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Rhox13 is required for a quantitatively normal first wave of spermatogenesis in mice

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Abstract

We previously described a novel germ cell-specific X-linked reproductive homeobox gene (*Rhox13*) that is upregulated at the level of translation in response to retinoic acid (RA) in differentiating spermatogonia and preleptotene spermatocytes. We hypothesize that RHOX13 plays an essential role in male germ cell differentiation, and have tested this by creating a *Rhox13* gene knockout (KO) mouse. *Rhox13* KO mice are born in expected Mendelian ratios, and adults have slightly reduced testis weights, yet a full complement of spermatogenic cell types. Young KO mice (at ~7–8 weeks of age) have a ≈50% reduction in epididymal sperm counts, but numbers increased to WT levels as the mice reach ~ 17 weeks of age. Histological analysis of testes from juvenile KO mice reveal a number of defects during the first wave of spermatogenesis. These include increased apoptosis, delayed appearance of round spermatids, and disruption of the precise stage-specific association of germ cells within the seminiferous tubules. Breeding studies reveal that both young and aged KO males produce normal-sized litters. Taken together, our results indicate that RHOX13 is not essential for mouse fertility in a controlled laboratory setting, but that it is required for optimal development of differentiating germ cells and progression of the first wave of spermatogenesis.

Keywords

Differentiating spermatogonia; testis; fertility; homeobox; *Rhox* genes; apoptosis

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Declaration of Interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Introduction

Spermatogenesis begins in the neonatal mouse testis with the transition of quiescent prospermatogonia into stem, progenitor, and differentiating type A spermatogonia (Busada and Geyer 2015, Drumond, et al. 2011, Niedenberger, et al. 2015, Yang and Oatley 2014, Yoshida, et al. 2006). These three types of spermatogonia provide the foundation for the 'first wave of spermatogenesis' that occurs during the first ~6 weeks of postnatal life, and after that the spermatogonial stem cells (SSCs) supply subsequent waves during steady-state spermatogenesis. SSCs divide asymmetrically to either produce another SSC (self-renewal) or an undifferentiated progenitor spermatogonium that will proliferate before eventually differentiating in response to retinoic acid (RA) (Busada and Geyer 2015, Yang and Oatley 2014). Differentiating type A spermatogonia will then proceed through 6 divisions (A_1, A_2, A_3, A_4, In, B) before entering prophase I of meiosis as preleptotene spermatocytes. Following meiosis, haploid round spermatids undergo a dramatic morphological transformation during the process of spermiogenesis that culminates in the release of testicular sperm into the lumina of the seminiferous tubules.

The first cohort of differentiating spermatogonia proceed through these above steps with remarkable precision during the first 5 weeks of life in mice such that specific advanced germ cell types appear in the testis in consistent numbers on particular days of postnatal development (Bellve, et al. 1977), with minor strain variation. For example, in testes of C57BL/6 mice these germ cell types first appear on the following postnatal (P) days: preleptotene spermatocytes = P8, leptotene spermatocytes = P10, zygotene spermatocytes = P12, pachytene spermatocytes = P14, round spermatids = P20, and testicular sperm ≈P35. The first wave of spermatogenesis results in the appearance of functional sperm in the epididymis by 6 weeks of age in mice, which ensures their reproductive competence at an early age. It also provides researchers with an excellent model system for studying spermatogenesis, as relatively pure populations of specific germ cell types can be isolated for study, and perturbations in germ cell development are readily identified.

The 'reproductive homeobox X-linked', or *Rhox*, genes are located on the X chromosome in mammals and encode homeodomain-containing transcription factors. The *Rhox* genes are expressed primarily in reproductive tissues including the testis, ovary, epididymis, and placenta (Geserick, et al. 2002, Maclean, et al. 2005, MacLean and Wilkinson 2010, Song, et al. 2013, Wayne, et al. 2002). Their expression is regulated, at least in part, at the level of transcription by DNA methylation (Jackson-Grusby, et al. 2001, Maclean, et al. 2011, Oda, et al. 2006, Richardson, et al. 2014). The genomes of multiple mammals contain *Rhox* genes that are located on a syntenic region on the X chromosome and are likely expressed in reproductive tissues (Maclean, et al. 2005, Song, et al. 2013, Wang and Zhang 2006, Wilming, et al. 2015). There are only three genes in humans (*RHOXF1, RHOXF2*, and *RHOXF2B*), and there is evidence that these genes are rapidly evolving in primates (Niu, et al. 2011). In mice, an evolutionary expansion generated a collinear cluster of 33 *Rhox* genes discovered in 2005 by the Wilkinson laboratory (Maclean, et al. 2005). The cluster contains 13 unique *Rhox* genes (named *Rhox1-13*), as well as multiple duplicated copies of *Rhox2-4* (MacLean, et al. 2006). A few of the *Rhox* genes have been targeted in mice by knockout (KO) or RNAi (KD) approaches to determine their roles in fertility. Inactivation of the

Sertoli-expressed *Rhox5* (by gene KO) and *Rhox8* (by siRNA KD) resulted in male subfertility, with increased germ cell apoptosis, reduced cauda epididymal sperm counts, and impaired sperm motility (Maclean, et al. 2005, Welborn, et al. 2015).

After the initial reports of the *Rhox* gene cluster (in which 12 unique genes were described), we discovered another gene just downstream of *Rhox12* that we designated *Rhox13* that is only expressed in male and female germ cells (Geyer and Eddy 2008). In the female, *Rhox13* mRNA is present in the fetal ovary, with RHOX13 protein detectable in oogonia and preleptotene oocytes (Geyer and Eddy 2008). Based on the coincidence of mRNA and protein solely in the fetal ovary, we concluded that *Rhox13* expression is under transcriptional control in female germ cells. In contrast, *Rhox13* mRNA is present in all stages of testis development from embryonic day (E) 12.5 through adulthood, including in pachytene spermatocytes and round spermatids (our unpublished data). However, RHOX13 protein is only readily detectable in spermatogonia and preleptotene spermatocytes. We found that *Rhox13* mRNAs are repressed in prospermatogonia through a direct interaction with the RNA binding protein NANOS2, and this repression is apparently alleviated in response to RA (Chappell, et al. 2013, Geyer, et al. 2012). This translational control in the testis results in a protein expression pattern for RHOX13 that is remarkably similar to that in the female (oogonia/spermatogonia and preleptotene oocytes/spermatocytes), although they seem to be determined by distinct mechanisms. Therefore, we hypothesize that RHOX13 plays an essential role in germ cell differentiation in both sexes prior to meiotic initiation.

In this report, we examine the requirement for RHOX13 in male germ cell development by generating *Rhox13* KO mice and analyzing their reproductive phenotype. Testes of *Rhox13*$^{-/y}$ males exhibit delayed appearance of germ cell types and a significant increase in germ cell apoptosis during the first wave of spermatogenesis, which results in reduced numbers of epididymal sperm in young adult males. These defects are not seen in subsequent waves of spermatogenesis as *Rhox13*$^{-/y}$ males age, and their fertility is not significantly altered, at least in a controlled laboratory setting. Altogether, our results indicate that RHOX13 is required for a quantitatively normal first wave of spermatogenesis in the mouse.

