

Title Page

5 **Mice lacking Raf Kinase Inhibitor Protein-1 (RKIP-1) have altered sperm
capacitation and reduced reproduction rates with a normal response to testicular
injury**

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Running title:

RKIP deficiency in mice

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35 **Abstract**

Raf Kinase Inhibitor Protein (RKIP-1) belongs to the phosphatidyl ethanolamine-binding family of proteins (PEBP), which are highly conserved throughout evolution and widely expressed in tissues of mammalian organisms. RKIP-1 is a modulator of ERK, NF- κ B, and GPCR signaling cascades and is implicated as a factor in numerous physiological processes and disease states including metastasis. Testicular germ cells also express high levels of RKIP mRNA during spermatogenesis, particularly from late pachytene spermatocytes through step 15 elongate spermatids. Therefore, the sensitivity of spermatogenesis to injury was compared in wild type and RKIP-1^{-/-} mice. Unlike what has been described with tumor suppressors such as p53, RKIP-1^{-/-} and wild type mice were equally sensitive to germ cell toxicity by x-irradiation as assessed by TUNEL positivity 9h after a 5 Gy exposure and testicular spermatid head counts 15.5 days after 0.5 Gy exposure. Recent findings also indicate that RKIP is a decapacitation factor receptor on sperm. The present study demonstrates that sperm from RKIP deficient mice are precociously capacitated compared to their wild type counterparts. Data from mating experiments indicate decreased reproduction rates between crosses of RKIP-1^{-/-} mice and either heterozygous or RKIP-1^{-/-} females. Furthermore, RKIP immunolocalization of epididymal sperm supports transfer of the protein from germ cell cytoplasm to the sperm via the cytoplasmic droplet during epididymal transport. Overall, these studies indicate an important role for RKIP in reproduction as a modulator of capacitation, but not in the regulation of testicular injury.

Keywords: RKIP, PEBP, capacitation, sperm, testis

Introduction

Raf Kinase Inhibitor Protein (RKIP-1) is an evolutionary conserved member of the phosphatidyl ethanolamine-binding protein (PEBP) family. The expression of RKIP-1 is associated with numerous biological processes, most notably in the regulation of cancer cell metastasis and apoptosis (reviewed by Odabaei *et al.*, 2004; Keller *et al.*, 2005). Decreased expression of RKIP-1 in cancer cells is thought to contribute to chemotherapeutic resistance by modulating survival pathways (Chatterjee *et al.*, 2004), such as Raf/MEK/ERK (ERK) and NF- κ B signaling cascades (Yeung *et al.*, 2000; Yeung *et al.*, 2001; Park *et al.*, 2006). Experiments with prostate and breast cancer cell lines indicate a distinct correlation between RKIP-1 expression and the onset of apoptosis, with down regulation of RKIP-1 conferring resistance to chemotherapy-induced apoptosis (Chatterjee *et al.*, 2004).

Spermatogenesis is a complex process, in which germ cell proliferation is balanced by apoptosis, optimizing sperm output (reviewed by Holdcraft and Braun, 2004). Furthermore, testicular toxicants such as mono-(2-ethylhexyl) phthalate (MEHP) and x-irradiation are known to activate testicular apoptosis through NF- κ B and other pathways (Rasoulpour and Boekelheide, 2005; Rasoulpour and Boekelheide, 2007) in which RKIP-1 is involved (Yeung *et al.*, 2001). Notably, significant RKIP-1 mRNA expression occurs in the testis during spermatogenesis, particularly from late pachytene spermatocytes through step 15 elongate spermatids (Hickox *et al.*, 2002). Therefore, it is plausible that RKIP-1 may have a role in regulating testicular apoptotic events by modulating ERK and NF- κ B pathways.

80 In addition to functioning as a metastasis inhibitor gene, RKIP-1 is emerging as
an important regulator of male reproduction. Upon release from the male reproductive
tract, sperm are incapable of fertilization. Sperm undergo a series of post-ejaculatory
maturational events that culminate in the ability to fertilize an oocyte. This series of
biochemical modifications, collectively referred to as capacitation, is a reversible process
85 mediated by binding of decapacitation factors to sperm. As decapacitation factors are
lost, numerous molecular changes occur including a rapid influx of Ca^{2+} , hyperpolarizing
the sperm membrane. Uncapacitated sperm maintain low intracellular Ca^{2+} levels
through a calmodulin-sensitive Ca^{2+} -ATPase, primarily located in the post-acrosomal
region of the sperm head (Adeoya-Osiguwa and Fraser, 1996). The Ca^{2+} -ATPase
90 becomes inactivated in capacitated sperm, resulting in a rapid increase in intracellular
 Ca^{2+} . Gradients of Ca^{2+} differ between uncapacitated, capacitated, and acrosomal reacted
sperm, resulting in distinct banding detectible by chlortetracycline labeling (DasGupta *et*
al., 1994).

