

·Review·

Protein phosphatase PP1 γ 2 in sperm morphogenesis and epididymal initiation of sperm motility

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Abstract

The serine/threonine phosphatase (PP1) isoform PP1 γ 2, predominantly expressed in the testis, is a key enzyme in spermatozoa. High PP1 γ 2 catalytic activity holds motility in check in immature spermatozoa. Inhibition of PP1 γ 2 causes motility initiation in immature spermatozoa and motility stimulation and changes in flagellar beat parameters in mature spermatozoa. The PP1 γ 2 isoform is present in all mammalian spermatozoa studied: mouse, rat, hamster, bovine, non-human primate and man. We have now identified at least four of its regulatory proteins that regulate distinct pools of PP1 γ 2 within spermatozoa. Our studies provide new insights into biochemical mechanisms underlying development and regulation of sperm motility. We hypothesize that changes in sperm PP1 γ 2 activity as a result of phosphorylation and reversible binding of the regulatory proteins to the catalytic subunit are critical in the development and regulation of motility and the ability of sperm to fertilize eggs. Targeted disruption of the *Ppp1cc* gene, which encodes the PP1 γ 1 or PP1 γ 2 isoforms, causes male infertility in mice as a result of impaired spermiogenesis. Our observations suggest that, in addition to motility, the protein phosphatase PP1 γ 2 might play an isoform-specific function in the development of specialized flagellar structures of mammalian spermatozoa. (*Asian J Androl* 2007 July; 9: 445–452)

Keywords: protein phosphatase; epididymis; sperm motility; spermatogenesis

1 Introduction

The spermatozoon is a highly differentiated cell designed to carry out its special function. The spermatozoon is composed of a head containing the acrosome and a condensed nucleus, and a flagellum consisting of a middle piece, a principal piece and an end piece. Flagellar beat propels spermatozoa through the female reproductive tract and through the external investments of the egg prior to fertilization. Spermatozoa leaving the testis are immotile and cannot fertilize or bind to eggs. Testicular spermatozoa acquire motility and fertilizing ability

during their passage through the epididymis.

The epididymis can be divided anatomically into at least four segments: the initial segment, the caput, the corpus, and the caudal epididymidis. Secretions from these segments are composed of distinct proteins. Specific modifications are thought to occur in spermatozoa as they pass through these segments. Spermatozoa removed from the caput epididymidis are immotile and infertile, whereas spermatozoa from the caudal region possess forward motility and the ability to bind and fertilize an egg. Several modifications occur during the passage of sperm through the epididymis. These include remodeling of the sperm plasma membranes, changes in composition and localization of proteins, acquisition and alteration of glycoproteins, and changes in membrane lipid composition [1–3]. The relationship between these changes and the acquisition of sperm motility is not known.

The levels of the intracellular factors known to be

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involved in regulation of kinetic activity of the flagellum, calcium ions (Ca²⁺), pHi and cyclic adenosine monophosphate (cAMP), change during epididymal sperm maturation [1, 4–7]. The levels of calcium fall, whereas intracellular pH and cAMP levels rise. The capacity for motility already exists in immotile testicular and epididymal sperm because motility can be induced in demembrated immotile spermatozoa [1, 8, 9]. Therefore, it is believed that changes in the intracellular levels of the mediators, cAMP, H⁺ and calcium, are responsible for initiation of motility. The mediators are thought to regulate flagellar activity through changes in protein phosphorylation.

The steady-state phosphorylation status of a protein is determined by the relative activities of the protein kinases and phosphatases acting on it. Increases in sperm protein phosphorylation have been implicated in the regulation of sperm function. Research in sperm protein phosphorylation, until recently, was largely focused on protein kinases: protein kinase A (PKA) in particular [10–14]. It is well known that motility stimulation can be affected by cAMP-mediated PKA activation [13, 15, 16]. A role for PKA necessarily implies a function for a protein phosphatase. Protein phosphatases can significantly modify and restrict PKA action. Inclusion of protein phosphatases in the reactivation media prevents motility initiation in demembrated sperm [17, 18]. Inhibition of phosphatase activity results in initiation and stimulation of motility [19, 20], suggesting that phosphatases have an important role in regulation of sperm kinetic activity. Although the role of protein phosphatases as components of signalling and regulatory pathways in other cell types is well known, relatively little is known about phosphatases in spermatogenesis and spermatozoa.

