BOR Papers in Press. Published on May 6, 2009 as DOI:10.1095/biolreprod.109.076398

Expression of transgenic PPP1CC2 in the testis of *Ppp1cc*-null mice rescues spermatid viability and spermiation but does not restore normal sperm tail ultrastructure, sperm motility, or fertility

David C. Soler[ac], Suraj Kadunganattil[a], Shandilya Ramdas[a], Kimberly Myers[a], Joaquim Roca[c], Theresa Slaughter[b], Stephen H. Pilder[b], Srinivasan Vijayaraghavan [a*].
[a] Biological Sciences, Kent State University, Kent, OH 44242
[b] Department of Anatomy and Cell Biology, Temple University School of Medicine, 3400 N. Broad Street, Room 618 OMS, Philadelphia, PA 19140
[c] Institut de Biologia Molecular de Barcelona-CSIC, Parc Científic de Barcelona 08028 Barcelona, Spain
*Corresponding author: Fax: +1 330 672 3713
E-mail address: svijayar@kent.edu (S. Vijayaraghavan)

Abstract
Two isoforms of phosphoprotein phosphatase 1, PPP1CC1 and PPP1CC2, are translated from alternatively spliced transcripts of a single gene, *Ppp1cc*, and differ only at their extreme C-termini. While PPP1CC1 expression is nearly ubiquitous, PPP1CC2 is largely restricted to testicular germ cells and mature spermatozoa. Targeted deletion of *Ppp1cc* leads to sterility of -/- males due to a combination of gross structural defects in developing spermatids resulting in apoptosis, and faulty spermiation. Because PPP1CC2 is the only PP1 isoform that demonstrates high-level expression in wild-type meiotic and post-meiotic male germ cells, we have tested whether its loss in *Ppp1cc* -/- males is largely responsible for manifestation of this phenotype by expressing PPP1CC2 transgenically in the testis of *Ppp1cc* -/- mice (rescue mice). Here we demonstrate that PPP1CC2 expression in the *Ppp1cc* -/- testis is anti-apoptotic, thus re-establishing spermatid development and spermiation. However, because aberrant flagellar morphogenesis is incompletely ameliorated, rescue males remain infertile. Since these results suggest that expression of PPP1CC2 in developing germ cells is essential but not sufficient for normal spermatogenesis to occur, appropriate spatial and temporal expression of both PPP1CC isoforms in the testis during spermatogenesis appears to be necessary to produce structurally normal, fertility-competent spermatozoa.

Keywords: Phosphoprotein phosphatase 1 catalytic subunit C 2, sperm, PP1γ2, knock out, transgene, testis specific, spermatogenesis

Introduction

Spermatogenesis is the complex sequence of events occurring within the seminiferous tubules of the testis to produce spermatozoa [1, 2]. The fourth and final phase of this process (spermiogenesis) encompasses the morphological transformation of haploid products of meiosis, called spermatids, into highly differentiated spermatozoa. Spermatids are evident by day 22 post-partum in male mice. Within this phase, round spermatids differentiate morphologically into elongating spermatids that undergo radical condensation to become mature testicular spermatozoa. The mature testicular spermatozoa consist of (1) a streamlined head containing a membrane-bound acrosome and a nucleus with highly condensed, transcriptionally inactive chromatin; and (2) a highly evolved, compartmentalized flagellum through which a central axonemal engine, anchored peripherally to unique outer dense fibers (ODFs), runs its entire length. By virtue of flagellar compartmentalization, the axoneme and surrounding ODFs are exposed to successive, differentially structured, regulatory environments. These include a proximal, mitochondrial sheath (MS)-bound compartment known as the midpiece, then more distally, a long, fibrous sheath (FS)-encapsulated principal piece, and finally most distally, a tiny end piece. The somatic cells of the testis, Sertoli and Leydig, play an essential role in supporting spermatogenesis through complex signaling networks.

Morphologically mature sperm, released into the lumen of the seminiferous tubule by a process called spermiation, are transported through the efferent ducts into the epididymides where they undergo additional maturation steps to acquire their competency to swim and to bind and fertilize eggs. Epididymal sperm maturation is an added feature of gamete development in mammals, involving a series of biochemical and physiological modifications which include: (1) remodeling the sperm plasma membrane, (2) changes in the composition and cellular localization of proteins, (3) acquisition and alteration of glycoproteins, and (4) changes in pH and in the levels of the second messengers, Ca^{2+} and cAMP [1, 2, 3, 4, 5]. These mediators of cellular regulation are thought to modulate sperm function through changes in protein phosphorylation. Curiously, until recently, investigations of the role of protein phosphorylation on male germ cell development and function have focused mainly on the protein kinases, especially cAMP-dependent protein kinase A (PKA) [6], while little attention has been paid to the influence of protein phosphatases on these processes [7, 8].

The predominant phosphoprotein phosphatase catalytic subunit in both testicular germ cells and epididymal spermatozoa is phosphoprotein phosphatase 1 catalytic subunit C2 (PPP1CC2, previously called PP1γ2), one of four highly conserved type 1 serine/threonine protein phosphatases (the other three being PPP1CA, PPP1CB and PPP1CC1, previously called PP1α, PP1β, and PP1γ1, respectively) [9]. The PPP1CC isoforms, PPP1CC1 and PPP1CC2, are products of alternatively spliced transcripts of a single gene, *Ppp1cc*. These polypeptides differ from each other at their extreme C-termini with PPP1CC2 having a unique 21-amino acid carboxyl-terminal extension [9]. The PPP1CC2 isoform with its virtually unaltered C-terminus is only found in mammals. Inhibition of PPP1CC2 in sperm by treatment with PPP1C inhibitors results in initiation and stimulation of motility [10, 11]. These highly correlative results have led to the suggestion that PPP1CC2 plays an important role in the regulation of mammalian sperm motility. However, in mice, targeted disruption of *Ppp1cc*, which eliminates both PPP1CC1 and PPP1CC2 expression, results in male infertility due to aberrant spermiogenesis. Disruption of

this phase is highlighted by impaired sperm head and tail morphogenesis, leading to the absence of germ cells in the epididymides, due to a combination of spermatid apoptosis and dysfunctional spermiation [12]. Mutant female mice are fertile and appear normal, suggesting that the other PPP1C proteins, PPP1CA and PPP1CB, can substitute for the loss of the PPP1CC isoforms in all cells and tissues where either is expressed, except testis. Thus, at least one, and possibly both, of the PPP1CC isoforms is indispensable for normal spermatogenisis. Since PPP1CC2 expression is predominant in testicular meiotic and post-meiotic germ cells [13], it was of interest to determine whether the expression of PPP1CC2 in *Ppp1cc*-null mice could re-establish normal spermiogenesis in the mutant testis, and thus, restore fertility to these males. A transgene construct encoding PPP1CC2 driven by the *Pgk2* promoter, the same promoter which was used to fully rescue fertility in *Ace* (angiotensin converting enzyme) -/- mice [14], was therefore utilized in an attempt to rescue fertility in *Ppp1cc* -/- males.

Materials and Methods

Generation of the PPP1CC2 rescue plasmid

The Sac1-XbaI fragment of the human *Pgk2* promoter was amplified from the plasmid pCR2.1 (a kind gift from Dr. John McCarrey, Department of Biology, University of Texas at San Antonio) by PCR with forward and reverse primers (5'-CTCGAGCTCGAGGTTTTTACATATCA-3' and 5'-CTCTCTAGAGACAATATAAAGACATA-3', respectively). This fragment was subsequently inserted between the Sac1-Xba1 sites of pBluescript SK+ (Stratagene, CA). A 1kb fragment comprising the start to the stop codon of rat testicular *Ppp1cc2* cDNA was ligated between the BamHI-XhoI sites (see Fig. 1A). The SV40 poly A signal was amplified from a pcDNA4.0 plasmid using the forward and reverse primers, 5'-CTCCTCGAGTCTCATGCTGGAGTTCT-3' and 5'-CTCGGTACCACCATGATTACGCCAAG-3', respectively, followed by ligation between the XhoI-KpnI sites of pBluescript SK+. The DNA fragment containing the *Pgk2* promoter, the *Ppp1cc2* cDNA, and the SV40 poly A signal was excised from the vector by digestion with BamH1 and KpnI, and the 1.7-kb fragment was gel-purified. The purified fragment was then microinjected into the pronuclei of fertilized B6/SJL eggs, and the injected eggs were implanted into the uteri of pseudo-pregnant mothers. Both microinjection and embryo implantation were carried out at the Transgenic Facility of Case Western Reserve University (Cleveland, Ohio). Transgenic mouse production and use at Kent State University follows approved Institutional Animal Care and Use Committee protocols adapted from the National Research Council publication, *Guide for the Care and Use of Laboratory Animals*.