The identification of decapacitation factors has greatly contributed to a
95 mechanistic understanding of the capacitation process. Removal of decapacitation
factors from uncapacitated sperm results in the rapid acquisition of fertilizing ability,
which is reversible upon reincubation with decapacitation factors (Fraser, 1984).
Although the exact identity of decapacitation factors has remained elusive, partially
purified factors are isolated through gentle centrifugation of uncapacitated sperm (Fraser
100 *et al.*, 1990; Fraser, 1998). Recently, two laboratories independently identified RKIP-1
as a sperm surface protein acting as a decapacitating factor (Nixon *et al.*, 2006) or
decapacitation factor receptor (Gibbons *et al.*, 2005). Binding experiments performed

using recombinant RKIP-1 demonstrated that decapacitation factors can be competed out from the sperm, resulting in capacitated sperm (Gibbons *et al.*, 2005). RKIP-1

105 immunolocalization studies indicate extensive staining along the sperm, with prominent staining in the post-acrosomal region at the junction of the midpiece and head. These studies clearly demonstrate the importance of decapacitation factors that interact with RKIP-1 in the regulation of sperm capacitation and may have significant implications in reproduction.

110 The present study utilizes wild type and RKIP-1^{-/-} mice to examine the importance of RKIP-1 in the testis following x-irradiation, examining affects on germ cell apoptosis and spermatogenesis. Furthermore, the reproductive implications of RKIP-1 deficiency were explored in these knockout mice by examining sperm capacitation states, reproduction rates, and localization of the RKIP-1 protein on sperm.

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Materials and Methods

Chemicals. All chemicals were purchased from Sigma-Aldrich Corporation (St. Louis, MO) and were of reagent grade or better.

Animals. Adult wild type and RKIP-1^{-/-} mice were generated, bred, and maintained at 120 Brown University as previously described (Theroux *et al.*, 2007). The mice were housed in community cages within a temperature and humidity controlled vivarium with a 12h alternating light-dark cycle having free access to water and Purina Rodent Chow 5001 (Farmer's Exchange, Framingham, MA). The Brown University Institutional Animal Care and Use Committee approved all experimental animal protocols in compliance with 125 National Institute of Health guidelines. Testes were either preserved in 10% neutral

buffered formalin for histopathological analysis or placed in 154 mM saline supplemented with 247 nM thimerosal and 0.05% triton X-100 for determining spermatid head counts. For X-Gal staining, testes were dissected out, briefly prefixed in 5% formaldehyde, and embedded in 3% low melting temperature agarose before making
130 vibratome sections (Theroux *et al.*, 2007).

Staining with X-Gal. Whole testes or vibratome sections were stained as described previously (Theroux *et al.*, 2007). Briefly, testes were dissected out from CO₂ euthanized animals, fixed for 1-2 hours in 2% paraformaldehyde and exposed to 2mM MgCl₂-0.01%, deoxycholate-0.02% NP-40-100mM phosphate buffer (pH.8.0), 5 mM
135 K₄Fe(CN)₆, 5mM K₃Fe(CN)₆, and 1 mg/ml of X-Gal for 5 hours at 32°C. Vibratome sections of fixed testes were stained with X-Gal reagent for 10 hours.

Irradiation Exposure

Unanesthetized, male wild type and RKIP-1^{-/-} mice were exposed to lower body x-irradiation using a Philips 250-kVp X-ray machine. Single exposures were administered
140 of 0.5 or 5.0 Gy at a rate of 0.89 Gy/min, as calculated by a Radcal radiation meter (Monrovia, CA). Animals were restrained in polypropylene chambers and the upper two-thirds of the body were shielded with 3 mm of lead. At designated time points, animals were killed by CO₂ asphyxiation, and testes were immediately excised.