Eukaryotic protein phosphatases are classified into two distinct gene families: serine threonine/phosphatases (PPP) and phosphotyrosine phosphatases (PTP) [21]. Protein phosphatase 1 (PP1) belongs to a family of protein phosphatases, PPP, which include PP1, PP2A and PP2B (calcineurin). The serine/threonine phosphatase, protein phosphatase 1 (PP1), is a highly conserved protein in all eukaryotes. It controls a variety of processes, such as cell division, transcription, translation, muscle contraction, glycogen and lipid metabolism, neuronal signalling and embryonic development [22–24]. In mammals, there are four catalytic subunit isoforms of PP1, encoded by three genes: PP1 α , PP1 β , PP1 γ 1 and PP1 γ 2 [22, 25, 26]. The enzymes PP1 γ 1 and PP1 γ 2, alternatively spliced variants generated from a single gene [26, 27], are identical in all respects except that PP1 γ 2 has a unique 21-amino-acid carboxy-terminus extension. Although PP1 α , PP1 β and PP1 γ 1 are ubiquitous, PP1 γ 2 is predominantly expressed in the testis and appears to be the only PP1

isoform in spermatozoa [19, 20]. Targeted disruption of the *Ppp1cc* gene, resulting in the loss of PP1 γ 1 and PP1 γ 2, causes infertility in male mice as a result of impaired spermiogenesis [26, 28]. This indicates that one or both of the isoforms are involved in sperm development and possibly spermiation (the release of mature spermatozoa from the seminiferous epithelium into the lumen). It is intriguing that the other isoforms of PP1, owing to their high level of amino acid sequence conservation, are able to substitute for the absence of PP1 γ 1 and PP1 γ 2 in all tissues except the testis.

Sperm formation in mammals is characterized by a well-defined sequence of mitotic and meiotic divisions, followed by a long period of complex morphogenetic differentiation, leading to the production of mature spermatozoa [29]. Mammalian sperm development, taking place in the seminiferous tubules of the testis, can be divided into three distinct stages: proliferative, meiotic (spermatogenesis) and spermiogenic (post-meiotic differentiation and morphogenesis). Although previous reports have shown that PP1 γ 2 is the predominant PP1 isoform expressed in the testis [19, 20], its distribution within specific cell types in the murine testis relative to the other PP1 isoforms is unknown. Using isoform-specific antibodies against PP1 γ 1, PP1 γ 2 and PP1 α , a distinct differential distribution of these proteins in wild-type testicular cells and spermatozoa from mice was observed [30]. To elucidate further the involvement of PP1 isoforms in spermatogenesis, we have also analyzed the morphology of developing spermatids from *Ppp1cc*-null mice. Our results indicate that PP1 γ 2 loss has a profound effect on sperm structure and morphogenesis.

This review briefly summarizes our present understanding of PP1 γ 2 and its regulation in mammalian spermatozoa in terms of motility. In addition to motility, we also suggest a role of PP1 γ 2 in development of specialized flagellar structures of mammalian spermatozoa.

2 Expression of PP1 γ 2 in the testes and spermatozoa and its role in motility

Enzyme activity and Western blot analyses showed that PP1 γ 2 is a predominant serine/threonine protein phosphatase in testes [19, 20]. Various murine tissue extracts analyzed by Western blots showed that PP1 γ 2 is a testis-predominant isoform (Figure 1). Western blot analysis also showed that all three PP1 isoforms were present in murine testes [30]; whereas PP1 γ 2 was the only PP1 isoform detected in spermatozoa. These observations in murine spermatozoa and testes are identical to those found with bovine, human and rhesus monkey spermatozoa [19, 20, 31]. Expression profiles of PP1