DNA samples isolated from ear punches of 289 twenty-one day-old progeny were analyzed by PCR for the presence of the transgene using the *Pgk2* forward primer, 5'-GCGCACACCTCAGGACTATT-3', and the SV40 reverse primer 5'-CTCGGTACCACCATGATTACGCCAAG-3'. The transgenic positive B6/SJL founder mice were mated either with *Ppp1cc*-null CD1 females or *Ppp1cc* +/- males, (*Ppp1cc*-null mice were obtained from Dr. Susan Varmuza, University of Toronto, Toronto, Canada). PPP1CC2 transgene-positive-*Ppp1cc* -/- males were obtained by crossing PPP1CC2 transgene positive-*Ppp1cc* +/- males to *Ppp1cc* -/- females.

Expression of the transgene
Reverse transcription-PCR (RT-PCR) was used to detect expression of the transgene in the testis. Briefly, 50-100 mg of testes were homogenized in 1 mL of TRI REAGENT (Sigma-Aldrich, MO), and DNA-free RNA was isolated according to the manufacturer's directions. The RNA samples were reverse transcribed at 42°C for 20 min using the RT Primer Mix (Qiagen, MD) followed by a 3 min incubation at 95°C to inactivate the reverse transcriptase. PCR was then performed using the same conditions employed to detect the transgenic *Ppp1cc2* gene using the forward primer, 5'-ATGGCGGATATCGACAA-3' and reverse, 5'-CGAACAACTCCAGCATGAGA-3'. The 1040 bp product was detected by ethidium bromide staining following electrophoresis through 1% agarose.

Protein extract preparation and western blot analysis
Mouse testes and brain were homogenized in 1ml of HB+ buffer using a Model Pro 200 tissue homogenizer (Pro Scientific Inc., CT) as described previously [13]. Epididymal sperm extruded (as described in Sperm extrusion methods) in PBS was centrifuged for 3 min at 300x*g* and the sperm pellet resuspended in 1% SDS, boiled for 5 min followed by centrifugation at 16000x*g* for 20 min. The supernatant was boiled after addition of 6X sample buffer and separated by 12% SDS-PAGE, and gels were blotted to Immobilon-P PVDF membranes (Millipore, MA). These were probed with a 1:1000 dilution of an affinity purified rabbit polyclonal anti-PPP1CC2 antibody directed against the PPP1CC2 unique C-terminal region, followed by a peroxidase-conjugated goat anti-rabbit secondary antibody [13]. Blots were developed by enhanced chemiluminiscence.

Sperm extrusion methods from testis, caput, and cauda epididymis
Testicular sperm from the rescue animals were isolated using the methodology described by Rumela *et al.* [13] In brief, testes were decapsulated in PBS. Seminiferous tubules were untangled manually using fine forceps. Dark regions of the tubule, as observed by transillumination, containing mature sperm were teased open and the suspension was fixed in 3.7% paraformaldehyde in PBS.

Sperm from both caput and caudal epididymis were isolated by carefully removing both regions separately, followed by squeezing sperm from both regions after piecing both the caput and caudal epididymis with a fine tip needle. The sperm were extruded into PBS, followed by fixation in two volumes of 3.7% paraformaldehyde in PBS. The fixed sperm were then observed under a Leica microscope (Leica Microsystems, Germany) using the DIC optics.

Statistical analysis of testicular, caput, and cauda epididymal sperm head and flagellar bends from rescue mice
Two slides each of extruded and fixed testicular, caput, and cauda epididymal sperm were prepared from a single rescue male from line A. From each slide 15 randomly selected fields were observed by light microscopy using DIC optics, and the numbers of straight sperm, sperm with 180 degree hairpin bends at the connecting piece, and sperm with 180 degree hairpin bends at the midpiece/principal piece junction were determined. The significance of statistical differences of the means of each morphological phenotype between testicular, caput, and caudal sperm were determined by the Tukey HSD test following a one-way ANOVA.

Sperm count and motility assessment
Caudae epididymides were lightly minced and incubated in KSOM medium [15] for 15 min at 37°C in 5% CO_2 in air to allow the sperm to swim out and disperse into the medium. A measured portion of the sperm suspension was briefly centrifuged at 600xg, resuspended in medium containing 0.02% sodium azide, and counted with a hemocytometer. Sperm motility was assessed by CASA and other higher resolution videotape analyses [16].

Fertility analysis
Experimental rescue and wild-type (control) male mice were mated with wild-type CD1 females over a period of five weeks, and the number of offspring in each litter was recorded. CD1 females that failed to become pregnant when mated with experimental males were subsequently tested for fertility by mating to wild-type CD1 (control) males.

Histology and immunohistochemistry
Tissues were embedded in paraffin and sectioned, then stained with hematoxylin and eosin. For immunostaining, paraffin was removed from sections by sequential washings in citrosol, alcohol, and PBS. Sections were blocked by incubating in 10% goat serum and 2.5% BSA overnight, then were washed and incubated with the aforementioned anti-PPP1CC2 antibody (1:100 dilution) for five hours at room temperature. After washing, sections were incubated with a Cy3-conjugated secondary antibody (dilution 1:250) for 1 h at room temperature. After washing, the sections were mounted, viewed, and photographed using an Olympus Fluorview 500 fluorescence microscope (Olympus, PA).

Ultrastructure analysis of epididymal spermatozoa by transmission electron microscopy
Caudae epididymides were fixed by immersion in 2.5% glutaraldehyde in 0.1 M sodium cacodylate. Following fixation, the epididymides were minced, and immersed in the same fixative for 15 min at 4°C. After washing in 0.1 M sodium cacodylate buffer, the tissue blocks were post-fixed in 1.33% OsO_4 for 90 min at 4°C, then dehydrated through a graded series of ethanols and infiltrated with and subsequently embedded in Epon/Araldite (Structure Probe, Inc./SPI Supplies, West Chester, PA). Ultra-thin sections 85 nm thick were cut and placed onto grids, followed by staining for 5 min in 10% uranyl acetate in methanol and then in Reynold's lead citrate for 2 min. Sections were viewed and photographed with a Philips 400 transmission electron microscope [16].

Northern Blot
A 30µl mixture containing 20µg of total RNA, 2µl 10xMOPS buffer, 4µl HCHO (37% solution, Amresco, OH, USA), 10µl deionized formamide (Amresco, OH, USA) and 1µl Ethidium Bromide (200µg/ml), was heated at 85°C for 10 min, and chilled on ice for another 10 min. A 2µl aliquot of 10X gel loading buffer (50% Glycerol, 10mM EDTA pH 8.0, 0.25% w/v bromophenol blue, 0.25% w/v xylene cyanol FF) was added to each sample, and samples were vortexed and briefly centrifuged. Samples were loaded onto 1% agarose/MOPS gel (SeaKem GTG Agarose, Cambrex, NJ, USA), and electrophoresed at 70V. The gel was denatured in a solution of 0.05M NaOH and 1.5M NaCl for 30 min, neutralized in a solution of 0.5M Tris-HCl, pH 7.4, 1.5M NaCl, and then equilibrated in 20xSSC for 45 min. The gel was transferred to Hybond-XL nylon membrane (GE Healthcare, NJ, USA) in 10xSSC for 16 hours. The membrane containing RNA was then baked at 85°C for 2 hours, after which it was prehybridized

in 8ml modified Church buffer (1mM EDTA, pH 8.0, 0.5M $NaHPO_4$, pH 7.2, and 5%SDS) for one hour at 65°C in a water bath. Fresh Church buffer containing a probe of *Ppp1cc2* cDNA between exon5 and exon 7 and actin cDNA labeled with P^{32}-dCTP [MP Biomedicals, OH, USA] was used for hybridization overnight at 65°C in a water bath. The membrane was then washed in 1XSSC/0.1%SDS three times for 2 min each at room temperature, followed by washing in 0.1XSSC/0.1%SDS twice for 5 min in a 65°C water bath. After washing, the membrane was dried at room temperature, covered with plastic wrap, exposed to film in a cassette with an intensifying screen overnight, and was then developed in a Typhoon automated film developer (GE Healthcare, NJ, USA).