Detection of apoptosis. Terminal Deoxynucleotidyl Transferase Biotin-dUTP Nick End
145 Labeling (TUNEL) staining was conducted using paraffin embedded testis sections (7µm), and stained using an ApopTag Peroxidase *In Situ* Apoptosis Detection Kit (Chemicon, Temecula, CA) as directed by the manufacturer. Sections were counterstained with methyl green. Percent of seminiferous tubules with 0, 1-3, or >3

TUNEL positive nuclei were assessed by blinded counting all seminiferous tubules with a
150 major:minor axis less than 1.5:1 in two cross-sections using a Zeiss Standard microscope
(Karl Zeiss, New York, NY). At least 50 tubules were scored per animal for 0, 1–3, or
>3 TUNEL-positive nuclei (wild type, n = 6; RKIP-1^{-/-}, n = 4).

Testicular Spermatid Head Counts

The rate of sperm production (spermatid head counts per g of testis per day) was assessed
155 as a sensitive measurement of germ cell dysfunction. Both testes from each animal were
homogenized individually and spermatid heads were counted on a hemocytometer as
previously described (Blazak *et al.*, 1993). Counts from the two testes of each animal
were averaged for statistical analysis (n = 3 per genotype).

Sperm Capacitation Evaluation

160 Epididymal sperm were freshly isolated by mincing two caudal epididymides in 0.8ml of
154 mM saline. Following a 5 min incubation at 37°C, 100µL aliquots of sperm were
fixed with 8µL of 12.5% (w/v) paraformaldehyde and treated with 100µL
chlortetracycline (CTC) solution as previously described (DasGupta *et al.*, 1993).
Briefly, the CTC solution (750µM CTC, 130mM NaCl, 5mM cysteine, and 20mM Tris-
165 HCl, pH 7.8) was freshly prepared and stored in the dark at 4°C. Fixed sperm (10µL)
were imaged on a microscope slide following the addition of an equal volume of 220 mM
1,4-diazabicyclo[2,2,2]-octane diluted in 154mM saline (9:1) to resist fluorescence
quenching. A coverslip was applied and firmly pressed between two Kimwipes to
remove excess liquid. Sperm were analyzed by fluorescence microscopy using a Carl
170 Zeiss Axiovert 35 microscope (New York, NY) with the appropriate fluorescence filters
(BP 546, FT 580, LP 590). The CTC banding patterns were compared with previously

published images of uncapacitated, capacitated, and acrosomal reacted sperm (Ward and Storey, 1984; DasGupta *et al.*, 1993). Three hundred sperm were counted per mouse (n = 3 mice per genotype).

175 *Sperm Immunolocalization*

Sperm were freshly isolated from caput and cauda epididymides as described above and streaked on a Fisher Super+ glass slides (Fisher Scientific, Hampton, NH). After air drying at room temperature, the slides were fixed in a 2% formaldehyde solution, followed by a 0.1% Triton X-100 in PBS wash. The sperm were blocked in 5% Normal
180 goat serum (NGS) in PBS supplemented with 0.1% Triton X-100. Antibody raised against RKIP-1 (Upstate Biotechnology, Lake Placid, NY) was diluted in 1% NGS in PBS with 0.1% Triton X-100. Detection of the primary antibody was conducted with a TRITC secondary antibody (Sigma, St. Louis, MO) incubated in 1% NGS in PBS with
185 0.1% Triton X-100. Sperm were analyzed by fluorescence microscopy as described above.

Statistical Analysis. Results are expressed as mean \pm standard error of the mean (SEM). Statistical differences for all figures were determined by ANOVA and Bonferroni's post hoc analysis. Statistical differences for breeding studies were assessed using a log linear model with adjustment for over-dispersion of Poisson distribution. Values were
190 considered significantly different with $p < 0.05$.

Results

Testis RKIP-1 Expression

The mouse strain used in these studies carries a gene-trapped allele expressing β -*geo* reporter controlled by the native RKIP-1 promoter (Theroux *et al.*, 2007). The β -*geo*

195 is a fusion protein composed of the neo resistance domain and bacterial β -galactosidase activity, which is detectable upon exposure to X-Gal. The tissue expression pattern of β -*geo* can thus be followed in these animals, and is believed to reflect the expression of the native gene into which the gene-trap integration has occurred.

