The PP1 gamma isoforms restore spermatogenesis but not fertility in PP1 gamma null mice.

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SUMMARY

Two isoforms of protein phosphatase 1, PP1γ1 and PP1γ2, are translated from alternatively spliced transcripts of a single gene, Ppp1cc, and differ only at their extreme C-termini, where PP1γ2 has a 23-amino acid extension that PP1γ1 lacks of. While PP1γ1 expression is ubiquitous, PP1γ2 is largely restricted to testicular germ cells and is the only isoform present in mature spermatozoa. Targeted deletion of Ppp1cc leads to sterility of +/- males due to a combination of gross structural defects in developing spermatids and faulty spermiation.

The transgenic expression of PP1γ1 or PP1γ2 driven by the testis specific promoter in Ppp1cc +/- mice -rescue mice- was designed in order to test whether either isoform could restore fertility per se.

Transgenic PP1γ2 expression in Ppp1cc +/- testis enabled spermatid development and spermiation and as a result, epididymis of rescue mice contained abundant spermatozoa albeit with several morphological abnormalities and low motility. Transgenic PP1γ1 produced a similar spermatogenetic phenotype but PP1γ1-rescue spermatozoa displayed wider heterogenic abnormalities and no motility compared to the PP1γ2-rescue mice. Neither the combined expression of PP1γ1 and PP1γ2 under PGK2 managed to reverse the virtually immotile spermatozoa and produce fertility competent sperm.

These results leave the door open to several interpretations. The PP1gamma endogenous promoter may be crucial in order to correctly orchestrate and increase the expression levels on both isoforms, or PP1gamma isoform expression is also required in the adjacent nurturing Sertoli cells.
Clarification:

For reading easiness, I will use the forms PP1γ1 and PP1γ2 instead of the standard nomenclature forms PPP1CC1 and PPP1CC2 respectively when referring to the protein isoforms.

INTRODUCTION

1. Spermatogenesis in mice

Spermatogenesis is the process of cellular differentiation in which diploid progenitor cells in the testis differentiate into haploid spermatozoa. Is one of the most productive self-renewing systems supported by stem cells in adult animals and is assisted by the complex network of nurturing and supporting cells present in the testis and known as Sertoli and Leydig cells (Figure 1).

A large number of spermatozoa can be produced in individuals of mammalian species—on the order of $1 \times 10^8$ sperm/ml or approximately 1,000 spermatocytes produced per heart beat. This high sperm cell productivity depends on spermatogonial stem cells (SSCs) that self-renew and produce daughter spermatogonia in a continuous fashion through adulthood till death in the male gonads. In turn, spermatogonia proliferate, undergo meiosis and morphologically differentiated into the fertility-competent sperm.

Figure 1 illustrates the overview on the differentiating process with the main cell types identified, while Figure 2 is an actual picture of a seminiferous tubule cross-section stained with hematoxylin-eosin stain.
Figure 1. Theoretic drawing of mammalian spermatogenesis. SC, Sertoli cell; Ap type A spermatogonia; AD type A dark spermatogonia; B type B spermatogonia; MP middle pachytene spermatocytes; ES early round spermatids; LS elongating spermatids; LM basal membrane of the seminiferous tubules. After MA Handel, Genetic Control of Spermatogenesis in mice; 1987, vol.15
Figure 2. Cross-section of a mouse seminiferous tubule. Nurturing cells and the main cells within the spermatogenetic lineage are identified with hematoxylin-eosin staining.

One of the central events of taking place in spermatogenesis is the meiosis, which comprises the reduction of the diploid number of chromosomes into the haploid gametes, the spermatozoa. This step takes approximately 11 days and during it chromosomes condense, pair, the synaptonemal complexes are formed and genetic recombination occurs between them in the functionally tetraploid pachytene spermatocyte cell type. Another feature to be highlighted during spermatogenesis is the early termination of transcription in the male germ cells, which makes translational regulation of the stored mRNAs an essential step in order to synthesize many of the proteins that will appear later during the haploid phase of spermatogenesis.
In rodents spermatogenesis takes approximately 22 days to complete, (Russell LD, Ettlin RA, Sinha-Hikim Amiya P., Clegg ED., 1990)(Meistrich ML, van Beek MEAB, 1993) and strict control of self-renewal and differentiation of SSCs is crucial to maintain a continuous spermatogenesis. Elucidation of the regulatory mechanisms involved in this control is one of the key questions awaiting to be answered in sperm biology.

Overall, spermatogenesis can be divided into three phases: the mitotic proliferation of spermatogonia, the meiotic division of spermatocytes, and the morphologic remodeling of the haploid spermatid cells into spermatozoa during the spermiogenesis.

1.2 Spermatogonia

Spermatogonia are classified into several subtypes according to the differentiation status. In rodents, like rats and mice, the spermatogonia are grouped into type A, intermediate, and type B spermatogonia. This mitotic cell passage takes approximately 10 days to complete in spermatogenesis in mice (Eddy, 1998).

The type A spermatogonia are further divided into $A_0$ (undifferentiated) and $A_1, A_4$ (differentiating) spermatogonia. $A_0$ spermatogonia are again differentiated into $A_{\text{single}} (A_s)$, $A_{\text{paired}} (A_{pr})$, and $A_{\text{aligned}} (A_{al})$ spermatogonia, which are defined primarily by position and cellular association within the seminiferous tubules.

Each $A_s$ spermatogonium is present in an isolated manner, while $A_{pr}$ and $A_{al}$ spermatogonia stay in a paired or aligned fashion, respectively, connected by cytoplasmid bridges. Every spermatogonium resides in the basement membrane of the seminiferous tubules. They divide and differentiate at that location. The mitotic kinetics of these cells are complex (Bellve AR, 1979), but just to describe their potential, it is estimated that one rat spermatogonia can produce 1,024 spermatocytes and which its sequential meiotic cell divisions,
this results in the production of 4,096 spermatozoa (Russell LD, Ettlin RA, Sinha-Hikim Amiya P., Clegg ED., 1990). It has been estimated, however, that 75-90% of spermatocytes disappear through apoptotic cell death (Barrat CLR, 1995),(Tegelenbosch & de Rooij, 1993). Figure 3 illustrates this process with the different spermatogonial populations identified in a sequential manner.

