleptotene spermatocytes entering meiosis^{147,148,217}, however its expression is not required for either process. Indeed, it was shown in *Stra8* KO mice on a mixed genetic background that male germ cells arrest in early pachynema, while on those on a congenic C57Bl/6N background arrest in preleptonema following meiotic DNA replication^{147,148}. Thus, STRA8 is required for progression through meiosis, and serves as an effective protein marker (as demonstrated here) to identify RA-responsive germ cells.

Here, we observed that ~25% of ZBTB16+ A_{Undiff}s remained STRA8- in response to a pulse of exogenous RA in the adult. This number closely approximates the percentage of spermatogonia that were GFRA1+^{261,262}. Since we did not observe any GFRA1+/STRA8+ spermatogonia post-RA, this indicates that GFRA1 can be a reliable marker of RA-insensitive spermatogonia. These results further suggest, as in the neonatal testis, there are distinct differences in how adult spermatogonia respond to RA – SSCs and early A_{Undiff}s cannot respond to RA (=RA-insensitive), mid-late A_{Undiff}s poised to differentiate (=RA-responsive), or A_{Diff}s that respond again (=RA-responded). Several studies have investigated sub-categories of A_{Undiff}s based on their expression of specific markers (e.g., ‘neurogenin 3’ (*Neurog3/Ngn3*) and ‘developmental pluripotency-associated 3’ (*Dppa3*)^{133,263,264}. In particular, *Dppa3* has recently been shown to reliably identify late-stage A_{Undiff}s into cohorts that preferentially differentiate in response to RA²⁶³.

We also noted differentiating spermatogonia became STRA8+ post-RA during steady-state spermatogenesis, both in the adult testis and the synchronized testis model. Despite having the capacity to respond to RA, A_{Diff}s did not expedite their progression through differentiation and into meiosis. These findings contrast with prior studies that reported the requirement for RA for meiotic initiation^{118,119,156,217}. To confirm our findings, adult mice were administered RA daily over the length of one seminiferous cycle and found no apparent differences compared to vehicle-treated

mice in the progression of these already differentiating spermatogonia into meiosis. In light of these findings, we propose the following: 1) A_{Diff} must complete the 8.6-day differentiation program prior to entering meiosis, regardless of additional exogenous RA; 2) the germ cell ability to respond to RA is acquired by A_{Undiff} , retained throughout differentiation, and then lost as they enter leptotema.

Our results support the existence of both cell autonomous and non-cell autonomous mechanisms governing male germ cell fate during steady state spermatogenesis. Considering very little circulating RA is used by germ cells²⁶⁵, and the criticality of the RA response for spermatogenesis, it makes sense that multiple systems are in-place to ensure proper RA response and thus homeostasis within the testis. The addition of exogenous RA drove widespread response (indicated by STRA8 reactivation) of multiple germ cell types, while advancement of spermatogenesis only occurred in a specific cell type – A_{Undiff} . Our results also revealed the adult testis does employ some level of CYP26-mediated catabolism to control RA levels. However, this RA catabolism was not the singular mechanism regulating RA response during steady-state spermatogenesis. Considering the degree of RA response in terms of the number of RA responding A_{Undiff} was reduced in talarozole- versus RA-treated mice, this further supports the concept that multiple mechanisms regulate the RA response during steady-state spermatogenesis. This markedly differed from our results seen in the developing testis, where RA catabolism conferred a near-complete degree of protection of A_{Undiff} from RA⁷⁴. This aligns with results from KO mice lacking CYP26B1, which is likely the CYP26 enzyme active in the testis – those mice had severe testicular phenotype in younger mice that improved as the mice aged^{138,245}.

There were remarkable similarities in germ cell responsiveness to RA both *in vivo* and *in vitro*, where the testis architecture is disrupted. This supports the concept that germ cells

themselves have intrinsic mechanisms regulating RA responsiveness. The molecular nature of this intrinsic response is currently unclear – a primary determinant of RA response would be predicted to be RA reception. Indeed, *in vivo* RARG was predominantly undetectable in GFRA1+ spermatogonia but readily detectable in RA-responsive A_{Undiffs} and A_{Diffs}. RARG expression was also evident throughout the testis in spermatogonia 12 hrs post-RA treatment, suggesting RARG is the primary receptor for RA responsiveness. Confounding this straightforward mechanism, however, is that spermatogonia in *Rarg* KO mice could differentiate and complete spermatogenesis in nearly all tubules in young adult mice, although progressive defects were not observed as these mice aged¹²⁷. The authors proposed *RARA* is acting in a compensatory mechanism at early ages, despite consistent, low *RARA* expression observed in the seminiferous tubule over time. These collective findings suggest RARG is sufficient, but not required for inducing spermatogonial differentiation in the murine testis. Alternatively, RARG may be the primary receptor for RA responsiveness, even at levels deemed undetectable for the assay. Noteworthy, *RARG* mRNA and protein are both abundantly expressed in the human testis^{266,267}, but to the best of our knowledge the RA response in human spermatogonia has not been examined.

A recent approach treating subfertile and infertile men with retinoids has yielded excited results. The testicular concentrations of RA were significantly lower in men with abnormal sperm production compared to their fertile counterparts²⁶⁸, and infertile men had significantly lower levels of RA synthesis enzymes (e.g., RALDH2), which corresponded with reduced germ cell numbers in the testis²⁴⁴. Men diagnosed with azoospermia (no sperm in ejaculate) and oligoasthenozoospermia (low sperm count and poor sperm motility) were given long-term treatments with isotretinoin, an isomer of RA, and they experienced significant improvements in fertility parameters^{241,242}. These treatments increased numbers of motile sperm in men with

oligoasthenozoospermia, resulting in pregnancies and live offspring. With these clinical results in mind, our results revealed that daily RA administration increased numbers of differentiating spermatogonia, resulting in significantly more spermatocytes both nine and seventeen days later. These RA-treated mice, if aged for another few weeks, would likely have increased sperm counts over their vehicle-treated control counterparts, matching the results from these clinical studies. Considering the remarkable conservation between mouse and human spermatogenesis⁶², the results presented here in mice provide an explanation for those from isotretinoin-based human clinical trials.

In designing long-term therapies, quality of health and the potential for therapy-induced toxicity are important considerations. Fortunately, the half-life of RA in the mammalian testis is <90 mins^{112,113}, therefore RA is either utilized by the cell or catabolized. In the human clinical trials, all men receiving long-term isotretinoin treatments experienced minimal side effects (e.g., dry skin, chapped lips) that were easily managed^{241,242}. Additionally, the isotretinoin concentrations administered to patients was several magnitudes lower than the dose range associated with teratogenicity, and the exposure of a pregnant female partner to isotretinoin from the treated partner's semen was negligible²⁴¹. At this time, we propose an alternative therapeutic approach, where high amounts of RA are given for a limited time (~ 2-3 weeks for patients) instead of the full spermatogenic cycle (~2-3 months). This shortened treatment regimen would increase numbers of spermatogonia undergoing differentiation in one seminiferous cycle while minimizing any potential side effects associated with long-term RA exposure. Considering SSCs are RA non-responsive, this temporary treatment regimen could be re-implemented as deemed necessary without hindering spermatogenesis long-term or inducing systemic side effects.

In summation, the findings here reveal the adult testis employs a complex and multilayered control system to modulate RA levels that limits spermatogonial progression through spermatogenesis in response to high levels of RA. This ensures normal germ cell development and ultimately preserves long-term fertility in male mammals. The addition of exogenous retinoids temporarily overcomes this system and presents an attractive strategy to increase chances of successful fertilization for men struggling with subfertility or infertility.

F. Acknowledgements

The authors thank Joani Zary-Oswald for histological assistance and Cindy Kukoly and Jim Aloor for technical assistance with imaging.

Table 3.1 Antibodies

<i>Antigen</i>	<i>Host</i>	<i>Source</i>	<i>Dilution</i>	<i>Catalog #</i>
GFRA1	Goat	R&D Systems	1:800	AF560
H1F6	Guinea Pig	Gift from <i>Handel</i>	1:500	Inselman et al 2003
KIT	Goat	R & D Systems	1:1000	AF1356
Lectin-488	Peanut	Thermo Fisher Scientific	1:1000	L21409
Lectin-594	Peanut	Thermo Fisher Scientific	1:1000	L32459
RARG	Rabbit	Cell Signaling	1:500	8965T
STRA8	Rabbit	Abcam	1:3000	Ab49602
SYCP3-488	Mouse	Abcam	1:200	Ab205846
TRA98	Rat	Abcam	1:1000	Ab82527
ZBTB16/PLZF	Goat	R & D Systems	1:1000	AF2944

Table 3.2 QRT-PCR Primers

<i>Primer Name</i>	<i>Sequence (5' to 3')</i>
<i>Rpl19-F</i>	GAAATCGCCAATGCCAACTC
<i>Rpl19-R</i>	TCTTAGACCTGCGAGCCTCA
<i>Stra8-F</i>	GAGGTCAAGGAAGAATATGC
<i>Stra8-R</i>	CAGAGACAATAGGAAGTGTC

G. Chapter 3 Figures

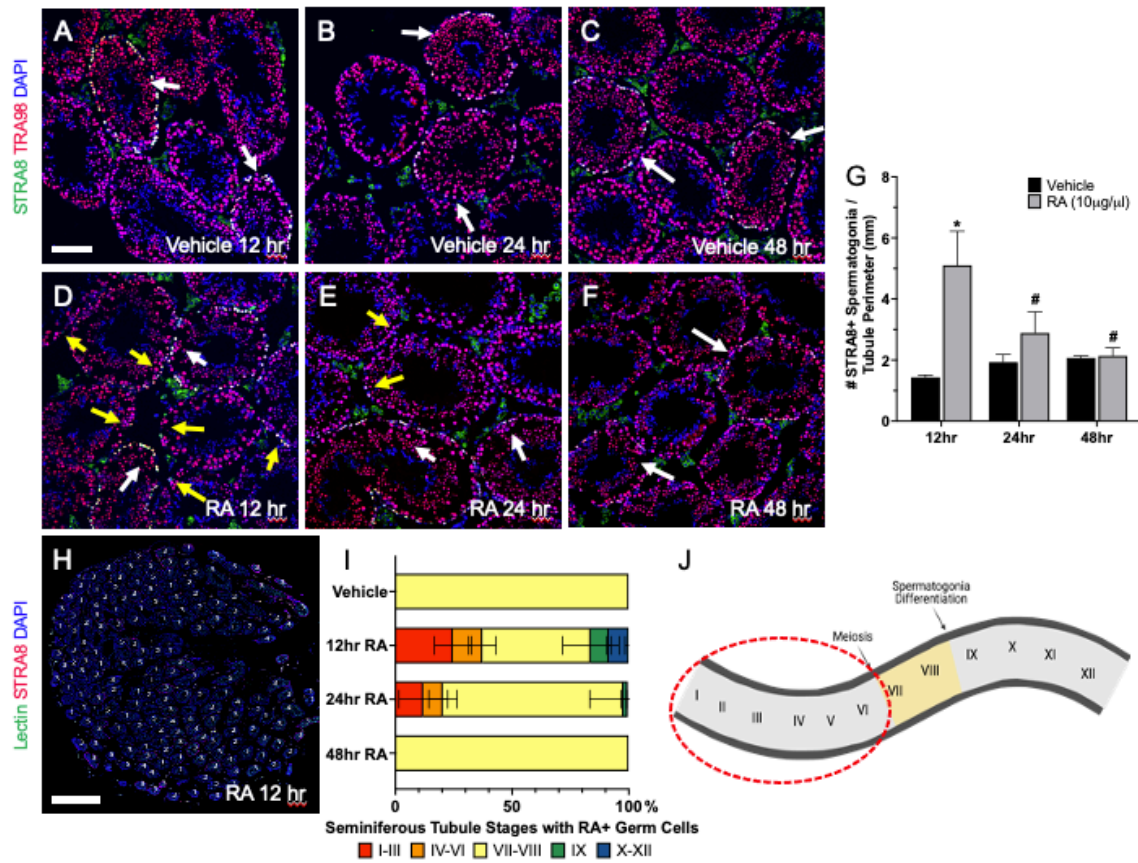


Fig. 3.1 Exogenous RA drives transient STRA8 expression in the adult testis.

(A-G) IIF for RA response marker (STRA8) in green, a pan germ cell marker (TRA98) in red and pan nuclei marker (DAPI) in blue in vehicle-treated **(A-C)** and RA-treated testes **(D-F)**. Treatment and time-of-sacrifice post-treatment indicated in each image. White arrows indicating tubules where STRA8⁺ spermatogonia normally reside and yellow arrows indicate tubules with spermatogonia responding to exogenous RA. Scale bar = 100 μ m. **G)** Quantitation of the number of STRA8⁺ spermatogonia per tubule perimeter (mm). *P<0.05 vs 12 hr vehicle-treated and #P<0.05 vs 12 hr RA-treated. **(H, I)** Staging of seminiferous tubules using lectin (green) and DAPI (blue) to identify locations of STRA8⁺ germ cells (red). Scale bar = 500 μ m. **I)** Distribution of seminiferous tubule stages containing STRA8⁺ germ cells per treatment using criteria established in literature and grouped by like characteristics: Group 1 = Stage I-III; Group 2 = Stage IV-VI; Group 3 = Stage VII-VIII; Group 4 = Stage IX; Group 5 = Stage X-XII. **J)** Location of spermatogonia responding to exogenous RA are concentrated in stages prior to onset of meiosis and differentiation. Diagram created using BioRender.com.

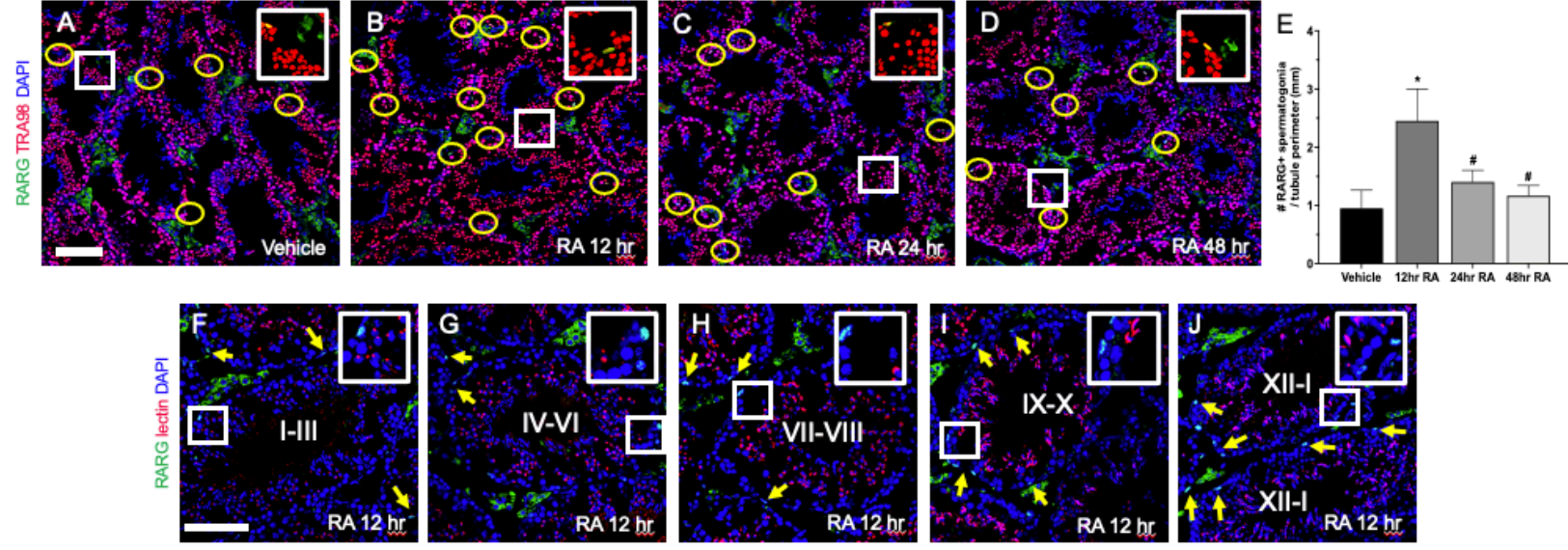


Fig. 3.2 Spermatogonia respond to RA through RARG signaling during steady-state spermatogenesis.

(A-E) IIF was done to quantify the number of germ cells (labelled in red) expressing the RA receptor RARG (cells in green). DAPI labels all cell nuclei in blue. Treatment and time-of-sacrifice post-treatment indicated in each image. Yellow circles encompass RARG⁺ germ cells. White box corresponds to zoomed in region (see insert). DAPI has been removed from insert. **E)** Quantitation of the number of RARG⁺ spermatogonia per tubule perimeter (mm). *P<0.05 vs vehicle-treated, and #P<0.05 vs 12 hr RA. **(F-J)** Staging of tubules was performed by co-immunostaining RARG (green), lectin (red), and DAPI (blue) to identify the location of RARG⁺ spermatogonia RA-treated (F-J). Stage of tubule is indicated in each image. Yellow arrows indicate RARG⁺ spermatogonia. White box corresponds to zoomed in region (see insert). Scale bar for all images = 100µm.

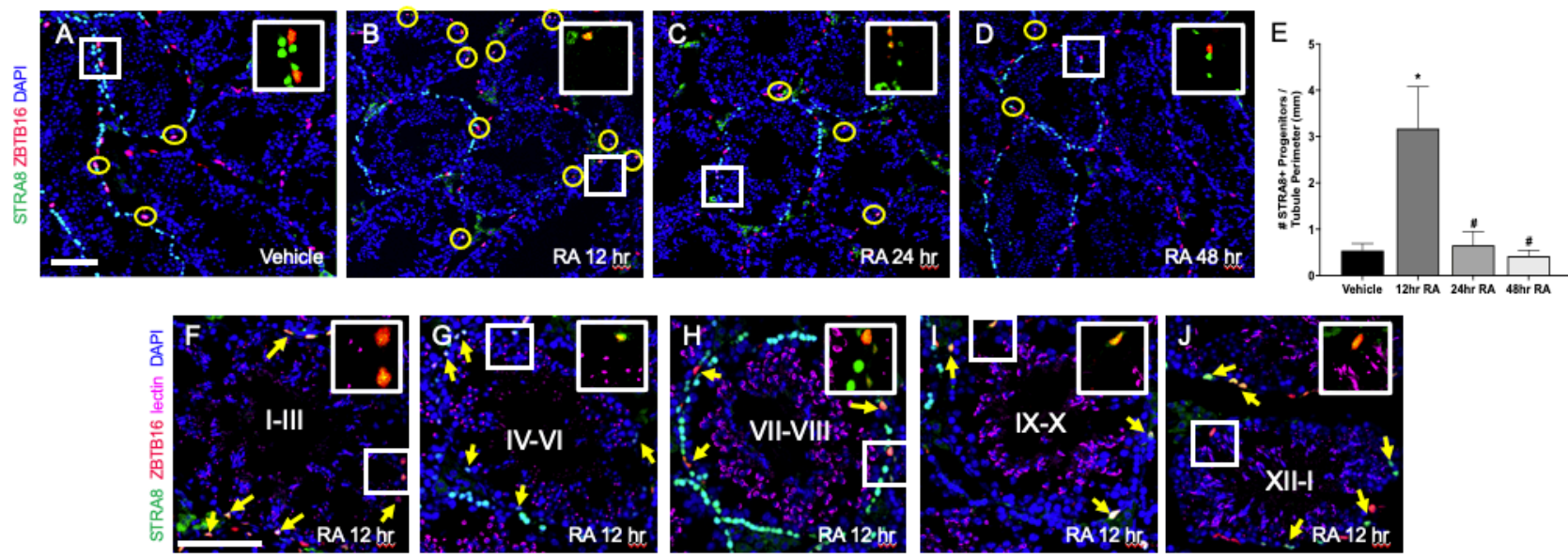


Fig. 3.3 Transient RA-responsive spermatogonia are predominantly undifferentiated progenitors.

(A-E) IIF was done to quantify the number of progenitors (ZBTB16+ cells in red) responding to RA (STRA8+ cells in green). DAPI labels all cell nuclei in blue. Treatment and time-of-sacrifice post-treatment indicated in each image. Yellow circles encompass STRA8+ ZBTB16+ cells. White box corresponds to zoomed in region (see insert). DAPI has been removed from insert. **E)** Quantitation of the number of STRA8+ progenitors spermatogonia per tubule perimeter (mm). * $P < 0.01$ vs vehicle-treated, and # $P < 0.01$ vs 12 hr RA. **(F-J)** Staging of tubules was performed through co-immunostaining of ZBTB16 (red), STRA8 (green), DAPI (blue) and lectin (magenta) to identify the location of STRA8+ progenitors in RA-treated testes sacrificed 12 hr post-treatment. Stage of tubule is indicated in each image. Yellow arrows indicate STRA8+ progenitors. White box corresponds to zoomed in region (see insert). DAPI has been removed from insert. Scale bar for all images = 100 μm .

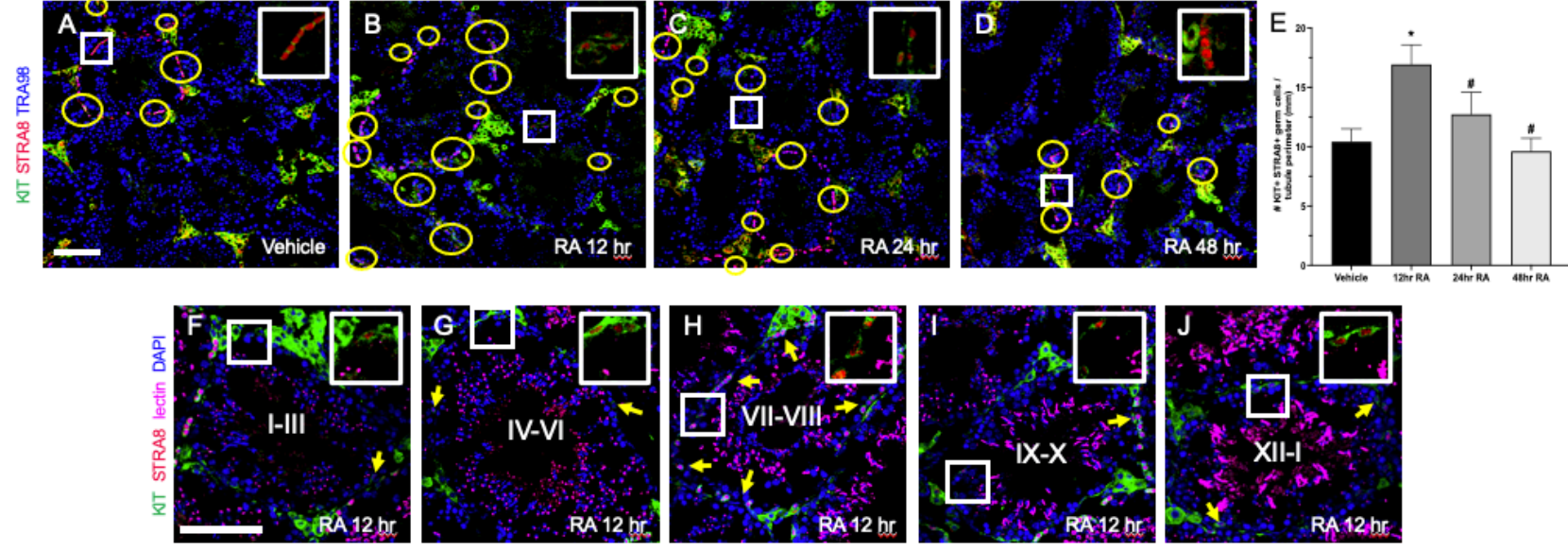


Fig. 3.4 Transient STRA8⁺ spermatogonia throughout the testis can enter differentiation in response to exogenous RA.

