AKAP9 Is Essential for Spermatogenesis and Sertoli Cell Maturation in Mice

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ABSTRACT Mammalian male fertility relies on complex inter- and intracellular signaling during spermatogenesis. Here we describe three alleles of the widely expressed A-kinase anchoring protein 9 (*Akap9*) gene, all of which cause gametogenic failure and infertility in the absence of marked somatic phenotypes. *Akap9* disruption does not affect spindle nucleation or progression of prophase I of meiosis but does inhibit maturation of Sertoli cells, which continue to express the immaturity markers anti-Mullerian hormone and thyroid hormone receptor alpha in adults and fail to express the maturation marker p27^{Kip1}. Furthermore, gap and tight junctions essential for blood–testis barrier (BTB) organization are disrupted. Connexin43 (Cx43) and zona occludens-1 are improperly localized in *Akap9* mutant testes, and Cx43 fails to compartmentalize germ cells near the BTB. These results identify and support a novel reproductive tissue-specific role for *Akap9* in the coordinated regulation of Sertoli cells in the testis.

REPRODUCTION in male mammals relies on sex- and age-dependent signal transduction events. Sertoli cells undergo maturation from birth through puberty, with changing gene expression and morphology as they halt proliferation (for review see (Sharpe *et al.* 2003). This differentiation includes the formation of the blood–testis barrier (BTB), separating the mitotic/spermatogonial compartment from the meiotic/spermatocyte compartment (Petersen and Soder 2006; Mital *et al.* 2011). Preleptotene spermatocytes must traverse the BTB, requiring Sertoli cell-regulated disassembly and reassembly of tight and gap junctions (Griswold 1995, 1998; Cheng and Mruk 2002; Wong and Cheng 2005).

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Connexin43 (Cx43, also known as GJA1) organizes BTB gap junctions between Sertoli cells and those between Sertoli and germ cells (Juneja *et al.* 1999; Plum *et al.* 2000; Roscoe *et al.* 2001; Mruk and Cheng 2004; Pointis and Segretain 2005; Weider *et al.* 2011). Cx43 is localized to Sertoli cells at the basal region of the seminiferous tubule and participates in germ and Sertoli cell migration, differentiation, and survival (Sridharan *et al.* 2007; Gilleron *et al.* 2009). Basal tight junctions between Sertoli cells form the principal structural component of the BTB by day 16 (Nagano and Suzuki 1976). The expression of the tight-junction protein, zonula occludens 1 (ZO-1, also known as TJP1), in the testis is restricted primarily to the inter-Sertoli cell tight junction interface by puberty (Byers *et al.* 1991). In Sertoli cells, ZO-1 is known to interact with Cx43 to regulate gap junction integrity (Giepmans and Moolenaar 1998; Toyofuku *et al.* 1998; Hunter *et al.* 2005) and proliferation (Rhett *et al.* 2011) and thus is an important functional marker for Sertoli cell tight junctions and maturation.

Protein kinase A (PKA) signaling cascades *in vivo* are numerous and insulated from one another via A-kinase anchoring protein (AKAP) scaffolds (Wong and Scott 2004). AKAPs localize protein kinase A and other cAMP-responsive proteins to specific subcellular compartments, thereby organizing combinations of enzymes to respond specifically to

intracellular second messengers (reviewed in Colledge and Scott 1999; Feliciello *et al.* 2001; Wong and Scott 2004). AKAPs play critical roles in spermatogenesis, and multiple AKAPs have been identified in varying stages of spermatogenic development and in sperm function (Carrera *et al.* 1994; Turner *et al.* 1999; Vijayaraghavan *et al.* 1999; Reinton *et al.* 2000; Furusawa *et al.* 2001; Miki *et al.* 2002).

One major AKAP family member, AKAP9, was first described as an *N*-methyl-D-aspartic acid (NMDA) receptor anchor in the brain (Lin *et al.* 1998) and was further characterized in the brain and heart (Feliciello *et al.* 1999; Kurokawa *et al.* 2004; Ciampi *et al.* 2005; Chen *et al.* 2007; Piggott *et al.* 2008; Chen and Kass 2011; Chopra and Knollmann 2011). Mutations in human *AKAP9* have been implicated in breast cancer (Milne *et al.* 2011), sporadic papillary thyroid carcinomas (Lee *et al.* 2006), and long QT syndrome (Chen *et al.* 2007). In rat Sertoli cells, AKAP9 coordinates PKA and phosphodiesterase 4D3 (Tasken *et al.* 2001). Large isoforms of AKAP9 are associated with centrosomal function and signaling, microtubule organization, and nucleation at the *cis*-Golgi (Sillibourne *et al.* 2002; Takahashi *et al.* 2002; Keryer *et al.* 2003a; McCahill *et al.* 2005; Rivero *et al.* 2009). To date, no functional role has been described for AKAP9 in spermatogenesis.

Previously, we conducted forward genetic screens to identify mutant alleles of novel genes necessary for gametogenesis in *Mus musculus* (Ward *et al.* 2003; Lessard *et al.* 2004). Here, via positional cloning of two such alleles and complementation analyses with a targeted null allele, we report that mutation of *Akap9* selectively disrupts male fertility and somatic cell-germ cell organization. These findings underscore the importance of highly regulated intracellular and intercellular signaling between these compartments for spermatogenesis in mammals. Furthermore, the results are significant with respect to the major physiological requirement of the widely studied AKAP9 protein, which hitherto had been demonstrated to be very important for nonreproductive functions.

Materials and Methods

Animal care and use

All animal care and use related to this study was done in strict accordance with procedures and recommendations in the National Research Council *Guide for the Care and Use of Laboratory Animals* and was approved by the institutional animal care and use committees of all affiliated institutions.

Genetic mapping

Linkage was implicated by the association of phenotype with homozygosity for marker loci of the mutagenized parental strains, 129S1/SvImJ and C57BL/6J for *mei2.5* and *repro12*, respectively. Heterozygous animals of both strains were crossed to wild-type CAST/Ei animals to take advantage of the higher degree of polymorphism between parental strains and CAST/Ei. Separate intersubspecific intercrosses were conducted between animals heterozygous for *mei2.5* and *repro12*. Resulting progeny were phenotyped with respect to infertility or testis histology, and genotyped with microsatellite or SNP markers in the proximal region of Chr 5, as indicated in Supporting Information, Figure S1.