Whole testis isolated from RKIP-1^{-/-} and wild type males were stained with X-
200 Gal. Seminiferous tubules from RKIP-1^{-/-} testis stained more intensely compared to wild type (Fig. 1A). Within the same tubule certain regions stained stronger than others, suggesting stage-dependent expression of the reporter during spermatogenesis (Fig. 1B). In contrast, wild type testis revealed some degree of non-specific staining detectable within the interstitium, but not in seminiferous tubules (Fig. 1C). This staining can be
205 attributed to the weak activity of endogenous β -galactosidase. Higher power magnification (Fig. 1D) revealed that the layer of cells adjacent to the basal lamina did not stain at any stage of spermatogenesis. This layer consists of spermatogonia and early spermatocytes. More advanced spermatocytes and spermatids revealed various levels of reporter expression. The overall stage-dependent expression was very similar to that
210 reported previously (Frayne *et al.*, 1998; Hickox *et al.*, 2002). These staining experiments confirm previously reported high expression levels of RKIP-1 in male testis and suggest its potential role in spermatogenesis (Jones *et al.*, 1983; Araki *et al.*, 1992; Rankin *et al.*, 1992; Frayne *et al.*, 1998; Theroux *et al.*, 2007).

Germ Cell Susceptibility to Ionizing Radiation-Induced Apoptosis

215 Wild type and RKIP-1^{-/-} mice were exposed to 5 Gy of lower body x-irradiation and killed 9h post-exposure (Figure 2). This dosing regimen is associated with significant increases in germ cell apoptosis (Hasegawa *et al.*, 1997). Based on the

quantification of seminiferous tubules with 0, 1-3, or >3 TUNEL-positive cells, no differences in susceptibility to apoptosis were detected between wild type and RKIP-1^{-/-} mice. These results suggest that RKIP-1 does not regulate apoptotic changes in murine germ cells following x-irradiation.

Meiotic Germ Cell Sensitivity to Ionizing Radiation Exposure

Wild type and RKIP-1^{-/-} mice were exposed to 0.5 Gy lower body irradiation and killed 15.5 days post exposure (Figure 3). The x-irradiated meiotic germ cells were allowed to develop over 15.5 days into elongate spermatids, and the apoptotic effect of the exposure was quantified by spermatid head counts as a sensitive measure of spermatogenesis. The similarity in testicular spermatid head counts between wild type and RKIP-1^{-/-} mice indicated that the meiotic spermatocytes in these two genotypes were equally susceptible to ionizing radiation.

RKIP-1^{-/-} Mice Undergo Premature Capacitation

Given the recent findings that RKIP-1 may act as a decapacitation factor (Nixon *et al.*, 2006) or as a receptor for decapacitation factors (Gibbons *et al.*, 2005), epididymal sperm from wild type and RKIP-1^{-/-} mice were evaluated for capacitation status using chlortetracycline imaging (Ward and Storey, 1984; Lee *et al.*, 1987; DasGupta *et al.*, 1994; Fuller and Whittingham, 1997). Wild type sperm were evenly distributed between uncapacitated (44.9%) and capacitated (46.8%) states after a 5 min incubation in saline (Figure 4). However, RKIP-1^{-/-} mice had a significantly higher percentage of capacitated (80.3%) sperm and fewer uncapacitated (10.3%) sperm. There were no appreciable differences in acrosome reacted sperm measured between genotypes (8.3% wild type;

240 9.4% RKIP-1^{-/-}). These results indicate that sperm from RKIP-1^{-/-} mice undergo precocious capacitation.

RKIP-1^{-/-} Mice Exhibit Reduced Reproduction Rates

Since premature capacitation would be expected to lead to reduced reproduction efficiency, the breeding rate was evaluated within the RKIP-1 colony. The lack of
245 embryonic lethality from RKIP-1 deficiency was previously demonstrated by the normal Mendelian distribution of genotypes (Theroux *et al.*, 2007). Comprehensive analysis of the progeny genotypes of 10 breeding pairs per possible genotype pairing revealed that RKIP-1^{-/-} male mice exhibited a reduction in reproduction rates when mated with females lacking either one ($p = 0.02$) or both RKIP-1 wild type alleles ($p = 0.0002$) (Table 1).
250 Interestingly, heterozygous male mice mated to RKIP-1^{-/-} females demonstrated normal reproduction rates compared to wild type.

Sperm acquire RKIP-1 from the Cytoplasmic Droplet

Immunostaining of caput epididymal sperm showed that RKIP-1 was exclusively localized within the cytoplasmic droplet (Figure 5A). The cytoplasmic droplet is residual
255 germ cell cytoplasm, which is reabsorbed during maturation of sperm within the epididymis. Analysis of caudal epididymal sperm (Figure 5B) showed that nearly all sperm had complete resorption of the cytoplasmic droplet with extensive RKIP-1 staining in the post acrosomal region and along the tail. Thus, these results suggest that RKIP may be transferred from germ cell cytoplasm to mature sperm via the cytoplasmic
260 droplet.