(A-E) IIF was done to quantify the number of spermatogonia that undergo differentiation (KIT⁺ germ cells in green) due to the RA signal (STRA8⁺ cells in red). TRA98, a pan germ cell marker, is shown in blue. Treatment and time-of-sacrifice post-treatment indicated in each image. Yellow circles encompass STRA8⁺ KIT⁺ germ cells. White box corresponds to zoomed in region (see insert). TRA98 has been removed from insert. **E)** Quantitation of the number of STRA8⁺ progenitors spermatogonia per tubule perimeter (mm). *P<0.05 vs vehicle-treated, and #P<0.05 vs 12 hr RA. **(F-J)** Staging of tubules was performed through co-immunostaining of KIT (red), STRA8 (green), DAPI (blue) and lectin (magenta) to identify the location of STRA8⁺ germ cells in RA-treated testes sacrificed 12 hr post-treatment. Stage of tubule is indicated in each image. Yellow arrows indicate STRA8⁺ KIT⁺ germ cells. White box corresponds to zoomed in region (see insert). DAPI has been removed from insert. Scale bar for all images = 100 μ m.

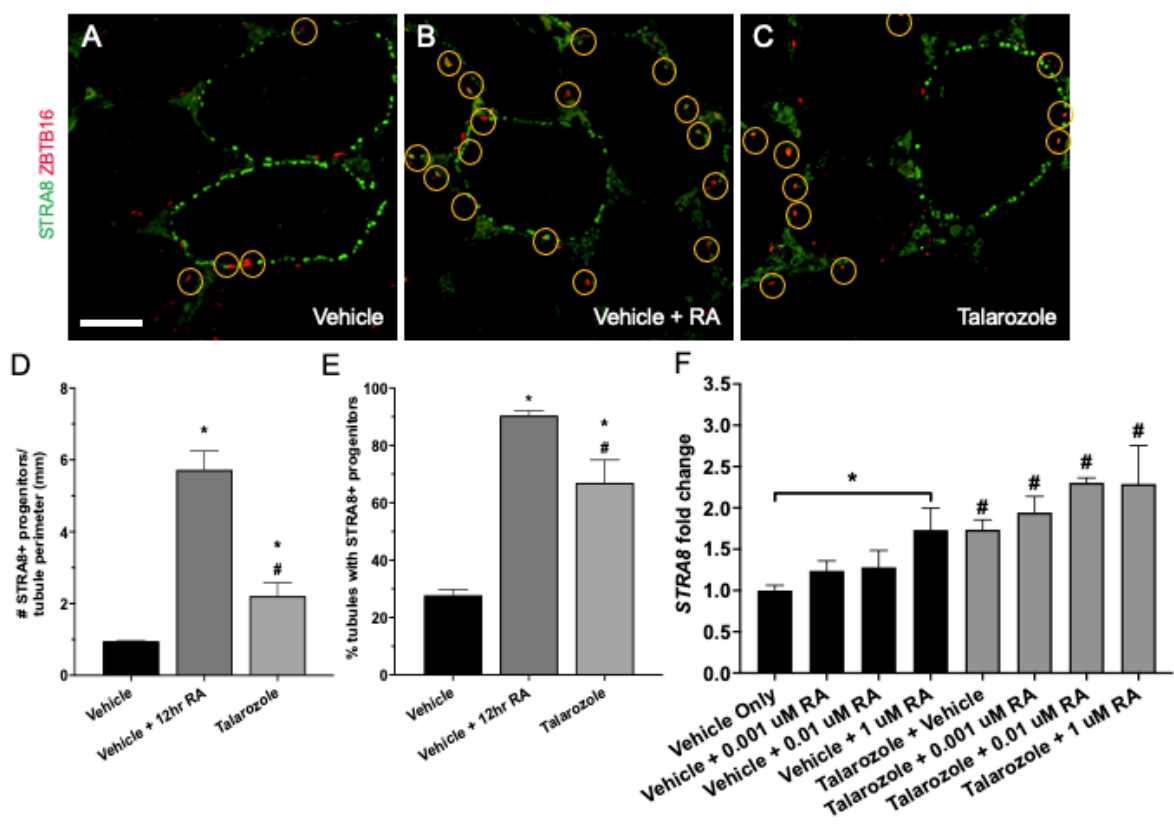


Fig. 3.5 RA catabolism limits the number of progenitors able to respond to RA during steady-state spermatogenesis.

(A-E) IIF was done to quantify the number of STRA8⁺ progenitors post-treatment. STRA8⁺ cells are labelled in green and ZBTB16⁺ progenitors are labeled in red. Treatment denoted in each image. Talarozole = CYP26B1/RA catabolism inhibitor. Yellow circles indicate STRA8⁺ progenitors. Scale bar for all images = 100µm. (D, E) Quantitation for the number of STRA8⁺ progenitors per tubule diameter (D) and the percentage of tubules that contain STRA8⁺ progenitors (E). * P<0.01 vs vehicle-treated testes and # P<0.05 vs vehicle + RA-treated testes. F) Whole testis single cell suspensions were treated with Vehicle or Talarozole with increasing concentrations of RA. Samples were collected after 12 hours for qRT-PCR of Stra8 mRNA expression. *P<0.05 vs Vehicle only, #P<0.01 vs same treatments without Talarozole (e.g. Talarozole + Vehicle vs Vehicle only).

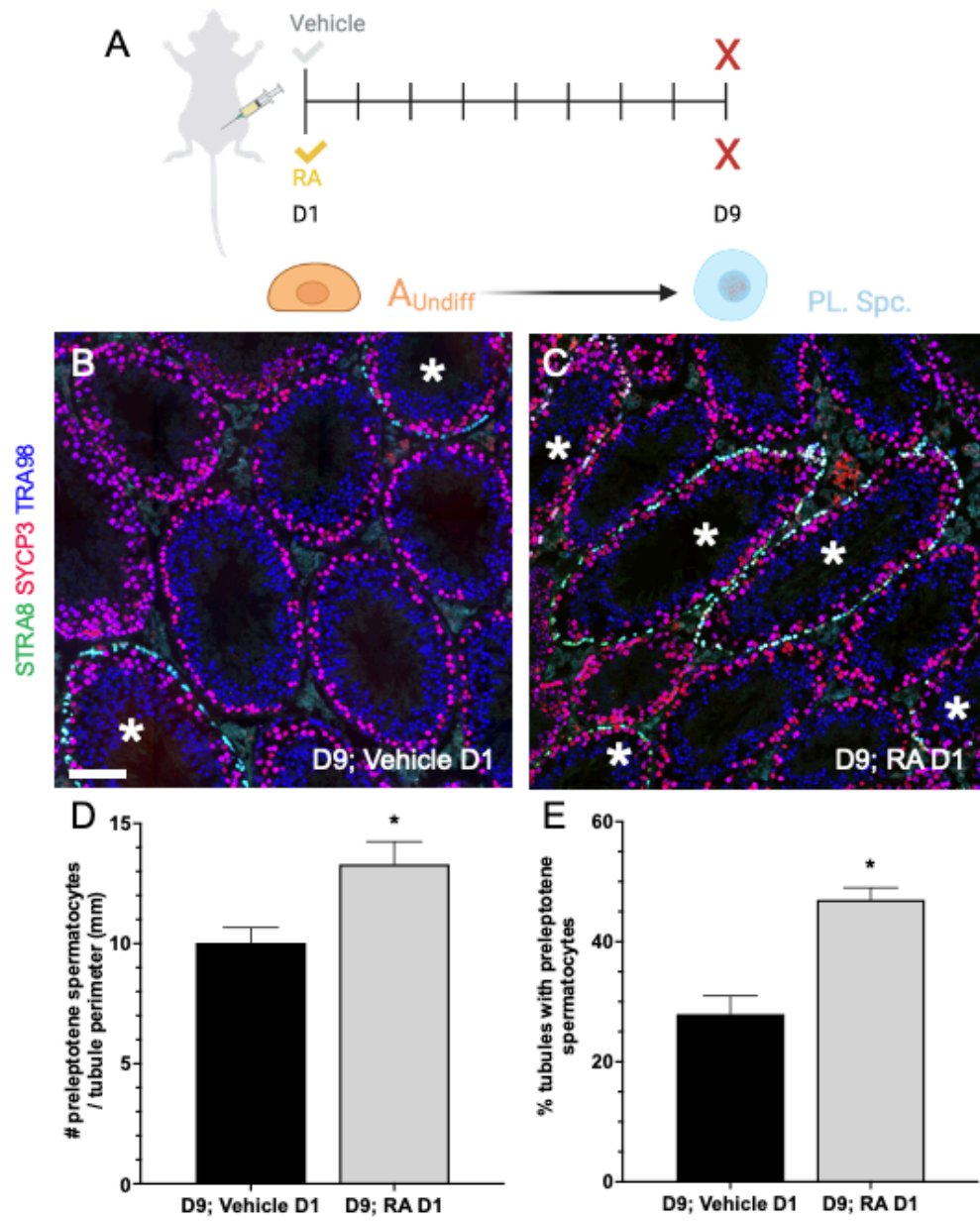


Fig. 3.6 RA-responsive progenitors progress through differentiation and enter meiosis.

(A) Mice were treated once with Vehicle or RA (D1) and sacrificed on D9. A_{Undiff} = undifferentiated progenitors and PL. Spc. = preleptotene spermatocytes. **(B, C)** Immunofluorescence was performed to identify preleptotene spermatocytes, which co-express STRA8 (green) and SYCP3 (red). TRA98, a pan germ cell marker, is in blue. White asterisks indicate tubules with preleptotene spermatocytes. Scale bar for all images = 100 μm . **(D, E)** Quantitation of the number of preleptotene spermatocytes per tubule perimeter (mm) **(D)** and the percentage of tubules with preleptotene spermatocytes **(E)**. * $P < 0.05$ vs vehicle-treated testes.

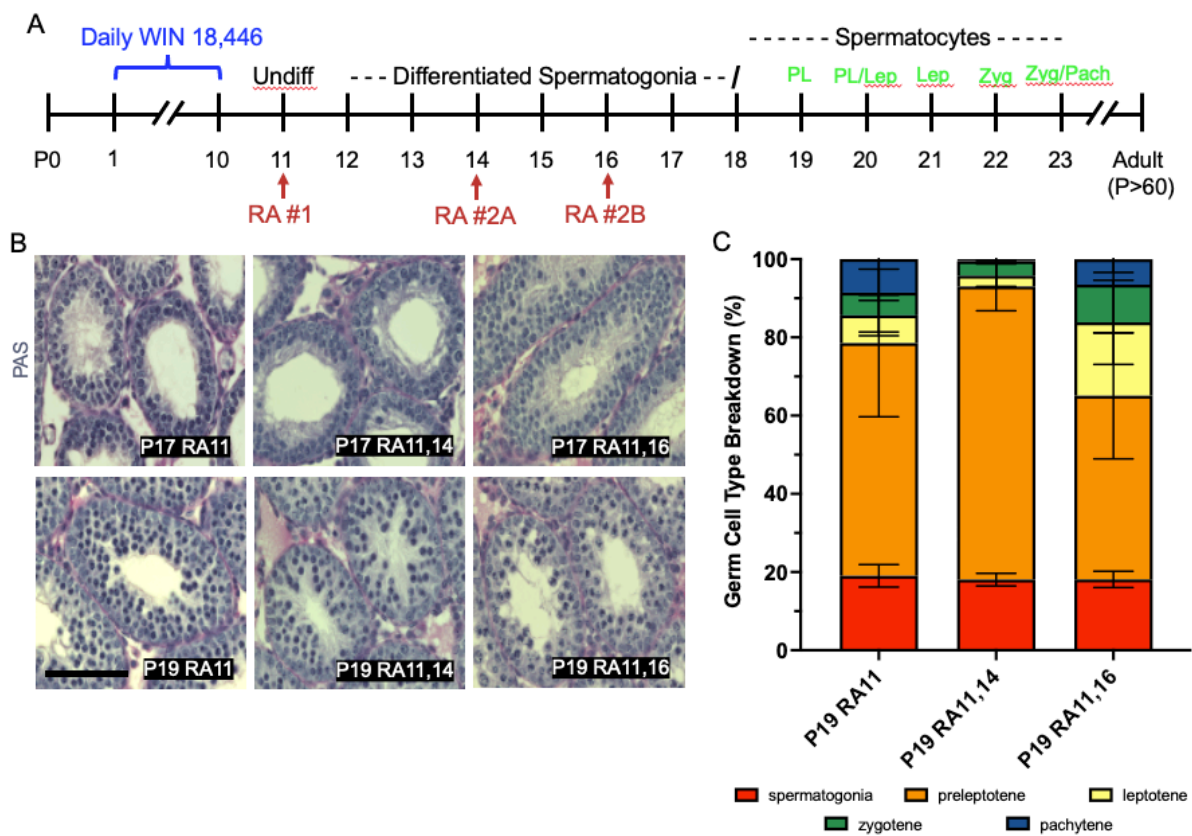


Fig. 3.7 Exogenous RA does not shorten the differentiation timeline in the synchronized steady-state model.

(A) Timeline denoting treatment schedule and germ cells that predominate at each postnatal age. WIN 18,446 = RA synthesis inhibitor. Undiff = Undifferentiated progenitors. PL = preleptotene spermatocytes. Lep = Leptotene spermatocytes. Zyg = Zygotene spermatocytes. Pach = Pachytene spermatocytes. RA was exogenously administered on P11 only, P11 and P14, or P11 and P16. **B)** Periodic acid Schiff's histological staining was performed to phenotype spermatocytes. Age and treatment schedule are denoted in the image. Scale bar for all images = 100 μm . **C)** Quantitation of the germ cell type breakdown at P19. Colors correspond to legend below the X-axis.

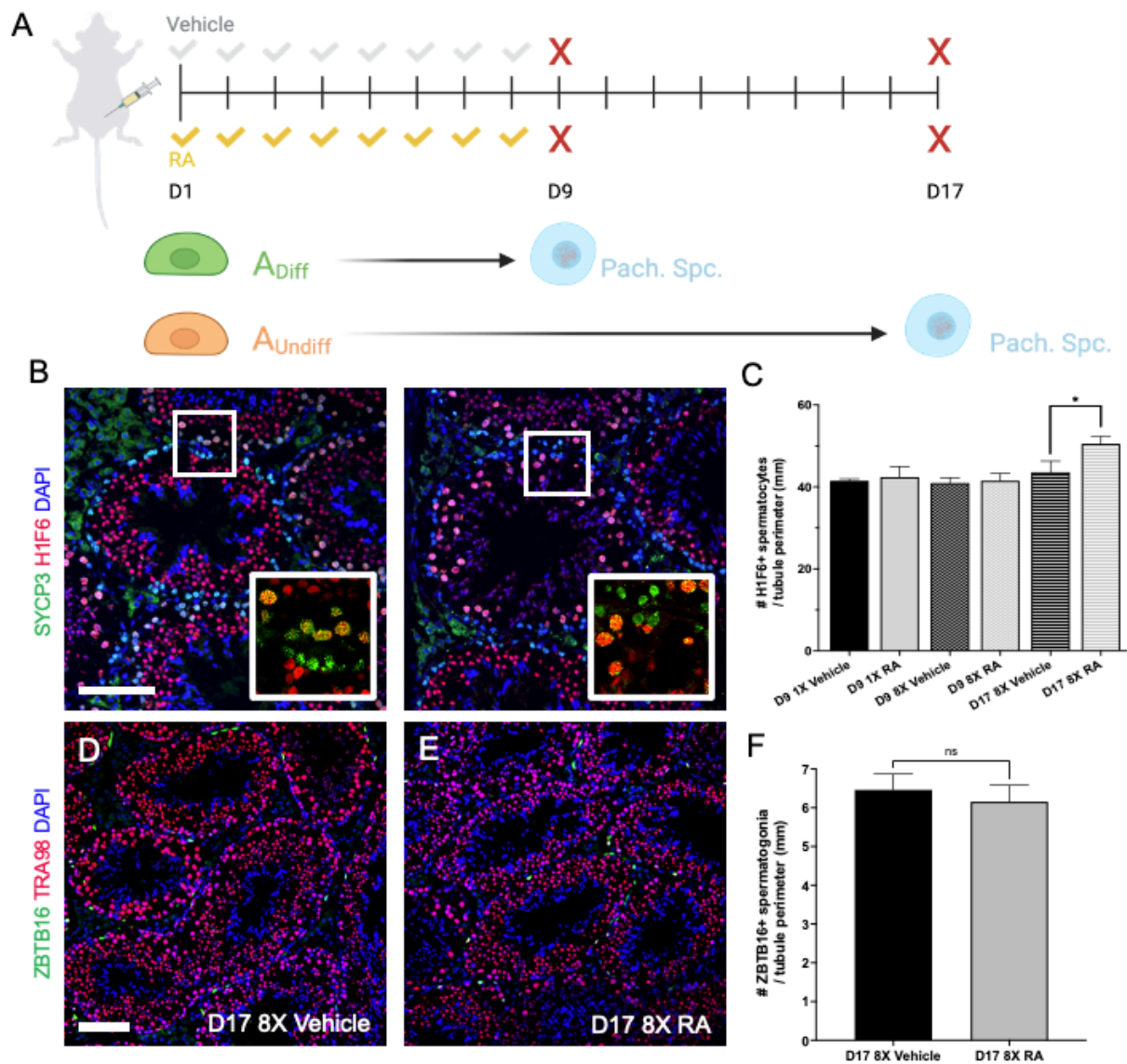


Fig. 3.8 Consistent RA treatment enriches a generation of germ cells proceeding through spermatogenesis.

(A) Mice were treated daily for 8 days with Vehicle or RA (D1-8) and sacrificed on D9 or D17. A_{Undiff} = undifferentiated progenitors, A_{Diff} = differentiated spermatogonia, and Pach. Spc. = pachytene spermatocytes. (B, C) Mid-and late-stage pachytene spermatocytes were quantified by co-immunostaining for SYCP3 (green) and H1F6 (red). DAPI stains all nuclei in blue. (C) Quantitation for H1F6⁺ spermatocytes per tubule perimeter. *P < 0.05 vs vehicle. (D-F) Immunofluorescence to quantify the number of progenitors (ZBTB16⁺ germ cells) on D17 after consistent vehicle (D) or RA (E) treatments. ZBTB16⁺ cells are labelled in green, TRA98⁺ germ cells in red, and all nuclei (DAPI) in blue. (F) Quantitation of the number of ZBTB16⁺ progenitors per tubule perimeter. NS = not-significant (P<0.05). Scale bar for all images = 100μm.

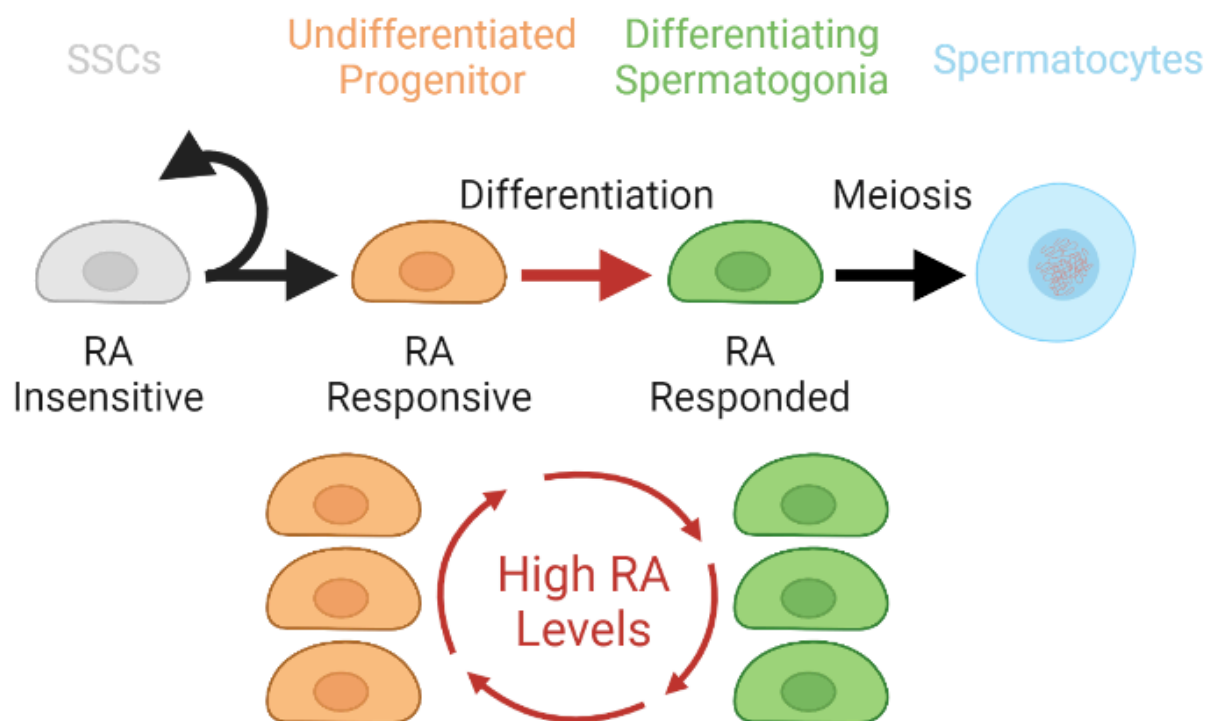


Fig. 3.S0 Abstract synopsis figure.

Image created using BioRender.com

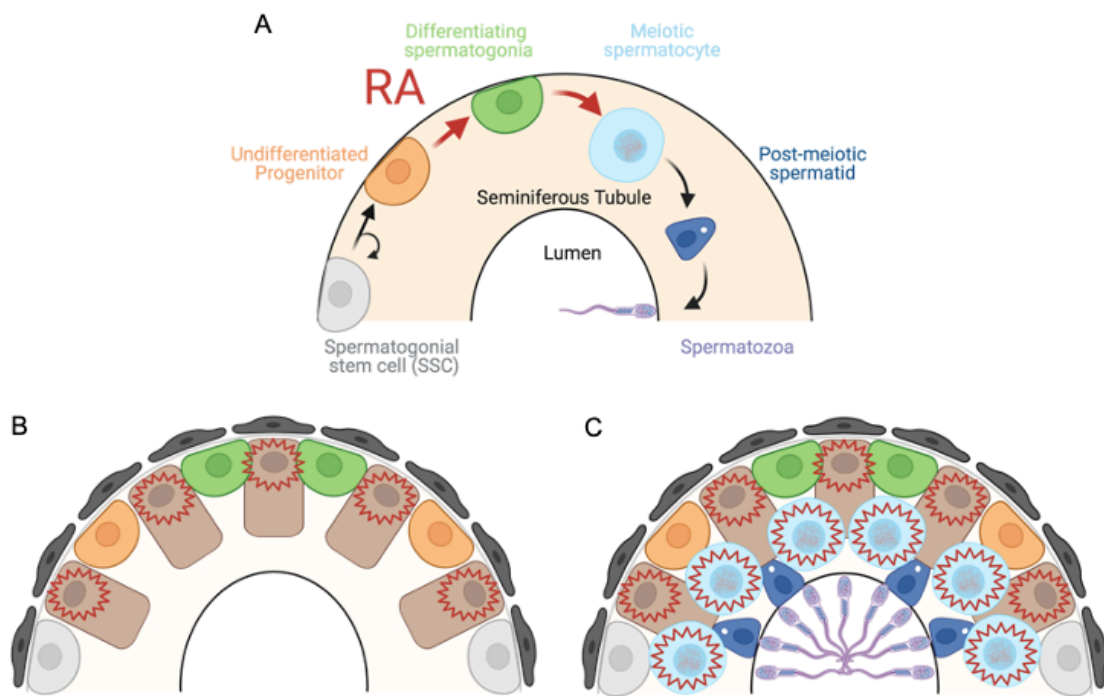


Fig. 3.S1 Germ cell progression and composition of the mouse seminiferous tubule.