Materials and Methods

Animal Care

All animal procedures were performed in accordance with the National Research Council Guide for the Care and Use of Laboratory Animals and approved by the Animal Care and Use Committees of East Carolina University (AUP #A181) and the National Institute of Environmental Health Sciences. C57BL/6NCrl mice were obtained from Charles River Laboratories, Inc. (Raleigh, NC, USA). Euthanasia of neonatal and juvenile mice up to P12 was performed by decapitation, and euthanasia of mice older than P12 was done by CO_2 asphyxiation followed by cervical dislocation.

Generation of Rhox13 KO mice

The mouse *Rhox13* gene (MGI:1920864) was targeted in E14Tg2a.4 (129/Ola) mouse embryonic stem (ES) cells derived from male embryos (BayGenomics, MMRRC-015890) using homologous recombination. The *Rhox13*$^{tm1a(KOMP)Wtsi}$ vector, (CDS79660), a pre-conditional 'knockout first' vector containing loxP and FRT sites, was obtained from the KOMP repository (www.komp.org/geneinfo.php?geneid=3451). The vector was linearized and electroporated into ES cells grown and selected as described previously (Cho, et al. 2001). Recombinant clones that correctly incorporated the *Rhox13* targeting construct into the X chromosome were identified by PCR and Southern blot analysis. One correctly targeted ES cell clone was electroporated with the circular pCAGGS-FLPe-IRES Spuro construct (Gene Bridges SSRF001) to excise the sequence flanked by FRT sequences, and then re-cloned. The resulting clones were re-screened to verify excision of the FRT-flanked regions and selected sub-clones were injected into C57BL/6N blastocysts to create chimeras. Male chimeras were bred to female C57Bl/6N mice, and pups were screened by coat color and genotyping to confirm germline transmission. Because only female pups carrying the mutant allele were produced (since *Rhox13* is X-linked), some were used to establish a colony of mice carrying the deleted *Rhox13* allele and others were mated to mice dizygous for the FLP deleter allele (B6.Cg-Tg (ACTFLPe) 9205 Dym/J, JAX Stock No: 005703). Resulting offspring with the FRT flanked region deleted were mated with *Hspa2*-Cre mice (Inselman, et al. 2010) to produce mice with exon 2 of *Rhox13* deleted (*Rhox13*$^{-/y}$ males and *Rhox13*$^{-/x}$ females). The colony was maintained by mating *Rhox13*$^{-/y}$ males with C57Bl/6N females.

PCR Genotyping

The genotypes of *Rhox13* WT and KO mice were determined using PCR to amplify genomic DNA isolated from tail tip tissue. The WT or deleted alleles were amplified using forward (5'-AGGGTTCTCCTAGGGTTGAGACC) and reverse (5'-TCCCTGGAAATGTCCAGAGCAG) primers. Amplification of DNA from wild type and KO mice should yield single 915 and 267 nt bands when run on a 1.5% agarose gel.

Breeding studies

We performed both long-term and short-term breeding studies. For the long-term breeding study, WT and KO male mice aged ≥P60 were paired with a single WT C57BL/6N female. Mice were paired for 92 days, and then males were euthanized. Female mice were monitored for another 22 days for the appearance of any final litters. For the short-term breeding study, P35 WT males and KO littermates were paired separately with a single female WT C57BL/6N female for 15 days. Male mice were euthanized at P50, and reproductive parameters analyzed. Female breeders continued to be monitored for 22 more days for the appearance of any final litters.

Cell quantitation from testis sections

Identification of preleptotene spermatocytes, zygotene spermatocytes, and round spermatids was performed during the first wave of spermatogenesis based on characteristic differences in position within the seminiferous cords and tubules, nuclear diameter, and chromatin

appearance (Bellve, et al. 1977). Paraffin sections from Bouin's-fixed testes isolated from P8, P12, and P20 mice were stained with hematoxylin and eosin using standard methods. Male germ cells were manually counted from photomicrographs captured with an Axio Observer A1 microscope (Carl Zeiss Microscopy GmbH, Jena, Germany) equipped with an XL16C digital camera and Exponent version 1.3 software (Dage-MTI, Michigan City, IN, USA). Photomicrographs were analyzed using Zeiss Axiovision software. Seminiferous cords were outlined manually, and the total germ cell numbers were divided by total seminiferous cord surface area and multiplied by 1,000 to obtain cells/mm^2. Cell counting was performed on ~30 round seminiferous cords or tubules in duplicate on testes from at least 4 different animals.

Cauda epididymal sperm counts

Sperm obtained from one cauda epididymis were counted in duplicate from each mouse. The cauda epididymides were removed from each mouse immediately after euthanasia and placed into 1 ml of 1× PBS at room temperature. Each epididymis was cut into small pieces and gently mixed by repeated passage through a P1000 pipet tip. The solution was diluted with distilled water to immobilize the sperm, and counts were obtained using a hemocytometer.

Daily sperm production counts

Whole testes were weighed and snap-frozen in liquid nitrogen. A portion of the frozen testis was cut off, weighed, detunicated, and homogenized in 1 ml of lysis solution (0.9% NaCl and 0.05% Triton X-100). The solution was further homogenized by multiple passages through a 26-gauge needle. The solution was then diluted and the numbers of step 14–16 spermatid heads were counted. The total number of spermatid heads was multiplied by the dilution and then by 2,500 and then divided by the weight of the testis fragment, multiplied by the weight of the whole testis, and divided by 4.84 to calculate daily sperm production per testis (Ashby, et al. 1999, Joyce, et al. 1993, Robb, et al. 1978).

Immunohistochemistry (IHC) and Indirect Immunofluorescence (IIF)

IHC was performed as previously described (Geyer, et al. 2012). Briefly, Bouin's-fixed testis sections were deparaffinized and then RHOX13 antiserum (generated previously, (Geyer and Eddy 2008)) was applied at 1:500 for 1 h at room temperature without antigen retrieval. Primary antibody was omitted as a negative control. Detection was performed using a Vectastain ABC kit (Vector Laboratories, Burlingame, CA, USA), and sections were counterstained with hematoxylin. Images were captured as described above. IIF was performed as before (Busada, et al. 2015a, Busada, et al. 2014, Busada, et al. 2015b, Niedenberger, et al. 2015). Briefly, 4% PFA-fixed and O.C.T.-embedded 5 μm frozen sections were incubated in blocking buffer for 30 min at room temperature and then incubated in primary antibody for 1 h at room temperature. Primary antibodies were against RHOX13 (1:500, (Geyer and Eddy 2008)), cleaved poly(ADP-ribose) polymerase 1 (PARP1, 1:100, #9544 Cell Signaling Technology, Danvers, MA, USA), BrdU (1:50, #B35130 ThermoFisher Scientific, Waltham, MA, USA), and STRA8 (1:3,000, #ab49602 Abcam, Cambridge, MA, USA). Following stringency washes, sections were incubated in secondary antibody (1:1,000 Alexa Fluor-488 donkey anti-rabbit or donkey anti-goat IgG,

Invitrogen, Carlsbad, CA, USA) plus phalloidin (1:500, Invitrogen, Carlsbad, CA, USA) for 1 h at room temperature. Blocking and antibody incubations were done in 1× PBS containing 3% BSA + 0.1% Triton X-100, and stringency washes were done with 1× PBS + 0.1% Triton X-100. Primary antibodies were omitted as negative controls. Coverslips were mounted with Vectastain containing DAPI (Vector Laboratories, Burlingame, CA, USA), and images obtained using a Fluoview FV1000 confocal laser-scanning microscope (Olympus America, Center Valley, PA, USA).