Discussion

Given the abundant presence in epididymal fluids (Jones *et al.*, 1983) and high
265 expression during spermatogenesis (Frayne *et al.*, 1998; Hickox *et al.*, 2002), RKIP-1 is
suspected to play an important role in the male reproductive tract. Spermatogenetic germ
cell proliferation is associated with apoptotic changes that regulate sperm output
(reviewed by Holdcraft and Braun, 2004). Upon exposure to testicular toxicants,
apoptosis occurs through induction of NF- κ B (Rasoulpour 2005, Rasoulpour 2007),
270 which also interacts with RKIP-1 (Yeung *et al.*, 2001), and through activation of other
cell signaling systems (Embree-Ku *et al.*, 2002). In tumor cell lines, RKIP-1 sensitizes
cells to apoptotic death upon exposure to chemotherapeutic drugs (Chatterjee *et al.*,
2004). Therefore we investigated the involvement of RKIP-1 in germ cell apoptosis
following x-irradiation in wild type and RKIP-1^{-/-} mice. Lower body exposure of 5 Gy
275 irradiation produced similar levels of germ cell apoptosis in both mouse genotypes, 9h
post exposure. Similarly, wild type and RKIP-1^{-/-} mice exposed to 0.5 Gy irradiation
showed no differences in testicular spermatid head counts 15.5d post exposure, a
sensitive measure of germ cell loss. Taken together, these results indicate a similar
susceptibility of wild type and RKIP-1^{-/-} germ cells to acute or delayed onset, irradiation-
280 induced apoptosis. These findings were somewhat surprising, given the known
importance of RKIP-1 in sensitizing cancer cells to drug-induced apoptosis (Chatterjee *et al.*, 2004), and indicate that RKIP-1 does not participate in the complex regulation of
germ cell apoptosis following ionizing radiation in the testis (Embree-Ku *et al.*, 2002;
Rasoulpour and Boekelheide, 2007). Another explanation is that RKIP-2, an RKIP-1

285 homolog that is prominently expressed within the testis (Hickox *et al.*, 2002), may provide some compensatory sensitization to apoptosis in the RKIP-1^{-/-} mice.

Capacitation is a highly regulated process in sperm maturation, requiring removal of surface decapacitation factors within the female reproductive tract. The loss of decapacitation factors influences changes in sperm motility and enables the acrosomal reaction required for fertilization. Recent reports indicate that RKIP-1 is either a
290 decapacitation factor or a surface receptor for sperm decapacitation factors (Gibbons *et al.*, 2005; Nixon *et al.*, 2006), suggesting that sperm from RKIP-1^{-/-} mice may undergo premature capacitation. Indeed, using the CTC staining technique, significantly higher levels of capacitated sperm were seen in cauda epididymides from RKIP-1^{-/-} mice
295 compared to wild type. Although many functional aspects of the capacitation process remain controversial, a significant body of evidence suggests that capacitation influences sperm motility, chemotactic responsiveness, and fertilization (Cohen-Dayag *et al.*, 1995; Jaiswal *et al.*, 1998; Zhu *et al.*, 2006). Therefore, we examined the affects of RKIP-1 deficiency on mating success. Conceivably, if capacitation is a critical regulator of sperm
300 maturation, functional differences between genotypes may result in different mating efficiencies. Interestingly, reproduction rates for all mating combinations were unremarkable except for RKIP-1^{-/-} males mated to either heterozygous or RKIP-1^{-/-} females.

An unanswered question has been the source of RKIP-1 protein expression on
305 sperm. Mature sperm lack the cellular machinery to produce proteins; therefore RKIP-1 must be acquired either from an earlier germ cell developmental stage or from another source within the male reproductive tract. Previous reports surmised that the

"phosphatidyl binding protein" may be transferred from the residual germ cell cytoplasm (Vierula *et al.*, 1992; Saunders *et al.*, 1995). The present study confirms the localization of RKIP-1, demonstrating probable transfer of RKIP-1 from the cytoplasmic droplet to the post acrosomal region of the sperm head and midpiece during maturation in the epididymis. This staining differs slightly from a previously published report (Gibbons *et al.*, 2005), which showed more extensive RKIP staining on the sperm head. The present RKIP staining differences are likely explained by the use of different RKIP antibodies or perhaps the method of epididymal sperm isolation.