(A) Diagram indicating the progression of germ cells from spermatogonial stem cells (SSCs) to spermatozoa. Red arrows indicate transitions traditionally associated with high levels of RA and black arrows indicate transitions not associated with RA. Composition of the neonatal testis at the onset of first-wave spermatogenesis **(B)** and the adult testis during steady-state spermatogenesis **(C)**. Black cells encircling the tubule represent peritubular myoid cells and brown cells represent Sertoli cells. Red “shockwaves” indicate cell types within the tubule known to synthesize RA for use in spermatogenesis. Macrophages (not included in image) are located between peritubular myoid cells and do contain RA-synthesizing enzymes as well²³⁷, however the degree to which their RA contributes to all the transitions during spermatogenesis is unknown. Images created using BioRender.com.

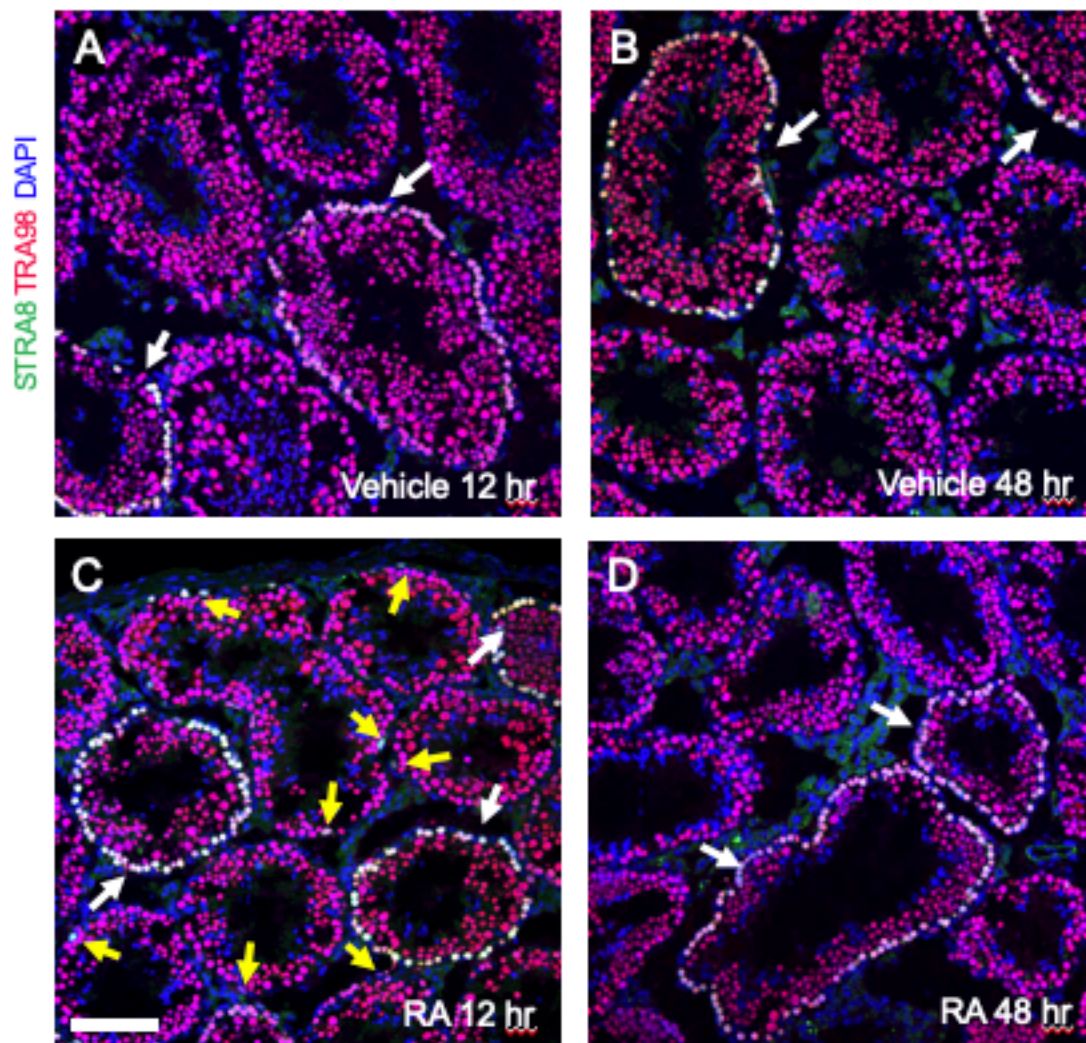


Fig. 3.S2 The transient STRA8 signal is conserved across genotypes.

(A-D) IIF was done to examine responsiveness of germ cells to RA in C57Bl/6N mice. Immunostaining revealing STRA8⁺ (RA responsive) cells in green, TRA98⁺ germ cells in red and nuclei for all cell types in blue (DAPI). Treatment and time-of-sacrifice post-treatment indicated in each image. White arrows indicating tubules where STRA8⁺ spermatogonia normally reside and yellow arrows indicate tubules with spermatogonia responding to exogenous RA. Scale bar = 100µm.

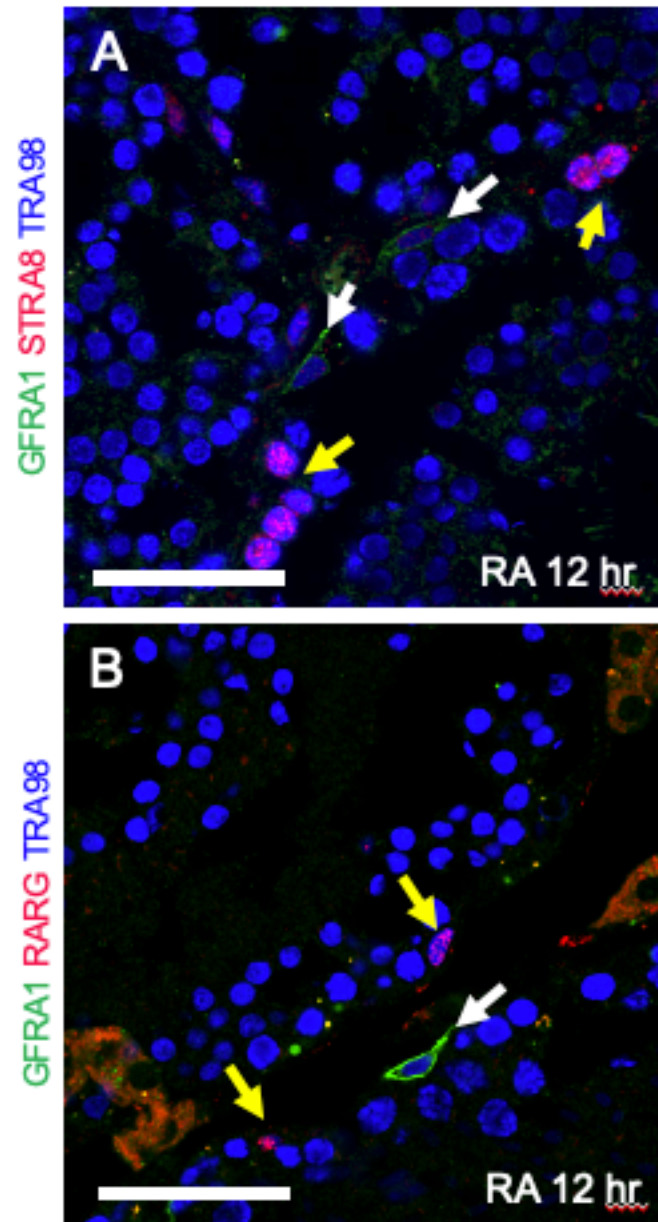


Fig. 3.S3 SSCs are RA non-responsive during steady-state spermatogenesis.

(A) IIF was done to identify RA responsive spermatogonial stem cells (SSCs). STRA8+ (RA responsive) cells are in red, GFRA1+ SSCs are in green and nuclei for all germ cell types (TRA98) are in blue. **(B)** Additional IIF was done to identify RARG+ SSCs 12 hrs post-RA treatment. RARG+ cells are in red, GFRA1+ SSCs are in green and nuclei for all germ cell types (TRA98) are in blue. For both figures, white arrows indicate SSCs, and yellow arrows indicate germ cells responding to RA (STRA8+ or RARG+). Scale bar = 50 μ m.

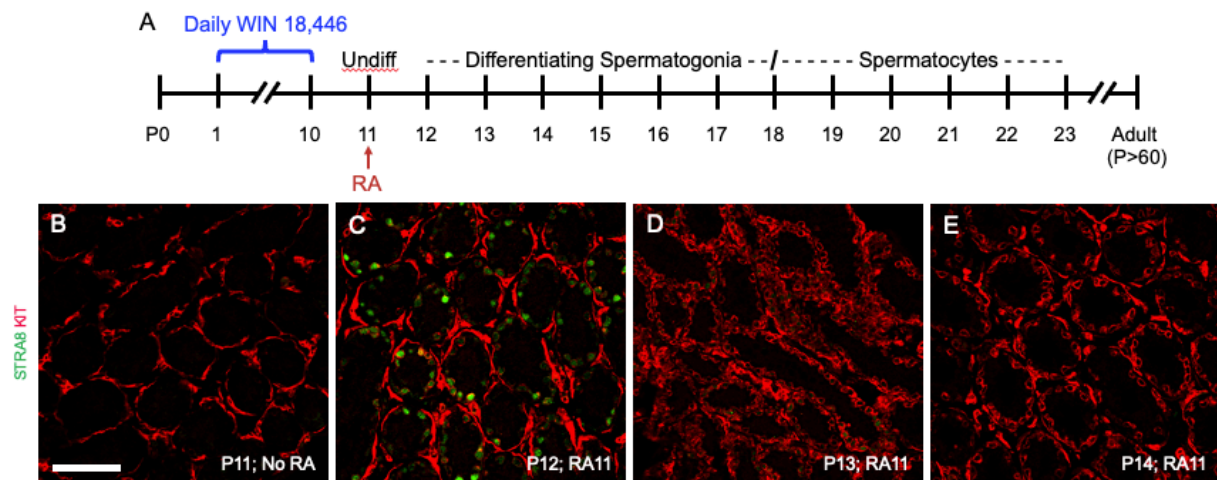


Fig. 3.S4 Modeling steady-state spermatogenesis by synchronizing germ cells using an RA synthesis inhibitor.

(A) Timeline denoting treatment schedule and germ cell stages that predominate at each postnatal age. WIN 18,446 = RA synthesis inhibitor, and Undiff = undifferentiated progenitors. RA was exogenously administered on P11 to initiate differentiation. **(B-E)** IIF was done to observe the transition from undifferentiated to differentiating spermatogonia. STRA8⁺ (RA responding) germ cells are in green and KIT⁺ differentiating spermatogonia are in red. Note that KIT is also expressed by peritubular myoid cells and interstitial cells at all ages. Ages and treatment are denoted in each image. Scale bar = 100 μ m.

A

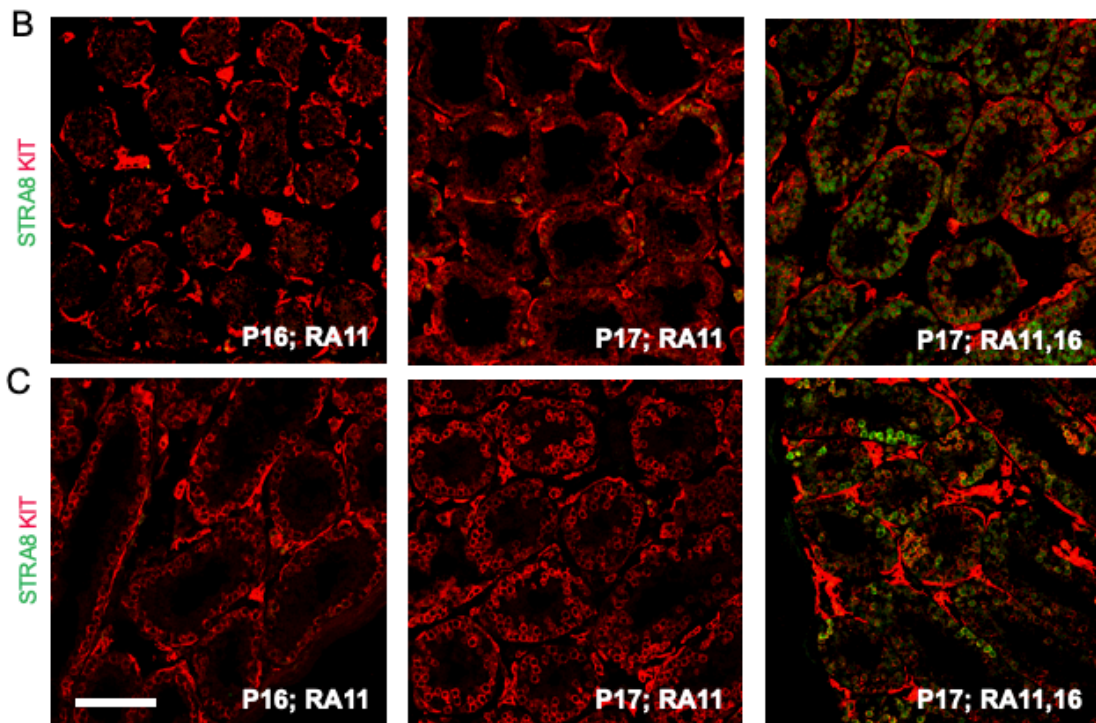
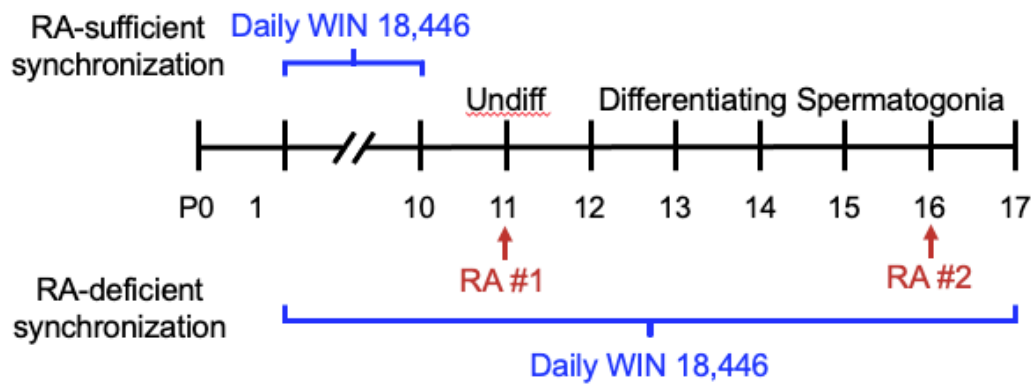
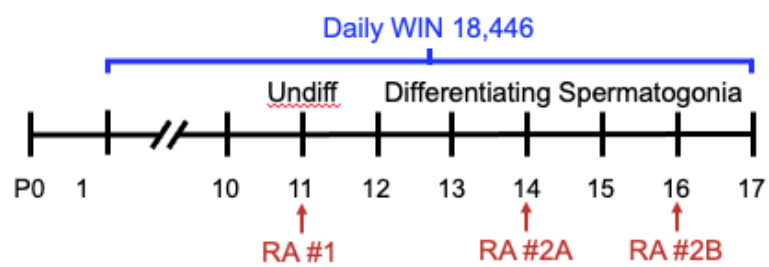


Fig. 3.S5 Exogenous RA elicits STRA8 expression in differentiating spermatogonia in the steady-state synchronized testis.

(A) Timeline denoting treatment schedule and germ cell stages that predominate at each postnatal age. Mice were subjected to either RA-sufficient synchronization (endogenous RA available during differentiation) or RA-deficient synchronization (no RA within the testis) via daily 18,446 treatments. RA was administered on P11 only or P11 and P16. (B, C) Immunostaining to detect RA-responsive differentiating spermatogonia during RA-sufficient (B) and RA-deficient (C) synchronization. STRA8⁺ (RA responsive) germ cells are in green and KIT⁺ differentiating spermatogonia are in red. Note that KIT is also expressed by peritubular myoid cells and interstitial cells at all ages. Ages and treatment are denoted in each image. Scale bar = 100 μ m.

A



B

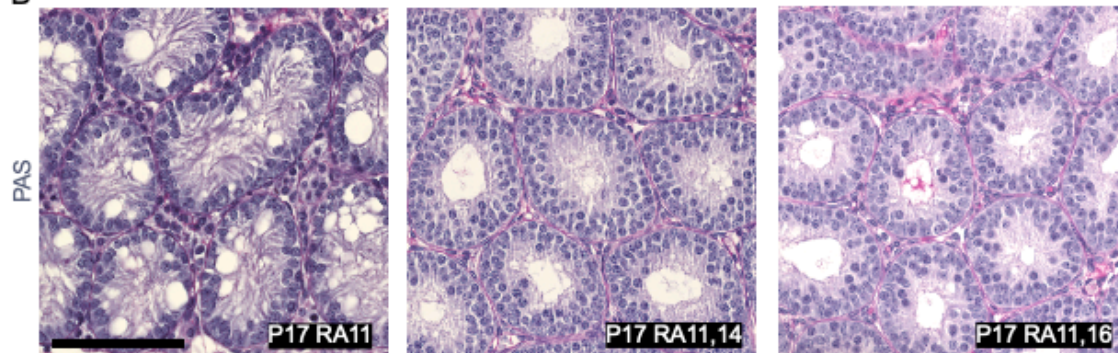


Fig. 3.S6 The differentiation program cannot be shortened in response to RA within an RA-deficient environment.

(A) Timeline denoting treatment schedule and germ cells that predominate at each postnatal age. Mice were subjected to RA-deficient synchronization (no RA within the testis) via daily WIN 18,446 treatments and RA was administered on P11 only, P11 and P14, or P11 and P16. **(B)** Periodic acid Schiff's staining was done to evaluate histology of testes. Age and treatment are depicted in each image. Scale bar = 100 μ m.

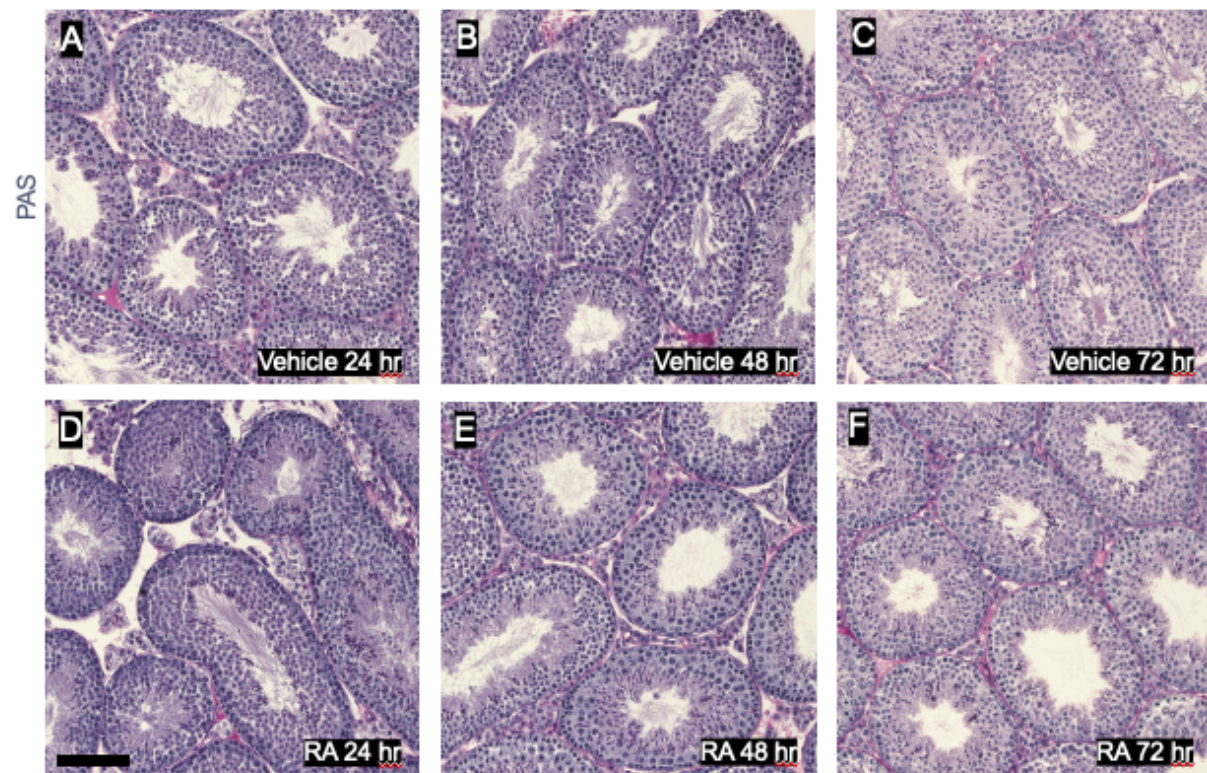


Fig. 3.S7 RA does not enrich the testis with spermatocytes at the onset of meiosis.

Mice were treated once with vehicle or RA and sacrificed 24 (**A, D**) 48 (**B, E**) and 72 (**C, F**) hours later. Periodic acid Schiff's staining was done to evaluate histology of testes. Treatment and time-of-sacrifice post-treatment are indicated in each image. Scale bar = 100 μ m.

UNCOVERING REQUIREMENTS FOR SPERMATOGENIAL PROGRESSION

Retinoic acid (RA) is absolutely required for spermatogonial differentiation and is presumably required for other transitions in spermatogenesis. Findings from chapter three reveal the three different spermatogonial sub-populations – stem cells, undifferentiated progenitors, and differentiated spermatogonia – each respond differently to RA. The different responses ensure germ cells progress through spermatogenesis in a controlled manner and the germline can be replenished safely over time. RA signaling has historically been leveraged as a potential mechanism for impeding spermatogenesis (e.g., WIN 18,446) and only recently have RA analogs been proposed as an option to improve fertility (e.g., Isotretinoin). Due to potential systemic side effects that may arise with manipulating RA signaling, there remains substantial interest in germ cell specific proteins as targets for future treatment options. In collaboration with researchers at the University of Pennsylvania, an RNA binding protein RBM46 was identified and explored as a potential regulator of germ cell progression. This protein was attractive for several reasons – RBM46 is exclusive to the gonad, is expressed specifically in spermatogonia and spermatocytes, and was not studied in mammals. The generation of *Rbm46* knockout and *Rbm46* FLAG-tagged mice not only revealed an essential role of this RNA binding protein in post-transcriptional regulation, but position RBM46 as an exciting target for understanding requirements of spermatogonia progression, which could be used as a foundation for a fertility treatment.

CHAPTER IV: THE GERM CELL-SPECIFIC RNA BINDING PROTEIN RBM46 IS ESSENTIAL FOR SPERMATOGONIAL DIFFERENTIATION IN MICE

*This chapter is modified from a **published** manuscript in PLOS Genetics.*

A. Summary

Male fertility relies upon continuous daily production of millions of fertilization-competent sperm. These sperm are created in the testis during spermatogenesis, the developmental program founded upon spermatogonial stem cells (SSCs). SSCs divide to produce progeny spermatogonia that either remain in the stem cell pool or commit to differentiate and enter meiosis and ultimately form sperm. The balance between stem cell self-renewal and production of gametes is controlled by changes in the expression of a large complement of genes. An emerging concept in control over gene expression is the essential role of proteins that bind to mRNAs and regulate their stability, storage, and/or translation into proteins. Here, we identify such an RNA binding protein – RBM46 – that is only expressed in the male and female germline and required for gamete production and thus fertility in both sexes. In male mice with a specific deletion of *Rbm46*, spermatogenesis is arrested at spermatogonial differentiation. RBM46 binds a specific cohort of mRNAs encoding factors essential for differentiation and meiosis and is thus positioned to play a critical role in post-transcriptional control over gene expression in mammalian spermatogonia.