Statistics

Statistical analyses were performed using Student's t-test, and the level of significance was set at $P<0.05$.

Results

Generation of the disrupted Rhox13 allele

The germ cell expressed X-linked *Rhox13* gene has a rather unique expression pattern. In the male, it is transcribed solely in germ cells throughout testis development, and mRNAs are detectable from E12.5 through adulthood (Geyer and Eddy 2008, Geyer, et al. 2012). However, RHOX13 protein is only readily detectable in spermatogonia and preleptotene spermatocytes, is decreased dramatically in leptotene spermatocytes, and is undetectable in pachytene spermatocytes and spermatids (despite both cell types containing abundant *Rhox13* mRNA) (Geyer and Eddy 2008, Geyer, et al. 2012). To test the hypothesis that RHOX13 plays an essential role in male germ cell development, we took a reverse genetics approach to disrupt the *Rhox13* gene. We introduced loxP sites flanking the second exon of the *Rhox13* gene, which encodes a portion of the homeodomain. Since *Rhox13* is only expressed in germ cells, we decided to convert the floxed allele into a deleted allele. This was accomplished by breeding $Rhox13^{fl/fl}$ female and $Rhox13^{+/y}$;*Hspa2*-Cre male mice, which express Cre recombinase in spermatocytes (Inselman, et al. 2010). We successfully deleted the floxed allele, and therefore one half of all resultant male pups are $Rhox13^{-/y}$ (KO) and the other half are $Rhox13^{+/y}$ (WT), and we distinguished between them by PCR genotyping (Fig. 1A–B). Deletion of *Rhox13* was confirmed at the protein level by immunostaining, as no RHOX13 was detectable in male germ cells of any of the *Rhox13* KO mice (Fig. 1C–F).

Analysis of phenotypic traits of Rhox13 KO mice

Rhox13 WT and KO mice were generated in equivalent ratios throughout this study. We analyzed males from litters from the first generation after constitutively deleting the *Rhox13* allele and found a relatively high degree of phenotypic heterogeneity both within and between *Rhox13* KO males. Some had extremely low cauda sperm counts and abnormal testis histology, while others had apparently normal testis histology, sperm counts, and motility. Most KO mice had a mixture of normal-appearing tubules adjacent to those missing layers of spermatogenic cells, including basal spermatogonia or adluminal spermatocytes and spermatids (Fig. 2A–D). In addition, there were many vacuolated spaces within the seminiferous epithelia of these KO tubules, suggesting germ cell loss by apoptosis.

To determine whether these histological abnormalities caused differences in reproductive performance, we entered adult (P>60) male WT and KO mice (n=4 for each genotype) into a 5-month breeding study. Males of both genotypes exhibited apparently normal breeding behavior, and we found there were no statistically significant differences in either the average numbers of pups per litter or in the time taken for birth of the first litter. However, there was a statistically significant increase in the time between litters sired by *Rhox13* KO males when paired with WT females (Fig. 3, P=0.008).

The *Rhox13* KO mice were produced by injecting 129/Ola ES cells into C57BL/6 blastocysts. Based on the high variability in testis histology and sperm counts, we chose to backcross the *Rhox13* KO line onto the C57BL/6N genetic background in order to remove any confounding genetic modifiers and obtain a stable phenotype. *Rhox13*$^{-/+}$ females were bred with WT males for 7 generations, and the resulting 'incipient congenic' pups were predicted to have a >99% C57BL/6-derived genome. All subsequent analyses were performed using 7th or later generation mice. Males were euthanized at P56 (unpaired, or virgin males) and at P50 and P120 (after short-term and longer-term breeding studies) for reproductive phenotypic assessment, and the results are summarized in Table 1. As expected, there were no differences in body weight at any of the ages examined. Testis weights were ~10–15% lower in KO mice at P56 and P120, but this difference did not reach the level of statistical significance. The size and weight of paired seminal vesicles are under androgen control, and therefore differences would suggest differences in testosterone levels (reviewed in (Dohle, et al. 2003)). Although seminal vesicle weights vary considerably between even WT mice, there were no significant differences between genotypes in the 3 groups of mice. We counted cauda epididymal sperm from WT and KO males at P50, P56, and P120 to measure the output of spermatogenesis. These ages are significant because sperm in P50 and P56 epididymides should be products of the first wave of spermatogenesis, while those in P120 epididymides arise from subsequent rounds of adult SSC-derived steady-state spermatogenesis. There were no statistically significant differences in cauda sperm quantity between P120 WT and KO males. In contrast, young KO males had reduced numbers of cauda epididymal sperm as compared to WT control littermates at both P56 (~1.6-fold decrease, P<0.001) and P50 (~1.5-fold decrease, not statistically significant). Importantly, P50 males were actively breeding until the day of euthanasia, while P56 males were never paired with females.

Histological abnormalities during the first wave of spermatogenesis

The reduced sperm counts in younger KO males (P50 and P56) suggested that the absence of RHOX13 had an effect during the first wave of spermatogenesis. We examined the histology of testes from WT and KO mice at P8, P12, and P20 because these ages coincide with the appearance of preleptotene spermatocytes, pachytene spermatocytes and round spermatids, respectively (Bellve, et al. 1977). We found that at P8 there were similar numbers of emergent preleptotene spermatocytes in WT and KO testes (Fig. 4A–B). At P12, there was no difference in the number of leptotene or zygotene spermatocytes (Fig. 4C–D). Round spermatids are the most advanced germ cell type present in the P20 testis, and in WT mice are found adjacent to the lumina of the seminiferous tubules (Bellve, et al. 1977). In KO testes, there was a 3.8-fold reduction in the number of round spermatids per area of

seminiferous tubules (Fig. 4E–F), and an apparent difference in the number of tubules containing round spermatids. By P22, both WT and KO males had similar numbers of round spermatids (data not shown), indicating that this developmental delay was temporary.

Germ cell proliferation is reduced in Rhox13 KO mice during the first wave of spermatogenesis

The preceding observations suggested an approximate 1-day delay in the appearance of specific germ cell types in *Rhox13* KO testes during the first wave of spermatogenesis. We assessed whether this might be explained by a change in the extent of germ cell proliferation. We injected juvenile mice with the nucleotide analog 5-bromo-2-deoxyuridine (BrdU), which is only incorporated into DNA of cells that are mitotically active and have passed through S phase. Mice were euthanized 20 h after injection, and testis sections were stained with an antibody recognizing BrdU. We counted BrdU+ proliferating germ cells, and found there were no significant differences in the numbers of BrdU+ germ cells in *Rhox13* KO mice at P8 and P20 (Fig. 5).