The apparent capacity of sperm to accept and integrate RKIP-1 from the cytoplasmic droplet is consistent with our observations of reproductive efficiency. Since the reproduction rates are only decreased in crosses between RKIP-1^{-/-} males and either heterozygous or RKIP-1^{-/-} females, the RKIP-1 deficient sperm could be acquiring RKIP-1 protein within the female reproductive tract when insufficient RKIP-1 protein is available to the male sperm during spermatogenesis. A biological role for RKIP-1 secretion in the female could be to provide a dynamic equilibrium for the removal of decapacitation factors from the sperm. As sperm travel within the female reproductive tract, RKIP-1 generated by the female (Frayne *et al.*, 1999) could facilitate the disassociation of decapacitation factors away from the sperm, thus initiating capacitation. Conversely, the equilibrium could also favor sperm re-acquiring decapacitation factors in the case of RKIP-1^{-/-} sperm in a wild type or heterozygous female, facilitating a dynamic process of cycling between capacitated and uncapacitated sperm as previously demonstrated *in vitro* (Gibbons *et al.*, 2005). Although this unique form of protein

330 transfer fits with the current data, further research is necessary to confirm this potential
mechanism.

The present study demonstrates similar susceptibility between wild type and
RKIP-1 deficient mice to ionizing radiation in the testis. However, important differences
exist between these genotypes with regard to sperm capacitation status. These findings
335 suggest that RKIP-1 is an important component in male reproduction and may be an
important therapeutic target for improving success rates of male infertility or providing
male contraception.

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460

Figure Legends

Figure 1. X-Gal staining.

465 A. Whole testes from 10-week old RKIP-1^{-/-} (left) and wild-type (right) males. Note that the seminiferous tubules of RKIP^{-/-} testicle stain to differing degrees, suggesting stage-dependent expression of the reporter.

B. Low magnification cross-section of 10-week old heterozygous testis stained for LacZ.

C. Low magnification cross-section of 10-week old wild type testis stained for LacZ.

470 Arrows indicate minor staining within the interstitial space.

D. Higher power magnification of a cross-section through the reporter positive testis. Arrows indicate cells adjacent to basal lamina that do not stain with X-Gal.

Figure 2. Quantification of seminiferous tubules with 0, 1-3, or >3 TUNEL-

475 positive cells following exposure to 5 Gy ionizing radiation. Wild type (gray bar, n=6) and RKIP-1^{-/-} (black bar, n=4) mice were sacrificed 9h after lower body x-irradiation to assess susceptibility to germ cell apoptosis. No statistical differences were detected between the genotypes (p > 0.05).

480 **Figure 3.** Analysis of wild type and RKIP-1^{-/-} meiotic germ cell sensitivity to ionizing
radiation exposure as measured by sperm production. Wild type (gray bar, n = 3) and
RKIP-1^{-/-} mice (black bar, n = 3) were exposed to 0.5 Gy lower body ionizing radiation
and spermatocytes were allowed to age 15.5 days post-exposure, developing into
elongate spermatids. No statistical differences were detected between the
485 genotypes ($p > 0.05$).

Figure 4. Sperm capacitation states differ in wild type and RKIP-1^{-/-} mice.

A. Epididymal sperm were imaged with chlortetracycline to identify sperm banding
patterns consistent with uncapacitated, capacitated, and acrosome reacted states. Arrow
490 indicates darker banding pattern associated with capacitated sperm.

B. The percent of capacitated sperm was significantly increased in RKIP-1^{-/-} mice (black
bar, n = 3) compared to wild type mice (gray bar, n = 3). Asterisks (*) indicate
significant differences from wild type ($p < 0.05$).

495 **Figure 5.** RKIP-1 immunostained caput and cauda epididymal sperm from wild
type mice. Merged fluorescent and differential interference contrast digital images of
sperm are shown.

A. Caput epididymal sperm with RKIP-1 staining localized to the cytoplasmic droplet.
Magnified representative image of a caput epididymal sperm is shown below.

500 B. Cauda epididymal sperm with RKIP-1 staining localized to the cytoplasmic droplet or

diffuse staining along the sperm flagellum. Immunostaining of sperm from RKIP-1^{-/-} mice produced no detectible fluorescence (data not shown). Bar = 20μm. Magnified representative image of a cauda epididymal sperm is shown below.