B. Introduction

The foundation of mammalian spermatogenesis is provided by the regenerative pool of spermatogonial stem cells (SSCs). SSCs are dispersed throughout the normal testis and, upon

division, progeny of SSCs either replenish the SSC pool or proliferate as transit-amplifying undifferentiated progenitor spermatogonia. These progenitor spermatogonia commit to meiosis by differentiating in response to retinoic acid (RA). The essential differentiation program in the mouse lasts 8.6 days, culminating in entry into meiosis as preleptotene spermatocytes. Disruption of spermatogonial fate diminishes male fertility by ultimately impairing sperm production; indeed, a block in differentiation of undifferentiated progenitor spermatogonia (A_{Undiffs}) results in maturation arrest, while overactive differentiation can lead to eventual germline loss. Spermatogonia that commit to the lengthy differentiation program have but two fates – either initiating meiosis as spermatocytes or dying by apoptosis. Indeed, we are unaware of any pharmacologic-treated or mutant or knockout (KO) mouse models with testes containing stable populations of differentiating spermatogonia (A_{Diffs}). Despite the critical nature of the differentiation program, the underlying molecular mechanisms remain largely undefined. One reason for this is the relative paucity of transcriptome-wide changes^{149,219,269–271}. In line with this, recent studies from our lab revealed RA activates the mTORC1 kinase signaling complex, leading to enhanced translation of differentiation-required proteins such as KIT, STRA8, and SOHLH1/2^{67,72,73,272}. Taken together, this reveals a critical reliance upon post-transcriptional control mechanisms for gene regulation during spermatogonial differentiation.

Gene expression can be profoundly controlled at the post-transcriptional level, by regulating pre-mRNA splicing, polyadenylation, mRNA stability, translation, and/or localization^{273–275}. These regulatory events are largely directed by sequence-specific RNA binding proteins (RBPs). RBPs are expressed in many tissues and cell types, but male germ cells express an especially high number of unique RBPs. Exemplary germ cell specific RBPs include MSY2, DAZL, BOLL, NANOS2, NANOS3, PIWIL1, DND1, RBMXL2, and DDX4, all of which play

essential roles during spermatogenesis, as evidenced by mouse KO studies^{167,168,276–280}. These RBPs have specialized functions at distinct steps of spermatogenesis, indicating the critical importance of RBPs in regulating gene expression to ensure maintenance of male fertility.

While performing a functional screen for a collection of cDNAs, we observed mRNAs encoding the predicted RBP ‘RNA-binding motif protein 46’ RBM46 were restricted to testes in mouse and human transcriptome databases²⁸¹. Based on this highly restricted expression pattern, we predicted an essential role for RBM46 in spermatogenesis. To test this hypothesis, we generated Rbm46 KO (*Rbm46*^{-/-}) male mice and discovered loss of RBM46 blocked the completion of spermatogonia differentiation, preventing sperm formation and resulting in infertility. The results presented here position RBM46 as a critical regulator of post-transcriptional gene expression in differentiating mammalian spermatogonia that is essential for completion of spermatogenesis and male fertility.

C. Materials and methods

C.1 Ethics statement

All animal procedures and experiments were approved by the Institutional Animal Care and Use Committees (IACUC) at the University of Pennsylvania (protocol #803164) and East Carolina University (approval A3469-01).

C.2 Mouse strains

Rbm46^{FLAG/FLAG} mice and *Rbm46*^{-/-} mice were generated in the Penn Transgenic and Chimeric Mouse and CRISPR-Cas9 Mouse Targeting Core Facilities (supported by NIH grant P30DK050306). To create *Rbm46*^{FLAG/FLAG} mice, Alt-R CRISPR-Cas9 crRNA (Integrated DNA

Technologies (IDT: Iowa City, IA)) targeting the sequence 5'-ATCAGTGTTTCTTCATTCA-3' (anti-sense) and a rescue donor oligo were created containing two tandem copies of the FLAG Tag in-frame after the ATG start codon with a 5' 91 nt homology arm and 3' 37 nt homology arm. The crRNA and donor oligos were microinjected in fertilized eggs together with an mRNA encoding Cas9 protein.

For *Rbm46*^{-/-} mice, two crRNAs were generated *in vitro* using T7 polymerase to target the following sequences in *Rbm46* exon 2 (5'- ATGAATGAAGAAAACACTGA-3' and 5'- ATAATTGTTAAGAATCCGGA-3' (anti-sense)). The two crRNAs were microinjected together into fertilized eggs along with Cas9 mRNAs. Resulting pups were screened by PCR for heterozygous KI or deletion and founder mice were confirmed by DNA sequencing. Mice were humanely euthanized by CO₂ asphyxiation followed by cervical dislocation. Mice were on a B6SJLF1/J hybrid genetic background (strain #100012, The Jackson Laboratory).

C.3 Tissue collection, fixation, and immunostaining

For cryosections or paraffin embedding, testes were fixed for 4 hrs – overnight in fresh 4% paraformaldehyde or Bouin's solution, respectively, at 4°C and prepared as described previously²⁸². Bouin's-fixed testes were stained with periodic acid Schiff's (PAS) using standard methods. For immunohistochemistry (IHC), immunostaining was performed on Bouin's-fixed sections as described²⁸². Brightfield images were captured on an Axio Observer A1 inverted microscope outfitted with a Zeiss Axiocam 503 color digital camera and Zen software (Carl Zeiss Microscopy, LLC).

For indirect immunofluorescence (IIF), immunostaining was performed on cryosections as described²⁸². Alexa-Fluor conjugated secondary antibodies (Thermo Scientific) raised against the

animal host of the primary antibody were incubated for 1 hr at room temperature at a 1:500 dilution. Coverslips were mounted for IIF with Vectastain containing DAPI (Vector Laboratories). Sections were imaged using a Fluoview FV1000 confocal laser scanning microscope (Olympus America).

C.4 RNA-seq

Testes from P8 mice were flash frozen in liquid nitrogen and ground using a mortar and pestle. Ground tissue was homogenized in TriZol reagent by passing samples through 18- and 26-gauge needles, and RNA was extracted with RNeasy minikit (Qiagen) using manufacturer's instructions. Total RNA was then submitted to Genewiz and Illumina libraries were prepared after rRNA depletion using the Illumina Ribo-Zero kit. Sequencing was performed using Illumina HiSeq for 150 bp paired end sequencing using four replicates each from wild type control and *Rbm46*^{-/-} samples. Adapters were trimmed from RNA-Seq samples using BBDuk, aligned to the mouse GRCm38 genome assembly using STAR v.2.5.1B, and sorted and indexed using samtools v.1.9. For gene expression quantification, salmon v.0.14.0 was used in mapping-based mode with selective alignment on trimmed fastq files using GENCODE vM23 annotation to create the index. Differential gene expression analysis was performed with DESeq2 v.1.22.2. Differential splicing analysis was performed with MAJIQ v.2.1 using GENCODE vM23 reference transcriptome annotation without intron retention quantification. We identified differentially spliced junctions by keeping junctions that had a delta PSI of at least 15 with a probability that the delta PSI is above 15 of at least 95%. Gene ontology analysis was performed with enrichR v.1.0 using a 2018 release of the GO Consortium annotations.

C.5 eCLIP-seq

Testes were harvested from mice and rinsed in phosphate buffered saline (PBS). Testes were detunicated, triturated, dounced in PBS, and tissue material was crosslinked three times at 400 mJ/cm² using a Stratalinker 2400 (Stratagene). Samples were then flash-frozen in liquid nitrogen and stored at -80°C until use. Each replicate was derived from a pair of testes from a single mouse. Samples were lysed, and crosslinked RNP complexes were treated with 5 U/ml RNase I, immunoprecipitated, and used to generate eCLIP libraries and control input libraries as previously described²⁸³. In brief, to extract RBM46-specific interactors, cleared immunoprecipitants were resolved on 4-12% Bis-Tris protein gel and transferred to a nitrocellulose membrane. The RNA: RNP complex was extracted from the nitrocellulose membrane by cutting a region that included the RNA binding protein, RBM46 (size ~62 kDa) and a region of the membrane ~50 kDa above the RBM46 band. The RNA was isolated from the membrane following proteinase K and urea treatments. An Illumina Nova-Seq was used for 50 bp paired end sequencing. Raw data from *Rbm46* eCLIP experiments and input controls were processed using CLIP Tool Kit (CTK)²⁸⁴. Unique tags were identified after stringent mapping to the reference genome (mm10) and collapsing of PCR duplicates. Only read2, which corresponds to the 5' end of CLIP tags, was used for analyses.

C.6 Statistics

Experimental groups were compared using one-way analysis of variance (ANOVA) and Student's T-tests. Differences were considered statistically significant at $P < 0.05$.

D. Results

D.1 RBM46 is specifically expressed in spermatogonia and spermatocytes in mouse testes.

In a search for novel RBPs expressed in the male germline, we identified a putative candidate encoded by the *Rbm46* gene that was testes-specific in transcriptomic datasets²⁸¹. Analysis of single cell RNA-seq data¹⁴⁹ revealed *Rbm46* mRNAs were detectable in adult testes in undifferentiated and differentiating premeiotic spermatogonia, increased in preleptotene, leptotene/zygotene, and pachytene meiotic spermatocytes as well as secondary spermatocytes, declined in early postmeiotic round spermatids, and were undetectable in mid- and late round spermatids, as well as somatic cells of the testis (Fig. 4.1A). We next sought to define the expression pattern of RBM46 protein in mouse testes. Since none of the commercially available antibodies yielded consistent results in immunostaining, CRISPR/Cas9 technology was used to generate mice with tandem copies of the FLAG epitope tag inserted at the N-terminus of RBM46 (Fig. 4.1B). Male mice with homozygous insertion of sequences encoding the FLAG tag (*Rbm46*^{FLAG/FLAG}) appeared normal and were fertile; their histologically normal testes (Fig. 4.S1) suggested the FLAG tag did not adversely affect RBM46 function. Immunostaining these adult testes using anti-FLAG antibodies revealed RBM46 protein was specifically expressed in cytoplasm of A_{Undiffs} and A_{Diffs} as well as spermatocytes, but not in spermatids, sperm, or somatic cells (Fig. 4.1C-G).

D.2 RBM46 is essential for fertility in both sexes.

To define the requirement for the RNA binding protein RBM46 in spermatogenesis, CRISPR/Cas9 technology was used to generate *Rbm46*^{-/-} mice. A founder male was identified with a frameshifting deletion between exons 2-3 (Fig. 4.2A). This frameshift in the region encoding the

first RNA Recognition Motif (RRM) led to a premature termination codon that disrupted all three consensus RRM, giving high confidence for a functional null allele (Fig. 4.S2A-C). *Rbm46*^{-/-} mice were viable, healthy, and displayed no overt defects (not shown). However, neither *Rbm46*^{-/-} male nor female mice were able to produce pups when mated with WT counterparts, revealing a requirement for RBM46 in fertility. Compared to WT littermates, adult *Rbm46*^{-/-} ovaries lacked oocytes, revealing complete loss of the germline (Fig. 4.S3A-B).

In this study, we focused on the male infertility phenotype. Paired testis weights of *Rbm46*^{-/-} mice were considerably lower (42.5 ± 13.0 mg) than those from *Rbm46*^{+/+} (255.5 ± 67.9 mg) and *Rbm46*^{+/-} (227 ± 55.9 mg) littermate controls (Fig. 4.2B). This dramatic decrease in *Rbm46*^{-/-} testis size (Fig. 4.2C) suggested impaired spermatogenesis. Indeed, histological analysis confirmed that, as compared to *Rbm46*^{+/+} and *Rbm46*^{+/-} testes (which appeared normal, e.g., Fig. 4.2C-D), *Rbm46*^{-/-} testes had severe defects in spermatogenesis, with seminiferous tubules containing Sertoli cells and only a few apparent spermatogonia, but lacking spermatocytes, spermatids, or testicular sperm (Fig. 4.2E). This result was confirmed by staining adult testes for the pan germ cell marker TRA98 (also termed GCNA^{278,285,286}) along with the somatic Sertoli cell marker GATA binding protein 4 (GATA4) (Fig. 4.S4A-B). In comparison to normal-appearing *Rbm46*^{+/-} testes (Fig. 4.2F), most tubules in *Rbm46*^{-/-} adult mice lacked germ cells, although there were isolated populations of ZBTB16⁺/TRA98⁺ A_{Undiffs} (Fig. 4.2G). To confirm the absence of more advanced germ cells in *Rbm46*^{-/-} testes, co-immunostaining was done to detect differentiating spermatogonia markers KIT and STRA8, the latter of which is also highly expressed in preleptotene spermatocytes. As expected, tubule cross sections in control testes contained numerous KIT⁺ A_{Diffs} and STRA8⁺ preleptotene spermatocytes (Fig. 4.2H). In *Rbm46*^{-/-} testes, some TRA98⁺ spermatogonia were also STRA8⁺, revealing the capacity to respond to RA; however, none were KIT⁺ (Fig. 4.2I, Fig

S4F), suggesting an inability to undergo bona fide RA-induced differentiation. In agreement with this, there were no SYCP3+ meiotic spermatocytes in *Rbm46*^{-/-} testes, in contrast to controls (Fig. 4.S4C-F). Taken together, these findings suggest impaired spermatogonial differentiation and reveal an absence of meiotic spermatocytes in *Rbm46*^{-/-} testes.

Although *Rbm46* mRNA and protein were detectable primarily in germ cells, we tested the cell-autonomous requirement by generating germ cell-specific conditional KO mice. These mice were created by crossing *Rbm46*^{fl/fl} and *Stra8-iCre*, the latter of which is expressed beginning in A_{Undiffs}²⁸⁷. The testis phenotype of adult *Rbm46*^{fl/-};*Stra8-Cre* mice (Fig. 4.S5) was indistinguishable from those with conventional whole-body deletion (Fig. 4.2), confirming an essential cell autonomous role for RBM46 during male germ cell development.

D.3 Spermatogonial differentiation is impaired in developing *Rbm46*^{-/-} testes.

We next sought to precisely define the onset of the spermatogenic defect in *Rbm46*^{-/-} testes. To accomplish this, we examined *Rbm46*^{-/-} testes during the well-characterized first wave of spermatogenesis, when populations of progressively advanced germ cells predictably appear on successive days⁵⁷. In control testes, at P6, 8, 10, 15, and 21 the most advanced germ cell types were A_{Diffs}, preleptotene spermatocytes, leptotene spermatocytes, pachytene spermatocytes, and round spermatids, as expected²⁸⁸ (Fig. 4.3A-E). In stark contrast, *Rbm46*^{-/-} testes only contained apparent spermatogonia on each of these days (Fig. 4.3F-J, K-L), and there was no difference in numbers of spermatogonia as early as P6 (Fig. 4.S6).

To confirm the identity of the resident germ cells in developing *Rbm46*^{-/-} testes, we performed immunostaining for the bona fide spermatogonia differentiation protein marker KIT, which also is expressed in somatic cells in the interstitial compartment^{255,256,289–291}. At P8, 10, 15,

and 21 KIT was readily detectable in the membrane of A_{Diff}s, as expected (Fig. 4.4A-D). In *Rbm46*^{-/-} testes, significantly fewer KIT⁺ spermatogonia were present at each of these ages, with numbers remaining stagnant as the mice age (Fig. 4.4E-I). Thus, we conclude that although spermatogonia initiated the program of differentiation, it was not sustained, leading to stalled germ cell development and an absence of meiotic cells.

D.4 Rbm46 is required for activation of differentiation- and meiosis-associated gene expression in spermatogonia.

To begin to define underlying molecular defects in *Rbm46*^{-/-} spermatogonia, we performed bulk RNA-Seq on WT and *Rbm46*^{-/-} testes from P8 mice. This age was selected for analysis as it represented a time that, although there was some germ cell degeneration in *Rbm46*^{-/-} testes (Fig. 4.3G), WT and *Rbm46*^{-/-} testes had similar apparent numbers of germ cells (Fig. 4.3K-L). Quantitation revealed a ~21% decrease in numbers of TRA98⁺ germ cells in *Rbm46*^{-/-} testes. We reasoned differences in gene expression at the mRNA level would reveal key dysregulated genes due to either direct regulation by RBM46 on mRNA stability or indirect downstream consequences of *Rbm46* deletion. We used DESeq2 to identify differences in mRNA levels between WT and *Rbm46*^{-/-} testes. For protein coding genes (using a cutoff adjusted p-value <0.05), we identified 561 upregulated and 1,218 downregulated transcripts (Table 4.S1). Changes in mRNA abundance were modest, with only 167 downregulated genes and 33 upregulated genes showing >2-fold changes (Fig. 4.5A). Gene ontology (GO) analysis of downregulated genes identified numerous terms relevant to spermatogenesis, including several related to meiosis: ‘*spermatogenesis*’, ‘*synapsis*’, ‘*male gamete generation*’, and ‘*synaptonemal complex assembly*’ (Fig. 4.5B). Examples of meiotic genes with reduced mRNA levels in *Rbm46*^{-/-} testes included *Dmc1*, *H2afx*,

Meiob, *Spo11*, *Mov10l*, *Hormad1*, *Sycp2*, and *Sycp3*. We also identified reduced levels of several mRNAs encoding proteins involved with (e.g., *Stra8*) or required for (e.g., *Kit*, *Sohlh1*; Fig. 4.5C) spermatogonia differentiation. There were no significant changes in mRNA levels of most markers of undifferentiated spermatogonia (e.g., *Gfra1*, *Id4*, *Nanos2/3*, *Cdh1*, *Ret*, *Itga6*, *Itgb1*, and *Sall4*). GO analysis of upregulated genes did not identify terms with apparent relevance to spermatogenesis (Fig. 4.5B). We did, however, note increased levels of somatic cell markers (e.g., Sertoli cell mRNAs *Sox9* and *Clu* and Leydig cell markers *Cyp17a1*, *Hmgcs2*, and *Prlr*^{149,271,292}). Using the Majiq computational pipeline²⁹³, we only found few changes in alternative splicing (see Table 4.S2) and, although the splicing differences were important (Fig. 4.S7A) and mostly involved alternative first or last exon events (Fig. 4.S7A), all but eight genes (*Lrfl1*, *Apobec3*, *Zfp429*, *Chd1l*, *Prickle2*, *Selenbp2*, *Zfp697*, *Ndufs1*) affected at the splicing level were unaffected at the level of mRNA abundance. Genes with differential splicing were not enriched for any specific GO term. In summary, there was an apparent decrease in the mRNA abundance of genes encoding proteins required for spermatogonial differentiation and meiosis, which is likely due to indirect action of RBM46, in that A_{Diff}s and preleptotene spermatocytes were absent in P8 *Rbm46*^{-/-} testes.

D.5 RBM46-bound mRNAs are enriched for functions in RNA processing, meiosis, and translation regulation.

To identify mRNAs directly bound by RBM46 in the male germline, we used enhanced crosslinking coupled with immunoprecipitation and RNA-seq (eCLIP-Seq). This method provides unbiased genome-wide coverage from small amounts of cellular input, enabling identification of RBP binding sites at single nucleotide resolution²⁸³. We used testes from *Rbm46*^{FLAG/FLAG} mice

(Fig. 4.1B), as the FLAG-tagged RBM46 protein can be efficiently and specifically immunoprecipitated using FLAG antibodies. Because RBM46 is expressed in both spermatogonia and spermatocytes (Fig. 4.1D-F), we used eCLIP in testes from *RBM46^{FLAG/FLAG}* mice at P21, an age when they contain spermatogonia, spermatocytes, and the very first emergent round spermatids²⁸⁸. Immunoprecipitated material was separated by electrophoresis, transferred to a nitrocellulose membrane, and the region containing crosslinked RNAs excised and released from the membrane (Fig. 4.6A, Fig. 4.S8). eCLIP libraries were prepared and five replicate eCLIP samples were sequenced with corresponding inputs, processed, and mapped at $\sim 8 \times 10^6$ non-redundant reads to the genome (mm10)²⁸⁴. We anticipated enrichment of binding sites in mRNA 3' untranslated regions (3' UTRs), like reports of other cytoplasmic RBPs in male germ cells²⁹⁴⁻²⁹⁷. To our surprise, nearly equal percentages of CLIP tags were present in the 3' UTR and protein coding sequences (CDS), though when corrected for the percentage of these regions in the transcriptome there was a modest enrichment of binding sites in the 3' UTR over the CDS (Fig. 4.6B). CLIP tags also showed a relatively uniform distribution across mRNAs (Fig. 4.6C).

To determine the binding specificity of RBM46, we extracted sequences around CITS and performed *de novo* motif discovery using mCross, an algorithm developed to simultaneously model RBP binding specificity and the crosslink position in the binding motif²⁹⁸. After pooling all replicates, mCross was used to extract sequences around crosslink-induced truncation sites for *de novo* motif discovery, which identified 90,243 crosslink-induced truncation sites (CITS, $P < 0.001$)^{284,299}. This analysis revealed a U-rich motif with a UGAU core and predominant crosslinking at the U1 position of the core (Fig. 4.6D). The UGAU motif is highly enriched at the crosslink sites (with a 25-fold enrichment for crosslinking at U1 of the UGAU motif), while a moderate enrichment was observed in regions around CLIP tag peaks. Given the high signal-to-

noise ratio of CITS, we identified a stringent subset of RBM46 target transcripts based on the presence of CITS satisfying two criteria: 1) presence of the UGAU motif with crosslinking at the U1 position; and 2) ≥ 50 putative truncated tags at the crosslink sites. This allowed us to identify 1,349 CITS associated with 873 unique genes. Gene Ontology (GO) analysis of these genes was performed using the Database for Annotation, Visualization and Integrated Discovery (DAVID)³⁰⁰. RBM46 target transcripts were enriched for terms relevant to spermatogenesis using GO analysis (Fig. 4.6E). Of note, there was significant enrichment of genes involved in RNA processing that included several RBPs with functions in spermatogenesis (e.g., DAZL, BOLL, PABPC1, CELF1, CEBP1, PTBP2, and RBM46 itself) and translation initiation factors (e.g., EIF1A, EIF2S1, EIF4G1, and EIF4G2). RBM46 also showed enriched binding to mRNAs encoding essential meiosis proteins (e.g., SYCP1, SYCP2, SYCP3, MEIOC, SPO11, TEX15, HORMAD1, HSPA2, and BRCA2). A list of mRNAs that were bound by RBM46 at P21 and exhibited differential abundance in P8 *Rbm46*^{-/-} testes are presented in Table 4.S3.

E. Discussion

Here, we localized the germ cell specific RBP RBM46 to the cytoplasm of spermatogonia and spermatocytes, but not in other testes germ cell types nor in somatic cells. We generated KO mice and discovered a germ cell autonomous requirement for RBM46 in spermatogenesis and male fertility. Specifically, RBM46 was essential, in spermatogonia, to complete differentiation in both developing and adult testes. *Rbm46* KO testes had altered transcriptomes, with downregulation of transcripts encoding differentiation- and meiosis-associated genes. Using enhanced crosslinking immunoprecipitation²⁸³ followed by binding analysis with the CLIP Tool Kit²⁸⁴, we determined RBM46 directly bound, at a U-rich consensus sequence, to mRNAs

encoding proteins involved in spermatogenesis as well as in general translation regulation. In summary, RBP46 is required for spermatogonial differentiation and male fertility, and directly binds to mRNAs encoding genes essential for differentiation and meiosis in the male germline.

All stages of spermatogenesis, from survival of prospermatogonia to the maintenance of SSCs to meiosis and spermiogenesis, require post-transcriptional regulation by RBPs. Indeed, several essential RBPs have been identified that repress or activate the translation of select mRNAs, including NANOS2, NANOS3, DAZL, TIAR/TIAL1, PIWIL2/MILI, PIWIL4/MIWI2, DDX4/VASA, MSY2, and LIN28A^{163–167,294,295,301–303}. The functions of essential RBPs include regulation of mRNA splicing, polyadenylation, localization, stability/degradation, and translation^{304–306}. While the mechanistic functions and global regulatory targets have been identified for multiple essential RBPs, many RBPs necessary for spermatogenesis remain to be defined and characterized. Therefore, identification of a novel RBP and its genome-wide regulatory targets provides new insights into the molecular pathways that control germ cell gene expression during maintenance and differentiation.