Figure 3. The spermatogonia progeny through a complete spermatogeneic round.
1.3 Meiosis

After a finite number of mitoses, the type B spermatogonia give rise to primary spermatocytes, the preleptotene spermatocytes, that undergo the last cell cycle S phase replication of the DNA and then enter meiotic prophase (Figure 4). The meiotic prophase of primary spermatocytes is a lengthy and complex process which can take up to 11 to 12 days in mouse, and is characterized by intense RNA transcription, pairing of homologous chromosomes and repair synthesis of DNA presumably associated with recombination. It comprises the leptotene, zygotene, pachytene, diplotene and diakinesesis stages, where the pachytene spermatocytes are the largest of all the spermatogeneic cells (Figure 2), and this stage is the longest phase of spermatogenesis. Coincident with early meiotic function, the primary spermatocytes move away from the basement membrane of the seminiferous tubule (Figure 2) passing through the junctions of the Sertoli cells from the basal compartment to the adluminal compartment of the seminiferous tubule.
Figure 4. Meiosis Overview. Meiosis is composed of two critical divisions: reductional division and equational division. The reductional division occurs during Meiosis I when homologous chromosomes are separated producing two haploid cells. In Meiosis II is where the equational reduction occurs whereby the sister chromatids are split, creating a total of 4 haploid cells (spermatids) per daughter cell from the first division as seen in the scheme above.

The adlumnial compartment is a microenvironment sequestered from the body by the blood-testis barrier, the morphological basis of which is the specialized Sertoli cell junctions (Russell LD, 1980). The effect of this barrier is to make spermatogeneic cells relatively inaccessible physiologically in order to prevent the exposure of their unique antigens to the
immune system. By this mechanism, spermatogenetic cells are protected from immunological rejection, especially during and after meiotic division, which at this point they could be detected as foreign cells from the body.

The first meiotic division also accomplishes segregation of homologous chromosomes, producing the secondary spermatocytes. These cells rapidly undergo the second meiotic division and each secondary spermatocyte will produce four haploid, round spermatids. During this process, the round nucleus undergoes elongation and the chromatin becomes tightly compacted and condensed, with concomitant changes in the repertoire of chromosomal proteins. It is at this time when the nucleus of mouse spermatids also acquires a rostral curvature, resulting in the species – and sometimes strain- specific shape.

Finally, the nucleus becomes invested by a membranous derivate of the Golgi apparatus, called the acrosome. This structure contains enzymes that will subsequently digest a path for the sperm through the outer vestments of the egg.

1.4 Spermiogenesis

Spermiogenesis takes approximately 14 days to complete in the mouse, (NEBEL, AMAROSE, & HACKET, 1961) and is the step when the haploid round spermatids differentiate into the mature spermatozoon. Many unique morphological transformations occur this process, including: remodeling and condensation of the nucleus into species-specific shapes, assembly of the tail, reduction in number and localization of the remaining mitochondria of the cell toward the midpiece region of the developing sperm, formation of the acrosome in the nucleus, and topographical changes in the cell surface (Bellve AR, 1979)(Bellve AR and O’Brien D.A., 1983). Toward the end of spermiogenesis, most of the cytoplasm of the maturing male gametes are
eliminated in a structure called the residual body. Many of these differentiative changes in the developing germ cell occur concurrently with or after nuclear DNA condensation and termination of RNA transcription.

The complex tail formed during spermiogenesis is characterized by the typical eukaryotic axonemal complex of two inner singlet and nine outer doublet microtubules. Throughout much of its length, this axonemal complex is surrounded by nine outer dense fibers. These structures are unique to mammalian sperm cells and their function is not yet understood. Peripheral to the outer dense fibers the mitochondria form a helical coil in the anterior portion (or middle piece) of the tail. Another unique structure, the fibrous sheath, develops in the principal piece of the tail. Amazingly, almost nothing is known of the mechanisms controlling the synthesis and assembly of these specialized components of the mouse sperm tail.
Figure 5. The morphological steps involved in mammal (rat) spermiogenesis. During spermiogenesis, the spermatids undergo a remarkable sequence of differentiation that includes condensing the chromatin, reorganizing and shaping the nucleus, forming and acrosome replete with enzymes to aid fertilization, and assembling a tail with its microtubule doublets, structural fibers, and a gyre of mitochondria. Notice how the acrosome is formed until attaining its elongated distinctive form in step E. The manchette is a characteristic structure composed of a conically shaped array of microtubules that completely covers the nucleus of the spermatid.

After MA Handel, Genetic Control of Spermatogenesis in mice; 1987, vol.15.
1.5 Capacitation of the Sperm

Although morphologically mature and motile, ejaculated spermatozoa from mammals do not yet possess the ability to fertilize the egg inside the female tract. In this matter, ejaculated sperm require a finite period of residence in the female reproductive tract to become fertilization-competent. This time-dependent acquisition of fertilization competence has been defined as ‘capacitation’ by both (CHANG, 1951)(CHANG, 1955) and (AUSTIN, 1951; AUSTIN, 1952). Thus, capacitation was originally defined as the time interval of sperm incubation (either in vivo or in vitro) that is required to bring about this final functional maturation of the sperm (Chang, 1984). The definition of capacitation has also been modified over the years to include the acquisition of the ability of the acrosome-intact sperm to undergo the acrosome reaction in response to its interaction with the zona pellucida (ZP), the egg’s extracellular matrix (Ward & Storey, 1984); (Florman H.M., 1991); (Kopf G.S., 1991).

Capacitation has also been shown to be correlated with changes in sperm intracellular ion concentrations, plasma membrane fluidity, metabolism, and motility (Yanagimachi, 1994). Although these changes have been known for many years to accompany the process of capacitation, the molecular basis underlying these events is poorly understood. It appears that certain components of such media play an important role in promoting the capacitation process. Work in a variety of species has suggested that the presence of serum albumin (Go & Wolf, 1985), Ca\(^{2+}\) (Coronel & Lardy, 1987); (Fraser, 1987)(Ruknudin & Silver, 1990);) and NaHCO\(_3\) (Lee & Storey, 1986);(Neill & Olds-Clarke, 1987); (Boatman & Robbins, 1991) in the medias surrounding the spermatozoa are required for capacitation.
However, it must also be emphasized that since the strict endpoint of capacitation is the ability to fertilize an egg and that this property is dependent on many different sperm functions (i.e. bona fide motility, hyperactivated motility, ability to undergo the acrosome reaction, etc.) it is not yet well delineated which sperm function is affected by the abovementioned media components.

The transmembrane and intracellular signaling events regulating sperm capacitation are, likewise, poorly understood. As stated above some of these events may be coupled to changes in ionic movements within the sperm during this time. Changes in sperm cyclic nucleotide metabolism and protein phosphorylation have been implicated in a variety of sperm functions, including the initiation and maintenance of motility (Garbers & Kopf, 1980); (Tash & Means, 1983); (Lindemann & Kanous, 1989); (Yanagimachi, 1994), induction of the acrosome reaction (Garbers & Kopf, 1980); (Kopf G.S., 1991), and capacitation (Monks, Stein, & Fraser, 1986)). Changes in tyrosine phosphorylation of specific sperm proteins have also been demonstrated to occur under conditions that support capacitation (Leyton & Saling, 1989)(Duncan & Fraser, 1993), although a correlation or cause-and-effect relationship between these two parameters has not been examined.