Results

Transgenic Expression of PPP1CC2 in the Ppp1cc -/- Mouse

Ppp1cc2 transgenic mice carrying the construct shown in Fig. 1A were produced as described in *Materials and Methods*. The promoter of the testis-specific *Pgk2* gene was used to drive transcription of rat *Ppp1cc2* in the mouse testis based on the following rationale: first, the *Pgk2* promoter is well-characterized and has been used successfully to rescue fertility by driving germ cell transcription of transgenic *Ace* in sterile *Ace*-null males (14); second, the *Pgk2* promoter would drive transcription of the *Ppp1cc2* transgene in an appropriate fashion to allow for elevated translation of transgenic *Ppp1cc2* in cells in which the level of wild-type PPP1CC2 protein is normally high (late spermatocytes and spermatids); and third, because the *Ppp1cc* endogenous promoter expresses transcripts in most somatic tissues (PPP1CC2 is the product of an alternatively spliced transcript particularly abundant in developing male germ cells), its use would run the risk of expressing PPP1CC2 everywhere, possibly producing complicating phenotypes.

Transgene positive mice were identified by PCR of DNA isolated from ear punches as described in *Materials and Methods*. Five lines that transmitted the transgene were established. RT-PCR was used to detect *Ppp1cc2* transgene mRNA expression (Fig. 1B). These transgene-positive mice were crossed as described in *Materials and Methods* to produce *Ppp1cc2* transgene-positive *Ppp1cc -/-* (rescue) males. Several lines of rescue mice showed testicular *Ppp1cc2* mRNA expression equivalent to the level expressed in the testis of fertile *Ppp1cc +/-* mice (Supplemental Figure S1, all Supplemental Figures are available online at www.biolreprod.org).

Transgene-positive *Ppp1cc +/+* male and female mice exhibited a wild-type phenotype as did transgene positive *Ppp1cc -/-* females. In addition, the presence of the *Ppp1cc2* transgene did not appear to affect the development or life span of any male, and the general health characteristics of rescue mice were comparable to those of wild-type and *Ppp1cc* -/- mice. Surprisingly, wild-type (*Ppp1cc +/+*) and heterozygous (*Ppp1cc +/-*) mice with or without the transgene expressed approximately the same amount of PPP1CC2 in testis protein extracts as assessed by immuno-reactivity on western blots (data not shown). However, significant transgenic PPP1CC2 protein expression was evident in rescue testes in which endogenous expression was absent (Fig. 2A). These findings suggested that an upper boundary of PPP1CC2 protein expression exists and is tightly controlled. Moreover, as expected, transgenic *Ppp1cc2* mRNA expression was restricted to the testis (Fig. 2A), due to the use of the spermatocyte-specific *Pgk2* promoter to drive transcription of the transgene. Western blot analysis of testis extracts from rescue males showed that four transgenic lines expressed significant amounts of PPP1CC2 in the testis (Fig. 2B), with

the E-line expressing the greatest amount, approximately one-third of the steady state level of wild-type (Fig. 2C). Finally, immunohistochemical analysis of testis sections from these mice demonstrated that transgenic PPP1CC2 was mainly restricted to meiotic and post-meiotic germ cells, roughly comparable to the staining pattern in wild-type testis [13] (Fig. 2D).

Effects of Ppp1cc2 Transgene Expression in Ppp1cc -/- Mice.
Previous studies have demonstrated that spermiogenesis is clearly impaired in *Ppp1cc* -/- mice [12] where the lumen of the seminiferous tubules are nearly devoid of late elongated spermatids and testicular spermatozoa, and virtually no mature sperm are found in the epididymal lumen. In contrast, light microscopic analysis of hematoxylin stained rescue testis sections revealed that the testicular architecture is similar to that of wild-type mice, and the lumina of the seminiferous tubules contain what appear to be numerous mature sperm tails (Fig. 3A). Many spermatozoa were evident in the lumina of the caudae epididymides from all lines of rescue mice, as well (Fig. 3B). Thus the presence of PPP1CC2 in the testis of rescue mice restores spermiogenesis and testicular/epididymal sperm numbers to qualitatively normal levels.

Sperm Morphology Phenotypes and Sperm Motility Assessment in Transgene Positive-Ppp1cc -/- Mice.
Absence of PPP1CC1 and PPP1CC2 expression in the testis causes a drastic impairment of spermiogenesis, where mitochondrial sheath formation is disrupted or absent in the few testicular sperm of *Ppp1cc* -/- mice, and sperm head shape is highly irregular (Fig. 4A). In contrast, testicular sperm heads appear to be normally hook-shaped in rescue mice (Figs. 4B & C). However, most mitochondrial sheaths appear to contain irregularities, including gaps especially at either their proximal, distal, or both ends (Figs. 4B & C). In addition, most of these sperm contained residual cytoplasmic irregularities at the head/connecting piece junction (Figs. 4B & C), suggesting some abnormality in the condensation process.

Interestingly, when we examined caput (Fig. 4D) and cauda epididymal rescue sperm (Figs. 4E & F), we detected two phenotypes not observed previously in sperm extruded from the testes of the same animal: many of the epididymal sperm had heads that folded back at the connecting piece at a 180 degree angle (hairpinning), and these appeared attached to the proximal end of the midpiece within a common, membrane bound cytoplasmic pouch (Fig. 4E). In many of these cases, the mitochondrial sheath appeared gapped or absent at what is normally its proximal end (Fig. 4E). A smaller number of sperm also showed a similar hairpin phenotype at the midpiece/principal piece junction (Fig. 4F). In most of these cases, the mitochondrial sheath did not appear to extend to the distal end of the midpiece.

It is possible that many of the hairpin flagella are byproducts of shear forces acting on already weakened regions of the rescue sperm flagella during passive transit from the testis to the epididymis. If this is truly the case, then the numbers of head/connecting piece junction and midpiece/principal piece junction hairpins should not differ significantly between caput and cauda epididymal sperm, but should show significant differences between epididymal sperm from either the cauda or caput and testicular sperm. A quantitative analysis of sperm morphologies (see *Materials and Methods*) from the testes, caput, and caudae epididymides of a rescue male (Table 1) indeed demonstrated that the numbers of head/connecting piece junction and midpiece/principal piece junction hairpins in both the caput and cauda epididymis differed

significantly from the numbers of testicular sperm exhibiting either phenotype (in both cases, $p<0.01$). Unexpectedly however, the numbers of head/connecting piece junction and midpiece/principal piece junction hairpins seen in the caput were significantly lower ($p<0.01$) than the number of sperm displaying these phenotypes in the cauda epididymis. A highly significant increase in both hairpin phenotypes in caudal vs caput sperm suggested that a biological process that takes place in the cauda but not in the caput might increase mechanical shear in the flagellum. An example of a process that would increase shear force in the flagellum is the development of initial flagellar motility in the cauda epididymis.

To test whether rescue sperm were motile, lightly minced caudae epididymides were incubated briefly in a medium that supports progressive sperm motility *in vitro* to allow sperm to move into the medium. High resolution videotape studies demonstrated that a small percentage of the rescue sperm displayed motility of variably poor quality (data not shown). However, most of these motile sperm exhibited so little forward (progressive) movement that quantifiable parameters of progressive motility were below the detectable limit of the sperm motion analysis (CASA) system employed (data not shown).

Expression and localization of PPP1CC2 in spermatozoa from rescue mice

We also tested to see if PPP1CC2 protein expressed in testicular germ cells of rescue mice was properly incorporated into epididymal spermatozoa. Western blot analysis of caudal sperm protein extracts from these mice showed that immuno-reactive PPP1CC2 was indeed present, although, as expected, at lower levels than in extracts prepared from wild-type sperm (Fig 5A). Immuno-cytochemical analysis demonstrated that PPP1CC2 localization was distributed along the tail and the head in a similar fashion to the pattern seen in wild-type caudal sperm (Fig. 5B).

Ultrastructure analysis of cauda epididymal sperm from rescue mice

As demonstrated above, "hairpin" phenotypes rarely observed in testicular sperm were significantly more frequent in caput and cauda epididymal sperm of rescue mice. To examine these hairpin phenotypes in more detail, we employed transmission electron microscopy (TEM) of cauda epididymal sperm *in situ.*

When the ultrastructure of rescue sperm in the cauda epididymis was inspected by TEM, head/connecting piece hairpins were readily evident (Figs. 6A-C &E). Plasma membrane with some cytoplasmic content loosely surrounded both the heads and proximal midpieces in these sections. This residual cytoplasm was often appended asymmetrically around the outer circumference of the proximal midpiece, where extruded ODFs as well as other cytoplasmic remnants were observed (Fig. 6B). We also discerned that the heads of these sperm were elongated (Figs. 6A-E), in contrast to the rounded heads of *Ppp1cc -/-*testicular sperm (Fig. 4A), and that they contained nuclei with completely condensed chromatin (Figs. 6A-E). However, in some cases, concave deformities were observed in the proximal acrosome (Figs. 6A & E).