Once in the cytoplasm, mRNAs face three possible fates: translation, storage, or degradation. Transit between these fates is well-known to regulate key transitions during male germ cell development^{307,308}. Our RNA-Seq findings offer further support for a spermatogonial differentiation block in *Rbm46*^{-/-} testes. However, the changes in mRNA levels were rather modest, including markers of A_{Diff}s and meiotic genes. The RNA-seq experiment was performed using testes at P8, a time when there was a ~21% decrease in germ cells, notably A_{Diff}s and the first emergent preleptotene spermatocytes entering meiosis. The loss of these cells, and their transcriptomes suggest many, if not most changes in transcript levels in *Rbm46*^{-/-} testes were

indirect, due to the differentiation impairment and not due to changes in RNA posttranscriptional control.

RBM46 was recently discovered to be part of a complex containing several essential proteins. These include the disordered protein ‘meiosis specific with coiled-coil domain (MEIOC, required for mouse meiosis^{309,310}), the exoribonuclease XRN1 (*Drosophila pacman*, required for spermatogenesis and male fertility³¹¹), and the RNA helicase ‘YTH domain containing 2’ (YTHDC2, *Drosophila bgcn*³¹², required for progression through meiosis in the male mouse germline^{313–317}). This YTHDC2-containing complex bound in testes containing both spermatogonia and spermatocytes to a canonical U-rich binding motif^{318,319}. This sequence closely resembles the one identified here, in P21 testes, which contain spermatogonia, spermatocytes, and the first emergent spermatids²⁸⁸. YTHDC2’s function in gametogenesis was recently shown to be independent of its N6-methyladenosine (m6A)-modified RNA binding^{318,319}; therefore, it is possible that RBM46, as a resident in this RNA management complex, provides additional RNA binding function (allosterically or directly) through the U-rich binding sequence we identified. In *Drosophila*, *Bgcn* is required for translation control and expressed in a reciprocal pattern to the Nanos proteins³²⁰. Based on published reports and the present data, this arrangement appears to be conserved in mice – we discovered a requirement for RBM46 in spermatogonial differentiation, whereas others have shown NANOS2 and NANOS3 are required for SSC maintenance^{321–323}. Therefore, RBM46 may aid in target recognition for YTHDC2 functions in translational regulation.

The uniform binding observed across both coding sequences and UTRs of mRNA transcripts is somewhat uncommon among RBPs but resembles the diffuse mRNA binding pattern shown by CLIP of ‘Fragile X mental retardation protein’ (FMRP) and ‘lin-28 homolog A’ (LIN28A)^{302,324}.

FMRP is present in actively translating polysomes and regulates translation^{325–327}. Similarly, binding of LIN28A across the CDS and UTRs positively regulates translation of mRNAs, including meiotic transcripts in mouse testes^{328–330}. Thus, the atypical binding pattern of RBM46 is consistent with or permissive for a role in translation regulation.

RBM46 is a highly conserved RBP whose function has been examined in flies, fish, and now mice. In *Drosophila*, the mouse ortholog of RBM46 is encoded by the RBP ‘tumorous testis’ (Tut), which is required for spermatogenesis and male fertility³³¹. Interestingly, the phenotype of Tut mutant flies is like that reported here – germ cell development is blocked at differentiation, and thus contain only undifferentiated spermatogonia. In addition, in zebrafish (*Danio rerio*), *rbm46* is expressed in male germ cells and is required for spermatogonia to enter or progress through meiosis³³². Indeed, male *rbm46* mutants were sterile, with testes containing only spermatogonia that proliferated into >16 interconnected 3C-4C germ cells, suggesting incorrect meiotic entry. *Rbm46*-depleted gonads were sex-reversed to testes, and transcriptome analyses revealed many more changes in mRNA abundance (4,436 up and 3,571 down) than we observed here, including reduced levels of many meiotic mRNAs (e.g., *spo11*, *dmcl1*, *rad51*, *msh4*, *mlh1*, *rec8*, *smc1b*, *syce1-3*). These findings support a major role in directing meiotic gene expression. Here, we identified numerous mRNAs encoding essential meiosis proteins among the top RBM46 CLIP targets in P21 testes containing a mixture of spermatogonia and spermatocytes (e.g., SYCP1, SYCP2, SYCP3, MEIOC, SPO11, TEX15, HORMAD1, HSPA2, and BRCA2). These findings provide further support for RBM46 functions in meiosis while also suggesting that mRNAs highly bound by RBM46 support translation, or at least do not inhibit it.

In two previous studies from the same research group, a critical role for RBM46 was reported in embryonic stem cell (ESC) and trophectoderm differentiation^{333,334}. These studies found

RBM46 promoted *Cdx2* mRNA stability and degradation of beta-catenin (*Ctnnb1*) mRNAs in ESCs. However, the second manuscript was recently retracted by the authors³³⁵. It is notable that *Rbm46* expression is rather low in ESCs in available datasets, suggesting the primary roles of RBM46 are in male germ cell development and function. Furthermore, the fact that male *Rbm46*^{-/-} mice were otherwise normal, without any phenotypes other than infertility, is not compatible with an essential role of RBM46 outside of the germline.

Table 4.1 Antibodies.

<i>Antigen</i>	<i>Host</i>	<i>Source</i>	<i>Dilution</i>	<i>Catalog #</i>
TRA98	rat	Abcam	1:1000	Ab82527
ZBTB16/PLZF	Goat	R & D Systems	1:1000	AF2944
KIT	Goat	R & D Systems	1:1000 (IIF); 1:500 (IHC)	AF1356
SYCP3-488	Mouse	Abcam	1:200	Ab205846
FLAG	Rat	Novus	1:250	NBP1-06712SS
STRA8	Rabbit	Abcam	1:3000	Ab49602
GATA4	Rabbit	Cell Signaling Technology	1:400	36966S
Lectin-488	Peanut	Thermo Fisher Scientific	1:500	L21409

Table 4.S1 List of differentially expressed genes in *Rbm46*^{-/-} testes at P8.

Screenshot displayed below.

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S
1	Gene Name	baseMean	log2FoldChange	lfcSE	stat	pvalue	padj												
2	Synb	321.6533791	-1.406099386	0.154353712	-9.109592286	8.27E-20	1.36E-15												
3	Sec24a	1337.232208	0.606523854	0.068383476	8.869450535	7.35E-19	6.03E-15												
4	Ppp2r2c	476.5813593	-1.25218459	0.144309792	-8.677059049	4.06E-18	2.22E-14												
5	Gm4969	413.0684827	-2.527783598	0.304651391	-8.29729872	1.07E-16	4.37E-13												
6	Lhx8	75.65472729	-2.481015319	0.303410162	-8.177100269	2.91E-16	9.53E-13												
7	Lcorl	2651.578728	-0.920101257	0.114273251	-8.051764087	8.16E-16	2.23E-12												
8	Fgfr1op	2418.504754	-0.551710499	0.073732555	-7.482590288	7.29E-14	1.71E-10												
9	Dmc1	795.8408566	-1.312608536	0.176488835	-7.437346009	1.03E-13	2.11E-10												
10	Mpped1	82.94786566	-1.755124651	0.240576571	-7.295492848	2.98E-13	5.42E-10												
11	Rnf227	1025.912427	-0.989403897	0.137778246	-7.181132934	6.91E-13	1.13E-09												
12	Plekha4	577.4428262	-1.258050053	0.179696825	-7.000958717	2.54E-12	3.47E-09												
13	Rbm46	1232.254461	-1.258870195	0.179619386	-7.008543008	2.41E-12	3.47E-09												
14	Tacc3	3141.995629	-0.469497152	0.067201421	-6.98641702	2.82E-12	3.56E-09												
15	Fen1	2822.227265	-0.560902384	0.081074524	-6.918355551	4.57E-12	5.35E-09												
16	Nr6a1	1478.458347	-0.797091821	0.117285219	-6.796183107	1.07E-11	1.17E-08												
17	Dsn1	1596.445988	-0.581675607	0.086096105	-6.756119852	1.42E-11	1.45E-08												
18	Kntc1	2907.760037	-0.369592151	0.055190286	-6.696688449	2.13E-11	1.84E-08												
19	Plk5	94.98901041	-2.151332086	0.321054479	-6.700831866	2.07E-11	1.84E-08												
20	Synpo2l	94.1524931	-1.879591811	0.2806338	-6.697667247	2.12E-11	1.84E-08												
21	Crabp1	1430.931193	-1.376104376	0.20614624	-6.675379461	2.47E-11	2.02E-08												
22	Zfp697	400.1105075	1.040347787	0.156209592	6.659948176	2.74E-11	2.14E-08												
23	Ccnb3	391.2905003	-1.520265565	0.23247202	-6.539563615	6.17E-11	4.60E-08												
24	C1qtnf6	816.4783697	0.700188529	0.107901609	6.489138906	8.63E-11	6.00E-08												
25	Wdhd1	4634.379469	-0.727124836	0.112295345	-6.475111132	9.47E-11	6.00E-08												
26	Wdr90	1396.229934	-0.589631391	0.091070147	-6.47447503	9.51E-11	6.00E-08												
27	ENSMUSG00000100967	1525.144105	-0.808324393	0.124621732	-6.486223406	8.80E-11	6.00E-08												
28	Ccdc36	610.3331523	-1.315838251	0.203884497	-6.453841619	1.09E-10	6.62E-08												
29	Dlx1	125.2360224	-2.179262785	0.339446758	-6.420042998	1.36E-10	7.98E-08												
30	Prdm9	504.3725646	-1.403756224	0.219878521	-6.38423533	1.72E-10	9.74E-08												
31	Nlrc3	971.4527983	-0.843462691	0.132664487	-6.357863447	2.05E-10	1.12E-07												
32	Zfp541	194.5330125	-1.802966501	0.284116691	-6.345866179	2.21E-10	1.17E-07												
33	Kif7	1415.165502	-0.498981576	0.078917808	-6.322800783	2.57E-10	1.32E-07												
34	Kif4	2590.180293	-0.437554546	0.070192144	-6.233668318	4.56E-10	2.26E-07												
35	Mgat5b	56.74886797	-1.908025437	0.306598641	-6.223202518	4.87E-10	2.35E-07												
36	Ctip2	322.5563744	-0.897439795	0.144661101	-6.203739576	5.51E-10	2.55E-07												
37	Cdca7	3477.020715	-0.545188488	0.08791273	-6.201473744	5.59E-10	2.55E-07												
38	Mcm2	6612.454675	-0.585751654	0.095082516	-6.160456002	7.25E-10	3.13E-07												
	Table S1 DEGs in P8 KO Testes																		

Table 4.S2 List of differential splicing events in *Rbm46*^{-/-} testes at P8.

Screenshot displayed below.

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O
	Module ID	Gene ID	Gene Name	Chr	Strand	LSV ID(s)	Event ID	Complex	Collapsed Event Name	De Novo	Junction Name	Junction Coordinate	rbm46_wt_E(PSI)	rbm46_ko_E(PSI)	rbm46_wt-rbm46_ko_E(ΔPSI)
1															
2	ENSMUSG00000028089.5	1	ENSMUSG00000028089.5	3	-	ENSMUSG00000028089.5:97580979-97581177	ENSMUSG00000028089.5_1_AS_1	FALSE	Alt 5x1	FALSE	E1_E2_Proximal	97572709-97580979	0.781	0.001	-0.781
3	ENSMUSG00000050064.14	1	ENSMUSG00000050064.14	3	+	ENSMUSG00000050064.14:98382461-98382578	ENSMUSG00000050064.14_1_ALE_1	TRUE	Alt 3x1_ALEx1	FALSE	C_A_Proximal	98382578-98410491	0.717	0.018	-0.709
4	ENSMUSG00000041536.13	1	ENSMUSG00000041536.13	12	+	ENSMUSG00000041536.13:104118421-104118699	ENSMUSG00000041536.13_1_CE_1	TRUE	Cassette1_ALEx1_Multi Exon Splicingx1	FALSE	C2_A	104116612-104118421	0.834	0.285	-0.564
5	ENSMUSG00000021466.12	1	ENSMUSG00000021466.12	13	-	ENSMUSG00000021466.12:63563029-na	ENSMUSG00000021466.12_1_OR_1	TRUE	P_AFE1_Orphan Junctionx1	TRUE	Orphan	63560917-63563029	0.456	0.031	-0.42
6	ENSMUSG00000035125.10	1	ENSMUSG00000035125.10	6	+	ENSMUSG00000035125.10:81935906-81936021	ENSMUSG00000035125.10_1_pAFE_1	TRUE	ALEx1_P_AFE1	TRUE	C_A_Proximal	81935762-81935906	0.571	0.168	-0.404
7	ENSMUSG00000056260.15	1	ENSMUSG00000056260.15	3	+	ENSMUSG00000056260.15:106732914-106733144	ENSMUSG00000056260.15_1_CE_1	FALSE	Cassette1	FALSE	C1_A	106733142-106733337	0.702	0.32	-0.372
8	ENSMUSG00000030020.13	1	ENSMUSG00000030020.13	6	-	ENSMUSG00000030020.13:92705346-92705431	ENSMUSG00000030020.13_1_pAFE_1	FALSE	P_AFE1	TRUE	C_A_Proximal	92705431-92705996	0.552	0.169	-0.366
9	ENSMUSG00000036598.4	1	ENSMUSG00000036598.4	8	+	ENSMUSG00000036598.4:95538092-95538418	ENSMUSG00000036598.4_1_AS_1	FALSE	Alt 5x1	TRUE	E1_E2_Proximal	95538418-95540799	0.693	0.334	-0.35
10	ENSMUSG00000037270.18	1	ENSMUSG00000037270.18	3	+	ENSMUSG00000037270.18:36863104-36863348	ENSMUSG00000037270.18_1_A5_1	FALSE	Alt 5x1	FALSE	E1_E2_Proximal	36863348-36864387	0.529	0.2	-0.341
11	ENSMUSG00000078994.9	1	ENSMUSG00000078994.9	13	-	ENSMUSG00000078994.9:67396630-67396756	ENSMUSG00000078994.9_1_AFE_1	FALSE	AFE1	TRUE	C_A_Proximal	67396756-67397263	0.467	0.124	-0.334
12	ENSMUSG00000047146.17	1	ENSMUSG00000047146.17	10	-	ENSMUSG00000047146.17:62819473-62819623	ENSMUSG00000047146.17_1_pAFE_1	FALSE	P_AFE1	TRUE	C_A_Proximal	62819623-62819760	0.34	0.017	-0.316
13	ENSMUSG00000032329.4	1	ENSMUSG00000032329.4	9	+	ENSMUSG00000032329.4:56489822-56490325	ENSMUSG00000032329.4_1_A3_1	FALSE	Alt 3x1	TRUE	E1_E2_Proximal	56489964-56494526	0.617	0.326	-0.291
14	ENSMUSG00000014923.1	1	ENSMUSG00000014923.1	13	-	ENSMUSG00000014923.1:67637647-67637769	ENSMUSG00000014923.1_1_pALE_1	TRUE	P_ALEx1_Orphan Junctionx2	TRUE	C_A_Proximal	67627381-67637647	0.325	0.041	-0.279
15	ENSMUSG00000028820.13	1	ENSMUSG00000028820.13	4	+	ENSMUSG00000028820.13:127035808-127035854	ENSMUSG00000028820.13_1_pAFE_1	FALSE	P_AFE1	TRUE	C_A_Proximal	127032805-127035808	0.659	0.389	-0.269
16	ENSMUSG00000005699.16	1	ENSMUSG00000005699.16	8	+	ENSMUSG00000005699.16:105702174-105702396	ENSMUSG00000005699.16_1_AFE_1	FALSE	AFE1	FALSE	C_A_Proximal	105701803-105702174	0.548	0.27	-0.263
17	ENSMUSG00000037211.12	1	ENSMUSG00000037211.12	3	+	ENSMUSG00000037211.12:37640520-37640808	ENSMUSG00000037211.12_1_AFE_1	FALSE	AFE1	FALSE	C_A_Proximal	37640105-37640520	0.744	0.496	-0.248
18	ENSMUSG00000015839.6	1	ENSMUSG00000015839.6	2	-	ENSMUSG00000015839.6:75704364-75704641	ENSMUSG00000015839.6_1_ALE_1	FALSE	ALE1	FALSE	C_A_Proximal	75692372-75704364	0.31	0.071	-0.241
19	ENSMUSG00000029190.19	1	ENSMUSG00000029190.19	5	-	ENSMUSG00000029190.19:36613246-36613730	ENSMUSG00000029190.19_1_CE_1	FALSE	Cassette1	TRUE	C1_A	36611946-36613246	0.359	0.125	-0.228
20	ENSMUSG00000106797.1	1	ENSMUSG00000106797.1	5	-	ENSMUSG00000106797.1:36613246-36613739	ENSMUSG00000106797.1_1_CE_1	FALSE	Cassette1	TRUE	C1_A	36611946-36613246	0.359	0.125	-0.228
21	ENSMUSG00000044350.14	1	ENSMUSG00000044350.14	14	-	ENSMUSG00000044350.14:77034793-77035405	ENSMUSG00000044350.14_1_CE_1	FALSE	Cassette1	FALSE	C2_A	77035405-77035812	0.74	0.994	0.233
22	ENSMUSG00000037017.15	1	ENSMUSG00000037017.15	5	+	ENSMUSG00000037017.15:138126454-138126644	ENSMUSG00000037017.15_1_CE_1	FALSE	Cassette1	FALSE	C2_A	138125690-138126454	0.634	0.872	0.235
23	ENSMUSG00000073643.11	1	ENSMUSG00000073643.11	1	-	ENSMUSG00000073643.11:79724645-79724757	ENSMUSG00000073643.11_1_ALE_1	FALSE	ALE1	TRUE	C_A_Proximal	79722268-79724645	0.593	0.863	0.254
24	ENSMUSG00000044877.15	1	ENSMUSG00000044877.15	4	+	ENSMUSG00000044877.15:117267890-117268582	ENSMUSG00000044877.15_1_CE_1	FALSE	Cassette1	FALSE	C2_A	117265975-117267890	0.54	0.806	0.256
25	ENSMUSG00000040877.16	1	ENSMUSG00000040877.16	12	+	ENSMUSG00000040877.16:108897909-108898725	ENSMUSG00000040877.16_1_AFE_1	FALSE	AFE1	FALSE	C_A_Proximal	108897059-108897905	0.077	0.354	0.261
26	ENSMUSG00000026976.15	1	ENSMUSG00000026976.15	2	-	ENSMUSG00000026976.15:24436448-24436568	ENSMUSG00000026976.15_1_ALE_1	FALSE	ALE1	FALSE	C_A_Proximal	24435969-24436448	0.584	0.898	0.297
27	ENSMUSG00000052214.9	1	ENSMUSG00000052214.9	7	+	ENSMUSG00000052214.9:19228334-19229112	ENSMUSG00000052214.9_1_ALE_1	FALSE	ALE1	FALSE	C_A_Proximal	19228584-19244754	0.563	0.862	0.302
28	ENSMUSG00000073007.10	1	ENSMUSG00000073007.10	X	+	ENSMUSG00000073007.10:107862957-107863021	ENSMUSG00000073007.10_1_pAFE_1	FALSE	P_AFE1	TRUE	C_A_Proximal	107826945-107862957	0.325	0.646	0.303
29	ENSMUSG00000022973.18	1	ENSMUSG00000022973.18	16	-	ENSMUSG00000022973.18:90976599-90976751	ENSMUSG00000022973.18_1_A3_1	FALSE	Alt 3x1	FALSE	E1_E2_Proximal	90974075-90976599	0.657	0.965	0.304
30	ENSMUSG00000009585.18	1	ENSMUSG00000009585.18	15	+	ENSMUSG00000009585.18:79898111-79898225	ENSMUSG00000009585.18_1_CE_1	FALSE	Cassette1	FALSE	C1_A	79898225-79899020	0.313	0.634	0.317
31	ENSMUSG00000031594.9	1	ENSMUSG00000031594.9	8	-	ENSMUSG00000031594.9:41200445-41200604	ENSMUSG00000031594.9_1_pAFE_1	FALSE	P_AFE1	TRUE	C_A_Proximal	41200604-41209186	0.335	0.675	0.321
32	ENSMUSG00000032425.15	1	ENSMUSG00000032425.15	9	+	ENSMUSG00000032425.15:88545376-88545454	ENSMUSG00000032425.15_1_CE_1	FALSE	Cassette1	FALSE	C2_A	88552861-88545376	0.287	0.622	0.337
33	ENSMUSG00000022362.13	1	ENSMUSG00000022362.13	15	-	ENSMUSG00000022362.13:58048547-58048785	ENSMUSG00000022362.13_1_pAFE_1	FALSE	P_AFE1	FALSE	C_A_Distal	58048785-58069319	0.368	0.732	0.348
34	ENSMUSG00000028558.14	1	ENSMUSG00000028558.14	4	+	ENSMUSG00000028558.14:109235632-109235875	ENSMUSG00000028558.14_1_AS_1	FALSE	Alt 5x1	FALSE	E2_E1_Proximal	109235632-109235774	0.384	0.755	0.354
35	ENSMUSG00000025968.16	1	ENSMUSG00000025968.16	1	-	ENSMUSG00000025968.16:63175637-63175688	ENSMUSG00000025968.16_1_ALE_1	FALSE	ALE1	TRUE	C_A_Proximal	63172257-63175637	0.501	0.892	0.389
36	ENSMUSG00000028898.15	1	ENSMUSG00000028898.15	4	-	ENSMUSG00000028898.15:132325352-132325485	ENSMUSG00000028898.15_1_AFE_1	FALSE	AFE1	FALSE	C_A_Proximal	132325489-132325946	0.404	0.797	0.394
37	ENSMUSG00000068877.12	1	ENSMUSG00000068877.12	3	+	ENSMUSG00000068877.12:94696992-94697104	ENSMUSG00000068877.12_1_AFE_1	FALSE	AFE1	FALSE	C_A_Proximal	94696850-94696992	0.177	0.612	0.445
38															
39															
40															
41															
42															
43															
44															

Table 4.S3 List of genes bound by RBM46 at P21 whose mRNAs were differentially expressed in *Rbm46*^{-/-} testes at P8.

Screenshot displayed below.