2. The Spermatozoon

The spermatozoon is the final product of spermatogenesis produced in the seminiferous tubules of the male tract (Figure 2). It is a motile and terminally differentiated structure designed to safely transport the haploid genome of the male progenitor in order to fertilize the egg inside the female tract in mammals. Its structure is well defined and its two main components are the head and flagellum, joined by the connecting piece (Figure 6). The head
contains the nucleus, acrosome, cytoskeletal structures, and a small amount of cytoplasm. The nucleus also contains the highly condensed chromatin, and is capped anteriorly by the acrosome, a membrane-enclosed cytoplasmic vesicle containing hydrolytic enzymes and operational only during the final fusion step between the spermatozoa and the egg inside the female tract. From the connecting piece, the flagellum is divided successively into the midpiece, principal piece and end piece regions (Figure 6). It contains a central complex of microtubules forming the axoneme, surrounded in turn by outer dense fibers extending from the neck into the principal piece. The midpiece contains the mitochondrial sheath, a tightly wrapped helix of mitochondria surrounding the outer dense fibers and axoneme. The outer dense fibers and the fibrous sheath are cytoskeletal structures that only appear present in higher vertebrates and may have evolved with the development of internal fertilization (Baccetti, 1986). The flagellum, like the head, is tightly enclosed by the plasma membrane and contains a sparse amount of cytoplasm. While most mammalian spermatozoa have these general characteristics, there are substantial species-specific differences in the size and shape of the head, and in the length and relative amount of the different components of the flagellum. Sperm from invertebrates usually have a head with an acrosome and a flagellum containing an axoneme and mitochondria, but lacking accessory cytoskeletal structures (Roosen-Runge, 1977).
Figure 6. DIC picture of a mouse spermatozoa.

The specialized structural features of mammalian spermatozoa may reflect the unique functions of this terminally differentiated cell type. The acrosome contains enzymes essential for penetrating the zona pellucida that surrounds the egg in order to achieve fertilization, while the flagellum contains the energy sources and machinery to generate the propulsion needed in order to attain the rendez-vous with the egg. These functions are essential for delivery of the genetic material contained in the sperm nucleus to the cytoplasm of the egg, where combination of the haploid male and female pronuclei occurs to produce the zygote and initiate development. In most mammals, the sex chromosome carried in the haploid sperm nucleus determines the sex of the resulting animal (Segal, 1985). Both a maternal and a paternal
genome are required for normal development to proceed to term, usually due to differential imprinting of genes during gametogenesis in males and females (Surani et al., 1990), (Kelly & Trasler, 2004).

2.1 The Sperm Head

The mammalian sperm head contains the nucleus and the acrosome surrounded by moderate amounts of cytoskeletal components and cytoplasm. While the nucleus is the structure responsible to deliver the genetic information, the acrosome is the enabler of the fusion between the egg and the sperm (Figure 6). Accordingly, the acrosome caps the anterior end of the nucleus, while the cytoskeletal components lie in the narrow space between the acrosome and the nucleus and between the acrosome and the plasma membrane. The sperm shape of most mammalian species has a spatulate head (Figure 7), with the nucleus and acrosome flattened in the plane of the anterior-posterior axis of the sperm.
Figure 7. Sperm head in mammals. Comparison of the falciform-shaped heads of mouse, rat, hamster with the spatulate-shaped in human, rabbit and guinea pig.

There are some exceptions to this structural disposition, like in the guinea pig, where the distal part of the principal piece bends out of the flattened plane and in rodents, its sperm heads have falciform-shapes instead of spatulated ones. It is remarkable to mention that although sperm numbers are uniform in size and shape, approximately one third of human sperm presents abnormal sizes and shapes.

2.2 The Sperm Nucleus

The chromatin of the sperm nucleus is highly condensed in its volume represents less than 5% of the one found in somatic cells. The organization and the amount of DNA within the sperm nucleus are also unique characteristics of the sperm nucleus. As a result of the two meiotic divisions that occur during spermatogenesis, only one copy of each chromosome is
present in the nucleus. Although as much as 15% of the nuclear DNA is still associated with histones (Tanphaichitr, Sobhon, Taluppeth, & Chalermisarachai, 1978), protamines are the major nuclear proteins associated with the mammalian sperm DNA.

Protamines are relatively small, about 27-6 amino acids, they are highly basic and rich in arginine and cystein proteins. In order to cope with the absence of translation in the spermatozoa, the mRNA encoding mouse protamines are transcribed from the haploid genome at the round spermatid stage, and translation is delayed until spermatids are undergoing elongation (Hecht NB, 1999).

Another common structure with unusual characteristics is the sperm nuclear envelope. In the sperm nuclear envelope the customary nuclear pores are absent and their inner and outer membranes are 7-10nm apart, in comparison with most other cells where their separation is about 40-60nm (Fawcett, 1975),(Friend & Fawcett, 1974)(Koehler, 1970).

2.3 The Acrosome

The acrosome is a unique sperm organelle that originates from the Golgi complex and contains enzymes required for the sperm to penetrate through the zona pellucida of the egg and thus eventually achieve fertilization. Acrosome shaped and size varies widely between species and the distribution and relative prominence of these two segments differ accordingly (Fawcett, 1970).

The acrosome is a membrane-enclosed vesicle sitting as a cap over the nucleus in the anterior part of the sperm head. The acrosome has been highly conserved throughout evolution and along with the axoneme is a hallmark of spermatozoa of animals in many phyla. Most of the
acrosomal components are produced during spermiogenesis, but a few begin to be synthesized in late pachytene spermatocytes.

Overall, the acrosome consists of two segments, the acrosomal cap (anterior acrosome) and the equatorial segment (posterior acrosome), which corresponds in distribution to the plasma membrane domains with the same names.

The acrosome plays a critical role in the fertilization process in mammals. Multiple enzymes are present within it, including acid hydrolases commonly found in lysosomes, and other enzymes specific to spermatogeneic cells. Thus, the acrosome has been commonly referred as a specialized lysosome (Friend & Fawcett, 1974),(Mann, T., Lutwak-Man, C., 1981),(Allison & Hartree, 1970), but it also holds bona fide characteristics of a regulated secretory vesicle.