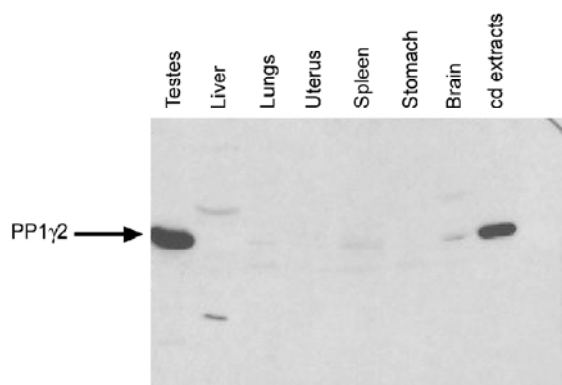


Figure 1. Immunoreactive PP1 γ 2 in mouse tissues. Protein extracts from testes, liver, lung uterus, spleen, stomach, brain and caudal epididymal sperm were separated by SDS/PAGE, transferred on to nitrocellulose membrane, and probed with PP1 γ 2 antibody. 50 μ g of protein was loaded per lane with the exception of caudal sperm extracts where 20 μ g was used.

isoforms in various cell types of the adult testis of wild-type mice using immunohistochemistry demonstrated that PP1 γ 2 was the only isoform abundant in secondary spermatocytes, round and elongating spermatids (Figure 2A), whereas PP1 γ 1 and PP1 α were restricted to spermatogonia, pachytene spermatocytes and interstitial cells (Figures 2B and 2C). Postnatal expression of PP1 γ 2 in developing testes increases with age [30]. The distinct expression patterns of PP1 α , PP1 γ 1 and PP1 γ 2 during post-natal testicular development and spermatogenesis suggest non-overlapping roles for the PP1 isoforms in cells within the testis.

Furthermore, antibodies against the PP1 γ 2 carboxy-terminus showed that the protein was present in spermatozoa from a wide range of mammalian species: immunoreactive PP1 γ 2 was detected in murine, hamster and bovine spermatozoa (Figure 3A), but was absent from *Xenopus* spermatozoa. The enzyme PP1 γ 2 is likely to be present in all mammalian spermatozoa. Surprisingly, the carboxy-terminus of PP1 γ 2, which is not essential for its catalytic activity [32], is conserved, as judged from Western blot analysis. In contrast, PP1 γ 1 which is present in *Xenopus* spermatozoa, is absent from mammalian spermatozoa (Figure 3B). Sperm from sea urchin and turkey contain immunoreactive PP1 resembling PP1 α (data not shown).

High protein phosphatase activity is correlated with low sperm motility, whereas low catalytic activity is associated with vigorous motility in bovine and monkey spermatozoa [19, 20, 31]. Soluble extracts of caput epididymal spermatozoa contain significantly higher PP1 γ 2 activity than caudal epididymal spermatozoa, in

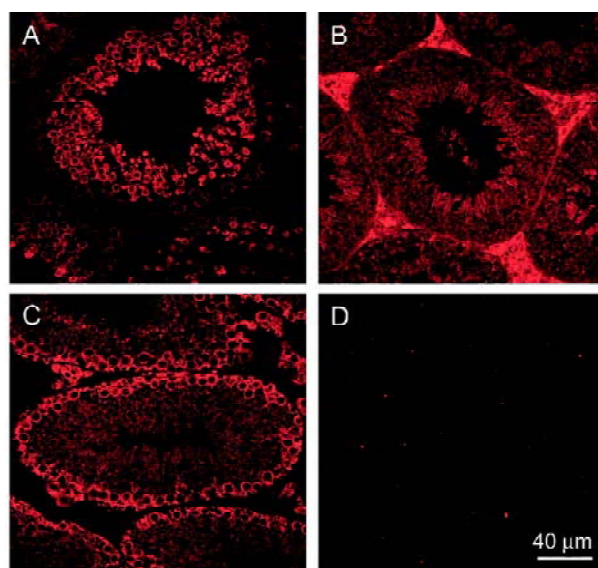


Figure 2. Localization of PP1 γ 1, PP1 γ 2 and PP1 α in wild-type mouse testes sections. (A): PP1 γ 2 is prominently expressed in the cytoplasm of germ cells ranging from secondary spermatocytes and round spermatids to elongating spermatids and spermatozoa. (B): PP1 γ 1 expression is observed predominantly in the interstitial cells of Leydig. (C): PP1 α showed strong expression in interstitial cells, spermatogonia, peritubular cells and pachytene spermatocytes. (D): No signal was seen when preimmune sera was used alone. Bar = 40 μ m.