	B	C	D	E	F	G	H	I	J	K	L	M	N
1	Genes with CITS (with truncated tag250) w/o UGAU at crosslink sites (PND21)	Genes Dysregulated Rbm46 KO (PND 8)	Genes in Common										
2	Gm20594	Eras	Pabpc1										
3	Pdzd2	ENSMUSG00000049235	Eif5										
4	Tcp1	Gcgr	Spc1										
5	Arntl2	8030474K03Rik	Setx										
6	Psmc4	Spo11	Pcbp2										
7	Pabpc1	Gm4969	Dld										
8	Rn45s	Lhx8	Cecr2										
9	Arhgap5	Syng3	Usp47										
10	Rmrp	Fer1l4	Stk33										
11	Trrap	Dppa3	Pcgf5										
12	Hist1h2aa	1700024B05Rik	Sycp2										
13	Setx	Glyf	Slc41a2										
14	Chst11	Dlx1	Taf9										
15	Ism1	PIK5	Smarca5										
16	2900060B14Rik	Gja3	Eif3i										
17	Rbm46	Trim61	Agtbp1										
18	Fyttd1	Grm5	Setd2										
19	1700112E06Rik	Tsk	Boll										
20	Csde1	ENSMUSG00000079374	Rnf17										
21	Canx	Mgat5b	Klu										
22	Snord104	Synpo2l	Taf2										
23	Hspa2	Kctd19	Strbp										
24	Morf4l1	Fibcd1	Fbxo47										
25	Rnu12	Prf1	Eps15										
26	Mapk15	Dmbx1	Rsp1										
27	Suz12	Zfp541	Glyr1										
28	Gtf2a1	Majin	Rbm46										
29	H3f3b	Mroh8	Aak1										
30	Rps3a	Atp12a	Eif5a										
31	Ddx6	Mpped1	Trim37										
32	Dazl	ENSMUSG00000079267	Ctcf										
33	Malat1	Hmx1	Dazl										
34	Dnahc8	Atg9b	Stk31										
35	Meig1	Otud6a	Lin52										
36	Hsp90b1	Dmrtc2	Hnrnp										
37	Usp32	Tmem63c	Naa15										
38	Atp5b	Glt1d1	Trim59										
39	Rpl8	L1td1	Heat1										
40	Trim17	Car8	Atr										
41	Gm11837	Ecel1	Morc2a										
42	Eif4g2	Zp3	Slc25a31										
43	Gm1564	Gje1	Mknk2										
44	Phospho2	Spata22	Larp1b										
45	Atr	Trim54	Atp13a3										
46	Trip12	Meiob	Tex101										
47	Psmc5	Kcnj4	Dpm1										
48	Skn1a	Tex13c2	Wdr11										

F. Chapter 4 Figures

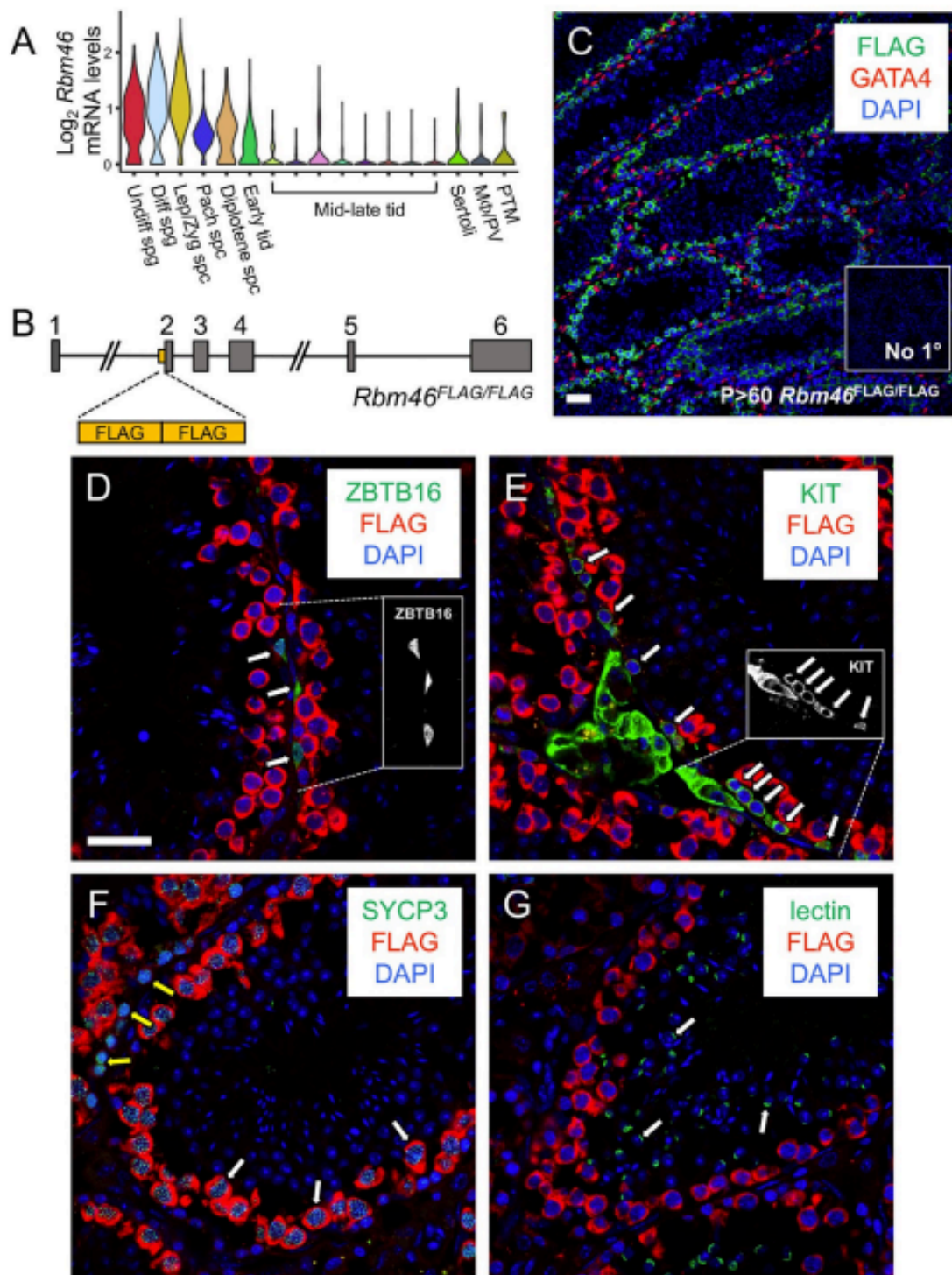


Fig 4.1 *Rbm46* expression is restricted to spermatogonia, spermatocytes, and early round spermatids in the adult testis.

(A) Violin plots showing relative mRNA levels from single cell (sc)RNA-seq data from adult mouse testes [4]. Undiff = undifferentiated; diff = differentiating; spg = spermatogonia; lep/zyg = leptotene + zygotene; pach = pachytene; spc = spermatocyte; tid = spermatid; MΦ = macrophage; PV = perivascular; PTM = peritubular myoid. (B) Diagram depicting insertion of the 2x FLAG tag upstream of exon 2 of the genomic *Rbm46* locus. (C-G) IIF was performed to localize the RBM46-FLAG (green in C, red in D-G) in testes from adult *Rbm46*^{FLAG/FLAG} mice. (C) RBM46-FLAG (green) was detectable in germ cells but not in GATA4+ (red) Sertoli cells. (D-G) RBM46-FLAG (red) was faintly detectable in ZBTB16+ (green) undifferentiated spermatogonia and KIT+ (green) differentiating spermatogonia, indicated by white arrows. Insets in D-E are single fluorescent channel images of individual ZBTB16+ undifferentiated and chains of KIT+ differentiating spermatogonia (in white), respectively. RBM46-FLAG (red) became readily detectable in SYCP3+ (green) spermatocytes (white arrows = pachytene, yellow = leptotene) and was undetectable in lectin+ (green) spermatids (white arrows). Nuclei were stained with DAPI (blue). Scale bars = 25 μm

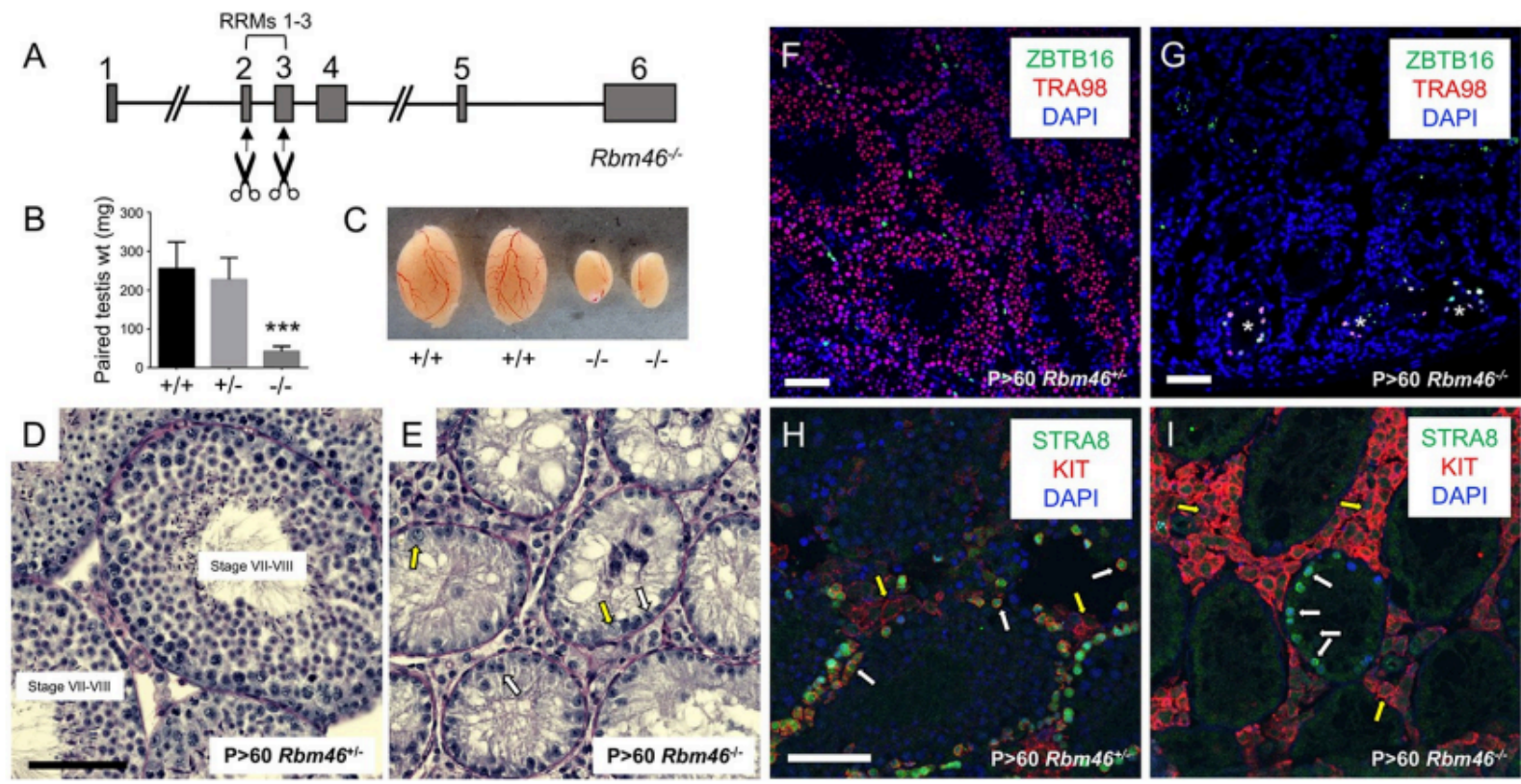


Fig 4.2 Adult *Rbm46*^{-/-} testes were dramatically reduced in size and contained only spermatogonia.

(A) Diagram showing the genomic *Rbm46* locus and the cut sites for deletion within exons 2-3. (B-C) Testes from *Rbm46*^{-/-} mice were significantly smaller than those from *Rbm46*^{+/+} littermate controls. (D-E) PAS-stained Bouin's-fixed testes from adult *Rbm46*^{+/+} (D) and *Rbm46*^{-/-} (E) mice, respectively. In contrast to control (D), *Rbm46*^{-/-} seminiferous epithelia (E) exhibited a single cellular layer, which included apparent Sertoli cells (white arrows) and spermatogonia (yellow arrows). (F-I) IIF was done to confirm the cellular identity of remaining cells in *Rbm46*^{-/-} seminiferous epithelia. Compared to controls (F), *Rbm46*^{-/-} seminiferous epithelia (G) contained ZBTB16⁺ (green) undifferentiated spermatogonia (in tubules marked with an asterisk), and germ cells were immunostained for the pan germ cell marker TRA98 (red). Compared to controls (H), some spermatogonia (indicated by white arrows) in *Rbm46*^{-/-} testes were STRA8⁺ (green, I), indicating response to RA, but none were KIT⁺ (red, I), revealing impaired differentiation. Interstitial cells (marked by yellow arrows, shown in H-I) are always KIT⁺. Nuclei were stained with DAPI (blue). Triple asterisks indicate statistical significance at P<0.001. Scale bars = 50 μ m.

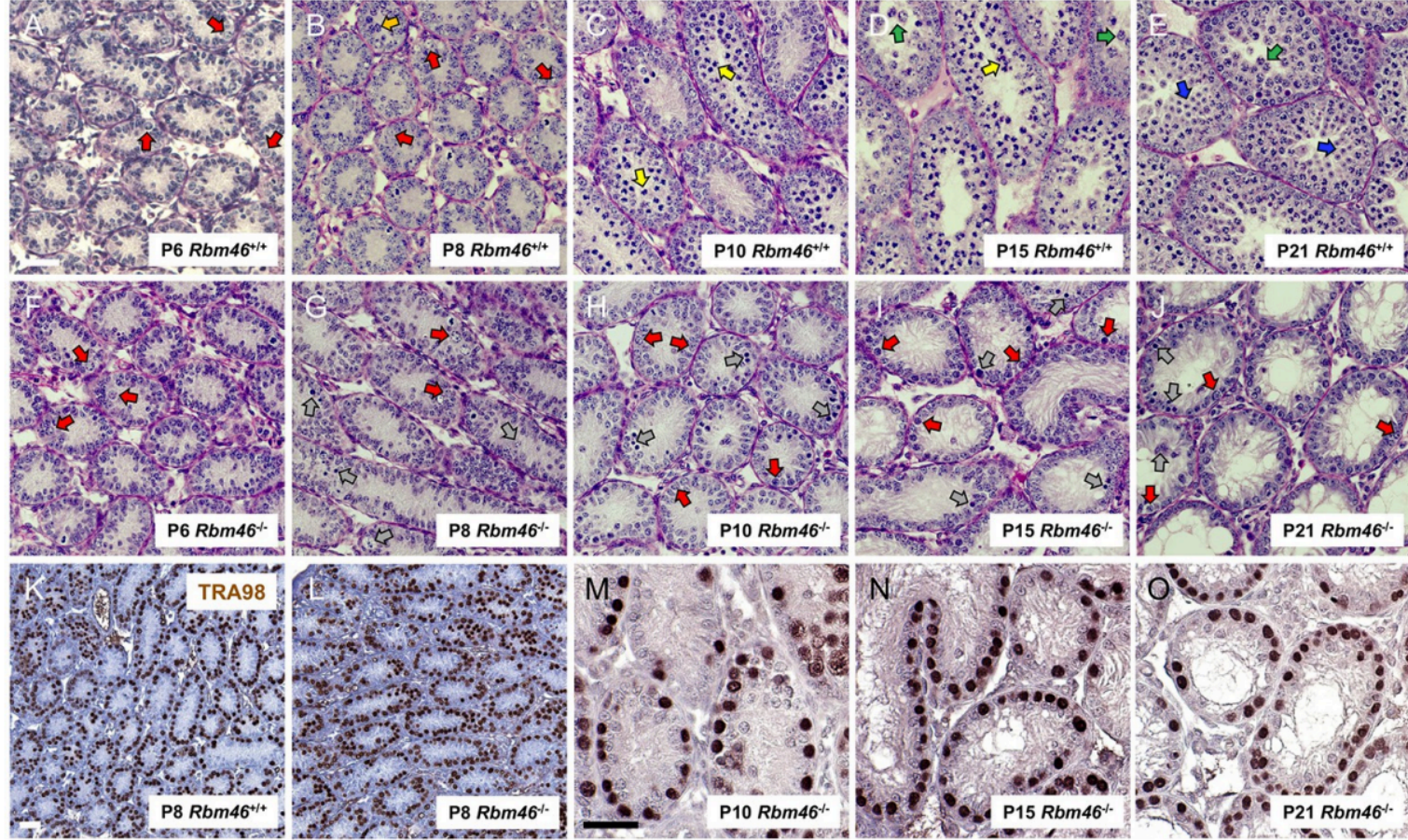


Fig 4.3 Germ cell loss was evident after P8 during the first wave of spermatogenesis in *Rbm46*^{-/-} testes.

(A-E) The first cohort of spermatogenic cells appear on predictable days during the first wave of spermatogenesis in control PAS-stained *Rbm46*^{+/+} testes. These include spermatogonia (red arrows), preleptotene spermatocytes (orange arrows), leptotene spermatocytes (yellow arrows), pachytene spermatocytes (green arrows), and round spermatids (blue arrows). (F-J) In contrast, *Rbm46*^{-/-} testes contained only spermatogonia (red arrows) and apparently degenerating germ cells (grey arrows). Meiotic spermatocytes were not observed at any age examined. (K-L) P8 testes contain abundant TRA98⁺ (brown) spermatogonia in both control and *Rbm46*^{-/-} testes. (M-O) P10, P15, and P21 *Rbm46*^{-/-} testes contained TRA98⁺ (brown) basally located spermatogonia. Scale bars = 50 μ m.

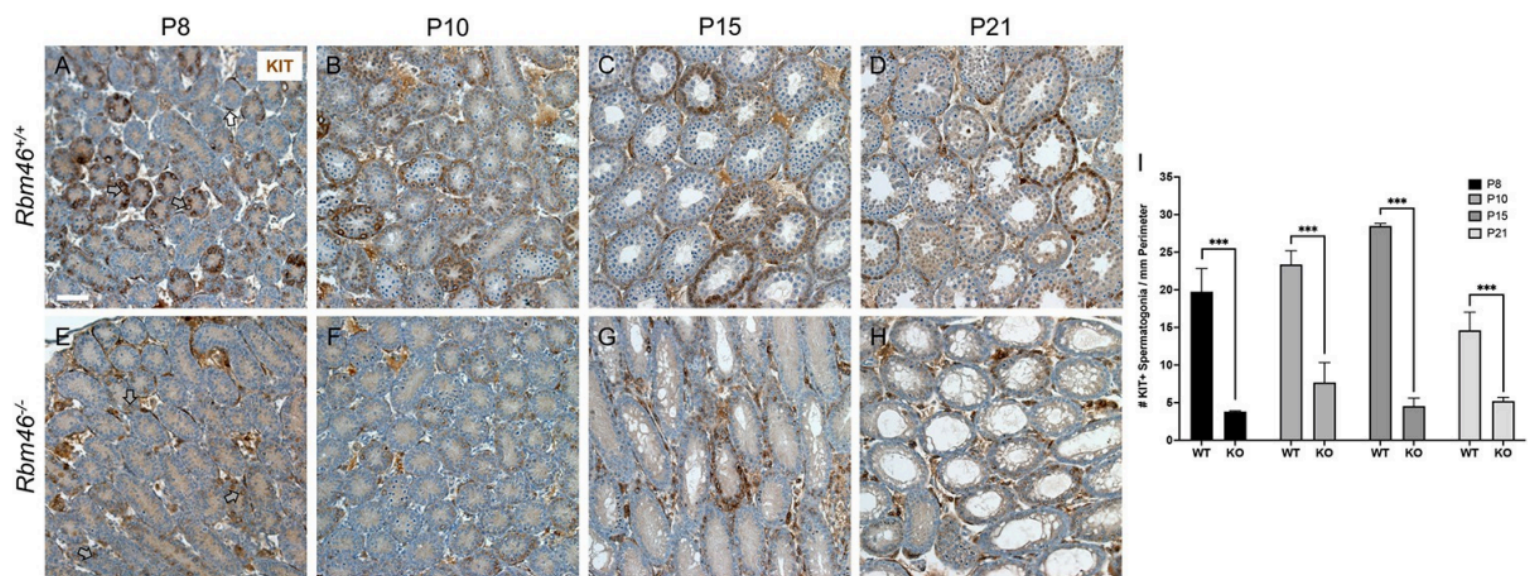


Fig 4.4 KIT⁺ differentiating spermatogonia were reduced dramatically in developing *Rbm46*^{-/-} testes.

(**A-D**) Darkly staining KIT⁺ (brown) spermatogonia (gray arrows, **A**) were numerous in *Rbm46*^{+/+} testes. KIT⁺ interstitial cells are indicated with white arrows. (**E-H**) At P8, there are scattered faintly staining KIT⁺ spermatogonia (gray arrows, **E**), and fewer are seen afterwards, from P10 (**F**) through P15 (**G**) and then at P21 (**H**). (**I**) Quantitation of KIT⁺ spermatogonia at each age. Scale bar = 50 μ m. Triple asterisks indicate statistical significance at $P < 0.001$.

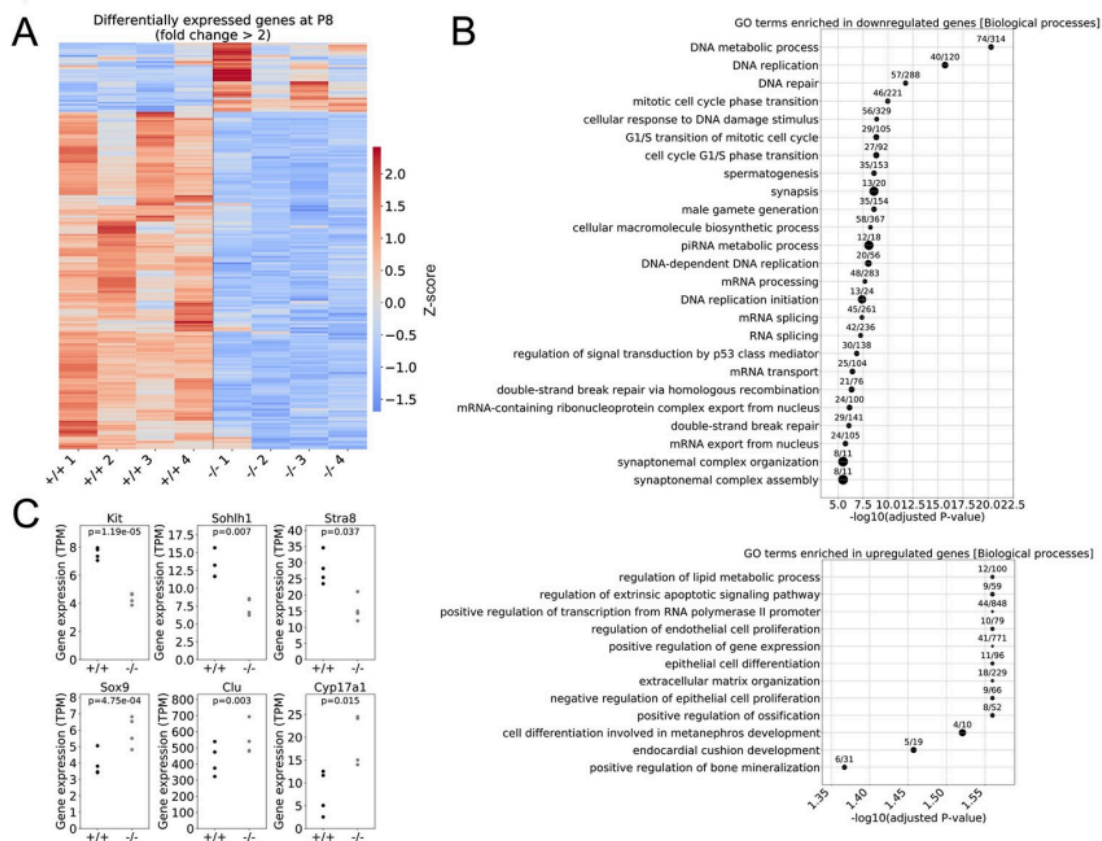


Fig 4.5 Genes involved in cell cycle regulation were deregulated in *Rbm46*^{-/-} testes at P8.

(A) Heatmap of 200 genes with >2-fold changes in *Rbm46*^{-/-} relative to *Rbm46*^{+/+} controls at P8.

(B) Gene ontology terms for biological processes enriched in genes that are downregulated (top) or upregulated (bottom) in *Rbm46*^{-/-}. Circle size and numbers correspond to the number of genes that are differentially expressed and represented in a GO term over to the total number of genes listed in the GO term. (C) Expression level of genes involved in spermatogonia differentiation and somatic cell markers. P-values are DESeq2 adjusted p-values comparing *Rbm46*^{+/+} to *Rbm46*^{-/-} testes.