The release of its contents is produced by exocytosis, and is calcium-mediated in response to specific signals. Following the release and activation of acrosomal enzymes, spermatozoa penetrate the zona pellucida surrounding the oocyte and syngenetesis is achieved.

Among the several enzymes that have been described within the acrosome, one of the best characterized constituents is the protein acrosin, a member of the serine protease superfamily that is present only in spermatogeneic cells. Acrosin is a trypsin-like protease which differs from similar enzymes in other tissues in its molecular weight, substrate specificity an inhibitor specificity (Polakoski & Parrish, 1977)(Tobias & Schumacher, 1977),(Brown & Harrison, 1978),(Mukerji & Meizel, 1979),(Mueller-Esterl, W., Fritz, H, 1981).
2.4 The Flagellum

The flagellum of the mammalian spermatozoon consists of four distinct segments: the connecting piece or neck, the middle piece, the principal piece, and the end piece (Figure 6). The main structural components within the flagellum of the mammalian sperm are the axoneme, the mitochondrial sheath, the outer dense fibers, and the fibrous sheath (Figure 6). The axoneme is a structure that extends throughout the full length of the flagellum and is composed of a “9+2” complex of microtubules. The outer dense fibers are adjacent to the axoneme and extend from the connecting piece to the posterior portion of the principal piece. The characteristic feature of the middle-piece segment is the mitochondrial sheath, while the fibrous sheath defines the extent of the principal-piece. While the mitochondrial sheath immediately surrounds the outer dense fibers in the middle piece, the fibrous sheath surrounds the outer dense fibers in the principal piece. The base of the flagellum abuts the nucleus at the junction between the connecting piece and the head (Zamboni & Stefanini, 1971).

The flagellum is the structure in the spermatozoon responsible for providing the motile force necessary for the sperm to reach the egg surface and achieve fertilization. The sperm length varies between species: in the rabbit it is 46um long, the mouse has 120um, the rat 190um and the Chinese hamster 250um. In humans the sperm length measures approximately 60um while the flagellum is 55um (Baccetti B, 1984).

2.5 The Mitochondrial Sheath

The mitochondria are helically wrapped around the outer dense fibers in the middle piece of the sperm tail (Figure 8).
Figure 8. The mitochondrial sheath.

They are generally arranged end-to-end, but the number of parallel helices, the number of gyres, and the length of the middle piece vary between species. In the mouse, the mitochondria are usually arranged in two parallel helices, with an average of 87 windings around the flagellum (Roy, Yan, Burns, & Matzuk, 2004). These mitochondria are usually of variable length and about end-to-end at random along the helix. Interestingly, the middle pieces differ considerably in length and the organization of mitochondria is quite variable between species with the mitochondrial gyres being proportional to the length of the middle piece as well. Underlying and adhering to the mitochondrial sheath is a complex network of filaments named the sub-mitochondrial reticulum (SMR), (Olson & Winfrey, 1985),(Olson & Winfrey, 1990). Briefly, the SMR consists of a network of ribbons of filamentous material that are laterally interconnected and fuse with the annulus at the junction between the midpiece and the principal piece. It has been hypothesized that the SMR may function in assembling and maintaining the ordered array of mitochondria in the midpiece (Olson & Winfrey, 1990).
2.6 The Axoneme

The organization of the axoneme in the mammalian sperm tail is similar as the one found in cilia and flagella of most plants and animals. The disposition of this structure consists of two central microtubules surrounded by nine microtubule doublets (Figure 9), (Fawcett & Porter KR, 1954).

![Figure 9. The axoneme in bull spermatozoa. An ultrastructural cross-cut of the axoneme with Outer Dense fibers numbered clockwise. Note the peripheral 9+2 doublets of the axoneme plus the central two. Arrow points at the fibrous sheath.](image)

Each doublet consists of a complete A microtubule, onto which is attached a “C-shaped” B microtubule. Radial spokes project helically towards the central pair of microtubules from the seven outer doublets of microtubules that surround the central pair (Bryan & Wilson, 1971). The doublets are numbered one through nine, with number one being the doublet situated on a
plane perpendicular to that bisecting the microtubules of the central pair (Figure 9). The microtubules are composed of α-tubulin and β-tubulin, closely related proteins of approximately 56 and 54 kDa, respectively.

During spermiogenesis, multiple α-tubulin and β-tubulin genes are expressed (Hecht NB, Klene KC, Distel RJ, & Silver IM, 1984), including testis-specific isoforms, and the diversity of these proteins are further expanded by posttranscriptional modifications (Kierszenbaum, 2002)(Distel, Kleene, & Hecht, 1984)(Hecht, Bower, Waters, Yelick, & Distel, 1986).

2.7 Outer dense fibers

The nine outer dense fibers surround the axoneme forming the “9+(9+2)” complex throughout the length of the middle piece and most of the principal piece of the flagellum of mammalian sperm (Figure 6, Figure 9). The outer dense fibers (ODF) are numbered corresponding to the adjacent microtubule doublet. Their basic disposition shows little variation in mammalian species, but they differ considerably in size and shape between species. ODF also differ among themselves in shape and size, with fibers 1, 5 and 6 being larger than the others. In human (Serres, Feneux, & Jouannet, 1986)), bull, and rat sperm ((Lindemann, Fentie, & Rikmenspoel, 1980)(TELKKA, FAWCETT, & CHRISTENSEN, 1961), the outer dense fibers occupy 60% of the length of the principal piece.

Their formation begins at the spermatid level and their composition contains cystein-rich proteins that are cross-linked by disulfide bonds during epididymal maturation (Calvin & Bedford, 1971), (J. M. Bedford & Calvin, 1974). Studies using radioautography also showed incorporation of proline accompanying a rapid protein synthesis during the growth of ODFs (Irons & Clermont, 1982). Some of the proteins best characterized are ODF2, ODF3 and ODF4.
ODF specific function remains unclear. It was first speculated ODF to be contractile because of their close association with the axoneme, but experiments with Chinese hamsters and rats (Phillips, 1972) suggest that the dense fibers might influence the form of the beat by determining the elastic properties of the sperm tail (Phillips, 1972). Additionally ODF could also provide added strength to protect sperm from damage by the shear forces encountered during epididymal transit or ejaculation (Baltz, Williams, & Cone, 1990).