Most of the proximal midpieces of these sperm contained additional, abnormalities, including disorganized, gapped mitochondrial sheaths, deformed mitochondria, disordered doublet microtubules (MT doublets), and/or misaligned outer dense fibers (ODFs) (Figs. 6A-C, & E). However, in contrast to *Ppp1cc -/-* testicular sperm, where the number of ODFs in the midpiece was usually highly increased [13], the number of ODF and MT doublets internal to the

mitochondrial sheaths of rescue sperm were generally normal or occasionally decreased by one (Figs. 6B , C, & F). In the latter case, it seemed probable that absent ODF/MT doublet complexes protruded through abnormal gaps in the more distal mitochondrial sheaths during initiation of motility (Fig. 6F).

In other cross sections through these more distal regions of the midpieces of rescue sperm, these and other abnormalities were evident (Figs. 6H, J, & K). First, extruded ODFs/MT doublet complexes were present in unshed, asymmetrical cytoplasmic bulges peripheral to the mitochondrial sheath (Fig. 6H). Also in this cross section, the shape of the circle of nine ODFs and attached MT doublets appears distorted by a gap, possibly created by the breakdown of extra MTs between ODF/MT doublet complexes three and four. Second, in sections where the mitochondrial sheath, ODFs, and axonemal MTs appeared normal, asymmetrical cytoplasm often surrounded the mitochondrial sheath (Fig. 6G). However, the appearance of some normal mitochondrial structures in rescue sperm was in stark contrast to the much larger number of mitochondrial anomalies seen in *Ppp1cc* -/- testicular sperm (Fig. 6I).

Tail disorganization appeared to be no less radical in the principal pieces of rescue sperm tails (Figs. 6J & K), where extruded ODFs associated with MT doublets and attached dynein arms were evident in abnormal asymmetric cytoplasmic appendages, and the axonemal MT doublets and associated periaxonemal ODFs were not only reduced in number, but dynein arms of different doublets sometimes faced in opposing directions (Fig 6J). It was also not unusual to see two different flagellar cross-sections bounded by a single membrane perhaps representative of the more distal flagellar hairpin bends. Within the principal piece portions of these cross sections, extrusion of ODFs 4-7 and associated MT doublets was common, while extruded ODFs were also observed in the midpiece portion of many of these sections (Fig. 6 K). The presence of these ultrastructural anomalies in the majority of caudal rescue sperm further increased the probability that hairpin phenotypes were exacerbated by the initiation of motility in the cauda epididymis. However, because normally high levels of PPP1CC2 activity in the wild-type caput epididymis are known to inhibit the initiation of sperm motility [11], we cannot rule out the possibility that the appearance of these phenotypes at a significantly higher level in caput vs testicular rescue sperm might indicate that lower catalytic activity of PPP1CC2 in the rescue caput epididymis leads to premature initiation of rescue sperm motility.

In order to test the possibility that the disorder we saw in caudal rescue sperm results from the initiation of axonemal motility in sperm with structurally unstable tails, we examined testicular rescue sperm by TEM (Supplemental Figure S2). While the results are based on a relatively small number of micrographs, they bear out the contention that more ultrastructural features of *Ppp1cc* -/- spermatids/testicular sperm appear to be rescued by expression of transgenic PPP1CC2 than is evident from examination of caudal rescue sperm ultrastructure. Thus, it seems likely that the initiation of motility in caudal (and perhaps caput) rescue sperm instigates the extrusion of structurally unstable ODFs and attached microtubule doublets through abnormal gaps between poorly developed mitochondrial gyres and/or fibrous sheath ribs.

Discussion

Targeted disruption of the gene ultimately responsible for expression of both PPP1CC protein isoforms, PPP1CC1 and PPP1CC2, results in male infertility stemming from impaired spermatogenesis, particularly in the spermiogenic phase, where, presumably, spermatid apoptosis is widespread [12]. Even though the other protein phosphatase 1 isoforms, PPP1CA and PPP1CB, are substantially upregulated in the *Ppp1cc* -/- testis [13], neither is able to substitute for the absent PPP1CC proteins in restoring spermatogenesis and male fertility. This is somewhat surprising, since the primary sequences of the four PP1 isoforms in mammals are not only nearly identical, but also are highly homologous to their counterparts in yeast and other organisms. In fact, any of the four mammalian PPP1C subunits can complement the single PPP1C isoform in yeast [17]. It is also interesting that although PPP1CC1 is expressed in nearly all tissues, while the PPP1CC2 protein is testis-restricted (and is the only PPP1C isoform present in mammalian spermatozoa), the absence of PPP1CC1 has no apparent detrimental effect on any process other than (possibly) testicular function. Because PPP1CC2 is more highly expressed than any other of the PP1 isoforms in testicular germ cells, we hypothesized that its spermatocyte- and spermatid-restricted expression in the *Ppp1cc* -/- testis might be able to restore spermatogenesis and fertility in males. Thus, our approach to test this hypothesis was to drive transcription of PPP1CC2 from the spermatocyte-specific promoter of *Pgk2* (Fig. 1) so that PPP1CC2 protein expression would be restored to secondary spermatocytes and spermatids. Previous studies have shown that the *Pgk2* promoter is able to drive transcription of the germinal isoform of angiotensin converting enzyme (*Ace*) in *Ace*-null mice, thus fully restoring their fertility [14].

Apparently, as a consequence of *Ppp1cc2* transgene expression in the *Ppp1cc* -/- mouse, PPP1CC2 spatial expression approximates wild-type protein expression in both the testis (Fig. 2D) and in caudal sperm (Fig. 5), while qualitatively normal testis architecture within the seminiferous tubules of PPP1CC2-rescue mice is restored (Fig. 3A). In addition, spermatozoa derived by swim-out from the cauda epididymis of the rescue mice were relatively abundant (~$2x10^6$ sperm/cauda epididymis compared to ~$1x10^7$ sperm/cauda epididymis of wild-type mice). Further, while the epididymis of the *Ppp1cc*-null mouse was virtually devoid of spermatozoa, the lumen of the epididymis of the rescue mouse appeared to have substantial numbers of spermatozoa in it (Fig. 3B). However, and contrary to our initial expectations, transgenic expression of PPP1CC2 in the *Ppp1cc* -/- testis failed to restore fertility of rescue males. Motility analysis showed that the great majority of caudal spermatozoa derived from PPP1CC2-rescue mice and incubated in medium that supports vigorous progressive motility in wild-type sperm, were immotile, while those that were motile lacked progressive movement. Additionally, high resolution light and electron microscopy of caudal rescue sperm provided a variety of structural causes for the lack of sperm motility (and thus, infertility).

Testicular rescue sperm have normal sickle-shaped heads, as opposed to deformed, rounded heads in *Ppp1cc* -/- testicular sperm (Fig. 4), strongly suggesting that PPP1CC2 activity regulates sperm head morphogenesis. However, one flagellar aberration seen in testicular sperm from *Ppp1cc* -/- mice, a malformed, abbreviated or gapped mitochondrial sheath, is only partially ameliorated in testicular rescue sperm, while irregularly shaped, residual cytoplasmic bulges are frequently observed at the head/connecting piece junction. (Fig. 4). These findings have provided obvious reasons for the loss of movement in rescue sperm, and suggest that either PPP1CC1 plays a major role in flagellar morphogenesis or PPP1CC2 activity is not sufficient in the rescue

testis, where the level of transgenic PPP1CC2 protein expressed is at best one-third of wild-type, to completely rescue flagellar morphogenesis.

Interestingly, approximately 44% and 75% of the heads of *Ppp1cc2* -rescue spermatozoa extruded from the caput and caudae epididymides, respectively, are bent backwards and partially encircle the circumference of the mitochondrial sheath. These are tethered to the proximal midpiece within unremoved residual cytoplasm, while another ~5% and ~17%, respectively, of these sperm exhibit a 180 degree hairpin bend at the junction of the midpiece and the principal piece (Fig. 4; Table 1). A strikingly similar phenotype to the head/connecting piece hairpin abnormality seen here has been reported recently in mutant sperm lacking SPEM1, a protein expressed in spermatids and localized to the cytoplasmic droplet [18]. This defect has been attributed to the improper removal of this residual cytoplasm in the newly formed spermatozoa during spermiation. Whether expression of SPEM1 or other still unidentified polypeptides required for proper removal of residual cytoplasm are compromised in rescue spermatozoa remains to be determined.