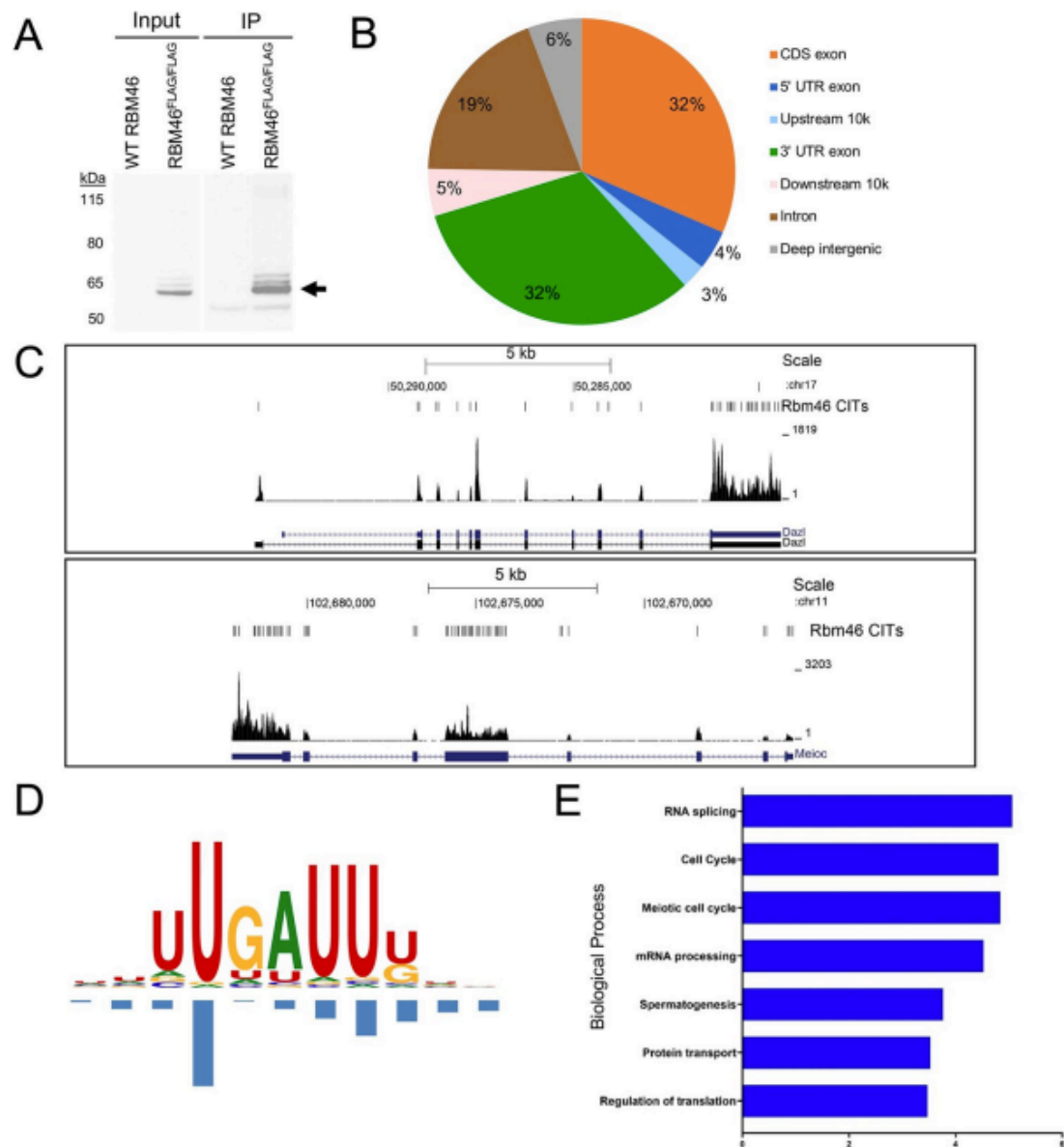


Fig 4.6 RBM46 sequence-specific binding to a specific cohort of mRNAs encoding factors required for RNA processing and meiosis.

(A) FLAG-stained western blot of lysates from *Rbm46*^{WT/WT} and *Rbm46*^{FLAG/FLAG} showing input and immunoprecipitated proteins, respectively. Band at the expected protein size is indicated by arrow. (B) Genomic distribution of RBM46 binding in the testis, displayed as percentages of binding to each sequence feature. (C) Representative UCSC genome browser view showing exonic binding of RBM46 across *Dazl* and *Meioc*. (D) WebLogo showing U-rich RNA binding sequence motif identified using mCross as the most enriched sequence at the RBM46 crosslink sites. (E) Gene ontology analysis performed using EnrichR showing the top biological processes of RBM46-bound mRNAs.

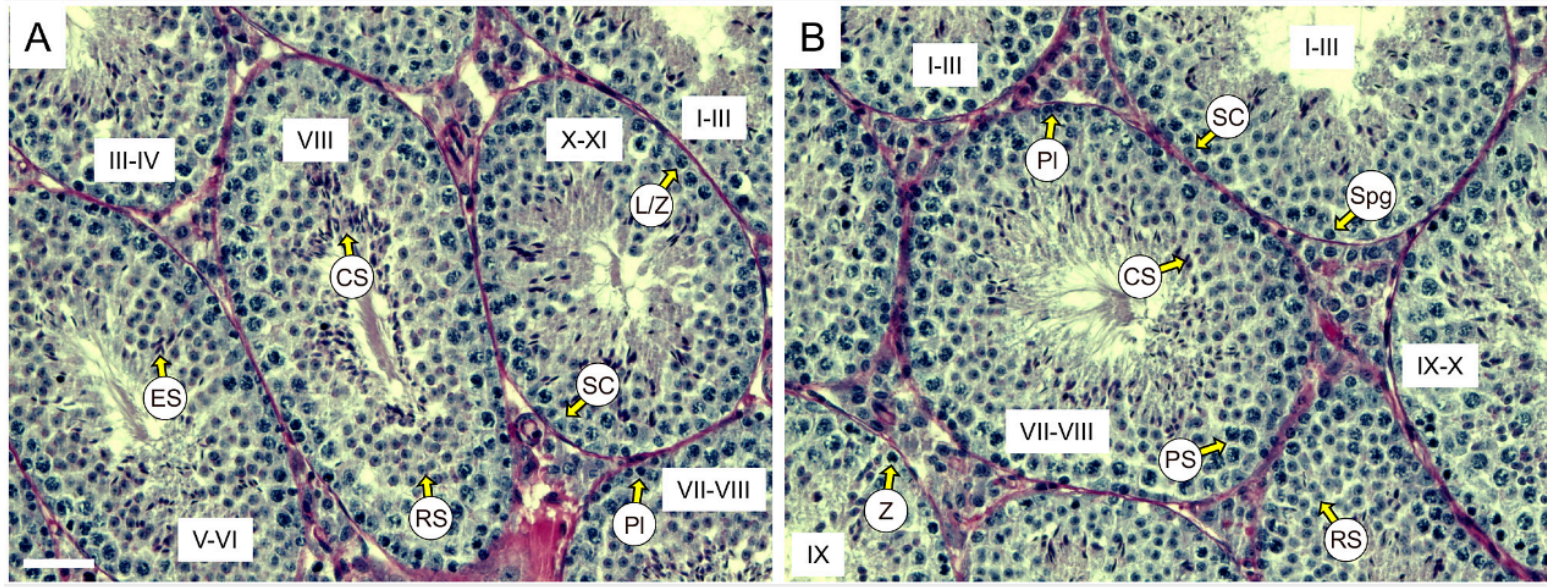


Fig 4.S1 Testes from adult *Rbm46*^{FLAG/FLAG} mice were morphologically normal.

(**A-B**) Similar to Bouin's-fixed and PAS-stained testes from adult (P>60) WT (**A**) mice, those from *Rbm46*^{FLAG/FLAG} mice (**B**) contained normal complements of male germ cells (Spg = spermatogonium; Pl = preleptotene spermatocyte; L = leptotene spermatocyte; Z = zygotene spermatocyte; PS = pachytene spermatocyte; RS = round spermatid; ES = elongating spermatid; CS = condensing spermatid; SC = Sertoli cell nucleus) within that appropriate seminiferous tubule stages, indicated on each cross section in Roman numerals. Scale bar = 50 μ m.

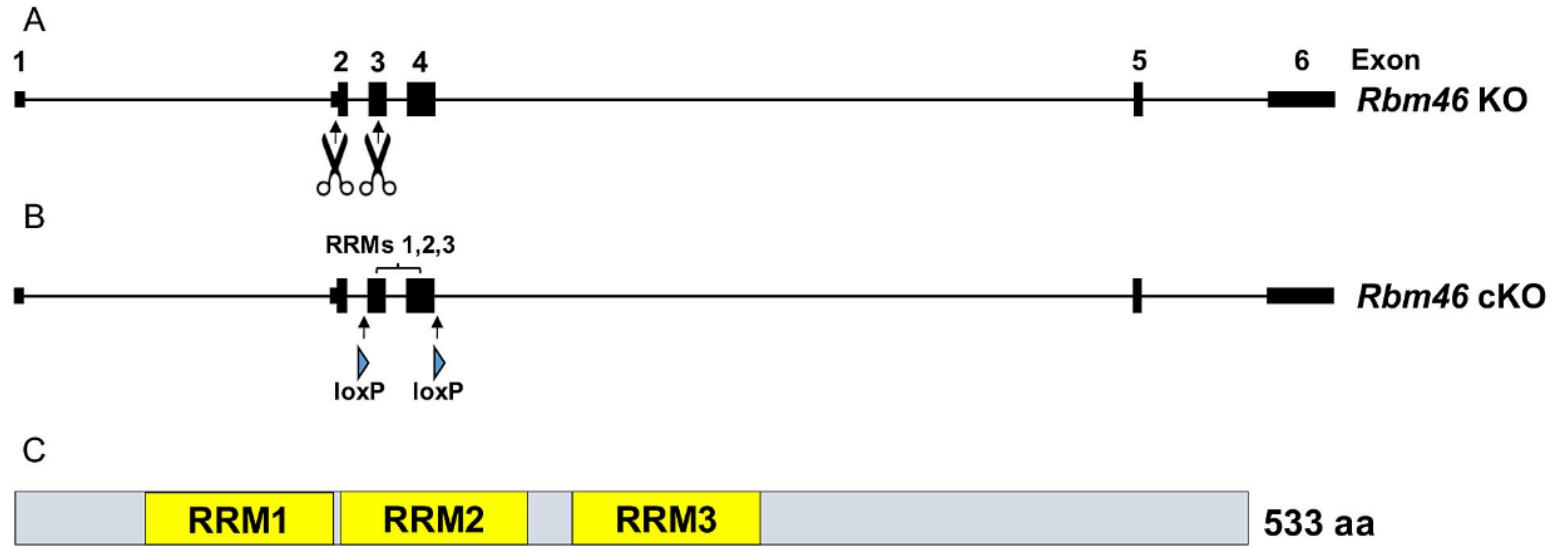


Fig 4.S2 Schematic of Rbm46 whole-body and conditional KO alleles.

(**A**) For whole-body KO allele, the deleted region is indicated by scissors. (**B**) For conditional KO allele, inserted loxP sites are represented by blue arrows. (**C**) RBM46 protein contains three RRM_s, indicated in yellow.

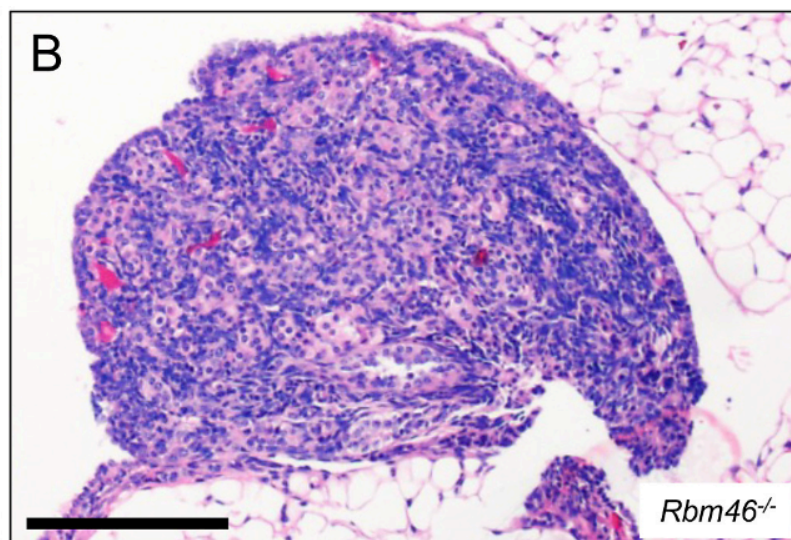
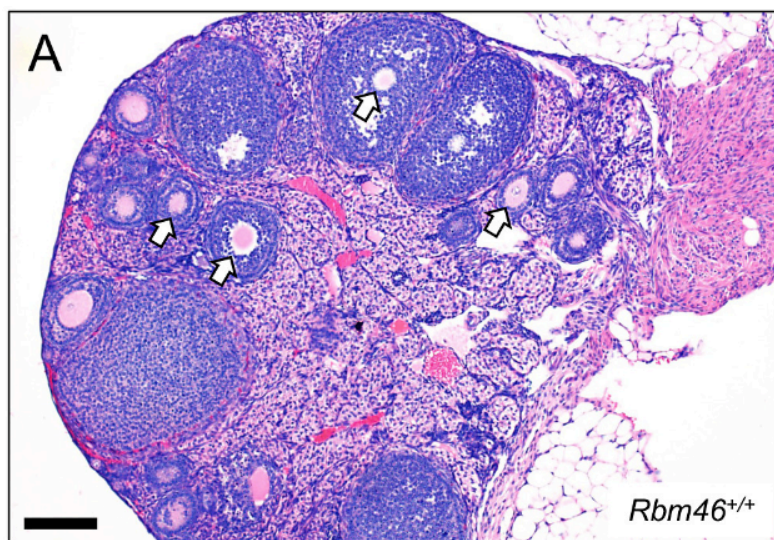


Fig 4.S3 Adult *Rbm46*^{-/-} ovaries lacked a germline.

(**A-B**) PAS-stained ovaries from *Rbm46*^{+/+} and *Rbm46*^{-/-} mice, with genotypes indicated on each image. The cortex of an *Rbm46*^{+/+} ovary (**A**) contained numerous oocytes (white arrows) in follicles at various stages of development. In contrast, the *Rbm46*^{-/-} ovary lacked oocytes or organized follicles (**B**). Scale bar = 200 μ m.

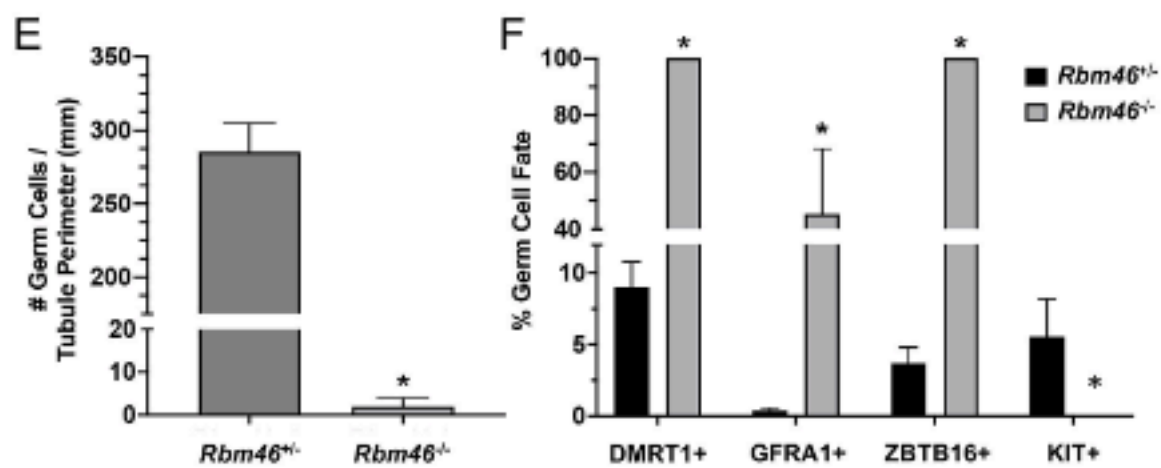
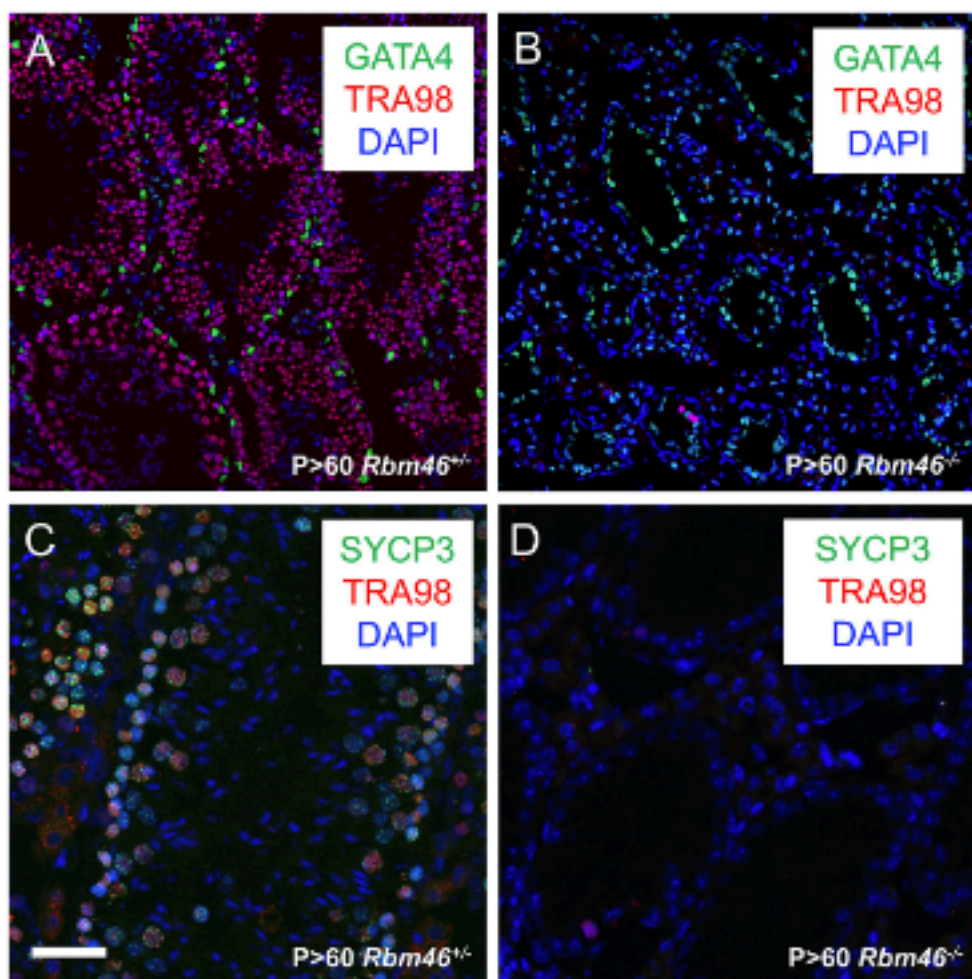


Fig 4.S4 Adult *Rbm46*^{-/-} testes contained abundant Sertoli cells but lacked SYCP3⁺ meiotic spermatocytes.

(**A-B**) GATA4⁺ Sertoli cells (green) were present in both *Rbm46*^{+/-} and *Rbm46*^{-/-} testes, but there were few TRA98⁺ (red) germ cells in *Rbm46*^{-/-} testes. (**C-D**) In contrast to *Rbm46*^{+/-} testes, there were no SYCP3⁺ (green) spermatocytes in *Rbm46*^{-/-} testes. (**E-F**) Using *Rbm46*^{+/-} and *Rbm46*^{-/-} testes, the numbers of germ cells (**E**) and % cell fate (**F**) were quantified. Nuclei were stained with DAPI (blue). Scale bar = 50 μ m.

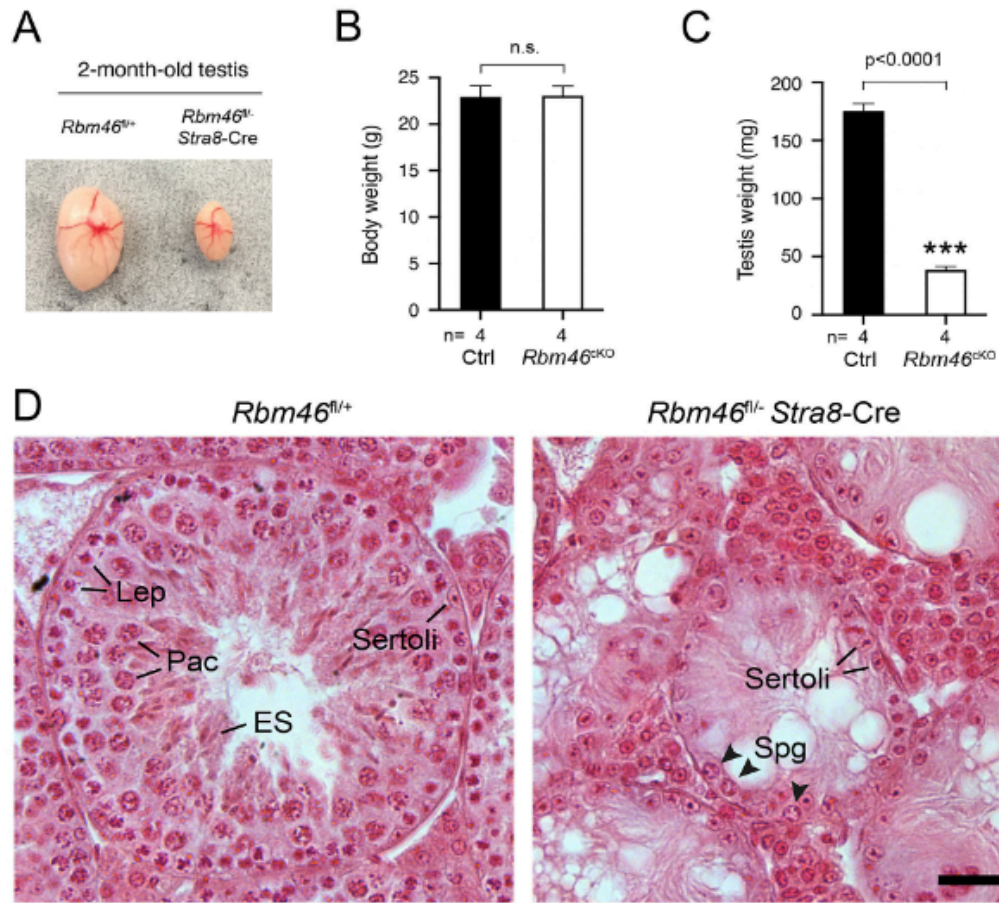


Fig 4.S5 Conditional deletion of *Rbm46* with *Stra8*-Cre resulted in an adult spermatogenesis phenotype resembling that of whole-body KO mice.

(A-C) Compared to controls, adult conditional KO testes were dramatically reduced in size. (D) Seminiferous epithelia from control mice (left panel) contained Sertoli cells as well as all advanced germ cell types, with examples marked including leptotene (Lep) and pachytene (Pac) spermatocytes as well as elongated spermatids (ES). In stark contrast, seminiferous epithelia of conditional KO testes contained only somatic Sertoli cells and a few spermatogonia (Spg). Scale bar = 50 μ m.