Generation of Akap9 knockout mice

Targeted ES cell clone D01 was acquired from the Knockout Mouse Project (KOMP) repository at the University of California Mouse Biology Program. This clone (from C57BL/6N) contains an exon-trapping cassette splice acceptor, beta galactosidase neomycin, poly adenylation (SA-β geo -pA) flanked by Flp-recombinase target (FRT) sites within the intron upstream of exon 8, and loxP sites on either side of exon 8 (see Figure 2). Generated at the Sanger Institute, it has the official allele name of $Akap9^{tm1a(KOMP)Wtsi}$ (abbreviated $Akap9^{Gt}$, where "Gt" stands for gene trap). These ES cells were injected into blastocysts of CD1 albino mice and the resultant male chimeras were backcrossed to C57BL/6J females. $Akap9^{Gt}$ mice were crossed to constitutive Cre mice obtained from The Jackson Laboratory (B6.FVB-Tg(EIIa-cre) C5379Lmgd/J) to create the exon-8-deleted allele referred to as $Akap9^{-}$, that still contains the vector insertion and exon trapping features (Table 1).

Genotyping and histology

DNA from 2-mm tail tips or ear tags was used for standard and allele-specific PCR reactions. Primer sequences are given below. For histology, testes were fixed in Bouin's solution for >24 hr, paraffin embedded, and 5-μm sections were cut and stained with hematoxylin and eosin (H&E).

Immunoblotting

Organ protein extracts were prepared with radioimmunoprecipitation assay (RIPA) buffer, separated on a 4–12% NuPAGE Bis-Tris gel (Invitrogen, Carlsbad, CA), transferred to nitrocellulose membrane, and blocked overnight. Immunoprobing was carried out at room temperature for 1 hr for primary and secondary antibodies followed by washes and chemiluminescent imaging. Antibodies to AKAP9 (a24, recognizes human *AKAP9* exons 24–27) were a kind gift of M. Bornens.

RT–PCR

RNA was isolated with the Qiagen RNeasy mini kit (Germantown, MD). cDNA was synthesized via reverse transcription of 1 μg of RNA using the Protoscript First-Strand cDNA Synthesis Kit (New England Biolabs, Ipswitch, MA). PCR using primers listed above was performed with Platinum Taq Hi-Fi (Invitrogen).

Immunohistochemistry and immunocytochemistry

For immunohistochemistry (IHC), testis tissue was fixed in neutral-buffered formalin, sectioned, mounted, deparaffinized, and rehydrated. Endogenous peroxidases were blocked

Table 1 List of primers used in genotyping and RT–PCR

Genotyping	Forward	5′-GGA TAG AGT AAT CTT GAC TCA-3′
	Wild-type reverse	5′-CGG CAG CAT GTG CAT ACC-3′
	Mutant reverse	5′-CGG CAG CAT GTG CAT ACT-3′
RT–PCR	Akap9E12F	5′-CCA TGA ACA ACA GAC AGA TGG T-3′
	Akap9E15R	5′-GCT ATT TCT TCT TCC AGT CG-3′
	AMH forward	5′-GCA GTT GCT AGT CCT ACA TC-3′
	AMH reverse	5′-TCA TCC GCG TGA AAC AGC G-3′
	THRA forward	5′-GGA GAT GAT TCG CTC ACT GCA G-3′
	THRA reverse	5′-CAG CCT GCA GCA GAG CCA CTT CCG T-3′

in 0.6% hydrogen peroxide in cold methanol and slides were autoclaved for 20 min in 10 mM citric acid (pH 6.0) to retrieve antigenicity. Sections were washed in PBS, blocked, and incubated with primary antibody overnight at 4°. Slides were washed in PBS and incubated 1.5 hr with secondary antibody at 25°, counterstained with Hoechst 33342 or DAPI (0.02 μg/mL) in PBS, and mounted with SlowFade Component A (Molecular Probes, S2828). For immunocytochemistry (ICC), testes from adult males (>60 days postbirth) were harvested, and meiotic chromosome spreads were prepared and immunolabeled as previously described (Peters *et al.* 1997; Reinholdt *et al.* 2004). For testis squashes, adult testes were harvested, and germ cell squashes were prepared and immunolabeled as previously described (Novello *et al.* 1996).

Antibodies

All antibodies used and their concentrations are described in Table 2.

Statistical analysis

To determine significance in Wilm's tumor-1 (WT-1)-positive Sertoli cell per tubule counts, ~20 tubular cross-sections were counted in each of three animals per genotype (wild type, $Akap9^{mei2.5/mei2.5}$, $Ccnb1IP1^{mei4/+}$, and $Ccnb1IP1^{mei4/mei4}$) and a one-tailed Student's *t*-test was conducted with a level of significance at $P < 0.01$. Levene's test was performed to verify equal variance using the statistical analysis software SPSS. For analyzing RT–PCR expression of anti-Mullerian hormone (AMH) and thyroid hormone receptor alpha (THRA), net band intensities of each sample were measured using Kodak 1D Image Analysis software (v3.6), and ratios between each Sertoli cell marker and age-matched β-actin controls were calculated. Assays were conducted in triplicate, and ratio cohorts averaged. Significance between age-matched groups was determined using one-tailed paired-sample Student's *t* tests with equal variance.

Results

Mutations in Akap9 disrupt spermatogenesis and alter transcript splicing and protein expression in multiple tissues

We previously described two male infertility mutations called *mei2.5* and *repro12* that exhibited abnormal testicular histology (Ward *et al.* 2003; Lessard *et al.* 2004, http://reproductivegenomics.jax.org/mutants/G1-448-151.html). Homozygous males display an incomplete failure of gametogenic progression at approximately, but not uniformly, metaphase I during epithelial stage XII (Figure 1, A *vs.* B and C *vs.* D). Spermatocytes are mislocalized in mutant animals, and round spermatids and luminal spermatozoa are rarely observed.

To identify the mutations responsible for the phenotypes of *mei2.5* and *repro12*, genetic mapping was performed. The analyses placed these alleles proximal to markers *rs13478098* and *D5Mit344*, respectively, on chromosome 5 (Figure S1A, gray box). Because *mei2.5* and *repro12* map to the same region and have similar phenotypes, we performed complementation tests. All males doubly heterozygous for the two mutations (Figure 1, F and G) were phenotypically indistinguishable from mutant homozygotes (Figure 1, B and D), indicating that these mutations are allelic.