2.8 Fibrous sheath

The fibrous sheath is an important structure which defines the extent of the principal piece, which is the longest segment of the flagellum (Fawcett, 1970). It is a cytoskeletal structure that is probably unique to the flagellum of sperm in mammals and some birds. It closely underlies the plasma membrane and is a tapering cylinder formed by two longitudinal columns connected by circumferential ribs (Figure 9 white arrow). Being the outermost surface in the sperm principal piece, one of its functions is that of blanketing the underlying structures and components. Accordingly, the fibrous sheath is highly resistant to acid solubilization (Wartenberg H. and Holstein F., 1975) and disulfide bonds cross-link the proteins and stabilizing it (Lalli & Clermont, 1981). Another classical view on its function is to modulate the plane of the flagellar beat by imposing a restraint to sliding of axonemal doublets and to flagellar bending (Eddy, Toshimori, & O'Brien, 2003). It also has become apparent that the fibrous sheath also serves as a scaffold for components involved in signal transduction, energy production, and other functions (Eddy et al., 2003). Several cAMP-dependent proteins kinase anchoring protein (AKAP) have been indentified (Eddy et al., 2003). An additional role for the fibrous sheath is to anchor enzymes of the glycolytic pathway, PGK2 (McCarrey & Thomas, 1987)) and GAPDS ((Welch, Schatte, O'Brien, & Eddy, 1992).
3. Protein Phosphatases


Thousands of proteins are expressed in a typical mammalian cell and about one third contain covalently bound phosphate. Approximately two hundred protein kinases and one hundred protein phosphatases have been already identified. Overall, 2-3% of all eukaryotic genes may code for protein kinases (Hanks, Quinn, & Hunter, 1988).

Until recently, protein phosphatases were perceived as rather unsophisticated components in the cellular tapestry of phosphorylation/dephosphorylation reaction and it was thought that there were only a few relatively unspecific phosphatases capable of resetting the work of numerous protein kinases. But this scenario is increasingly being regarded as far from being the real one.

Based on biochemical parameters, protein phosphatases holoenzymes were initially divided in two main principal classes: type-1 phosphatases (PP1) and type-2 (PP2). In turn, type-2 can be further subdivided into spontaneously active (PP2A), Ca\textsuperscript{2+}-dependent (PP2B) and Mg\textsuperscript{2+}-dependent (PP2C) classes, while PP1 can be divided in the PP1\(\alpha\), PP1\(\beta\), PP1\(\gamma\)1 and PP1\(\gamma\)2 isoforms. PP1\(\alpha\) and PP1\(\beta\) have their own genes and are ubiquitously expressed, while PP1\(\gamma\)1 and PP1\(\gamma\)2 are splice variants from the same gene PP1 gamma. Remarkably, all the PP1 isoforms
share a high-degree of homology (over 90%) and their differences reside in their C-terminal region (Figure 10).

![Figure 10](image.png)

**Figure 10. The PP1 isoforms share a high degree of homology.** The differences among the PP1 isoforms reside in their C-terminal, while the sequence homology comprising their catalytic domain is unchanged.

Type-1 can be inhibited and therefore classified by heat stable proteins Inhibitor-1 (I-1), Inhibitor-2 (I-2), its brain-specific isoform DARPP-32 (dopamine- and cAMP-regulated phosphoprotein) and NIPP1 (Oliver & Shenolikar, 1998), while type-2 are resistant to their action. Type-1 preferentially dephosphorylates the β-subunit of protein tyrosin kinases, while type-2 phosphatases does so to the α-subunit (Cohen, 1989a; Ingebritsen & Cohen, 1983). I-1 and DARPP32 are only active after their phosphorylation by cAMP-dependent protein kinases.
while I-2 is spontaneously active. The further use of okadaic acid—a specific phosphatase inhibitor—facilitates the distinction among the subclasses within Type-2.

**Table 1. Classification of Serine/Threonine phosphatases**

<table>
<thead>
<tr>
<th>TYPE 1</th>
<th>Inhibited by I-1 &amp; I-2</th>
<th>Specificity for phosphorylase kinase</th>
<th>Catalytic Subunit</th>
<th>Regulatory subunit</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP-Mg dependent</td>
<td>Yes</td>
<td>β</td>
<td>C1, α, γ1, γ2, δ</td>
<td>R1,2, RGL, RMr, NIPP1</td>
</tr>
<tr>
<td>Glycogen/ SR-associated</td>
<td></td>
<td>β</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Myofibril-associated</td>
<td></td>
<td>β</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nuclear</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TYPE 2A</td>
<td>No</td>
<td>α</td>
<td>C2 α, β</td>
<td>A α, β, B α, β, γ, δ</td>
</tr>
<tr>
<td>Phosphatase 2A2</td>
<td></td>
<td></td>
<td></td>
<td>B' α, β, γ, δ</td>
</tr>
<tr>
<td>2A1</td>
<td></td>
<td></td>
<td></td>
<td>B''</td>
</tr>
<tr>
<td>2A0</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PCS</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TYPE 2B</td>
<td>No</td>
<td>α</td>
<td>A α, β, δ</td>
<td>B, calmodulin</td>
</tr>
<tr>
<td>Calcineurin</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TYPE 2C</td>
<td>No</td>
<td>α</td>
<td>C α, β</td>
<td>none</td>
</tr>
<tr>
<td>Mg-activated</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

PP2A can be active in the absence of cations, while PP2B and PP2C require Ca$^{2+}$ and Mg$^{2+}$ respectively. Recently, the presence of inhibitor NIPP1 has been reported to be associated with a nuclear PP1. Phosphorylation of NIPP1 by the cAMP-dependent protein kinase renders the protein less effective and decreases its ability to bind to the catalytic subunit of PP1 ((Beullens, Van Eynde, Bollen, & Stalmans, 1993)). This effect is opposite to that produced by I-1. While I-1 and DARPP32 are activated by protein kinase A (PKA)-mediated phosphorylation and inactivated
by calcineurin-induced dephosphorylation, I-2 and NIPP1 are active when dephosphorylated but inactive by the phosphorylation of glycogen synthase 3 (GSK-3) and PKA.

Although still in use, the biochemical classification stated above has been blurred by phylogenetic analysis. According to these, PP2A turned to be more closely related to PP1 than to PP2C. Therefore, from a phylogenetic point of view would be more reasonable to group PP1, PP2A and PP2B into family I while PP2C into family II (Wera & Hemmings, 1995). Moreover, in the past few years many novel protein phosphatases have been identified that do not fit into the biochemical classification. Many of these protein phosphatases are closely related to the existing classes or are intermediates between classes.

A detailed comparison of the primary structures of 44 different protein Ser/Thr phosphatases, excluding PP2C, was carried out by Barton and colleagues (Barton, Cohen, & Barford, 1994). This revealed a common core structure that comprises two domains. The first domain is predicted to fold as a single β sheet flanked by α helices, whereas the second is predominantly helical. This multiple alignment also shows that family I can be subdivided into PP1-like, PP2A-like and PP2B-like enzymes (Barton et al., 1994).