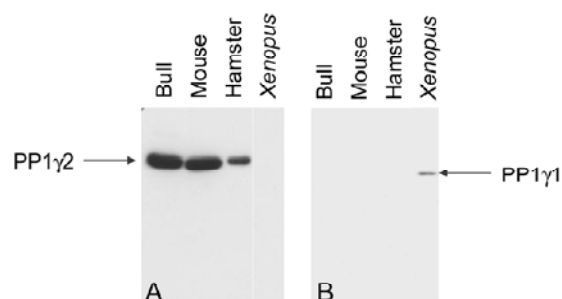


Figure 3. Immunoreactive PP1 γ 2 and PP1 γ 1 in spermatozoa from different species. Sperm extracts (20 μ g in each) were subjected to SDS-PAGE followed by Western blot analysis with PP1 γ 2 (A) and PP1 γ 1 (B) antibodies.

extracts containing the same amount of immunoreactive PP1 γ 2 [19, 20]. These data suggest that the high catalytic activity of PP1 γ 2 might be responsible for the lack of motility in immature caput epididymal spermatozoa. This suggestion is further supported by the observation that inhibition of protein phosphatase activity by calyculin A and okadaic acid induces motility in caput spermatozoa [20]. A lowering of PP1 γ 2 catalytic activity might be part of biochemical mechanisms underlying sperm motility development in the epididymis. Protein phosphatases in general are regulated by their binding and

targeting proteins [22, 25]. Following identification of PP1 γ 2, we expected that one or more somatic cell protein regulators of PP1 might be present in spermatozoa. PP1 γ 2 in extracts from caput and caudal epididymal spermatozoa can be chromatographically resolved into several distinct pools. The proteins associated with PP1 γ 2 in these chromatographic fractions were identified by micro-sequencing.

3 Protein regulators of sperm PP1 γ 2

3.1 Mammalian homologue of the yeast PP1 regulatory protein: *sds22*

One of the proteins that co-eluted with PP1 γ 2 in immuno-affinity chromatography and was identified by micro-sequencing of sperm extracts was *sds22* [33]. A homologue of this protein was identified as a PP1 binding protein in yeast and a nuclear protein in mammalian somatic cells. The enzyme PP1 γ 2 bound to *sds22* is catalytically inactive against the substrate phosphorylase *a* [33], a situation analogous to *sds22*-bound yeast PP1 [34]. Intriguingly, however, unlike in yeast and somatic cells, a substantial portion, if not all, of sperm *sds22* is cytoplasmic [33]. It appears that PP1 γ 2-*sds22* binding is regulated. What regulates *sds22* binding to PP1 γ 2 is not yet known. Phosphorylation of *sds22* or some intermediary protein might be involved [35]. It is possible that a cycle of PP1 γ 2 activation and inactivation, owing to its binding to and dissociation from *sds22*, might be part of the biochemical mechanisms regulating motility and other sperm functions. Studies are in progress to determine how the biochemical mechanisms underlying PP1 γ 2-*sds22* binding regulate sperm function.

3.2 Protein 14-3-3

Protein 14-3-3 isoforms are a highly conserved family of acidic proteins, expressed in a variety of eukaryotic cells. They bind to a wide variety of proteins to regulate and coordinate several cellular processes, such as cell cycle progression, apoptosis, protein trafficking, cytoskeleton rearrangements, metabolism and transcriptional regulation of gene expression [36–38]. The effect of 14-3-3 binding depends on the nature of its ligand. Binding might activate or inhibit the enzyme activity or change the localization and phosphorylation status of proteins [39]. More than 100 14-3-3 binding partners have been identified in somatic cells through affinity chromatography coupled with proteomic analysis [40–43]. We first documented the expression of 14-3-3 in mature spermatozoa and showed that it binds to a distinct pool of PP1 γ 2 in spermatozoa [44]. It appears that PP1 γ 2 bound to 14-3-3 is phosphorylated. The physiological

significance of this binding is yet to be unraveled. It is possible that 14-3-3 regulates PP1 γ 2 catalytic activity, phosphorylation or its interaction with other proteins. There is also evidence that, in addition to PP1 γ 2, at least three other 14-3-3 binding phosphoproteins exist. The identities of these proteins is not known [44]. Studies are underway to identify those and other 14-3-3 binding phosphoproteins and the biological relevance of their binding to PP1 γ 2. Protein 14-3-3 is present in spermatozoa isolated from species as diverse as *Xenopus*, turkey, mouse, bull and man, suggesting an essential role for this protein in male gamete function.