Table 2 List of antibodies used in immunohistochemistry analyses

Antigen	Host	Clonality	Concentration	Manufacturer	λ	Cat. no.
Primary						
SUMO-1	Mouse	Monoclonal	2.5 μg/mL	Zymed Laboratories		33-2400
SYCP3	Rabbit	Polyclonal	5 μg/mL	Abcam		ab15092
Cx43 (GJA1)	Mouse	Monoclonal	2.5 μg/mL	Zymed Laboratories		35-5000
ZO-1 (TJP1)	Rabbit	Polyclonal	1.25 μg/mL	Zymed Laboratories		40-2200
p27^{Kip1}(Cdkn1b)	Rabbit	Monoclonal	4 μg/mL	Abcam		ab7961
SMA (ActaA2)	Mouse	Monoclonal	Unknown, 1:50	Sigma		A2547
WT-1	Rabbit	Polyclonal	1 μg/mL	Santa Cruz Biotech.		sc-192
Secondary					λ	Cat. no.
Mouse IgG	Goat	Polyclonal	5 μg/mL	Molecular Probes	488	A-11001
Mouse IgG	Goat	Polyclonal	5 μg/mL	Molecular Probes	594	A-11005
Rabbit IgG	Goat	Polyclonal	5 μg/mL	Molecular Probes	594	A-11012
Rabbit IgG	Goat	Polyclonal	5 μg/mL	Molecular Probes	488	A-11008

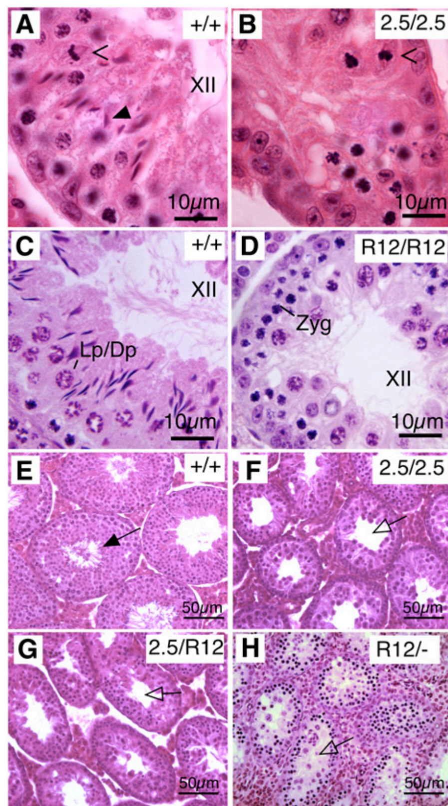

Figure 1 Mutations in *Akap9* disrupt spermatogenesis. H&E staining of wild-type stage XII seminiferous tubules (A and C) reveals differentiating germ cells, including metaphase spermatocytes (open arrowhead) and elongated spermatids (closed arrowhead). The $Akap9^{mei2.5/mei2.5}$ tubule (2.5/2.5, B) has metaphase structures (open arrowhead) but lacks consistent evidence of spermiogenesis. $Akap9^{R12/R12}$ germ cells in stage XII tubules (D) are not advanced beyond zygonema (Zyg). Lp/Dp, late pachynema/diplonema. Matings were organized between animals heterozygous for *mei2.5*, *repro12*, and $Akap^{-}$. (E–H). Seminiferous tubule cross-sections from a +/+ mouse (E) demonstrate wild-type spermatogenesis as evidenced by the mature spermatozoa in the lumen of the tubule (black arrow). In *mei2.5/mei2.5* animals, *repro12/mei2.5* heterozygotes, and *repro12/Akap9*$^{-}$ heterozygotes (F–H, respectively), spermatogenesis is severely impaired and the lumen is devoid of mature spermatozoa (open arrows).

The region proximal to *rs13478098* has 21 genes, (Table S1), but we prioritized *Akap9* as a candidate based on its large size and hence a large mutagenesis target. *Akap9* is composed of 48 predicted exons that yield a 12-kb transcript encoding 3779 amino acids. Sequencing of the *mei2.5* allele revealed a G-to-A transition in a conserved splice site at the last position of exon 13, causing inclusion of intron 13–14 and a stop codon (Figure 2A). The *repro12* allele was found to contain a T-to-A nonsense mutation in exon 14, changing amino acid 1353 from a cysteine to a stop codon (Figure 2A). To verify that these *Akap9* mutations underlie the spermatogenic defect, we generated mice bearing a gene trap vector insertion within intron 7 (Figure 2A, $Akap9^{Gt}$) and a derivative allele ($Akap9^{-}$) that both retains the gene trap functionality and deletes exon 8, generating a frameshift. The $Akap9^{-}$ allele failed to complement *repro12* (Figure 1H), further confirming a novel role for *Akap9* in spermatogenesis. Heterozygous intercrosses produced homozygotes at Mendelian frequencies ($N = 65$, $X^2 = 0.415$, $P = 0.8125$), and these $Akap9^{-/-}$ males were also sterile.

$Akap9^{mei2.5/mei2.5}$ animals demonstrate aberrant splicing and inclusion of intron 13–14 in all tissues examined, as expected due to the nature of the mutation (Figure 2B). Splicing is unaffected in *repro12* homozygotes, but these and $Akap9^{Gt}$ mice fail to express AKAP9 protein in testis or pancreas when probed using an AKAP24 (a24) antibody specific to human *AKAP9* exons 24–27 (Rivero *et al.* 2009) that normally detects several ~250-kDa protein species (Figure 2B).

Akap9 mutant spermatocytes exhibit normal spindle nucleation and progression through meiosis I

We hypothesized that defects in spindle formation could account for the gametogenic phenotype of *Akap9* mutants, particularly since isoforms of AKAP9 have been implicated in centrosomal biology and spindle nucleation (Schmidt *et al.* 1999; Witczak *et al.* 1999; Keryer *et al.* 2003b; Kim *et al.* 2007; Hurtado *et al.* 2011; Sehrawat *et al.* 2011). Indeed we confirmed that AKAP9 (Figure 3B, red) colocalizes at the centrosome (Figure 3A, green) in wild-type animals (Figure 3C, merge; marked by gamma tubulin), and is strikingly reduced in $Akap9^{repro12/repro12}$ mutants (Figure 3F, merge). Despite this, bipolar meiotic spindle nucleation was observed in both wild-type and $Akap9^{mei2.5/mei2.5}$ testis sections (Figure 3, G *vs.* H, respectively; white arrows). Therefore, the failed spermatogenesis phenotype is not likely to be caused by spindle defects in testis germ cells even though AKAP9 is nearly absent from the centrosome.