Surprisingly, neither hairpin phenotype is exhibited to a significant degree (~6% and ~0.4% for the head/connecting piece junction phenotype and the midpiece/principal piece junction phenotype, respectively) in testicular rescue sperm (Table 1). More importantly, caput and caudal rescue sperm are also significantly different from each other ($p<0.01$) for both hairpin phenotypes. The reason for this continuum of phenotypic variation along the male genital tract has several possible explanations. As is the case in the *Spem1*-null sperm, cytoplasm that is normally removed from the junction between the head and neck of the condensing spermatid is insufficiently loosened, so that the sperm head and proximal midpiece become abnormally encapsulated within this residual material in the epididymis, possibly due to mechanical shear forces arising first from transit of sperm from the testis to the caput epididymis, and exacerbated by the initiation of sperm motility in the cauda epididymis; or, transit of sperm from the testis to the caput epididymis might play little if any role in creating either flagellar bend phenotype: instead, lower than wild-type PPP1CC2 catalytic activity in caput rescue sperm (and possibly the testis) could permit limited flagellar activity in the testis and caput that is then amplified in the cauda epididymis as PPP1CC2 activity is further suppressed [11]. Electron micrographs of rescue spermatids and testicular sperm appear to reinforce these explanations for the incidence of flagellar bending phenotypes in testicular vs. epididymal rescue sperm, as the tail ultrastructue of testicular rescue germ cells is relatively improved compared to that of caudal rescue sperm (Supplemental Figure S2).

While these simple explanations remain to be more completely tested, it is notable that spermatozoa recovered from selenium deficient mice or mice lacking the serum seleno-protein P1 or the putative seleno-protein receptor, apolipoprotein E receptor 2 [19, 20, 21], display similar principal piece and midpiece ODF abnormalities to rescue mice. Striking similarities include thinning of the midpiece particularly at its distal end where the mitochondrial sheath is abbreviated distally, and hairpin bends of the flagellum at the midpiece/principal piece junction accompanied by peripheral extrusion of ODF/MT doublet complexes 4-7 through the gap created between the annulus and the prematurely terminated mitochondrial sheath. This suggests that *Ppp1cc2*-rescue mice may be defective in selenium transport and metabolism. It is possible that expression of the putatitive seleno-protein P1 (SEPP1) receptor in the Sertoli cells is

compromised or in some way functionally defective in rescue mice. The rare testicular spermatozoa that could be recovered from *Ppp1cc -/-* mice are characterized by disorganized or missing mitochondrial sheaths. Mitochondrial sheath abnormalities, while less prominent, still persist in rescue spermatozoa (Figs. 4 & 6). As stated above, thinning of the mitochondrial sheath at varying positions in the midpiece is frequently observed in spermatozoa from rescue mice. Studies in progress show that the level of the seleno-protein, phospholipid hydroperoxide glutathione peroxidase, an abundant protein in mitochondrial sheaths [19], is substantially reduced in both *Ppp1cc -/-* and *Ppp1cc2* -rescue testis (S. Vijayaraghavan, unpublished observations). Further work is required to examine whether either or both PPP1CC isoforms in Sertoli or Leydig cells plays a role in selenium import into and the expression of seleno-proteins in the testis.

Unless PPP1CC1 plays a major role in directing spermiogenesis, the exact reason for the lack of complete rescue of sperm structure and function in PPP1CC2 mice is not entirely clear. However, several testable possibilities exist. First it could be that relatively low levels of PPP1CC2 protein expression in *Ppp1cc2*-rescue testis are insufficient to sustain normal spermatogenesis and sperm maturation. However, it should be emphasized that the sperm phenotype is essentially the same in all lines of transgenic mice (expressing varying low levels of PPP1CC2 compared to wild-type). Surprisingly, the presence of the transgene fails to increase PPP1CC2 protein levels in +/+ and +/- backgrounds (data not shown). It appears that a homeostatic mechanism might operate to ensure no more than an optimum level of PPP1CC2 translation from its mRNA. Such a mechanism can be at the level of initiation of protein synthesis or in the break down of excess protein by proteolysis. However, the reason why mRNA derived from the transgene does not translate into higher protein levels in the *Ppp1cc -/-* background is puzzling, since several rescue lines express testicular levels of *Ppp1cc2* mRNA equivalent to fertile *Ppp1cc +/-* mice (Supplemental Figure S1). One possibility is that the stability of the mRNA derived from the transgene is less than that of the message derived from the endogenous gene. It should be noted that the transcript derived from the transgene lacks both the 5' and 3' UTRs present in the mRNA derived from the endogenous *Ppp1cc* gene. It also should be emphasized that only a limited number of genes are transcribed in spermatids. Generally, translation in spermatids is from pre-existing mRNA [22]. Thus, the reduced stability of the mRNA from the *Ppp1cc2* transgene may compromise PPP1CC2 protein levels in spermatozoa. In order to address this concern, we have engineered a new transgene under the same *Pgk2* promoter which includes the cDNA for both *Ppp1cc1* and *Ppp1cc2* and the bona fide 5' and 3' UTRs present in the *Ppp1cc* gene.

Another possibility for the lack of a complete rescue is that normal spermatogenesis may require expression of PPP1CC2 at stages of germ cell development earlier than when the *Pgk2* promoter becomes normally active. Transcription of the sperm-specific *Pgk2* gene apparently occurs in secondary spermatocytes [23]. Fluorescence immuno-histochemical analysis of testis sections shows that a strong signal for PPP1CC2 first appears in primary spermatocytes and remains strong through all further stages of germ cell development (Figure 2), although western blot analysis also has shown that a weak signal for PPP1CC2 is detected in the testis of 8-day old mice (corresponding to spermatogonial/somatic cell expression) [13]. Thus, PPP1CC2 may be essential for gene expression and for other biochemical processes in primary spermatocytes. Therefore, it is conceivable that a lack of PPP1CC2 at the primary spermatocyte stage (or earlier)

of germ cell differentiation in rescue mice could result in the abnormal assembly of the sperm flagellum during spermiogenesis. It may be noted that messenger RNAs for a number of proteins that are synthesized in haploid spermatids are transcribed earlier in primary and secondary spermatocytes (24, 22). Among these are proteins of the outer dense fibers which are unique features of the flagella of mammalian spermatozoa. It is possible that PPP1CC2, a PP1 isoform found only in mammals, may be involved in an isoform specific manner in the morphogenesis of the unique features of the mammalian sperm flagellum. Finally, as noted earlier, we cannot rule out the requirement of a function for the PPP1CC proteins in Sertoli and or Leydig cells. That is, one or both PPP1CC isoforms may be part of a signaling system that must operate between Sertoli and developing germ cells to ensure normal spermatogenesis.

Efforts are currently underway to create transgenic mice whose expression of PPP1CC2 is driven by its endogenous promoter and the development of conditional knock out mice where one or both of the PPP1CC isoforms can be deleted in specific cell types in the testis. These studies should shed further light on the role and requirement of the unique PPP1CC2 isoform in spermatogenesis in mammals.

Acknowledgments
We thank Dr. Gary E. Olson (Department of Cell and Developmental Biology, Vanderbilt University, Nashville, TN) for his comments regarding this manuscript, Dr. Douglas Kline (Department of Biological Sciences, Kent State University, Kent, OH) for his helpful expertise, Dr. Mike Model (Department of Biological Sciences, Kent State University, Kent, OH) for his assistance in confocal fluorescence microscopy and members of the Vijayaraghavan lab for useful discussions. This work was supported by NIH grants HD 38520 to S. Vijayaraghavan, and HD31164 to S. Pilder.