Rhox13 testes have higher levels of germ cell apoptosis

We noted an apparent increase in the number of apoptotic-appearing germ cells with pyknotic nuclei and brightly eosinophilic cytoplasm in H&E-stained *Rhox13* KO testis sections during the first wave of spermatogenesis (Fig. 4). To quantify the extent of apoptosis, we stained frozen testis sections with an antibody recognizing cleaved PARP1 (c-PARP1). At P8, there were similar numbers of c-PARP1+ germ cells in WT and KO testes (Fig. 6A–B). At both P12 and P20, there was a significant increase of approximately 3-fold more c-PARP1+ germ cells in KO versus WT testes (Fig. 6C–F, $P<0.05$). We did not observe significant differences in any of the reproductive parameters measured from P120 adult mice undergoing SSC-derived steady-state spermatogenesis (data not shown).

Breeding studies assess the fertilization ability and efficiency of first wave- and adult SSC-derived sperm

We could not rule out the possibility that RHOX13 performs an essential role in a process required for fertility that we could not discern from the analyses described above. To examine this, we performed a 5-month breeding trial with 4 adult males of each genotype (age = P60 at the beginning of the trial). There were no significant differences in the average time to first litter (WT = 22 days, KO = 22 days), average time between subsequent litters (WT = 25 days, KO = 20 days), or average numbers of pups per litter (WT = 6.75, KO = 7.5).

During the first wave of spermatogenesis, KO testes exhibited a variety of histological abnormalities, decreased proliferation, increased apoptosis, and 50% fewer cauda epididymal sperm. Therefore, we performed a short-term breeding study to assess the fertility of these young male KO mice. KO and WT males at P35 were housed with mature females (P>60) for 15 days, and were then euthanized at P50 (n=4 KO, n=3 WT). The female mice were monitored for the next 22 days to record any subsequent litters. Two out of the three WT males produced a litter, while two out of four KO males produced a litter. There were no statistically significant differences in average numbers of pups per litter or

days to first litter (Table 1). Thus, we conclude that deletion of *Rhox13* does not significantly impair mouse fertility, at least in a controlled laboratory setting.

Discussion

These studies examined the reproductive phenotype of male *Rhox13* KO mice during the first wave of spermatogenesis and during steady-state spermatogenesis in older adult mice. We found that, although RHOX13 is not essential for fertility, its loss led to a number of histological abnormalities and developmental delays that were particularly evident during the first wave of spermatogenesis. Compared to WT littermates, KO testes had delayed appearance of specific germ cell types, histological abnormalities, and an approximate 3-fold increase in germ cell apoptosis, culminating in a ~50% reduction in cauda epididymal sperm content in young adult males. These defects were not seen in older adult male mice, indicating a reduced role for RHOX13 in steady-state spermatogenesis.

The effect of genetic background on the reproductive phenotype

The first *Rhox13* KO mice that we analyzed were on a mixed 129/Ola x C57BL/6N genetic background. Adult males that we analyzed exhibited a remarkable variety of reproductive phenotypes; some had apparently normal histology and sperm counts, while others had disrupted spermatogenesis. There is precedence in the literature for genetic background effects on fertility phenotypes, and this is likely due to unlinked genetic modifiers. One example is provided from studies of *Stra8* KO mice, which exhibited an inconsistent array of spermatogenic defects on a mixed genetic background; the phenotype became consistent once the mutation was backcrossed onto a C57BL/6N background (Anderson, et al. 2008, Baltus, et al. 2006). We bred 129/Ola x C57BL/6N KO mice with WT inbred C57BL/6N mice for 7 generations to progressively dilute the 129/Ola-derived genome. Based on classical genetics, these ‘incipient congenic’ mice should have a genetic background that is >99% derived from the C57BL/6 strain (Berry and Linder 2007). These C57BL/6N *Rhox13* males had less of a phenotype than those on the mixed background, suggesting that defects might have been more severe had we chosen to backcross onto the 129/Ola background.

Altered timing and increased apoptosis in Rhox13 KO males during the first wave of spermatogenesis

We observed a consistent delay in the appearance of round spermatids accompanied by a significant increase in apoptosis beginning at P12. Spermatogenesis in rodents is a tightly coordinated and precisely timed process, and as a consequence the adult seminiferous tubule segments contain predictable combinations of germ cells in various stages of development. The stage is set for this during the first wave of spermatogenesis, as specific germ cell types appear on defined days of development accompanied by a series of coordinated events such as Sertoli cell terminal differentiation and formation of the blood-testis barrier (BTB). Therefore, it is logical to presume that if the progression of germ cell development were delayed, then the coordination between developing germ cells and their environment would be disrupted. This might contribute to the significant increase in apoptosis during the first wave of spermatogenesis. There are other examples of altered timing of spermatogenesis during the first wave of spermatogenesis. The first is provided by studies of the ‘follicle

stimulating hormone (FSH) receptor' KO mouse, which exhibited decreased testicular weight, reduced seminiferous tubule diameters, and a delay in the appearance of spermatids (Krishnamurthy, et al. 2001). Another is provided by studies of mice deficient for 'mediator complex subunit 1' (*Med1*). *Med1* KO spermatocytes appeared precociously, approximately 3 days earlier than in WT littermate controls (Huszar, et al. 2015). This interesting phenotype included a significant increase in the abundance of pachytene spermatocytes that persisted through at least P42, but did not result in reduced fertility.

The cauda epididymis is the main site for sperm storage in most mammals (Bedford 2015, Hinton, et al. 1996, Jones 1999, Moore 1998), and therefore quantifying sperm content provides a reasonable measure of the functional output of spermatogenesis. In young adult males (~P50), we observed a ~50% reduction in cauda epididymal sperm, the final product of the first wave of spermatogenesis. The fact that virgin males had not expelled any sperm likely explains why there were higher sperm numbers in their cauda epididymides.

A potential role of Rhox gene cluster in fertility

Rhox genes are present in all mammalian species examined to-date (MacLean and Wilkinson 2010). There are 3 *Rhox* genes in humans (*RHOXF1, RHOXF2*, and *RHOXF2B*) (Geserick, et al. 2002, Richardson, et al. 2014, Song, et al. 2013, Wayne, et al. 2002), and there are at least 8 polymorphic sites (Niu, et al. 2011). It will be important to determine whether any polymorphisms are associated with human male infertility. The 33 *Rhox* genes in mice exhibit unique expression patterns throughout the male and female reproductive systems (Maclean, et al. 2005, MacLean, et al. 2006, MacLean and Wilkinson 2010). It is apparent that the individual rodent *Rhox* genes exhibit a much more restricted expression pattern than the human *RHOX* genes, which suggests that their encoded proteins may play more refined or specialized roles within various reproductive tissues. In addition, the expansion of the *Rhox* gene cluster on the X chromosome may contribute to, among other things, regulation of the first wave of spermatogenesis. Indeed, the defects reported here in *Rhox13* KO mice are most evident during the first wave, and decrease or disappear during steady-state spermatogenesis in the adult testis.