Table 1. The number of pups obtained from 10 mating pairs with defined genotypes

| Male | Female | Number of Pups in 16 Weeks | Number of Litters | Average Number of Pups/ Litter |
|------|--------|----------------------------|-------------------|--------------------------------|
| +/+ | +/+ | 130 | 17 | 7.64 |
| +/+ | +/- | 139 | 17 | 8.17 |
| +/+ | -/- | 152 | 21 | 7.23 |
| | Total | 421 | | |
| +/- | +/+ | 131 | 17 | 7.70 |
| +/- | +/- | 130 | 21 | 6.19 |
| +/- | -/- | 121 | 17 | 7.11 |
| | Total | 382 | | |
| -/- | +/+ | 141 | 18 | 7.83 |
| -/- | +/- | 63 ^a | 10 | 6.30 |
| -/- | -/- | 11 ^b | 3 | 3.66 |
| | Total | 215 | | |

Letters indicate statistical differences compared to the number of pups born in 16 weeks from wild type x wild type crosses (+/+ x +/+) a, p = 0.02; b, p = 0.0002.

Figure 1.

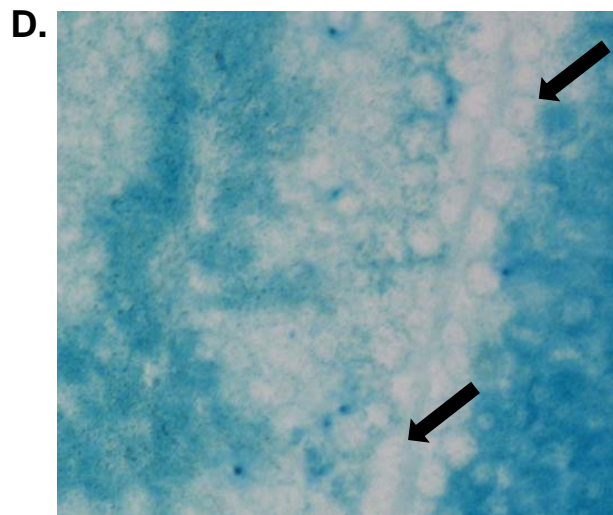
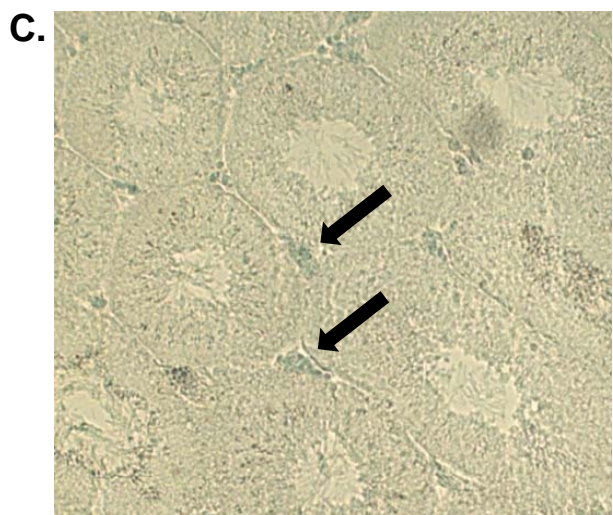
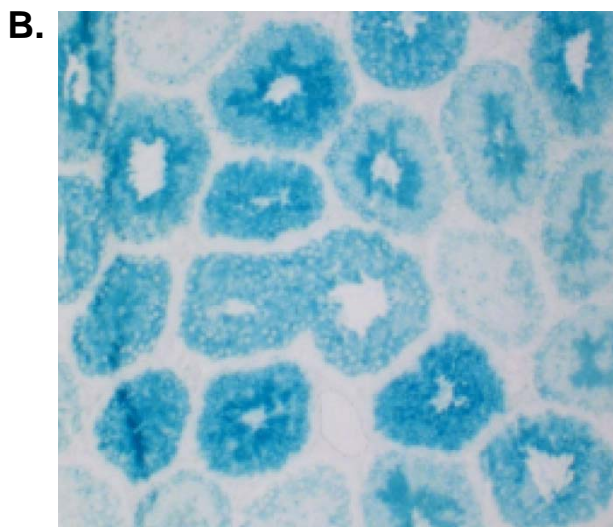


Figure 2.

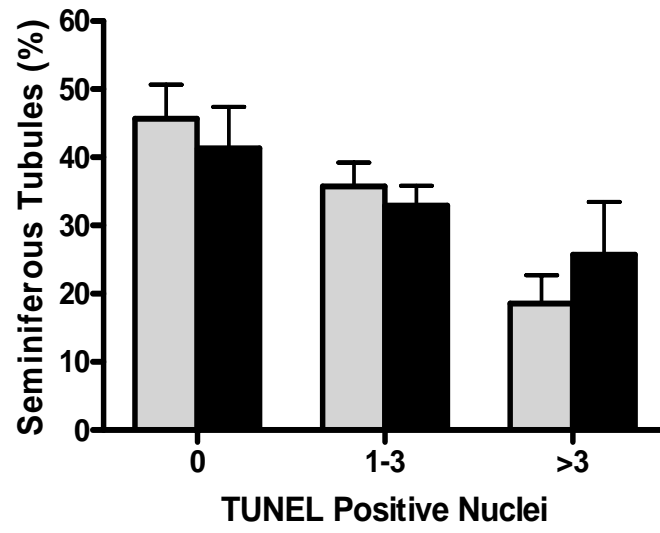


Figure 3.