3.3 Inhibitor 2 (I2) and glycogen synthase kinase-3 (GSK-3)

Our studies on sperm PP1 γ 2 first focused on identification of heat-stable inhibitors of the enzyme. The first candidate protein examined was the ubiquitously expressed PKA-regulated inhibitor 1 (I1). The established role of PKA in sperm function made I1 a logical candidate. Surprisingly, I1 activity was undetectable in bovine sperm extracts; however, substantial activity resembling inhibitor 2 (I2) was present in heat-stable sperm extracts [20, 45]. This activity was thought to be I2-like because inhibition could be reversed by glycogen synthase kinase-3 (GSK-3). We have now used specific antibodies to confirm the presence of inhibitor I2 in spermatozoa (Figure 4). Spermatozoa contain high levels of GSK-3 activity and GSK-3 is significantly less phosphorylated

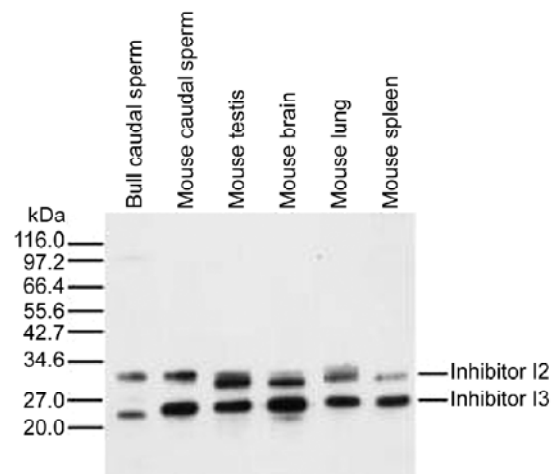


Figure 4. Presence of inhibitor 2 (I2) and inhibitor 3 (I3) in testes and spermatozoa. Heat stable tissue and sperm extracts following Western blot were probed with affinity purified PPP1R11 (I3) antibody (1:1 000) raised against the whole I3 protein (a gift from Dr Mathieu Bollen, Belgium), and affinity purified peptide I2 antibody (1:1 000) raised against amino acid residues 134–147, EKRRQFEMKRKLH, of I2.

(i.e. more active) in immotile caput compared to motile caudal epididymal spermatozoa [20, 45]. Furthermore, an increase or decrease in motility causes a corresponding increase or decrease in tyrosine and serine phosphorylation of GSK-3 [46]. It appears that GSK-3 is inactivated by a combination of tyrosine and serine/threonine phosphorylation. The upstream GSK-3-regulating enzymes, PI3-kinase, PDK1 and PKB, are also present in spermatozoa [46]. It is likely that one of the consequences of inactive GSK-3 (e.g. in caudal spermatozoa) is the lowering of PP1 γ 2 activity because inhibitor I2 is likely to be unphosphorylated and, hence, able to bind to PP1 γ 2. Therefore, low GSK-3 and PP1 γ 2 activities might be prerequisites for the optimum function of spermatozoa. However, the question of whether an extracellular signal activates sperm GSK-3 remains to be answered.

3.4 Inhibitor 3 (I3)

A potent heat-stable PP1 inhibitor was identified through yeast two-hybrid studies designed to identify PP1-binding proteins from human brain [47]. This protein is identical to the protein product of human hemochromatosis candidate gene *HCG V*, orthologous to the mouse *Ppp1r11*, also called *Tctex5*. This is localized to the *t* complex, a naturally occurring polymorphism of the proximal third of chromosome 17 and represented by a family of closely related *t* haplotypes that carry similar mutant alleles of genes implicated in sperm function [48]. The heavily mutated *t*-allele of *Ppp1r11* is thought to code for one of three tightly linked *t* haplotype proteins whose expression in sperm from *t/t* men coincides with a flagellar waveform phenotype, “curlicue”, which is strongly associated with the sterility of these men [49–51]. The protein product of *HCG V/Tctex5* is a protein phosphatase 1 regulatory subunit 11 (PPP1R11, TCTEX5, inhibitor I3). Like protein phosphatase inhibitor I1 and inhibitor I2, PPP1R11 is hydrophilic, heat stable, behaves anomalously on SDS-PAGE and is a specific PP1 inhibitor [47]. Additionally, I3 is extremely sensitive to the protease activity.