Three other *Rhox* genes have been disrupted in mice. *Rhox9* (previously termed *Gpbox*) is expressed in the placenta and in fetal germ cells of both sexes, and targeted disruption did not lead to observable defects in spermatogenesis (Takasaki, et al. 2000, Takasaki, et al. 2001). However, it has been proposed that the closely related *Rhox6* gene product could provide functional compensation, as it shares sequence similarity and expression pattern (MacLean and Wilkinson 2010). *Rhox5* and *Rhox8* are both expressed selectively in Sertoli cells, and their ablation resulted in male subfertility (Maclean, et al. 2005, Welborn, et al. 2015). It is likely that RHOX proteins may have overlapping functional roles, which would explain why the reproductive defects reported to-date are relatively minor. Future studies in our laboratory will examine the transcriptome-wide changes in gene expression in *Rhox13* KO mice in order to identify RHOX13-regulated genes, and it is possible that other *Rhox* genes are upregulated in the absence of RHOX13 to provide functional compensation in the adult testis.

The first wave of spermatogenesis occurs in rodents, and results in the production of functional sperm capable of fertilization as early as 7–8 weeks of age in mice. Most rodents become independent by 4–5 weeks of age and the vast majority are polygamous (Curtis 2010), relying on opportunistic breeding encounters while leading an existence fraught with the danger of being consumed by a wide variety of different predators. Therefore, the ability to successfully reproduce a week or more earlier because of first wave-derived sperm (rather than waiting on germ cell production from SSCs) would increase a male's chances of passing genes to the next generation before a potential early demise. Therefore, the role of the germ cell-expressed *Rhox13* gene may be to support early fertility and increased fecundity by providing additional support for the first wave of spermatogenesis, thus favoring the evolutionary expansion of the *Rhox* gene cluster.

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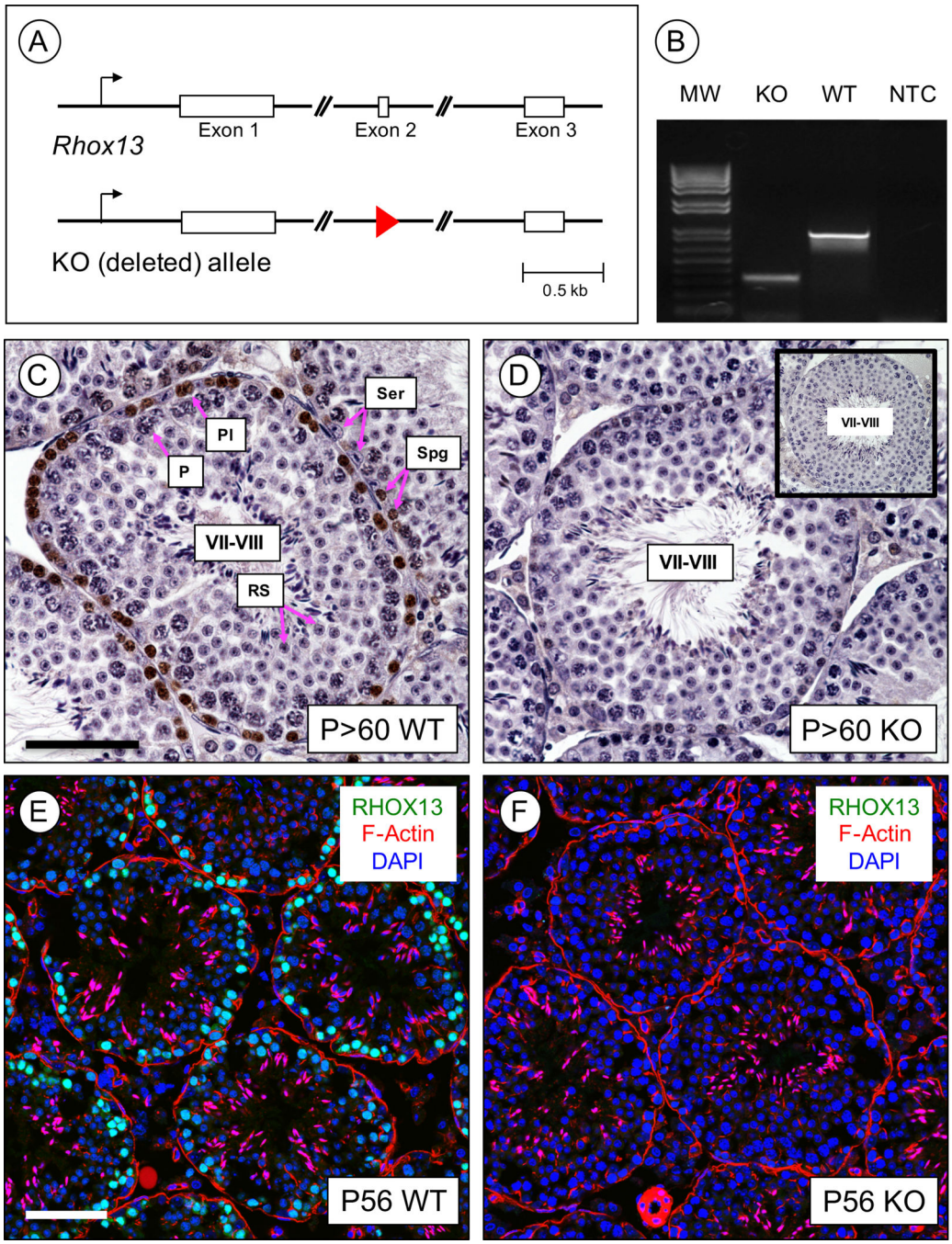

Figure 1. Generation of *Rhox13* KO mice

(**A**) The *Rhox13* gene is comprised of three exons (shown in boxes), as is typical of most homeobox genes. We created a KO allele by deleting exon 2, and a single loxP site remains (red triangle). (**B**) PCR amplification using primers on either side of exon 2 distinguishes KO from WT alleles. MW = molecular weight ladder, and NTC = no template control. (**C–D**) IHC was done to verify loss of RHOX13 protein (brown staining) in P>60 adult testes. Shown are stage VII-VIII seminiferous tubule sections from WT (**C**) and KO (**D**) mice. Sections were counterstained with hematoxylin, and inset (in **D**) is a no primary antibody

control. Spg = spermatogonia, Pl = preleptotene spermatocytes, P = pachytene spermatocytes, RS = round spermatids, Ser = Sertoli cells. (**E–F**) IIF was done to localize RHOX13 (in green) in WT (**E**) and KO (**F**) P56 testes. DNA is stained with DAPI (blue), and F-actin is stained with fluorescently-conjugated phalloidin (red). Scale bar = 50 μm.