References
1-Bedford, J.M., Hoskins, D.D., The mammalian spermatozoon: morphology, biochemistry and physiology. In: Lamming G.E. (Ed), Marshall's Physiology of Reproduction, Churchill Livingstone, Edinburgh, London, Melbourne, New York: 1990, pp. 379-568
2-Jones, R., 1998. Plasma membrane structure and remodeling during sperm maturation in the epididymis. J. Reprod. Fertil. Suppl. 53, 73-84.
3-Vijayaraghavan, S., Hoskins, D.D., 1986. Regulation of bovine sperm motility and cyclic adenosine 3',5'-monophosphate by adenosine and its analogues. Biol. Reprod. 34, 468-477
4-Vijayaraghavan, S., Bhattacharya, A., Hoskins, D.D., 1989. Calcium uptake by bovine epididymal spermatozoa is regulated by the redox state of the mitochondrial pyridine nucleotide. Biol. Reprod. 40, 744-751
5-Vijayaraghavan, S., Critchlow, L.M., Hoskins, D.D., 1985. Evidence for a role for cellular alkalinization in the cyclic adenosine 3',5'-monophosphate mediated initiation of motility in bovine caput spermatozoa. Biol. Reprod. 3, 489-500
6-Burton, K.A., McKnight, K.S., 2007. PKA, germ cells and fertility. Physiology. 22, 40-46
7-Chakrabarti, R., Cheng, L., Puri, P., Soler, D., Vijayaraghavan, S., 2007. Protein phosphatase PP1γ2 in sperm morphogenesis and epididymal initiation of sperm motility. Asian J Androl. 9, 445-452.

8- Vijayaraghavan, S., Chakrabarti, R., Myers, K., 2007. Regulation of sperm function by protein phosphatase PP1γamma2. Soc. Reprod. Fertil. Suppl. 63, 111-121.
9-Kitagawa, Y., Sasaki, K., Shima, H., Shibuya, M., Sugimura, T., Nagao, M., 1990. Protein phosphatases possibly involved in rat spermatogenesis. Biochem. Biophys. Res. Commun. 171, 230-235
10-Smith, G.D., Wolf, D.P., Trautman, K.C., daCruz e Silva, E.F., Greengard, P., Vijayaraghavan, S., 1996. Primate sperm contain protein phosphatase 1, a biochemical mediator of motility. Biol Reprod. 3, 719-727
11-Vijayaraghavan, S., Stephens, D.T., Trautman, K., Smith, G.D., Khatra, B., da Cruz e Silva, E.F., Greengard, P., 1996. Sperm motility development in the epididymis is associated with decreased glycogen synthase kinase-3 and protein phosphatase-1 activity. Biol. Reprod. 54, 709-718.
12-Varmuza, S., Jurisicova, A., Okano, K., Hudson. J., Boekelheide K., Shipp E.B., 1999. Spermiogenesis is impaired in mice bearing a targeted mutation in the protein phosphatase 1c gamma gene. Dev. Biol. 205, 98-110.
13-Chakrabarti, R., Kline, D., Lu, J., Orth, J., Pilder, S., Vijayaraghavan, S., 2007. Analysis of *Ppp1cc*-null mice suggests a role for PP1γamma2 in sperm morphogenesis. Biol. Reprod. 76, 992-1001.
14- Kessler, S.P., Gomos, J.B., Scheidemantel, T.S., Rowe, T.M., Smith, H.L., and Sen, G.C. 2002. The Germinal Isozyme of Angiotensin-converting Enzyme Can Substitute for the Somatic Isozyme in Maintaining Normal Renal Structure and Functions. J. Biol. Chem. 277, 4271-4276.
15-Summers, M.C., McGinnis, L.K., Lawitts, J.A., Raffin, M., Biggers, J.D., 2000. IVF of mouse ova in a simple optimized medium supplemented with aminoacids. Hum. Reprod. 15, 1791-1801.
16-Pilder, S.H., Olds-Clarke, P., Orth, J.M., Jester, W.F., Dugan, L., 1997. Hst7: a male sterility mutation perturbing sperm motility, flagellar assembly, and mitochondrial sheath differentiation. J Androl. 18, 663-671.
17-Gibbons, G.A., Kozubowski, L., Tatchell, K., Shenolikar, S., 2007. Expression of human protein phosphatase-1 in *Saccharomyces cerevisiae* highlights the role of phosphatase isoforms in regulating eukaryotic functions. J. Biol. Chem. 282, 21838-21847
18-Zheng, H., Stratton, C.J., Morozumi, K., Jin, J., Yanagimachi, R., Yan, W., 2007. Lack of Spem1 causes aberrant cytoplasm removal, sperm deformation, and male infertility. Proc. Natl. Acad. Sci. U. S. A. 104, 6852-6857.
19-Olson, G.E., Winfrey, V.P., Nagdas, S.K., Hill, K.E., Burk, R.F., 2005. Selenoprotein P is required for mouse sperm development. Biol. Reprod. 73, 201-211.
20-Andersen, O.M., Yeung, C.H., Vorum, H., Wellner, M., Andreassen, T.K., Erdmann, B., Mueller, E.C., Herz, J., Otto, A., Cooper, T.G., Willnow, T.E., 2003. Essential role of the apolipoprotein E receptor-2 in sperm development. J. Biol. Chem.278, 23989-23995
21-Olson GE, Winfrey VP, Hill KE, Burk RF. Sequential development of flagellar defects in spermatids and epididymal spermatozoa of selenium-deficient rats.2004.Reproduction..Mar;127(3):335-42.
22-Iguchi, N., Tobias, J.W., Hecht, N.B., 2006. Expression profiling reveals meiotic male germ cell mRNAs that are translationally up- and down-regulated. Proc. Natl. Acad. Sci. U.S.A. 103: 7712-7717.

23-Zhang, L.P., Stroud, J., Eddy, C.A., Walter, C.A., McCarrey, J.R., 1999. Multiple elements influence transcriptional regulation from the human testis-specific PGK2 promoter in transgenic mice. Biol. Reprod. 60, 1329-1337.
24-Hecht, N.B., 1990. Regulation of 'haploid expressed genes' in male germ cells. J. Reprod. Fertil. 88, 679-693.

Figure Legends

Figure 1. Design of PPP1CC2-rescue transgenic construct and genotyping transgenic mice. (A). The *Pgk2- Ppp1cc2* cDNA- *SV40* poly A transgene. (B). RT-PCR was used to detect the presence of transgenic mRNA of expected length (1.3 kb) in *Ppp1cc2* -rescue testis.

Figure 2. Presence of PPP1CC2 in rescue mouse testis. (A). Transgenic PPP1CC2 is restricted to testis and is not expressed in brain. (B). The amount of PPP1CC2 expressed in each rescue line testis is estimated by western blot analysis: line E expresses ~ 1/3 of wild-type PPP1CC2 expression, line D, ~ 1/6, line A, ~ 1/7, and line G, ~ 1/15. (C). Concentration curve/western blot comparing line E to wild-type protein expression. (D). Immunohistochemistry of wild-type, *Ppp1cc -/-* (Null), and line E (Rescue) testes. Sections were probed with rabbit anti-PPP1CC2 antibody followed by goat anti-rabbit cy3-labeled secondary antibody. Slides were viewed with an Olympus Fluorview 500 microscope at 20X magnification.

Figure 3. Hematoxylin-eosin staining of experimental mouse testes and caudae epididymides. (A)Testes cross sections from wild-type, null, and E-line rescue mice were stained with hematoxylin-eosin. The stained sections were viewed with an Olympus IX70 microscope at 20X and 40X magnifications. Note the seemingly wild-type architecture of the rescue testis at these magnifications. (B). Hematoxylin-eosin stained cross sections through the lumina of the caudae epididymides from wild-type, null, and E-line rescue mice were viewed with an Olympus IX70 microscope at 40X. Note the dirth of sperm in the null epididymis.

Figure 4. DIC imaging of testicular, caput, and cauda epididymal spermatozoan. (A). Typical testicular sperm from *Ppp1cc -/-* mouse. Note rounded, deformed head and mitochondrial sheath abbreviated at both ends. (B) Typical testicular sperm from rescue mouse, containing a hook-shaped head, a cytoplasmic remnant at the head/connecting piece junction, a distally shortened mitochondrial sheath, and a straight flagellum. (C). Testicular sperm from rescue mouse showing gross similarities to sperm in B, but displaying non-hairpin bending at the head/connecting piece junction and in the principal piece. Note the distally shortened mitochondrial sheath leaving a small gap between the mitochondrial sheath and the beginning of the principal piece. (D). Rescue sperm from the caput epididymis. Note ~ 90 degree sharp bend at gap in the mitochondrial sheath near the distal end of the midpiece. (E). Cauda epididymal sperm from rescue mouse. Note gap in mitochondrial sheath near the head/connecting piece junction of upper sperm, and the head/connecting piece junction hairpins, where the heads appear to be engulfed in cytoplasm with the proximal midpiece. (F.) Cauda epididymal sperm exhibiting hairpin bend at the midpiece/principal piece junction. All sperm were viewed at 100X magnification.