Because the *mei2.5* and *repro12* alleles primarily affect production of male postmeiotic cells, we next investigated hallmark aspects of spermatocyte development. Immunolabeling of surface-spread spermatocyte nuclei with the axial element protein SYCP3 revealed that $Akap9^{mei2.5/mei2.5}$ testes contained all stages of meiotic cells, with the meiotic chromosomes having a normal appearance through diplonema and metaphase I (Figure 4, A–H). Immunolabeling for the synaptomenal complex (SC) protein SYCP1 confirmed that $Akap9^{mei2.5/mei2.5}$ autosomes fully pair and are capable of successfully forming the SC central element during pachynema, indicative of synapsis (Figure 4, K and L). These data indicate that chromosomal defects in meiosis I are not a likely cause of failed spermatogenesis. Finally, we examined formation of the XY body (Figure 4, I and J), the disruption of which is hypothesized to cause meiotic arrest and male infertility in multiple mouse mutants (Cloutier and Turner 2010; Royo *et al.* 2010). Expression of small ubiquitin-related modifier 1 (SUMO-1) in $Akap9^{mei2.5/mei2.5}$ spermatocytes suggests that the XY body forms normally (Handel 2004) in the few midpachynema cells that are present (Figure 4, J *vs.* I, respectively; open arrowheads). However, XY body-containing cells localize abnormally to the adluminal compartment (Figure 4, J *vs.* I; dotted lines

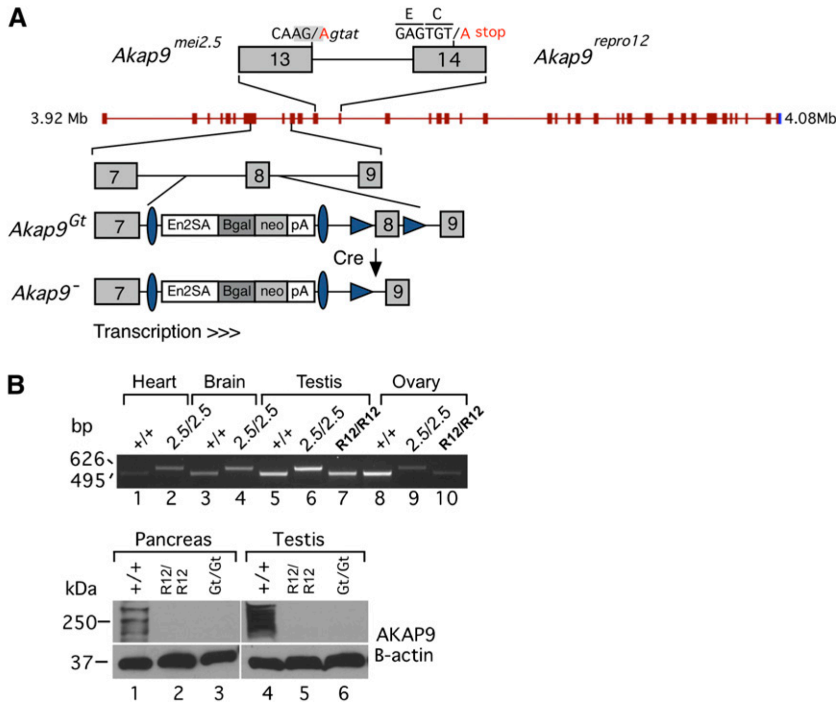

Figure 2 Mutation in *Akap9* causes failed splicing or protein expression in multiple tissues. (A) *mei2.5*, *repro12*, *Akap*^{Gt}, and *Akap*$^{-}$ mutations. Cre-lox recombination was used to remove exon 8 from the *Akap9* gene (− allele) as shown, with selection by β-galactosidase and neomycin (B gal; neo) in the exon-trapping cassette (SA – Bgeo-pA). Triangles, loxP sites; ovals, FRT sites. (B) RT–PCR of *Akap9* exons 12–15 in selected tissues shows a 495-bp amplimer in wild-type (+/+) and *Akap9*$^{R12/R12}$ (R12/R12) animals, whereas *Akap9*$^{mei2.5/mei2.5}$ (2.5/2.5) animals display a unique band (626 bp) corresponding to the inclusion of intron 13–14, indicating that *mei2.5* causes defective splicing at the 3′ end of exon 13. AKAP9 protein expression in pancreas and testis shows several protein species centered at 250 kDa in wild-type (+/+) animals, which is absent in R12/R12 and Gt/Gt mice.

indicate tubule boundary), indicating a potential defect in migration or structural support within seminiferous tubules.

Mutation of Akap9 leads to a high number of immature Sertoli cells

The absence of obvious meiotic defects coupled with the mislocalization of spermatocytes led us to examine Sertoli cell terminal differentiation at puberty, marked by basal localization and loss of proliferative ability (reviewed in Sharpe *et al.* 2003). Sertoli cell maturation may be characterized by the expression of a number of markers, including Wilm's tumor-1 (WT-1) (Kreidberg *et al.* 1993; Nordqvist 1995), anti-Mullerian hormone (AMH), thyroid hormone receptor alpha (THRA) (Arambepola *et al.* 1998; Holsberger *et al.* 2003, 2005), and the cell cycle inhibitor p27^{Kip1} (Beumer *et al.* 1999; Tan *et al.* 2005). Expression of these markers originates from and may initiate additional signal transduction events. Seminiferous tubules of *Akap9*$^{mei2.5/mei2.5}$ testes exhibit a significantly higher number of WT-1-positive Sertoli cells compared with wild type (Figure 5, A, B, and E; $P < 0.01$). The relative increase of Sertoli cells in *Akap9* mutants is not an indirect consequence of germ cell loss: similar analysis of a mutant (*Ccnb1ip1*$^{mei4/mei4}$) that undergoes late meiosis I arrest and lacks all germ cell types past meiosis I (Ward *et al.* 2007) revealed no such increase in Sertoli cells relative to controls (Figure 5, C–E).