4. **Regulation of Sperm Motility**

Sperm undergo two types of motility, progressive motility and hyperactivated motility. They acquire the capacity for progressive motility during epididymal maturation, but do not become motile until released from the epididymis. This type of motility is characterized by vigorous and relatively symmetrical flagellar motion and results in rapid forward movement. Once in the female reproductive tract, sperm display hyperactivated motility, characterized by high amplitude whip-like beating of the flagellum, asymmetrical flagellar bends, and circular or erratic swimming trajectory (Yanagimachi R., 1981),(Suarez & Ho, 2003). Hyperactivation occurs
during the process of capacitation, but many unexplained processes remains, such as whether hyperactivation is a consequence of capacitation or whether hyperactivation and capacitation are separate processes that occur in parallel. Capacitation is a process during which sperm gain the ability to undergo the acrosome reaction and is observable after it has occurred. Several mechanical paths contributing to both capacitation and sperm motility are shared, suggesting that different mechanisms contribute to the regulation of both processes.

Mammalian sperm motility is initiated when an extrinsic stimulus triggers a receptor to initiate a signal transduction cascade. Finding this primordial trigger is one of the holy grails of mammalian sperm biology.

The initiation of motility involves the downstream changes in protein conformation, phosphorylation and or localization of effector proteins in the pathways and in the cellular processes being activated. The key factors involved in the initiation of progressive motility and induction of hyperactivated motility in sperm are known. These include calcium ions (Ca\(^{2+}\)), bicarbonate (HCO\(_3\)\(^-\)), cyclic adenosine monophosphate (cAMP) and protein phosphorylation. It is also probable than other unidentified key factors are also involved in the regulation and coordination of sperm motility.

However, currently there is a limited amount of information regarding the receptors on the sperm surface that might be involved in triggering progressive or hyperactivated motility. Among candidates are olfactory receptors such MORE23 and hOR17-4 and a GABA receptor. As an example, when a cognate ligand binds to a olfactory receptor present in sperm, it triggers an increase in intracellular Ca\(^{2+}\) which changes the flagellar beating (Fukuda, Yomogida, Okabe, & Touhara, 2004) and swimming behavior (hypermotility). Interestingly the GABA receptor
mentioned has been associated with acrosome-related functions, though changes in motility parameters have also been recorded (Calogero et al., 1996).

Recent studies with knockout mice have also demonstrated that calcium plays an important role in mediating multiple aspects of ranging from sperm function to sperm motility. It has also been reported that the sperm from infertile male mice had reduced progressive motility (Qi et al., 2007).

Other ion movements across the sperm plasma membrane also have been shown to have important roles in sperm motility. pH, for example is an important factor. The sperm-specific sodium-hydrogen exchanger (NHE) which regulates intracellular pH has been found to be located in the principal piece and disruption of the gene Slc9a10 resulted in male infertility and markedly reduction in sperm motility (Wang, King, Quill, Doolittle, & Garbers, 2003).

The second class of modulators of sperm motility, as mentioned earlier, are cAMP and bicarbonate. cAMP is a key second messenger in the regulation of sperm motility. An increase in cAMP levels occurs when adenylyl cyclase is stimulated to convert ATP to cAMP. A major result of this in sperm is the activation of cAMP-dependent kinase A (PKA), which has the potential to phosphorylate serine and threonine residues and trigger a cascade of protein phosphorylation of proteins on tyrosine residues (Leclerc, de Lamirande, & Gagnon, 1996)(Si & Okuno, 1999). Sperm appear to depend primarily on a more recently discovered soluble adenylyl cyclase (sAC) that lacks a putative transmembrane domain and is not G-protein regulated as the other ordinary forms are (Sinclair et al., 2000). To this instance, studies in rat and human sAC have shown that HCO$_3^-$ acts directly on sAC (Chen et al., 2000)(Jaiswal & Conti, 2003),(Litvin, Kamenetsky, Zarifyan, Buck, & Levin, 2003).
The effect of HCO$_3^-$ on sperm motility is quite rapid. Stop-motion and waveform analysis indicated that upon addition of HCO$_3^-$ the flagellar beat frequency of mouse sperm began increasing within 5 seconds and was near maximal by 30 seconds (Wennemuth, Carlson, Harper, & Babcock, 2003). The addition of PKA inhibitors prevented the increase in flagellar beat frequency and Ca$^{2+}$ channel responses to HCO$_3^-$, strongly suggesting that HCO$_3^-$ influences these processes by raising cAMP levels and stimulating PKA-mediated protein phosphorylation (Wennemuth et al., 2003).

Lastly, the role of protein phosphatases has just recently begin to acquire significance. It has been shown that increases in sperm protein phosphorylation have implications in the initiation of progressive motility, hyperactivated motility, capacitation, the acrosome reaction, and fertilization (Cohen & Cohen, 1989).

Most sperm proteins which become phosphorylated between release from the epididymis and fertilization are in the flagellum and potentially involved in motility. PKA phosphorylates proteins on serine and threonine residues, which in turn activates a signaling cascade leading to robust tyrosine phosphorylation of flagellar proteins. A number of changes need to occur in the sperm before the tyrosine phosphorylation begins and the time course is relatively slow (Visconti & Kopf, 1998). Newly phosphorylated proteins are first seen in mouse sperm after 30-45 minutes in capacitation medium and the maximum level of phosphorylation is reached at about 90 minutes (Visconti et al., 1995). While some of the increases in phosphorylation might be involved in regulating hyperactivated motility, initiation of progressive motility occurs within a few seconds of exposure to medium containing HCO$_3^-$ (Wennemuth et al., 2003). This might suggest that rapid PKA-mediated serine/threonine phosphorylation events are involved in initiation of progressive motility. A few proteins have been identified that are serine/threonine
phosphorylated in response to HCO$_3^-$ such ODF2 (Harrison, 2004), AKAP4, AKAP3 and valosin-containing proteins (VSP) in human sperm (Ficarro et al., 2003). In addition, several other proteins that undergo changes in tyrosine phosphorylation during capacitation have been identified, such fibrous sheath proteins AKAP3 (Ficarro et al., 2003), AKAP 4 (Carrera, Gerton, & Moss, 1994) and CAXYR (Naaby-Hansen et al., 2002) among several others (Ficarro et al., 2003), (Geussova, Kalab, & Peknicova, 2002).