Figure 5. Transgenic PPP1CC2 detection in caudal spermatozoon from A-line rescue mouse (A). Immunocytochemistry of PPP1CC2 in rescue sperm shows presence of PPP1CC2 along the head and tail, similar to wild-type in (B). Slides were viewed using an Olympus Fluorview 500 at 60X magnification.

Figure 6. Ultrastucture of cauda epididymal *Ppp1cc2*-rescue sperm *in situ*. (A). Longitudinal section through sperm head abutting transverse section of midpiece (MP): (1) arrows point to plasma membrane extending from sperm head and surrounding one side of the MP; (2) note absence of intervening plasma membrane between head and MP, and (3) distorted mitochondrion. (B). Longitudinal section through sperm head abutting transverse section of MP: (1) arrows point to membranous structures extending from head and beginning to encircle MP containing poorly formed mitochondrial sheath. (2) arrow indicates disruption of axoneme along the longitudinal plane created by MT doublet/ODF pairs 3 and 8 connected to the central singlet pair of microtubules by radial spokes; however, none of the ODFs or associated MT doublets appear to be extruded at this level of section of the MP. (3) arrows pointing to ODFs external to the mitochondrial sheath. Their presence indicates that they were extruded into the peripheral cytoplasm distally, perhaps through weak spots or discontinuities in the mitochondrial and/or fibrous sheaths. (C.) Longitudinal section through sperm head abutting transverse section of MP: (1) arrows point to plasma membrane extending from sperm head and arching around one side of the MP; (2) arrow indicates absence of entire ODF/axonemal complex from within the highly disorganized mitochondrial sheath; the ODF/axonemal complex appears to have extruded through a large gap in the poorly formed mitochondrial sheath. (3) Arrow indicates unidentified cytoplasmic content. (D.) (1) Arrows indicate plasma membrane connecting center of sperm head to the MP (top). Note unidentified cytoplasmic content within the plasma membrane between the head and MP. (E). Longitudinal section through sperm head abutting oblique section of MP: (1) both single and double headed arrows show what appear to be thin membranous connections between the head and MP. (2) Arrow indicates disorganized/absent ODF/axonemal complex. (3) Arrow points to gap between nucleus and acrosome. (F). Longitudinal section through distal end of a shortened mitochondrial sheath; (1) distal extent of shortened mitochondrial sheath; (2) broken ODFs extruding through gap between principal piece (PP) and shortened distal end of mitochondrial sheath; (3) broken ODF internal to mitochondrial sheath on only one side of axoneme. (G). (1). Section through normal mitochondrial sheath and ODF/axonemal complex contained within asymmetrically surrounding cytoplasm (H). MP containing mitochondrial sheath circling full complement of ODFs and MT doublets. (1) Arrows indicate extruded ODFs, as in part B*;* (2) arrow indicates gap in circle of ODF/MT doublet complexes filled with debris. (I). Section through thoroughly unorganized mitochndrial sheath of *Ppp1cc -/-* testicular sperm. (J). Transverse section through PP of rescue sperm: (1) arrows indicate regions of asymmetric cytoplasmic bulge with granular content (left) and three extruded ODF/MT doublet-dynein arm complexes (right). (2) Arrows indicate opposite direction of dynein arms of adjacent MT doublets 3 (leftward) and 4 (rightward); note also that ODF associated with MT doublet 4 is present, but reduced in size. *K.* Cross section through MP and adjacent PP bounded by single plasma membrane. (1) Arrows point to extruded ODFs in cytoplasmic bulge around MP. (2) Long arrow points to missing ODF/MT doublet complexes 4-7 from PP section; short arrow indicates possible extruded ODF. (3) Arrow indicates continuity of plasma membrane extending around MP and PP cross sections.

TABLE 1
SPERM TAIL BEND PHENOTYPE

Sperm Type	Straight	Hairpin at Head/CP Junction	Hairpin at MP/PP Junction	Total
Testicular	218 (93.96%)	13 (5.6%)	1 (0.43%)	232
Caput	108 (50.9%)[1]	94 (44.33%)[3]	10 (4.7%)[3]	212
Caudal	13 (8.8%)[1,2]	109 (74.14%)[3,4]	25 (17%)[3,4]	147

[1]Significantly < Testicular ($p<0.01$)
[2]Significantly < Caput ($p<0.01$)
[3]Significantly > Testicular ($p<0.01$)
[4]Significantly > Caput ($p<0.01$)

Figure 1

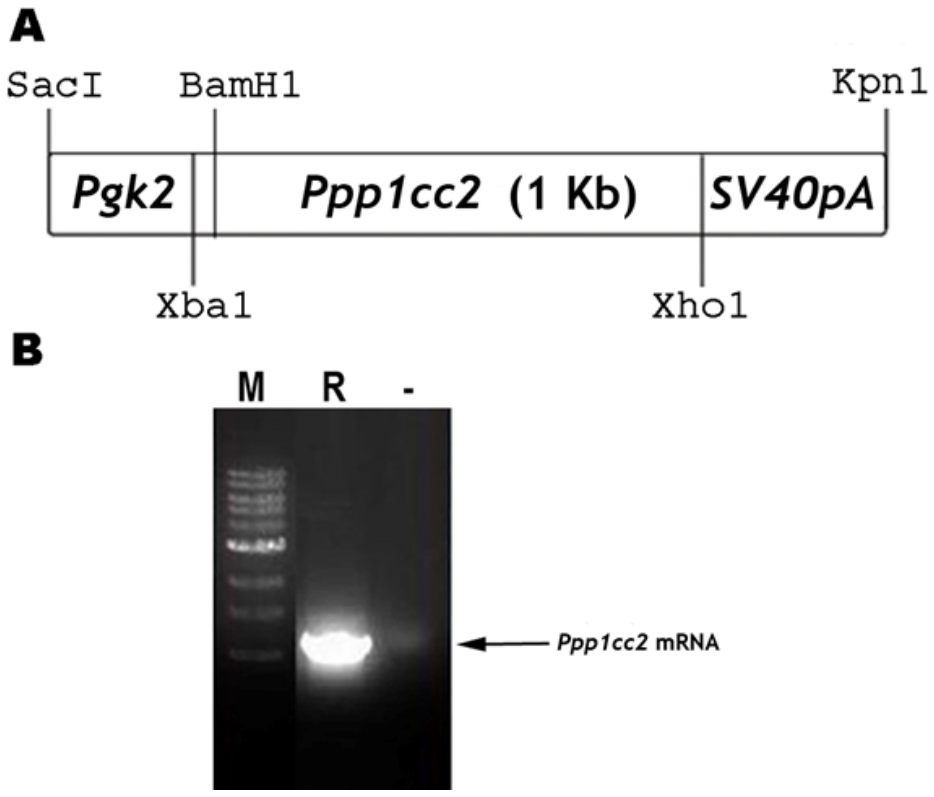
A
SacI
BamH1
Kpn1
Pgk2
Ppp1cc2 (1 Kb)
SV40pA
Xba1
Xho1
B
M
R
-
Ppp1cc2 mRNA

Figure 2

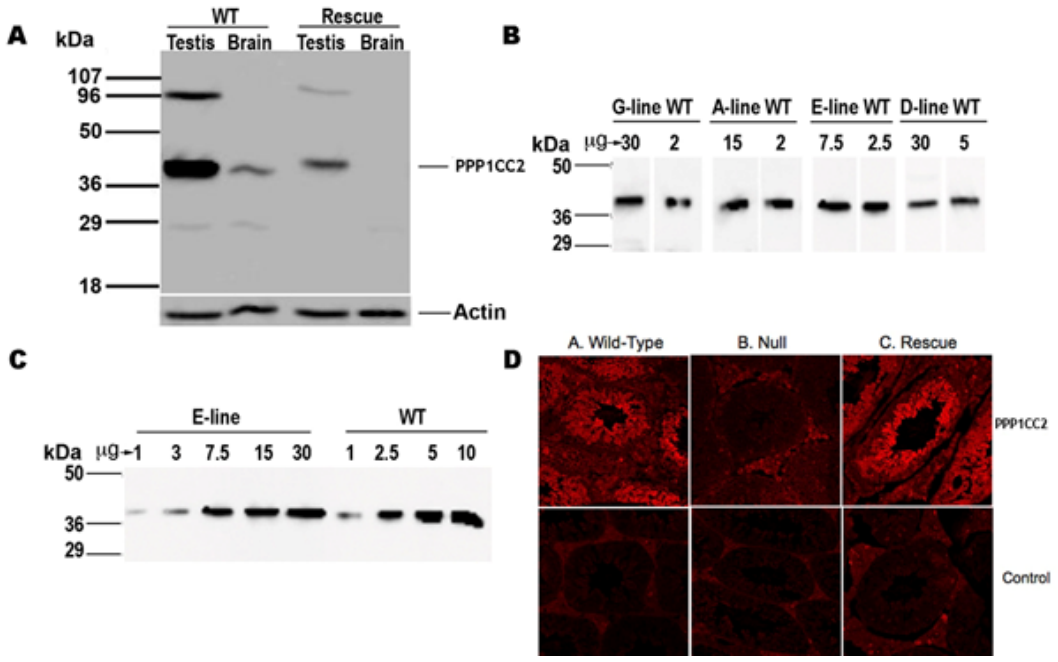
A
kDa
WT
Rescue
Testis
Brain
Testis
Brain
107
96
50
36
29
18
PPP1CC2
Actin
B
G-line WT
A-line WT
E-line WT
D-line WT
kDa
µg→30
2
15
2
7.5
2.5
30
5
50
36
29
C
E-line
WT
kDa
µg→1
3
7.5
15
30
1
2.5
5
10
50
36
29
D
A. Wild-Type
B. Null
C. Rescue
PPP1CC2
Control