To further interrogate Sertoli cell integrity, we examined the expression of the negative cell cycle regulator p27^{Kip1} and the prepubertal markers AMH and THRA. p27^{Kip1} expression is typically low in neonatal testes, high in adult testes, and when ablated, leads to abnormally high numbers of Sertoli cells (Beumer *et al.* 1999; Holsberger *et al.* 2005). Adult *Akap9*$^{mei2.5/mei2.5}$ Sertoli cells fail to express p27^{Kip1} (Figure 6, A *vs.* B), whereas p27^{Kip1} expression is unaffected in the *Ccnb1ip1*^{mei4} meiotic mutant (Figure 6, C *vs.* D) and hence is not dependent upon postmeiotic cell differentiation.

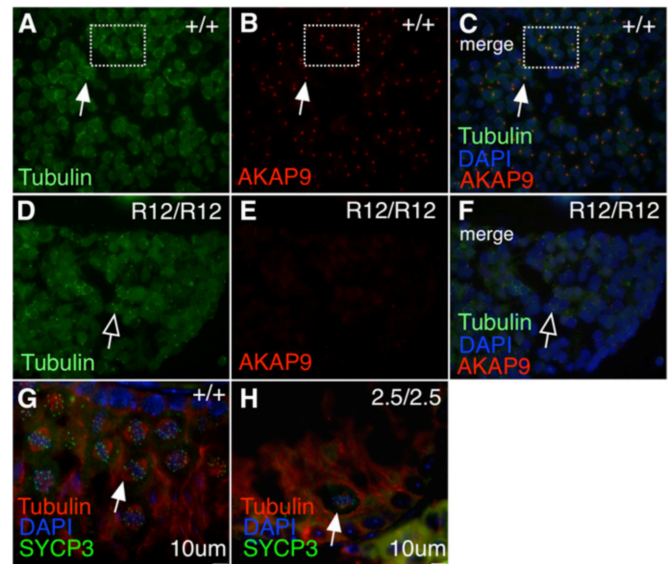

Figure 3 Spindle nucleation occurs in *Akap9* mutant homozygote germ cells despite minimal AKAP9 expression at the centrosome. (A–F) Seminiferous tubule squashes show strong colocalization of AKAP9 (a24 antibody, B, red) to the germ cell centrosomal marker gamma tubulin (A, green) in wild-type animals (C, merge, solid arrow; dotted box provided to aid colocalization pattern recognition) but greatly reduced centrosomal AKAP9 in *repro12* mutants (F, merge, open arrow). D and E are unmerged tubulin and AKAP9 labeling, respectively. (C and D) Immunolocalization of alpha tubulin (red) and SYCP3 (green) demonstrates successful chromosome arrangement on a bipolar spindle in adult wild=type (G) and *mei2.5/mei2.5* (H) testis sections.

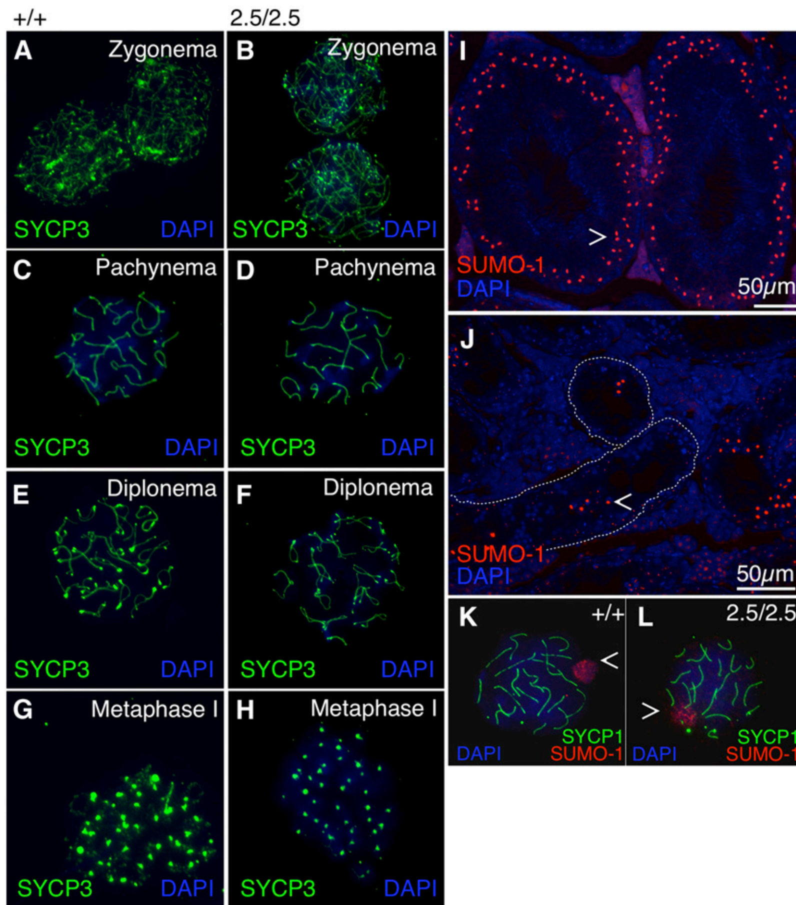

Figure 4 *Akap9* mutant males exhibit normal spermatocyte progression through prophase I but irregular primary spermatocyte localization but successful central element formation during pachynema. Chromosome spreads labeled for SYCP3 and counterstained with DAPI (A–H) show normal progression through zygonema, pachynema, diplonema, and metaphase I in wild-type (left) and in mutant (right) spermatocytes. SUMO-1 successfully localizes to the XY body in both wild-type (I, +/+) and mutant (J, 2.5/2.5) pachytene germ cells (white arrowheads), but mutant pachytene spermatocytes with XY bodies fail to localize at the tubule periphery (dashed white line) and are instead adluminal. Immunolabeling of the synaptomenal complex protein SYCP1 (K and L) demonstrates that homologous chromosomes in mutant tubules successfully pair during pachynema, indicative of synapsis. These are representative spread preparations and not directly derived from the tissue sections described above.