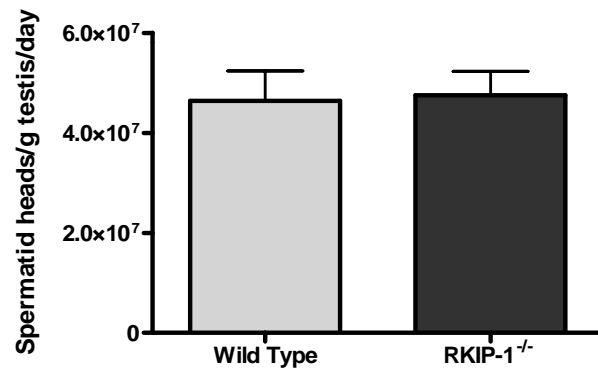


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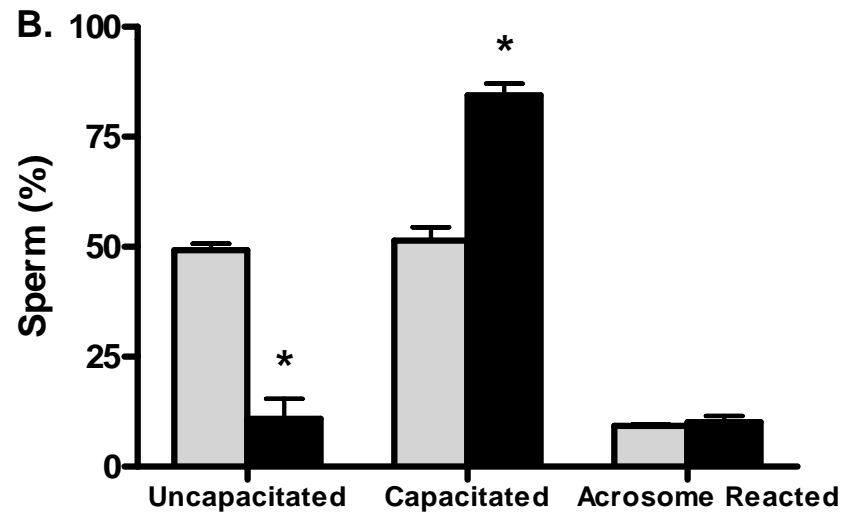
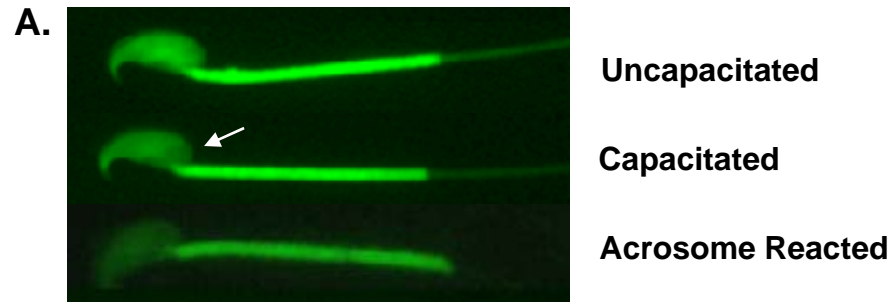
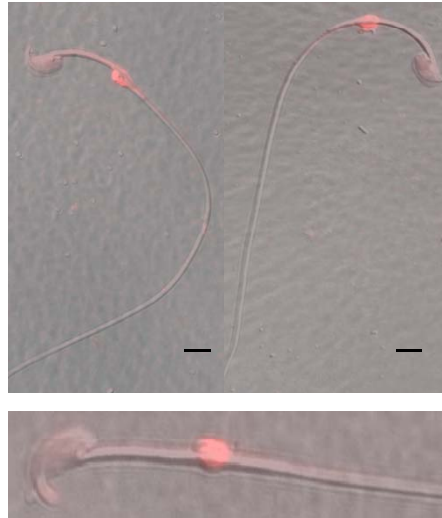


Figure 5.

A.



B.