In the other side of the coin, the inhibition of phosphatase activity results in the stimulation of motility, strongly suggesting that phosphatases also have an important role in regulating this and other processes in sperm. While phosphatases are important components of signaling and regulatory pathways in other cell types, little is known about phosphatases in sperm. In this matter, the sperm phosphates PP1γ2 has received some attention. Because of its proline-directed serine/threonine phosphatase activity it could serve to buffer the serine/threonine kinase activity of PKA.

5. Role of PP1 gamma isoforms in spermatogenesis

Based on the observation that protein phosphatases prevented motility initiation in demembranated spermatozoa in reactivation media, (Murofushi, Ishiguro, Takahashi, Ikeda, & Sakai, 1986),(da Cruz e Silva et al., 1995), it was long suspected that protein phosphatases were involved in the flagellar motility regulation. Later studies (Smith et al., 1996) supplied evidence that the Protein Phosphatase 1 -PP1- was not only present in sperm, but the sperm motility increased when PP1 activity decreased through caput to caudal epididymis (Huang & Vijayaraghavan, 2004). Further observations noted that PP1 gamma1 -PP1γ1- and PP1 gamma 2
-PP1γ2- were also present in sperm and testes and their roles were associated with sperm motility (Huang & Vijayaraghavan, 2004). Interestingly, the PP1γ2 isoform is present in all mammalian spermatozoa researched to date —mouse, rat, hamster, bovine, non-human primate, and human, sharing in all of them a nearly identical C-terminal region (Figure 11).

<table>
<thead>
<tr>
<th>Species</th>
<th>Amino Acid Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>mouse</td>
<td>GSGLNPSIQKASNYRNNTVLYE</td>
</tr>
<tr>
<td>dog</td>
<td>TSGLNPSIQKASNYRNNTVLYE</td>
</tr>
<tr>
<td>hedgehog</td>
<td>TSGLNPSIQKASNYRNNTVLYE</td>
</tr>
<tr>
<td>human</td>
<td>SGLNPSIQKASNYRNNTVLYE</td>
</tr>
<tr>
<td>elephant</td>
<td>ASGLNPSIQKASNYRNNTVLYE</td>
</tr>
<tr>
<td>macaque</td>
<td>ASGLNPSIQKASNYRNNTVLYE</td>
</tr>
<tr>
<td>opossum</td>
<td>TSGLNPSIQKASNYRNNTVLYE</td>
</tr>
<tr>
<td>bushbaby</td>
<td>TPSLNPSIQKASNYRNNTVLYE</td>
</tr>
<tr>
<td>chimp</td>
<td>ASGLNPSIQKASNYRNNTVLYE</td>
</tr>
<tr>
<td>rat</td>
<td>GSGLNPSIQKASNYRNNTVLYE</td>
</tr>
<tr>
<td>microbat</td>
<td>ASGLNPSIQKAPNYRNNNTVLYD</td>
</tr>
</tbody>
</table>

Figure 11. The C-terminal of PP1γ2 is highly conserved across mammalian species.
The isoform PP1γ2 is produced by the alternative splicing of the PP1γ gene that generates two isoforms, PP1γ1 and PP1γ2 (Figure 12) (Okano, Heng, Trevisanato, Tyers, & Varmuza, 1997). These two variants are identical in all respects, except that PP1γ2 has a unique 23-amino acid carboxy-terminus extension. This sequence is produced when the intron between the 7th and 8th exons is removed in the transcript of PP1γ2, while it is kept in the transcript of PP1γ1, retaining a stop codon that halts its transcription. Therefore, there is a 7 amino acid carboxy-terminus in PP1γ1 while there is a 23 in that of PP1γ2. This sperm-specific phosphatase is particularly enriched in testis and is expressed during male germ cell differentiation; its phosphorylated form is localized predominantly in the posterior region of the sperm head of mature spermatozoa. (Sasaki et al., 1990), (Kitagawa et al., 1990), (da Cruz e Silva et al., 1995).

**Figure 12. Map of PP1 gamma gene splicing.** Retention of Intron 7 leads to an early stop codon, transcribing PP1γ1 mRNA only. Skipping intron 7 leads to exon 8 stop codon, transcribing PP1γ2 mRNA with its unique C-terminal extension.
It is known that mammalian spermatozoa capacity for motility and fertilization becomes operational during the epididymal transit, undergoing changes in biochemical and physiological properties that progressively transform infertile spermatozoa into motile and thus fertile sperm cells (J. M. Bedford & Hoskins, 1990), (Cooper, Waites, & Nieschlag, 1986). These modifications include key changes in shape, metabolic patterns, enzymatic activities, chemical and physical properties, acquisition of ability to bind to specific epididymal proteins and finally ability to bind to the zona pellucida in the egg within the female tract. Despite all this known facts, the biological significance of those variables in relation to the development of mature spermatozoa is still unclear.

One of the puzzling factors is that PP1γ2 is found attached to three different protein pools – sds22, 14-3-3 and hsp90- in caput and caudal epididymal spermatozoa (Huang & Vijayaraghavan, 2004), but their relative contribution to sperm motility initiation is not known.

Recent Knock Out (KO) experiments trying to elucidate PP1 gamma role (Varmuza et al., 1999) showed that the removal of the entire PP1 gamma gene – and thus effectively removing its both PP1 gamma isoforms- induced male infertility only in mice (Varmuza et al., 1999). This prompted speculation to determine which isoform was key for sperm development and fertility.
Figure 13. PP1γ2 expression in the germ cells. This map shows the wild type cell expression of PP1γ2 compared to the secondary spermatocyte expression of PP1γ2 that usage of the PGK2 promoter will establish in the PP1γ2-rescue mice.

6. The PGK2 promoter

In order to understand the rationale behind our transgenic studies in mice, a close examination on the molecular biology of the Phosphoglycerate Kinase (PGK) promoter is needed. Briefly, the 500-bp PGK2 promoter was used in these transgenic studies because it is a testis-specific promoter that restricts transcription at the secondary spermatocyte level, thus minimizing potential undesired effects from the expression of transgenic PP1γ1 and Pp1γ2. Past
studies using this promoter to transgenically express the germinal Angiotensin-converting Enzyme (ACE) in KO male mice have reported a full rescue phenotype (Kessler et al., 2002). The transgenic male mice were as healthy as wild type and the males were fertile. Interestingly, the germinal ACE is a post-meiotically expressed protein, closely mimicking the expression pattern dictated by the PGK2 promoter.