Germ cell numbers in P6 testes

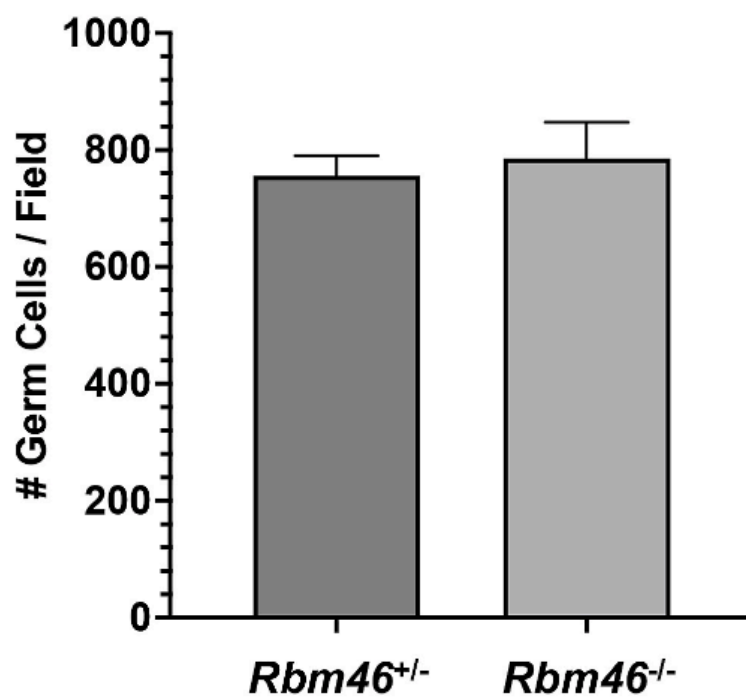
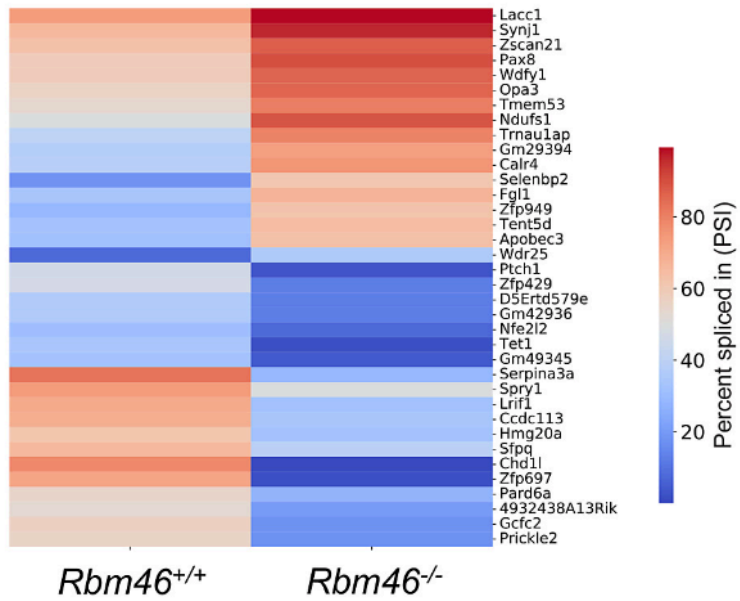


Fig 4.S6 Similar numbers of spermatogonia present in *Rbm46*^{+/-} and *Rbm46*^{-/-} testes at P6.

A

Differential splicing events



B

Types of differentially spliced events

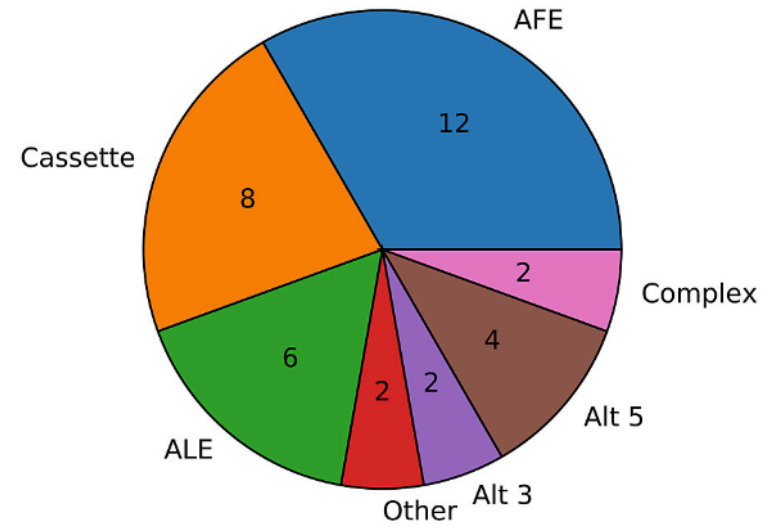


Fig 4.S7 Differential splicing events in *Rbm46*^{-/-} testes at P8.

(A) Heatmap depicts 36 splicing events with a change in percent spliced in (PSI) of at least 15% in *Rbm46*^{-/-} relative to *Rbm46*^{+/+} testes at P8. (B) Distribution of the types of altered splicing events in *Rbm46*^{-/-} testes. Absolute number of changing events for each type shown on the chart. ALE = Alternative Last Exon; AFE = Alternative First Exon; Alt 3 = Alternative 3' splice site; Alt 5 = Alternative 5' splice site.

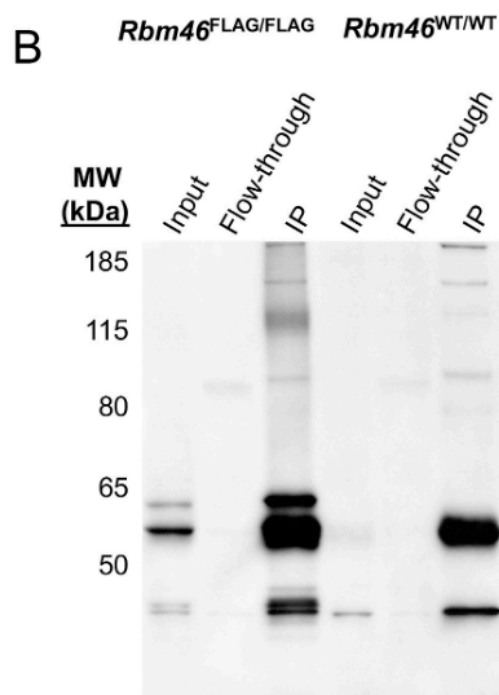
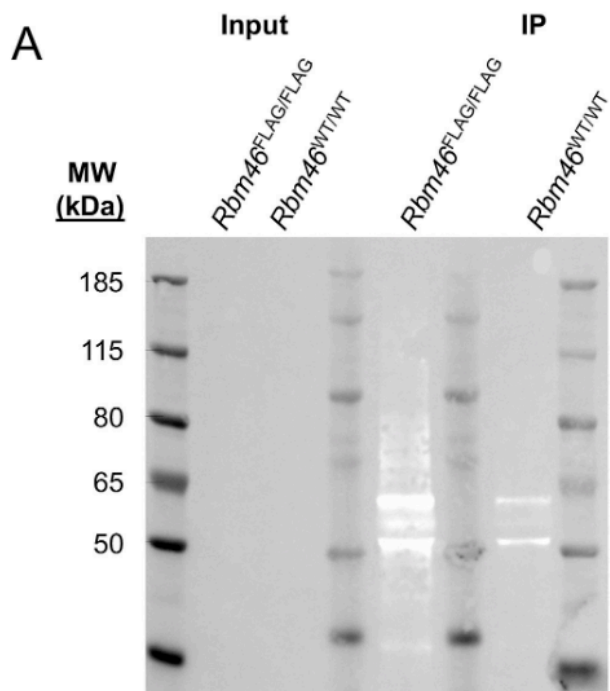


Fig 4.S8 CLIP of *Rbm46*^{Flag/Flag} in P21 mouse testes.

(A) SDS-PAGE of crosslinked immunoprecipitants and input from *Rbm46*^{FLAG/FLAG} and *Rbm46*^{WT/WT} testes. RNAs in the immunoprecipitants were ligated (on beads) with an RNA linker containing the IRDye 800CW fluorochrome to enable RNA visualization. (B) Corresponding anti-FLAG western blot of crosslinked immunoprecipitants and input from *Rbm46*^{FLAG/FLAG} and *Rbm46*^{WT/WT} testes following FLAG immunoprecipitation.

CHAPTER V. CONCLUSIONS AND GENERAL DISCUSSION

Mammalian male germ cells must proceed through numerous fate transitions to produce sperm. The three studies presented here focused on two of these transitions – spermatogonial differentiation and meiosis entry/initiation. These two transitions are indispensable for male fertility, yet the mechanisms governing spermatogonia through these transitions remains limited. In the second chapter, a presumed dogma in the field – that RA acts as the MIS – was emphatically disproven. In the third chapter, the differential responsiveness of spermatogonia subpopulations to RA was described in steady-state spermatogenesis. In the fourth chapter, a novel RNA binding protein was shown to be required for spermatogonial differentiation. These three projects expand our understanding of spermatogonial biology and simultaneously spotlight the criticality of spermatogonial differentiation.

Despite knowing that vitamin A is required for male fertility for nearly a century^{105,226}, our understanding of RA signaling in spermatogenesis remains limited. We and others have shown the essential role of RA for spermatogonial differentiation^{136,240,336}, however there remains little to no evidence supporting a requirement for RA in meiotic entry. The positioning of RA as the MIS is predicated on expression of the RA-responsive gene product STRA8 in preleptotene spermatocytes entering meiosis^{147,148,219,260}. Complicating the requirement of RA in meiotic entry is the progression of germ cells through differentiation and into meiosis in *Stra8* KO mice^{147,148}. Using a combination of *in vivo* and *in vitro* approaches, we formally examined the role of RA as the MIS. Our results detailed in chapter two decisively conflict with this presumed dogma. We showed differentiating spermatogonia did not require a second pulse of RA to initiate meiotic entry/initiation. In fact, germ cells progressed through multiple fate transitions following a singular pulse of RA every ~ 26 days in total in the mouse testis. Germ cells in both RA-sufficient and RA-

deficient testes completed differentiation, entered meiosis, expressed classical markers of meiotic events, completed meiosis, and progressed through spermiogenesis, on the same timelines. These results support the following conclusions – 1) STRA8 is not required, as the *meiotic gatekeeper* as reported^{119,147,148,217}; 2) progression through meiosis appears to be largely governed by intrinsic, or cell autonomous mechanisms; and 3) RA is not the MIS in the mouse testis as previously proposed^{52,53,156,223}. In support of the third conclusion, we noted that mRNA levels for many “meiotic genes” become upregulated midway through differentiation (in A3 differentiated spermatogonia) as well as towards the end of differentiation (in Type B spermatogonia). This reveals that a significant portion of the meiotic gene expression program is activated during differentiation, well in advance of the proposed second pulse of RA at meiotic initiation. These findings also support recent reports of 1) meiotic genes poised for activation in undifferentiated progenitors³³⁷; and 2) super enhancers within progenitors that control meiotic gene expression²³⁷.

Maintaining healthy pools of SSCs, undifferentiated progenitors, and differentiating spermatogonia is essential for spermatogenesis and preserving long-term male fertility. The concept of differential responsiveness of spermatogonia to RA in the neonatal testis was previously reported by our laboratory⁷⁴. The number of STRA8⁺ germ cells significantly increased post-RA treatment *in vivo* and *in vitro* – most germ cells responded to RA, but a cluster of germ cells (the SSCs) did not respond to RA. This differential response to RA was evidenced in testes at P1-2 (testis filled with prospermatogonia) and P5-6 (testis filled with SSCs, A_{Undiffs}, and A_{Diffs}). The concept of differential responsiveness to RA was further verified by inhibiting RA catabolism – treatment with the CYP26 inhibitor talarozole increased the number of STRA8⁺ germ cells like RA-treated testes. I followed up on these results, to determine whether the mechanisms mediating spermatogonial RA response in the developing testis were maintained in the adult testis (chapter

three). STRA8 expression in spermatogonia significantly increased after RA treatment, with the largest number of STRA8⁺ germ cells evidenced 12 hrs post-RA treatment. GFRA1⁺ SSCs were shown to be RA non-responsive, whereas RA-responsive spermatogonia – STRA8⁺/ZBTB16⁺ progenitors and STRA8⁺/KIT⁺ differentiating spermatogonia – were located throughout the testis. IIF using markers for tubule staging (lectin and DAPI) revealed RA-responsive germ cells are housed in all tubule stages, contrasting with reports of RA-responsive germ cells in specific tubule stages^{216,217}. Collectively, spermatogonial populations at all ages can be categorized as such: 1) SSCs remain non-responsive to RA, despite exposure to both high levels of RA and consistent pulses of RA; 2) A_{Undiffs} can respond to RA and precociously enter differentiation; 3) A_{Diffs} can respond to RA but cannot precociously advance into meiosis.

One noteworthy difference between the neonate and the adult testis is the protection conferred from RA catabolic enzymes – CYP26-mediated catabolism offers germ cells a greater degree of protection from RA exposure in the neonatal testis compared to the adult testis. In the adult testis, CYP26 inhibition via talarozole significantly increased the number of STRA8⁺ progenitors compared to vehicle treated, however this number was less than those in RA-treated testes. Equally interesting, the percentage of tubules with STRA8⁺ progenitors after talarozole treatment was significantly more than vehicle treated, but less than RA-treated testes. In considering reports of RA levels varying along the seminiferous epithelium^{216,217}, we posit the levels of CYP26 enzymes also vary. PTMs, found encircling the germ cells, are involved in RA catabolism due to high levels of CYP26B1 expression⁷⁴. The variability of RA levels at different tubule stages suggest other cell types may also secrete RA catabolic enzymes. We identified RA responsive and RA non-responsive tubules in the wild-type testis as early as P4⁶⁷ and preliminary stainings of whole mounted seminiferous tubules revealed explicit delineations between RA-

responsive (STRA8+) and RA non-responsive (STRA8-) tubule segments (data not shown). To the best of our knowledge, no one has measured protein levels of CYP26 at each tubule stage and it is unknown how these distinct borders between RA responsive and RA-nonresponsive regions are maintained. Variable levels of CYP26 enzymes along the length of the tubule, specifically low levels of CYP26 enzymes in tubule stages VII-IX, could explain why spermatogonia in those stages respond to RA and subsequently differentiate.

Several significant differences are evident between the neonatal testis and the adult testis:

- 1) The first wave of spermatogenesis in the neonatal testis originates from prospermatogonia, whereas steady-state spermatogenesis in the adult testis originates from SSCs;
- 2) the neonatal testis lacks the full complement of germ cells that are present in the adult testis;
- 3) the first wave of spermatogenesis begins with limited cellular sources of RA; and
- 4) the first wave of spermatogenesis begins in the immature seminiferous cord whereas steady-state spermatogenesis occurs within the mature seminiferous tubule.

Remarkably, the core concept of RA responsiveness in germ cells is preserved throughout testis development. Germ cells near one another are exposed to RA simultaneously and must respond differentially to ensure long-term fertility. Indeed, if none of the germ cells responded to RA then the testis would fill with undifferentiated progenitor spermatogonia. Conversely, if all spermatogonia responded to RA and differentiated, the germline would be quickly depleted. Fortunately, while acute or chronic exposure to RA can stimulate the precocious differentiation of A_{Undiffs}, RA-insensitive SSCs are unaffected and can maintain the germline to produce the next generation of progenitors.

Our lab has a long-standing interest in spermatogonial differentiation, using mice as a model organism^{67,71-74,206,251,282,336}. In mice, the spermatogonial differentiation program necessitates five cell divisions over ~8.6 days, followed by a subsequent division as preleptotene

spermatocytes in preparation to enter meiosis^{65,72,73,254}. Using both adult WT mice and synchronized mice, we discovered an additional pulse of RA does not shorten the 8.6-day differentiation program such that spermatogonia can conclude differentiation and precociously enter meiosis. These findings contrast with reports of differentiating spermatogonia appearing to ‘await’ the RA signal and enter meiosis thereafter^{119,216,217}. I believe these misinterpretations were made due to the increased levels of endogenous RA, increasing STRA8 expression in spermatogonia as they transition into meiosis as preleptotene spermatocytes. It appears the reactivation of STRA8 during this transition is a mechanism that is retained from the differentiation transition and is ultimately lost as spermatocytes proceed through meiosis. The timing of spermatogonial differentiation also appears to be intrinsically regulated as the timeline cannot be altered by the presence or absence of RA either *in vivo* or *in vitro*.

Our laboratory capitalized on an opportunity to collaborate with the Carstens laboratory at the University of Pennsylvania to evaluate the requirement for the germline-specific RNA binding protein RBM46²⁵¹. Our interest was rooted in the lack of known post-transcriptional regulators specifically involved in the activation and maintenance of spermatogonial differentiation. RBM46 is an evolutionarily conserved protein²⁸¹ that was first discovered in *Drosophila* (tumorous testis, tut). In *tut* mutant flies, spermatogonia accumulated and were unable to differentiate³³¹. Using novel *Rbm46*-FLAG tagged and *Rbm46* KO mice, we defined the expression pattern and requirement for RBM46 in mouse spermatogenesis and male mammalian fertility. RBM46 is expressed in both spermatogonia and spermatocytes, but not in somatic cells nor in post-meiotic germ cells (e.g. spermatids, sperm)²⁸¹. *Rbm46* KO mice were healthy and displayed no overt defects, however mice were unable to produce any pups. Tubules of adult *Rbm46* KO testes were devoid of any post-mitotic germ cells and spermatogonia were unable to differentiate, a similar

phenotype to *tut* KO flies³³¹. Impaired spermatogonial differentiation and germ cell loss was evidenced as early as P8²⁸¹. Bulk RNA-seq of P8 *Rbm46* KO testes revealed the downregulation of several genes associated with spermatogonial differentiation (e.g., *Kit*, *Sohlh1*) and meiosis (e.g., *Sycp3*, *Dmc1*, *Spo11*) and no significant differences in genes associated with the undifferentiated state (e.g., *Gfra1*, *Ret*). eCLIP-Seq of P21 *Rbm46*-FLAG tagged testes revealed mRNAs bound to RBM46 associated with meiosis (e.g., *Sycp3*, *Spo11*, *Hormad1*) and the initiation of translation (e.g., *EIF4G1*). Altogether, RBM46 has an essential role during mammalian spermatogonial differentiation in the preparation of germ cell entry into meiosis. The Geyer lab will continue to investigate the mechanisms by which RBM46 exerts posttranscriptional control during differentiation in addition to exploring both potential interacting partners and the positioning of RBM46 within RA signaling.

My personal goals for these studies were to decipher events in spermatogonial transitions that could be foundational pieces for future treatments that either repress or enhance male fertility. Men are more willing today than years past to use contraceptives^{171,172} – with the contingency that the contraceptive is safe, effective, reversible, non-hormonal and non-surgical. Reciprocally, ~1 in 7 couples worldwide struggles with infertility, and men contribute to 50% of the cases¹⁹². Treatments focused on manipulating spermatogonial progression at differentiation would capitalize on a key transition critical for germ cell advancement and division. For example, RA-centric therapies could enrich a generation of germ cells by precociously inducing differentiation of progenitors, resulting in significantly more spermatocytes (shown here in mice; chapter three) and spermatids later (shown in small scale clinical trials)^{112,241,242}. Targeting a germ cell specific RNA binding protein such as RBM46 provides an alternative path, independent of RA signaling, that could be leveraged for the potential repression or enhancement of male fertility.

In summation, my work here examines mechanisms essential for spermatogonial differentiation for entry into meiosis. I found SSCs are unaffected by precocious and consistent pulses of RA, allowing for continual replenishment of the progenitor pool. Undifferentiated progenitor spermatogonia are capable of precociously entering and completing differentiation in response to RA. Differentiated spermatogonia are equally capable of responding to RA, but this response does not shorten the fixed differentiation timeline, nor does RA induce precocious entry into meiosis. In fact, spermatogonia can enter and complete meiosis in the absence of RA – upending a dogma in the field. Finally, the RNA binding protein RBM46 is required for spermatogonia to undergo and complete differentiation. These collective findings highlight the importance of spermatogonial differentiation for germ cell progression and ultimately the long-term maintenance of male fertility.

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APPENDIX A: ANIMAL USE PROTOCOLS



Animal Care and Use Committee
212 Ed Warren Life Sciences Building | East Carolina University | Greenville, NC 27834-4354
252-744-2436 office | 252-744-2355 fax

November 5, 2019

Chris Geyer, Ph.D.
Department of Anatomy/ECDOI
ECHI 4th floor
East Carolina University

Dear Dr. Geyer:

Your Animal Use Protocol entitled, "Using the Mouse to Study Testis Development" (AUP #A198a) was reviewed by this institution's Animal Care and Use Committee on October 21, 2019. The following action was taken by the Committee:

"Approved as submitted"

Please contact Aaron Hinkle at 744-2997 prior to hazard use

A copy is enclosed for your laboratory files. Please be reminded that all animal procedures must be conducted as described in the approved Animal Use Protocol. Modifications of these procedures cannot be performed without prior approval of the ACUC. The Animal Welfare Act and Public Health Service Guidelines require the ACUC to suspend activities not in accordance with approved procedures and report such activities to the responsible University Official (Vice Chancellor for Health Sciences or Vice Chancellor for Academic Affairs) and appropriate federal Agencies. **Please ensure that all personnel associated with this protocol have access to this approved copy of the AUP and are familiar with its contents.**

Sincerely yours,

A handwritten signature in black ink, appearing to read "S. B. McRae".

Susan McRae, Ph.D.
Chair, Animal Care and Use Committee

SM/jd

Enclosure

APPENDIX B: BIOLOGICAL SAFETY PROTOCOLS



Office of Prospective Health/Biological Safety REGISTRATION FOR THE USE OF BIOHAZARDOUS AGENT

Version 4/14/2020

NOTE: This registration is project/biohazardous agent-specific. Use of a new/different agent, manipulation, or research protocol will require submission of a new registration.

Biological Safety Registration #: 11; 14; 17; 20 (assigned by Biological Safety)

Date approved:

Principal Investigator: Chris Geyer

Date: 7/30/2020

Department: Anatomy & Cell Biology

Phone: 252-744-3433

Laboratory Location(s): ECHI 4400, 4404

Building: ECHI

Renewal:

☒ Yes

☐ No

If yes, approval dates: 9/2017

Are there any changes? ☒ Yes

☐ No

AUP #: a198A

IRB #: 20-001862

******Please attach a summary of changes from previous registration and highlight the changes in this current registration.******

I. PROJECT TITLE / AGENTS (Principal Investigator, please complete pages 1-8)

Project Title: **The role of retinoic acid (RA) in male germ cell development**

A. What materials or agents will be used? (Check all that apply):

☒ Recombinant DNA or RNA use, genetic recombinant techniques, complete molecules, or organisms (If checked complete Appendix A)

☒ Transgenic organisms, plants, animals? (Complete Appendix A)

☐ Viral or host systems Vectors (specify _____) Complete Appendix A

☐ Infectious Agents; bacteria, viruses, fungal, prions, etc.

☐ Biotoxins, allergens or other Biohazardous Material (Complete Appendix E, if applicable)

☒ Human/Non-Human Primate Blood serum or fluid

☒ Human/Non-Human Primate tissue or cell lines

NOTE: All human blood/tissue, or cell cultures are to be potentially infectious for bloodborne pathogens regardless of ATCC designation

B. Is this agent/vector/material potentially infectious or hazardous to humans? ☒ Yes ☐ No

If yes, by what exposure routes? ☒ Injection ☒ Ingestion ☒ Contact ☒ Inhalation

C. Is this agent/vector/material a potential hazard to the environment, agriculture, wildlife, or other plants or animals?

☐ Yes ☒ No

D. Will this work use a Select Agent as defined in Biosafety Manual or CDC web site (most up-to-date)?

☐ Yes ☒ No

E. Status of Project: ☐ Contingent on funding, notification date _____ ☐ Contingent on other (specify) _____

REGISTRATION FOR THE USE OF BIOHAZARDOUS AGENTS

1

EAST CAROLINA UNIVERSITY

Certificate Of Completion

Envelope Id: E9182B4CC11448B29EF0A8D094B7B484

Status: Completed

Subject: Please DocuSign: Geyer Biohazard Registration - 2020 CG edits.docx

Source Envelope:

Document Pages: 29

Signatures: 5

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209 Cotanche St.

MS# 229

Greenville, NC 27858

taylor@ecu.edu

IP Address: 150.216.194.22