We found that PPP1R11 is ubiquitously expressed in various murine tissues and highly expressed in bovine and murine testis and spermatozoa (Figure 4); PPP1R11 from murine and bovine spermatozoa is bound to PP1 γ 2 both *in vitro* and *in vivo*, as shown by immunoprecipitation (IP), microcystin-agarose and GST/His-tagged recombinant-I3 proteins pull down assays. Protein phosphatase assays showed that PPP1R11 inhibited the catalytic activity of both recombinant PP1 γ 2 and PP1 γ 2 in sperm extracts, and that PP1 γ 2 bound to the GST-fusion protein in the pull-down assays was catalytically

inactive. Studies are underway to determine the role of inhibitor I3 in sperm motility.

4 Is the PP1 γ 2 isoform essential in spermatozoa?

The isoforms PP1 γ 1 and PP1 γ 2 are alternatively spliced products of a single gene consisting of eight exons [26, 27]. The two isoforms result from the inclusion (PP1 γ 1) or exclusion (PP1 γ 2) of an intron between exons 7 and 8. This last exon contributes the unique 21-amino-acid extension in PP1 γ 2. This carboxy-terminus sequence does not appear to be essential for catalytic activity, as truncated PP1 γ 2 lacking the 21-amino-acid C-terminus is able to complement the PP1-deficient yeast cell [32]. Male mice lacking the PP1 γ gene (i.e. both PP1 γ 1 and PP1 γ 2 isoforms) are sterile owing to arrest

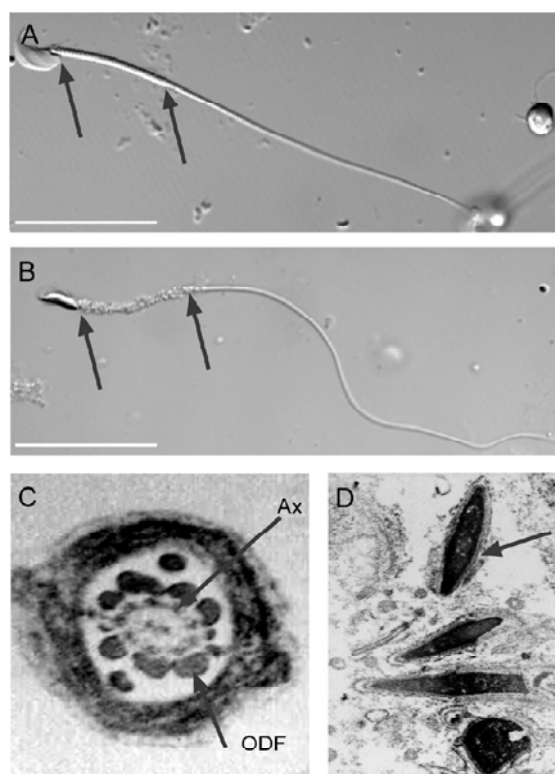


Figure 5. Aberrant morphology of testicular spermatozoa of *Ppp1cc*-null mouse. (A): Normal hook shape head and mid-piece of testicular spermatozoa of wild type mouse. Scale bar = 40 μ m. (B): *Ppp1cc*-null sperm display mid-pieces that have disorganized mitochondrial sheaths and aberrant sperm head structure. Scale bar = 40 μ m. (C): Transverse section through the principal piece of *-/-* developing sperm tail showing many extra outer dense fibers (ODF) (total of 13) and apparent developmental abnormality of the fibrous sheath, particularly the morphology of its longitudinal columns ($\times 80\ 000$). (D): An example of degeneration of a condensing spermatid from a *-/-* mouse ($\times 80\ 000$). Ax, axonemal complex.

of spermatogenesis at the spermatid stage [26, 28, 52]. In contrast, *Ppp1cc*-null females are all fertile [26]. There was a significant reduction in spermatids and testicular spermatozoa in *Ppp1cc*-null testes. Loss of spermatids occurred at the round spermatid stage and increased in severity resulting in a marked reduction in condensing and elongating spermatids and an almost complete absence of mature spermatozoa. Intriguingly, PKA knockout and casein kinase knockout mouse models do not show this phenotype. Therefore, our results suggest that PP1 γ 2, along with an unknown protein kinase, might be involved in protein phosphorylation and dephosphorylation events during spermiogenesis.