AMH expression, assayed in triplicate by RT–PCR at postnatal days 10, 20, 60, and 100, declined significantly in wild-type animals (+/+, Figure 6E), but remained high in *Akap9* mutants ($P < 0.05$) relative to wild-type controls of the same age. THRA likewise showed continued high expression in *Akap9*$^{mei2.5/mei2.5}$ animals relative to age-matched wild-type controls (Figure 6F). Taken together, these results indicate that AKAP9 deficiency causes Sertoli cells to retain a prepubertal character.

AKAP9-deficient mice show irregular tight and gap junctional protein localization

Organization of the blood–testis barrier is an important function of adult Sertoli cells (Smith and Braun 2012). We hypothesized that abnormal Sertoli cell numbers and fertility defects in males reflected a failure to establish junctions characteristic of the BTB, a prediction substantiated by the abnormal localization of germ cells (Figure 4). In prepubertal animals, testis ZO-1 is localized apicolaterally (Stevenson *et al.* 1986; Anderson *et al.* 1988; Byers *et al.* 1991), and shifts to associate almost exclusively with Sertoli cell basolateral tight junctions in peri- and postpubertal mice (Byers *et al.* 1991). In the wild-type adult testis, we observed ZO-1 proximal to the basement membrane (Figure 7A, red signal, white arrows; higher magnification in Figure 7C), whereas *Akap9*$^{mei2.5/mei2.5}$ testes exhibit ZO-1 expression towards the apical surface of Sertoli cells, with weak or no localization along the seminiferous tubule basement membrane (Figure 7B, open arrowheads; higher magnification in Figure 7D).

To investigate gap junctions, we examined the localization of Cx43 in the seminiferous epithelium (Figure 7, E and F). In wild-type sections, Cx43 is localized at the basal region of seminiferous tubules and forms distinct compartments around early meiotic cells identified by SYCP3 labeling (Figure 7E, arrows; higher magnification in Figure 7G). Cx43 expression is dissipated around preleptotene spermatocytes and forms gap junctions in the adluminal compartment, proximal to pachytene spermatocytes (closed arrowheads). In *Akap9*$^{mei2.5/mei2.5}$ testis, Cx43 localization is aberrant throughout the lumen without apparent relation to spermatocytes (Figure 7F, open arrowheads; higher magnification in Figure 7H), and fails to discretely compartmentalize early meiotic cells along the basal compartment. Combined, these data show the lack of appropriate tight and gap junction constituent localization, particularly at the basal portion of the seminiferous tubule of *Akap9*$^{mei2.5/mei2}$ testis.

Discussion

We present compelling evidence that mutations in *Akap9* disrupt the Sertoli cell terminal differentiation program,

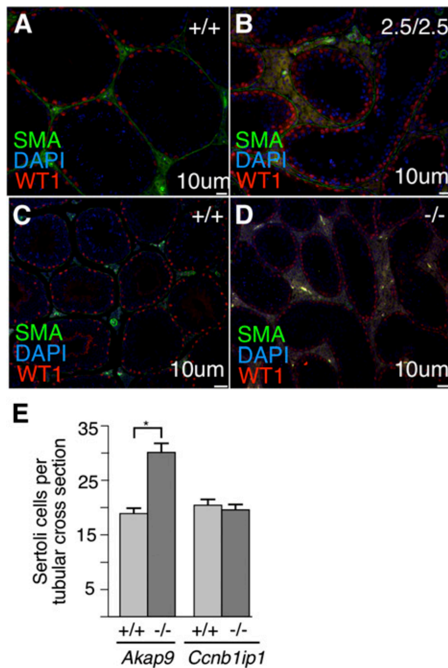

Figure 5 *mei2.5* homozygosity but not loss of germ cells disrupts Sertoli cell numbers in seminiferous tubules. Sertoli cell nuclei identified by the marker WT-1 (red) are observed adjacent to the basal membrane (smooth muscle actin, SMA; green) in +/+ (A) and *mei2.5/mei2.5* testes (B), but are significantly more numerous in *mei2.5/mei2.5* tubules (E, 30.2 ± 1.6 cells *vs.* 18.9 ± 1.1 cells) per tubular cross-section (*$P < 0.01$). The *mei4* mutation in mouse gene *Ccnb1ip1* prevents postmeiotic cell differentiation (*mei4*, C and D) (Ward *et al.* 2007). Sections from a heterozygote (*mei4*/+, C) and homozygote (*mei4/mei4*, D) were labeled with WT-1 to differentiate between mutation effects (*mei2.5*) and the consequences of failed meiosis and spermatogenesis (*mei4*). WT-1-positive Sertoli cell numbers are not affected by lack of postmeiotic germ cell differentiation (E).

Despite a strong decrease in centrosomal AKAP9 in mutant males, meiotic spindle formation and therefore microtubule organization in AKAP9-deficient mice appear normal, suggesting that cell division and spindle formation errors are not responsible for the reproductive phenotype. Proper XY body formation, SYCP1 and SYCP3 chromosome labeling, and rare postmeiotic spermatids indicate functional meiosis. Further, female mice carrying mutations in *Akap9* have no significant observed reproductive issues. It is unknown whether males homozygous for either *mei2.5* or *repro12* undergo homologous recombination, but since failure to recombine is normally associated with pachytene or metaphase I arrest in males (Morelli and Cohen 2005), it is unlikely that this pathway is affected. *Akap9* mutations appear instead to disrupt homeostasis of the seminiferous tubule by disturbing Sertoli cell function.

The abnormal localization of spermatocytes in $Akap9^{mei2.5/mei2.5}$ mice suggests that Sertoli cells have lost their proper structural organization along the seminiferous tubule periphery. Additionally, the significantly increased number of Sertoli cells and the absence of $p27^{Kip1}$ in adult *Akap9* mutants resulting in higher numbers and lack of maturation of Sertoli cells in adult mice. There is prolonged expression of juvenile Sertoli cell markers and a failure to form normal tight and gap junction morphologies, revealing that AKAP9 expression is essential for spermatogenesis but not the process of meiosis *per se*.