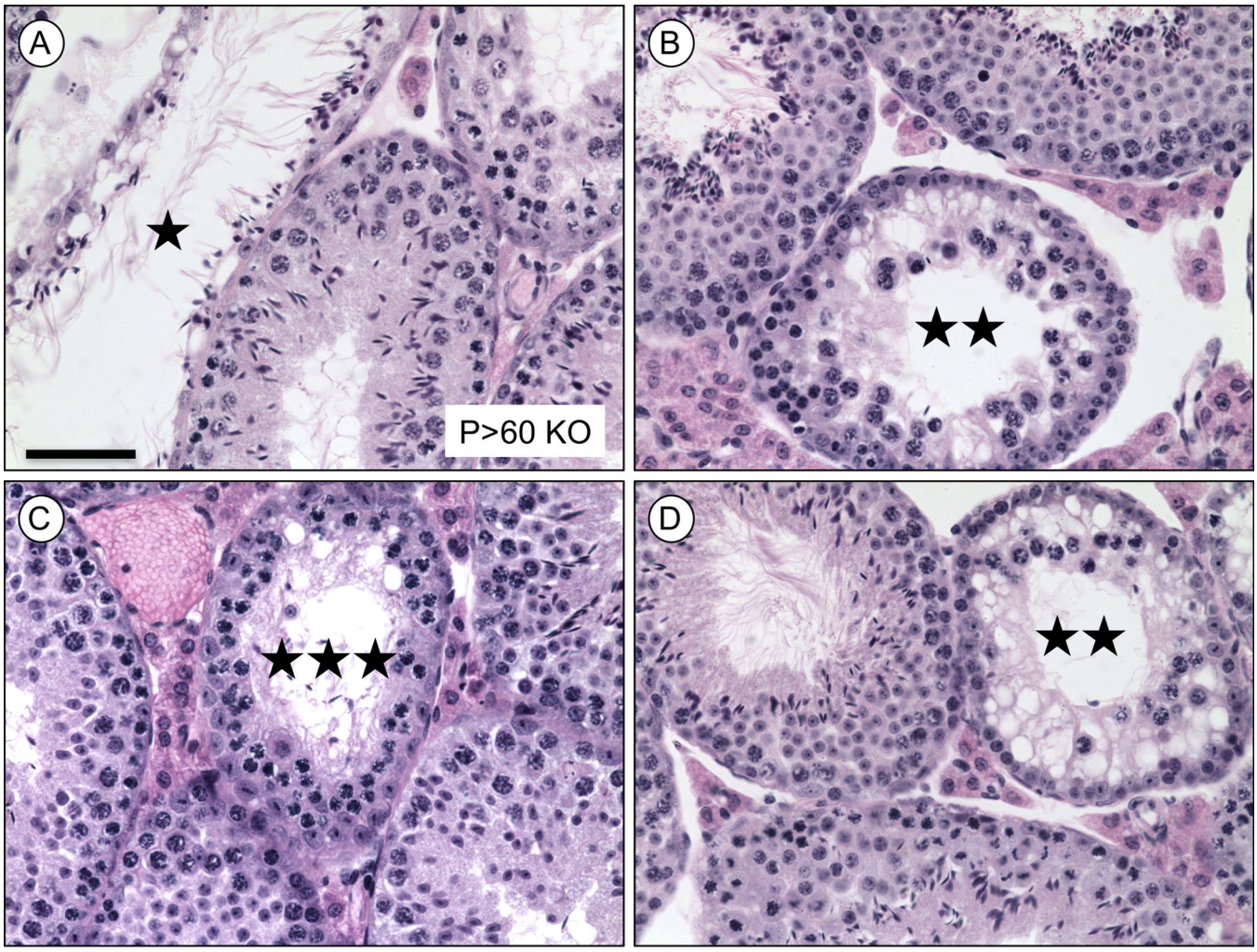

Figure 2. Histological abnormalities of adult (P>60) testes from *Rhox13* KO on a mixed genetic background

(**A–D**) Bouin's-fixed KO sections are stained with H&E and abnormal tubules are indicated by asterisks (★ = only containing condensed spermatids and Sertoli cells; ★★ = lacking spermatids; ★★★ = lacking spermatids and almost all spermatogonia). Scale bar = 50 μm.

A

Genotype	Average Pups Per Litter	Days to First Litter
WT	7.1 ± 3.1	24.2 ± 5.4
KO	7.6 ± 3.1	26.2 ± 4.7

B

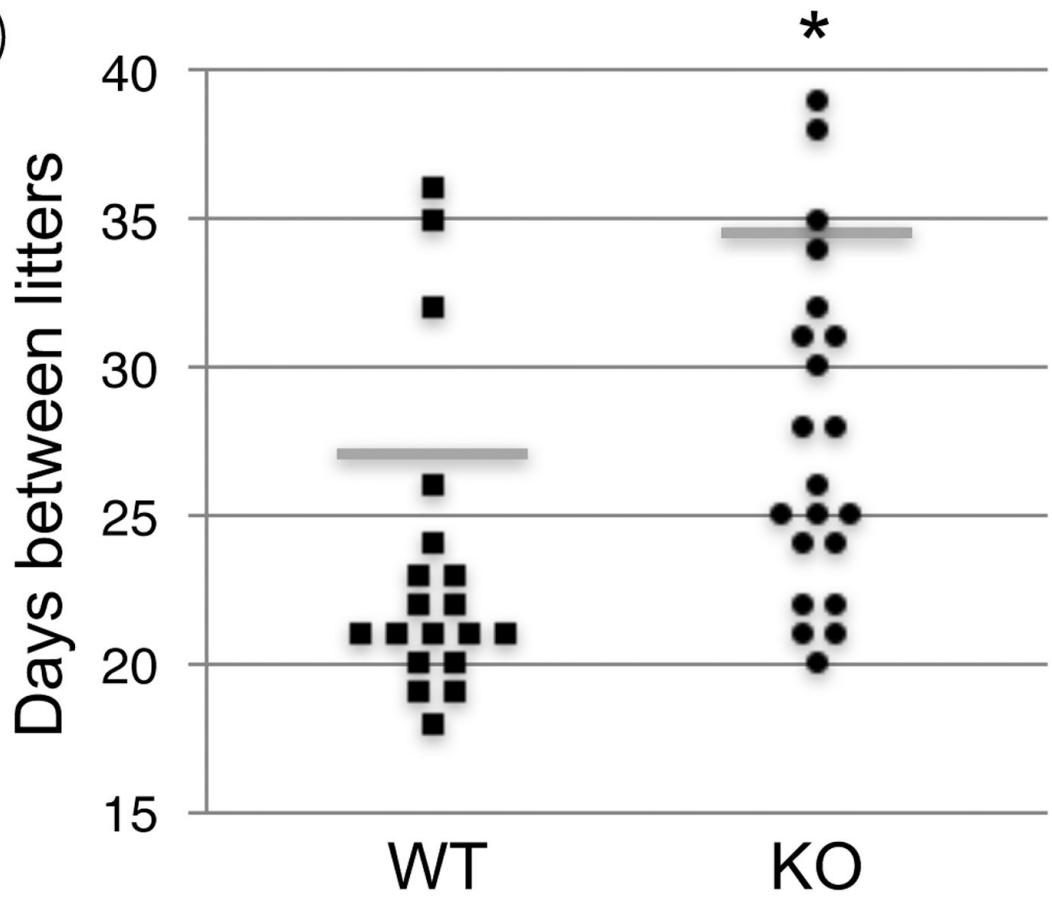

Figure 3. Fecundity results from long-term breeding trial of *Rhox13* WT and KO mice on mixed genetic background

(A) The average litter size and days to first litter were not statistically different between *Rhox13* WT and KO males. **(B)** In a graphical representation of time between litters, a horizontal gray line represents the average. Statistical significance is indicated by an asterisk (P=0.008).