The reduced number of spermatozoa in *Ppp1cc*-null mice might be a result of either increased apoptosis or cell death or a result of a defect in spermiation [30] leading to phagocytic activity of Sertoli cells [53, 54]. A detailed ultrastructural analysis using light and transmission electron microscopy showed numerous structural defects in elongating spermatids and testicular spermatozoa of the *Ppp1cc*-null male mice (Figure 5). Abnormal head shapes were also observed in agreement with a previous report [28]. Prominent defects observed were poorly developed or missing mitochondrial sheaths, and supernumerary, disorganized outer dense fibers (ODF) throughout the sperm tails [30]. We also detected frequent degeneration of condensing spermatids, indicated by fragmentation of tail structures and the presence of numerous vacuoles in the cytoplasm of elongating spermatids. A subtle abnormality in the development of the fibrous sheath was observed, specifically in the formation of distinct, triangular-shaped longitudinal columns and inward projections that replaced the ODF associated with axonemal microtubule doublets 3 and 8 in wild-type sperm [30]. These observations suggest that PP1 γ 2 is required for flagellar integrity and the development of flagellar structures. Defects in flagellar structures prompted us to determine the expression of some of the post-meiotic proteins. Western blot analysis and immunohistochemistry showed the absence of, or a sharp reduction in, the levels of post-meiotic proteins (AKAP4/AKAP82, *odf2*, *sds22*, FSII) in *Ppp1cc*-null testes [30]. Some of these proteins appear to be associated with sperm tail development and function [20, 33, 55–57]. This reduction of protein expression could be a result of a lack, or reduced number, of cell types expressing these proteins or a result of reduced protein expression in spermatids. Although studies using selected antibodies and immunofluorescence in sections suggest reduced protein expression in the null testes, further examination using immunocytochemistry of testicular spermatozoa has shown that AKAP4, *odf2*, FSII and *sds22* are present

in mutant spermatozoa [30]. This suggests that the apparent reduction in intracellular protein levels seen in Western blots could be a result of a reduced number of differentiating spermatids in the null testis where these proteins are synthesized. It is possible that defective tail development might be a result of reduced levels of these flagellar proteins. Additional studies are required to determine the exact role of PP1 γ 2 in protein synthesis in developing spermatids.

Our experiments show that the PP1 γ 2 isoform is present in all mammalian spermatozoa studied (mouse, hamster and bull), and absent from non-mammalian species, such as *Xenopus* (Figure 3A). In contrast, PP1 γ 1 is present in *Xenopus* sperm extracts (Figure 3B). Therefore, it is tempting to speculate that PP1 γ 2 might have an isoform-specific function in the development of outer dense fibers and the fibrous sheath, which are structures found in mammalian sperm. If this is true, then PP1 γ 2, but not PP1 γ 1, should restore sperm formation and fertility in *Ppp1cc*-null mice. Moreover, because spermatozoa lack PP1 γ 1, it is more likely that lack of PP1 γ 2 is responsible for the morphological defects in spermatozoa. To test this hypothesis, we made transgenic mice expressing either PP1 γ 1 or PP1 γ 2 under the testis-specific *Pgk2* promoter. Studies to determine which of the two isoforms might rescue the phenotype in *Ppp1cc*-null mice are underway in our laboratory.

In summary, the enzyme PP1 γ 2 is a key signalling protein in spermatozoa. The enzyme is regulated in novel ways: by phosphorylation and by proteins identified for the first time in spermatozoa. Understanding how regulation of the enzyme is essential in spermatogenesis and in mature sperm function has implications for clinical andrology and in the identification of novel targets for the development of male contraceptives.

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