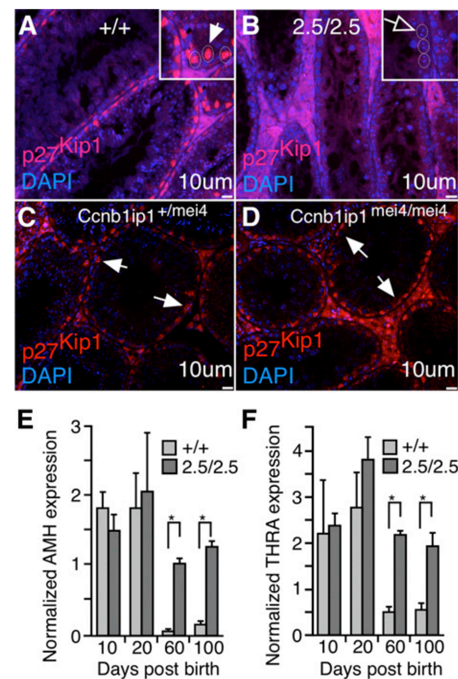

Figure 6 *mei2.5* homozygosity is responsible for Sertoli cell maturation defects. Mature, differentiated, wild-type Sertoli cells stain positively for the negative cell cycle regulator $p27^{Kip1}$ (A, arrow in inset), but this expression is absent in *mei2.5/mei2.5* testes (B, open arrowhead in inset). Relative to controls (C), *mei4/mei4* animals (D) retain $p27^{Kip1}$ expression (arrows), demonstrating that the absent phenotype is inherent to the *mei2.5* mutation and not a consequence of loss of postmeiotic cell populations. RT–PCR of Sertoli cell immaturity markers AMH (E) and THRA (F) at selected postnatal developmental intervals reveal high levels in prepubertal animals (10 and 20 days postbirth) and near absence in adult animals (60 and 100 days postbirth). In contrast, expression persists in adult *mei2.5* homozygotes ($P < 0.05$). All gene specific expression was normalized relative to age-matched B-actin controls, with three biological replicates at each timepoint.

Surprisingly, despite its widespread expression in many tissues, including the brain and heart (Lin *et al.* 1998; Kurokawa *et al.* 2004; Chen *et al.* 2007; Chen and Kass 2011; Chopra and Knollmann 2011; Kendler *et al.* 2011), overall animal viability is not markedly affected in AKAP9-deficient mice. This is even more remarkable considering that AKAP9 has been implicated in proper cell cycle progression via centriole formation and microtubule organizing center (MTOC) function (Keryer *et al.* 1993, 2003a,b; Takahashi *et al.* 1999; McCahill *et al.* 2005; Kim *et al.* 2007; Rivero *et al.* 2009; Hurtado *et al.* 2011). Although we have not conducted detailed physiological and morphological studies of the presumably null $Akap9^{-/-}$ mice, the lack of drastic somatic phenotypes suggests that additional AKAP family members could perform redundant functions in other tissues, but not those that impact sperm development.

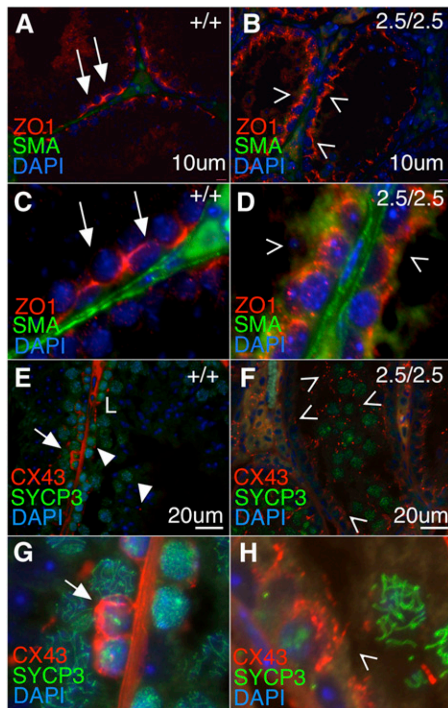

Figure 7 Junctional complexes characteristic of mature Sertoli cells are mislocalized in *Akap9* mutants. (A) ZO-1 (red) in wild-type mice localizes to the basal side of germ cells early in spermatogenesis (white arrows). (B) In *mei2.5*/*mei2.5* mice, ZO-1 is apically expressed (open arrowheads) in immature germ cells. (C and D) Higher magnification of representative cells from A and B. (E) Cx43 (red) in wild-type mice localizes at the basal region of seminiferous tubules, forming distinct compartments around spermatogonia (white arrows). Cx43 expression is also observed in the adluminal compartment presumably as part of gap junctions (white arrowheads). (F) In *mei2.5*/*mei2.5* mice, Cx43 is aberrantly expressed throughout the tubule lumen, and fails to discretely compartmentalize spermatogonia (open arrowheads). Spermatocytes in late prophase lose characteristic progression through the tubule lumen. (G and H) Higher magnification of representative cells from E and F. Smooth muscle actin (SMA; green) indicates the basement membrane of seminiferous tubules. L, leptonema.

signify that these cells may have retained their proliferative capabilities, implying partially failed maturation. The p27^{Kip1} knockout mouse exhibits two-fold larger testes, increased numbers of spermatogonia, and increased Sertoli cell proliferation, indicating that p27^{Kip1} participates in the halt of Sertoli cell mitogenesis at puberty (Kiyokawa *et al.* 1996; Beumer *et al.* 1999; Holsberger *et al.* 2005). Mice lacking p27^{Kip1} also contain prematurely sloughed off germ cells in the seminiferous epithelium (Beumer *et al.* 1999); we observed this and multilayering of premeiotic germ cells along the basal lamina of *Akap9* mutant tubules. However, because there are distinct differences between the *Akap9* mutants and p27^{Kip1} knockout phenotypes, the lack of p27^{Kip1} in *Akap9* mutants is likely not the exclusive cause of the infertile phenotype. Importantly, abnormal numbers of Sertoli cells were not observed in *Ccnb1ip1* mutants, suggesting that loss of differentiating germ cells is not the cause of Sertoli cell dysfunction. From these data, we conclude that AKAP9 deficiency causes Sertoli cell dysfunction either through intracellular signal transduction changes, aberrant intercellular regulation via neighboring cells, or possibly impairment of hormonal signaling from other sources in the body.