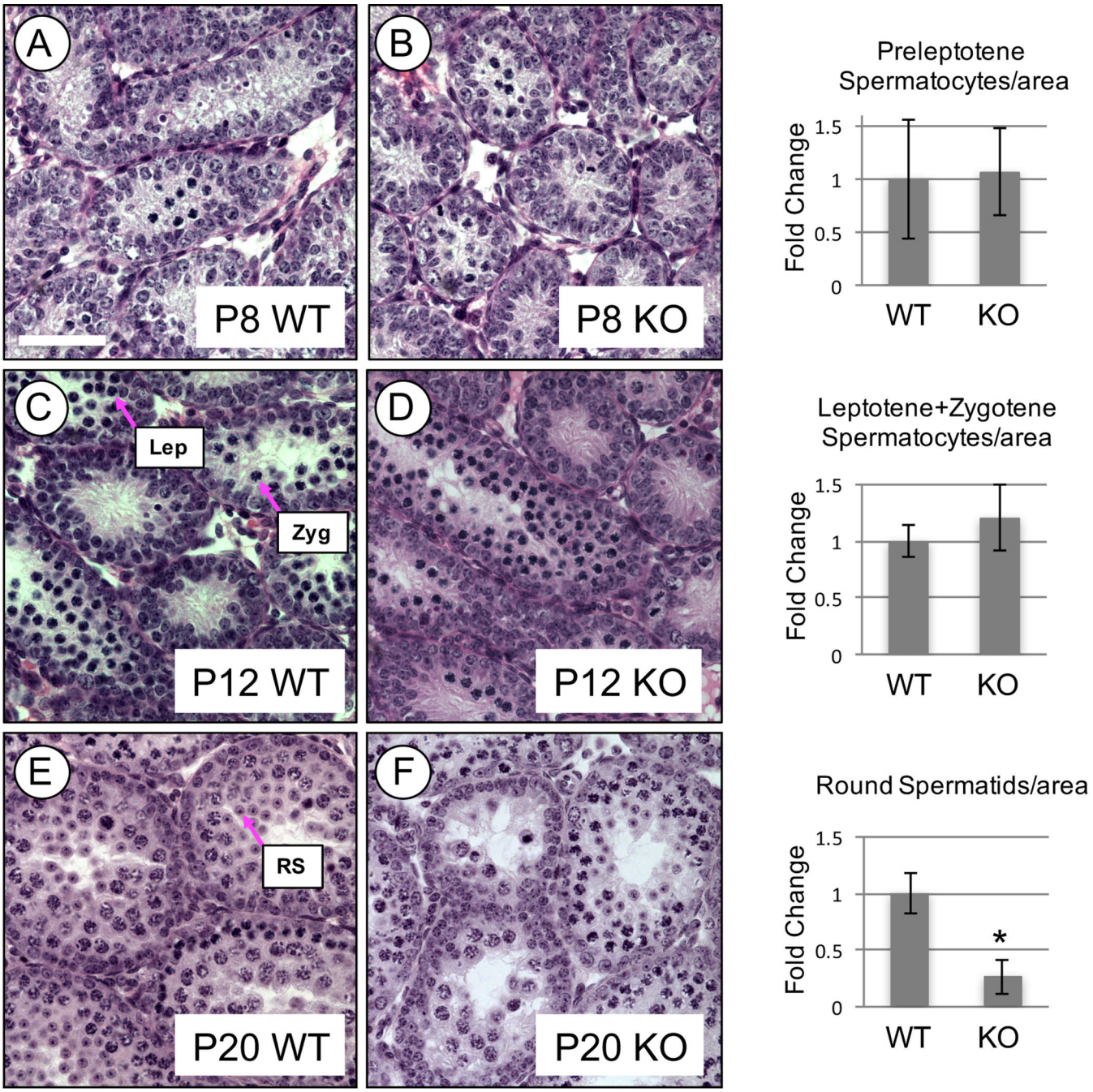

Figure 4. Developmental delays and histological abnormalities during the first wave of spermatogenesis in backcrossed *Rhox13* KO mice

There were no significant differences in the numbers of preleptotene spermatocytes at P8 (**A–B**) or leptotene (Lep) and zygotene (Zyg) spermatocytes at P12 (**C–D**). In contrast, P20 KO testes had significantly reduced numbers of round spermatids (RS) (**E–F**). Asterisk indicates significance at P=0.0002. Scale bar = 50 μm.

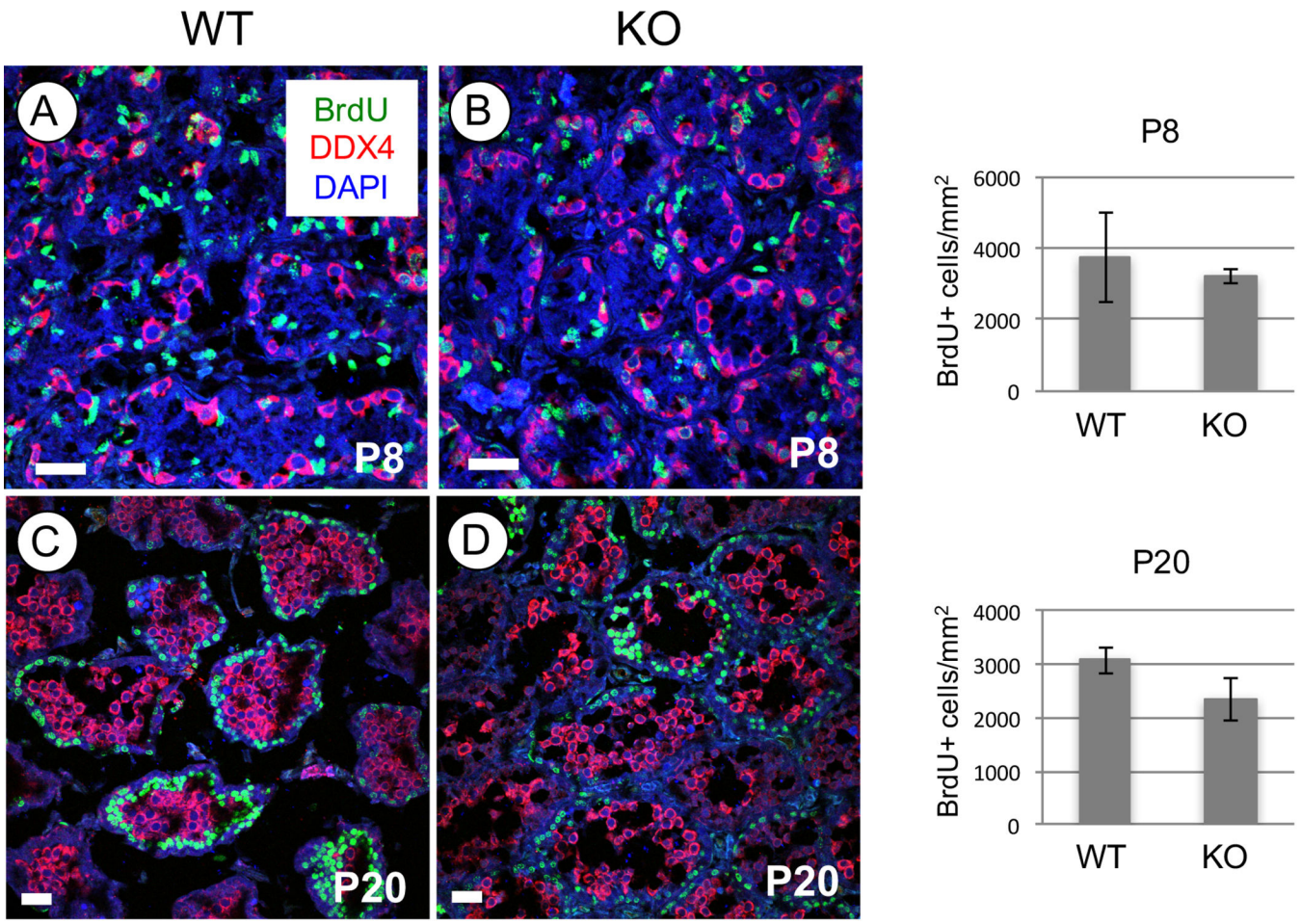

Figure 5. Germ cell proliferation decreases in backcrossed KO testes during the first wave of spermatogenesis