Figure 3

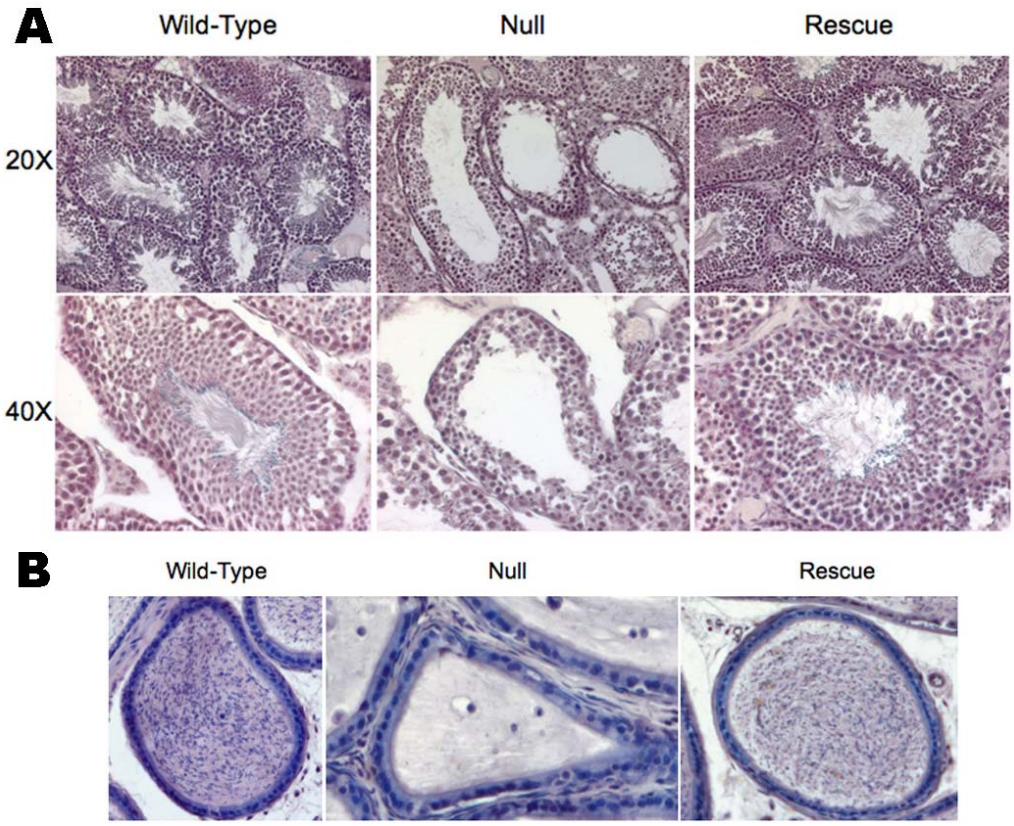

Figure 4

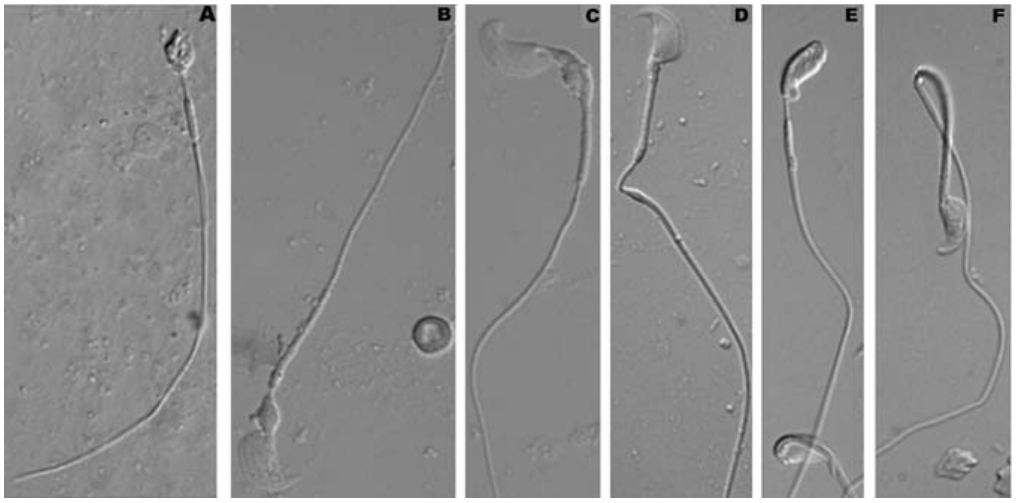

Figure 5

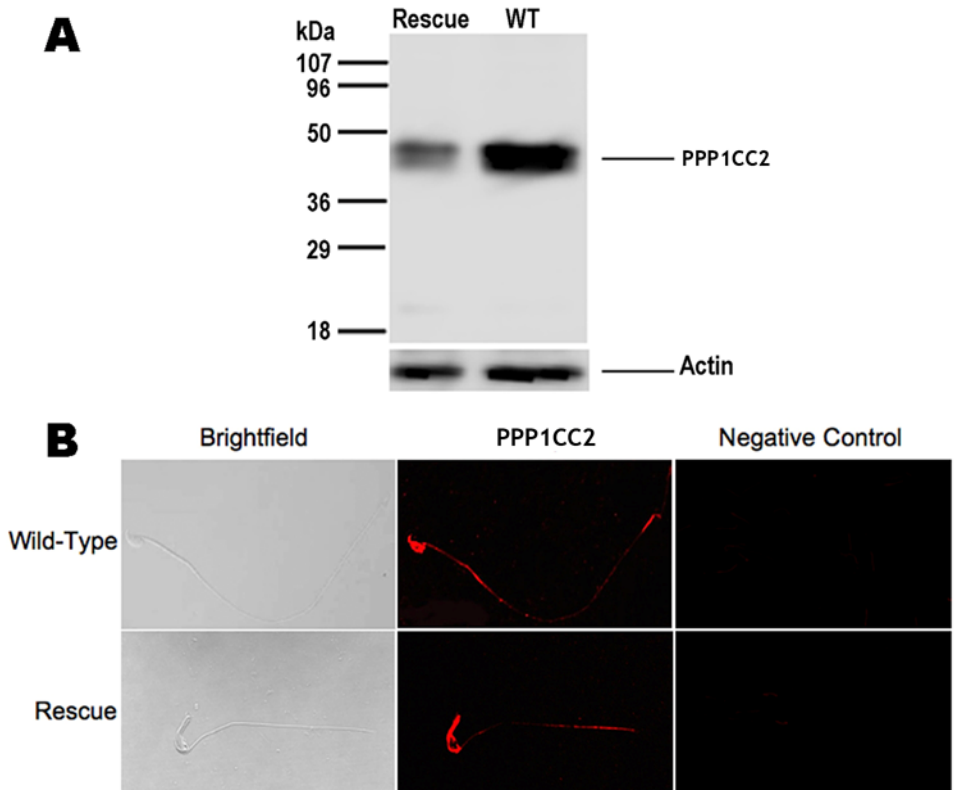
A
kDa
Rescue
WT
107
96
50
36
29
18
PPP1CC2
Actin
B
Brightfield
PPP1CC2
Negative Control
Wild-Type
Rescue

Figure 6