Dysfunctional and immature Sertoli cells in *Akap9* mutant mice provide a better explanation for the interrupted support of the later stages of germ cell development and may account for the failed structural management of germ cells with XY bodies. The loss of ZO-1 expression at the basal side of mutant Sertoli cells could indicate a disruption of cell polarity conferred by proper tight junction formation. Irregular tight junctions would impair the compartmentalization of the basal and adluminal environments, compromising Sertoli cell integrity. Similarly, Cx43 no longer distinctly compartmentalizes spermatogonia and preleptotene spermatocytes in mutants, showing promiscuous expression throughout the adluminal compartment of seminiferous tubules. This patterning is similar to Cx43 expression in immature tubules: during testicular maturation, Cx43 localization shifts from punctate and linear arrays throughout the adluminal compartment and near tight junctions to the basal region of seminiferous tubules (Risley *et al.* 1992; Pointis and Segretain 2005). We note the remarkable similarity of phenotype between *Akap9* mutant mice and Sertoli cell-specific Cx43 knockout mice (Brehm *et al.* 2007), suggesting a role for *Akap9* in the negative control of Sertoli cell proliferation and/or terminal differentiation.

We have not yet conducted physiological analyses of the hypothalamic–pituitary–gonadal (HPG) axis in *Akap9* mutants. Disruption of the luteinizing and follicle-stimulating hormone pathways causes severe fertility issues in males and females (Wang and Greenwald 1993a,b; O'Shaughnessy *et al.* 2009; Siegel *et al.* 2012) and could contribute to the *Akap9* mutant phenotype. Interestingly, *Akap9* mutant females are fertile but a detailed assessment of the HPG axis in these animals will be an important area for future investigation.

Based on the present data, however, we propose a specific role for *Akap9* in the coordinated regulation of blood–testis barrier dynamics in the testis. It is possible this occurs by a cell type- or junction-specific mechanism, and determining if the requirement for AKAP9 is autonomous to Sertoli cells or germ cells may be addressed in the future using methods such as spermatogonial transplantation. Importantly, the *Akap9*$^{mei2.5/mei2.5}$ phenotypes of multiple layers of spermatogonia along the basal lamina, sloughed off germ cells, and adluminal spermatocytes do not reflect the impairment of one specific junction. Binding of ZO-1 targets Cx43 to the plasma membrane, controlling gap junction size and distribution, and may shepherd signal molecules or actin filaments to Cx43 to integrate gap junction formation with the actin cytoskeleton and modulate intracellular signaling (Giepmans and Moolenaar 1998; Toyofuku *et al.* 1998; Wei *et al.* 2004). ZO-1 is additionally required for PKC-controlled disassembly of Cx43 from gap junctional plaques (Wu *et al.*

2003; Hunter *et al.* 2005; Akoyev and Takemoto 2007). The AKAP350 splice variant of AKAP9 associates with PKA, PP1, and PKC in a multiprotein complex and interacts with ZO-1 in cultured mammalian cells (Schmidt *et al.* 1999; Diviani and Scott 2001; Berryman and Goldenring 2003). Loss of a major regulator of Cx43 activity would impede proper gap junction formation and function in the testis, impairing spermatogenesis. However, these associations have not yet been investigated in the testis.

In conclusion, we find that AKAP9 is critical for male fertility but is not essential for basic cellular function or mouse development, contrary to prior evidence. Our phenotypic studies implicate a key role for this protein in the establishment of proper somatic cell–germ cell interactions in the seminiferous tubules, and formation of the blood–testis barrier.

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AKAP9 Is Essential for Spermatogenesis and Sertoli Cell Maturation in Mice

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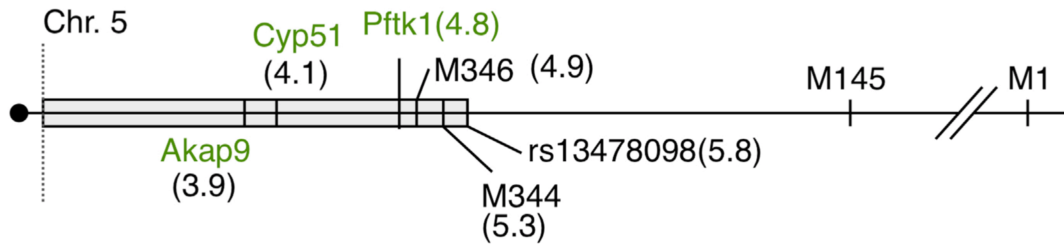

Figure S1 Genetic mapping of *Akap9* mutants. Separate intersubspecific intercrosses were organized between animals heterozygous for *mei2.5 and repro12*, and the number of meioses were scored for recombinants in particular intervals as a fraction of the total number of meioses. Parenthetical numbers indicate base pair position (in Mb) of the depicted loci. The data placed *mei2.5* and *repro12* in a region corresponding to a physical distance of ~5 mb (gray box). "M" = *D5Mit*.

Table S1 (NCBI m37)

Gene Start (bp)	Ensembl Gene ID	External Gene ID	RefSeq DNA ID
3350318	ENSMUSG00000040274	Cdk6	NM_009873
3549930	ENSMUSG00000058503	5830415L20Rik	NM_001042501
3577679	ENSMUSG00000008307	1700109H08Rik	NM_029843
3589984	ENSMUSG00000040302	C030048B08Rik	NM_172991
3602072	ENSMUSG00000005907	Pex1	NM_027777
3645975	ENSMUSG00000007415	Gatad1	NM_026033
3663010	ENSMUSG00000014529	4930511M11Rik	NM_029141
3696006	ENSMUSG00000040351	Ankib1	NM_001003909
3809195	ENSMUSG00000000600	Krit1	NM_030675
3851179	ENSMUSG00000040367	4932412H11Rik	NM_172879
3896588	ENSMUSG00000040429	Mterf	NM_001013023
3896588	ENSMUSG00000040429	Mterf	NM_172135
3934184	**ENSMUSG00000040407**	**Akap9**	**NM_194462**
4086680	**ENSMUSG00000001467**	**Cyp51**	**NM_020010**
4198373	ENSMUSG00000053178	ENSMUSG00000053178	NM_001042670
4761658	ENSMUSG00000044674	Fzd1	NM_021457
4809739	**ENSMUSG00000028926**	**Pftk1**	**NM_011074**
5511115	ENSMUSG00000046798	Cldn12	NM_022890
5585286	ENSMUSG00000040473	A330021E22Rik	NM_172447
5676944	ENSMUSG00000015653	Steap2	NM_028734
5742328	ENSMUSG00000015652	Steap1	NM_027399