(**A–D**) Immunostaining was done to detect BrdU (green) and DDX4 (red), and nuclei were labeled with DAPI (blue) in testes from P8 (**A–B**) and P20 (**C–D**) WT (**A, C**) and KO (**B, D**) mice. Ages are indicated on each panel. Scale bars = 30 μm.

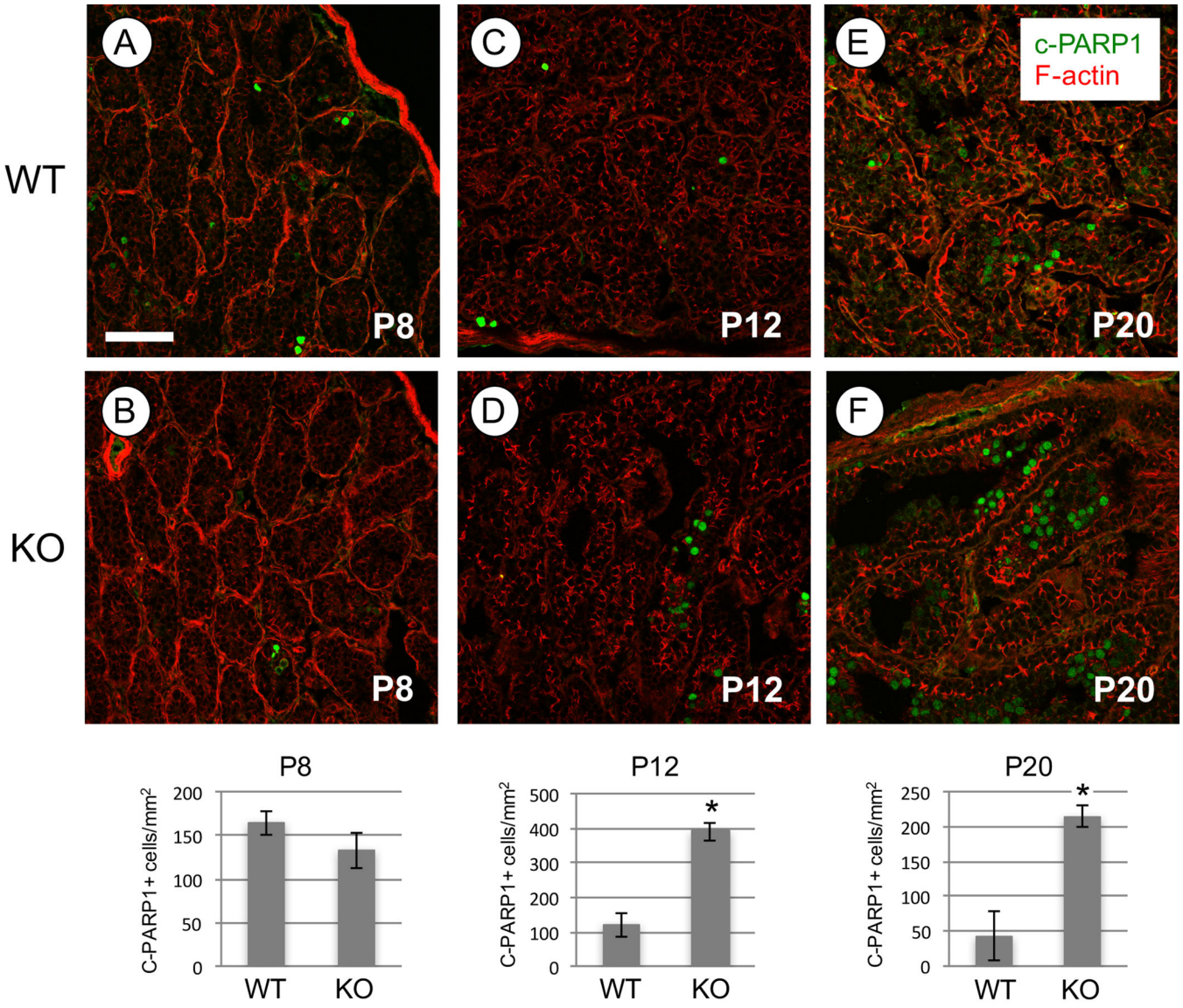

Figure 6. Increased germ cell apoptosis in backcrossed KO testes during the first wave of spermatogenesis

(**A–F**) Immunostaining was done to detect c-PARP1 (green), and phalloidin labeled F-actin (red). The c-PARP1+ cells were counted at each age and in each genotype, and the results are presented for each age below their respective panels as c-PARP1+ cells per mm^2 of seminiferous cord (P8, P12) or tubule (P20). Asterisk indicates statistical significance ($P<0.05$). Scale bar = 30 μm.

Table 1

Reproductive phenotype of backcrossed *Rhox13* KO males.

Genotype (Age)	Body weight (g)	Testis weight (mg)	Paired seminal vesicle weight (mg)	Cauda epididymal sperm (× 10^6/ml)
Rhox13$^{+/y}$ (WT, P50)	22.4 ± 0.9	86.4 ± 2.5	115.4 ± 12.0	2.5 ± 1.3
Rhox13$^{-/y}$ (KO, P50)	22.0 ± 1.3	82.5 ± 2.9	104.3 ± 35.2	1.7 ± 0.9
Rhox13$^{+/y}$ (WT, P56)	22.4 ± 0.2	87.5 ± 0.7	ND	9.41 ± 0.7 *
Rhox13$^{-/y}$ (KO, P56)	22.4 ± 1.3	78.7 ± 6.3	ND	5.71 ± 0.6 *
Rhox13$^{+/y}$ (WT, P120)	32.9 ± 4.0	100.9 ± 18.1	296.3 ± 30.7	12.5 ± 4.0
Rhox13$^{-/y}$ (KO, P120)	32.4 ± 1.3	92 ± 5.3	252.8 ± 151.8	13.9 ± 1.9

All values are presented as averages with standard deviation.

ND = not determined.

In comparisons of cauda epididymal sperm counts, asterisks indicate statistical differences, at P<0.001.

N = 3 WT, 4 KO (P50); N = 2 WT, 4 KO (P56); N = 4 WT, 4 KO (P120) WT, 4 KO (P50); N = 2 WT, 4 KO (P56); N = 4 WT, 4 KO (P120).