In mammals, PGK is encoded by two genes, Pgk-1 and Pgk-2 (PGK1 and PGK2 in human). Although the two genes produce proteins that are both structurally and functionally similar (McCarrey et al., 1996), they differ markedly in the manner in which their expression is regulated (McCarrey et al., 1996) (McCarrey, 1987) (McCarrey, 1990) (McCarrey et al., 1992). Pgk-1 is X-chromosome linked and expressed in somatic cells (VandeBerg, Cooper, & Close, 1976), (Kozak, McLean, & Eicher, 1974), (Beutler, 1969), whereas the autosomal Pgk-2 gene is expressed only in meiotic and haploid male germ cells (Erickson, Kramer, Rittenhouse, & Salkeld, 1980), (Gold, Fujimoto, Kramer, Erickson, & Hecht, 1983). Pgk-2 transcription is first seen in pachytene spermatocytes, with message levels increasing during later stages of spermatogenesis (Gold et al., 1983) (McCarrey & Thomas, 1987; Thomas, Wilkie, Tomashesfky, Bellve, & Simon, 1989). The PGK-2 protein is not detected until 7 days later in haploid round spermatids (VandeBerg et al., 1976) (Kramer & Erickson, 1981; Kramer, 1981; Kramer, 1981) and is coincident with the appearance of Pgk-2 message on polysomes (Gold et al., 1983), suggesting additional regulation at the translational level. Human (McCarrey & Thomas, 1987; Thomas et al., 1989) (Szabo, Grzeschik, & Siniscalco, 1984) (Tani, Singer-Sam, Munns, & Yoshida, 1985) and mouse ((Boer, Adra, Lau, & McBurney, 1987) Pgk-2 genomic sequences have been cloned, and both genes lack introns and share extensive homology. Several lines of evidence suggest that the PGK2 gene arose as a processed duplication of the PGK1 gene through RNA-mediated retroposition early during mammalian evolution (McCarrey & Thomas, 1987). Additional data
suggest that the original PGK2 retroposon carried a copy of the progenitor “PGK1-like” promoter that directed ubiquitous expression, and that this promoter subsequently evolved a tissue-specific regulatory function so that it now directs testis-specific transcription (McCarrey, 1990).

Transcription of the PGK2 gene is controlled by regulatory sequences located in the 59 flanking region of the gene, (Figure 14A) (Robinson, McCarrey, & Simon, 1989); (Gebara & McCarrey, 1992); (Kumari, Stroud, Anji, & McCarrey, 1996). This region includes both core promoter sequences and tissue-specific enhancer sequences (Figure 14B). Core promoter function has been demonstrated in the first 188 base pairs (bp) upstream from the translational start site in the human PGK2 gene, including a 70-bp 59 untranslated region. The sequences 5' to the coding region are 66% homologous between Pgk1 and Pgk2 (Boer et al., 1987), and both genes exhibit similar promoter motifs, consisting of the consensus sequences for the SP1 transcription factor (GC box) and for nuclear factor 1 (CAAT box) (Jones, Kadonaga, Luciw, & Tjian, 1986), (McKnight & Tjian, 1986) (Figure 14) rather than the TATA consensus sequence found adjacent to the start of the coding region in many genes (Breathnach & Chambon, 1981).

It has been shown that CAAT and GC-boxes act as binding sites for the ubiquitous transcription factors, CTF-1 and Sp1, respectively (Jones K. A., Yamamoto K. R., Tjian R., 1985); (Kadonaga, Carner, Masiarz, & Tjian, 1987). Cell line cotransfection experiments indicated that the transcription factor Sp1 is indispensably required for initiation of transcription from the PGK2 core promoter, and that the core promoter region alone is sufficient to direct basal transcription of a ligated reporter sequence in cultured cells. But appropriate expression was achieved only with an additional 327 bp of upstream sequence, indicating the presence of enhancer activities in this upstream region (Robinson et al., 1989). Tissue-specific protein binding activities within
this region were mapped by gel-shift experiments to a 40-bp subregion (E1/E4) located immediately upstream from the core promoter (Gebara & McCarrey, 1992) (Figure 14).

**Figure 14. The PGK2 promoter.** A) The nucleotide sequence of the 5' region of the human PGK2 is shown. The CAAT consensus Sequence (position -184bp) and the GC consensus sequence (position -114bp) are boxed. B) Map of the PGK2 promoter with the Core Promoter regions and the Positive Enhancers required for its testes-specific expression.

7. Overall goal

Since knocking out both isoforms of PP1 gamma arrests spermatogenesis, the intent of this research revolves around a very specific question: which PP1 gamma isoform has the capability to restore spermatogenesis and fertility in the Ppp1cc/- mice males?
Hypothesis

The overall hypothesis of this thesis is that PP1γ1 is involved in building the spermatozoa, while PP1γ2 is involved in providing the motility as a result of being the only PP1 isoform detected in spermatozoa.

In order to address the abovementioned goal, this dissertation contains two specific aims:

1. To determine if transgenic expression of the PP1γ2 isoform can fully rescue spermatogenesis and fertility, and analyze the effects of PP1γ2 expression in the PP1 null mice.

2. To determine if transgenic expression of the PP1γ1 isoform can also fully rescue spermatogenesis and fertility, and analyze the effects of its expression in the PP1 null mice.
MATERIALS AND METHODS

1. Generation of the PP1γ1 and PP1γ2 rescue plasmids

1.1 PP1γ1-rescue construct

The human PGK2 promoter was a gift from Dr. McCarrey. The sequence was digested out from a pCR2.1 plasmid using BamH1 and HindIII restriction enzyme sites and subcloned into a pBluescript SK+ plasmid. The PP1γ1 cDNA from mice was ordered from a EST databank and amplified by PCR with forward and reverse primers (5’- CTCAAGCTTATGGCGGATATCGACAA -3’ and 5’-CTCATCGATGCGTGCCATACAGTCCA -3’, respectively). This fragment was subsequently inserted between the HindIII and ClaI restriction sites of pBluescript SK+ (Stratagene, CA). The SV40 poly A sequence was amplified from a pcDNA4/to/myc-His A using forward and reverse primers (5’- CTCCCTCGAGTCTCATGCTGGAGTTCT -3’ and 5’- CTCGTTACCACCATGATTACGCCAAG -3’, respectively) and subcloned between the XhoI and KpnI restriction sites (Figure 15). The DNA fragment containing the Pgc2 promoter, the Ppp1cc1 cDNA, and the SV40 poly A signal was excised from the vector by digestion with BamHI and KpnI, and the 2.1-kb fragment was gel-purified (Figure 16). 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 from 168 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'.

Figure 15. The PP1γ1-rescue construct
1.2 PP1γ2-rescue construct

The Sac1-Xba1 fragment of the human Pgtk2 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 a hybrid human and rat c-terminal testicular Ppp1cc2
cDNA was ligated between the BamHI-XhoI sites (Figure 17) from the plasmid pTATAc provided by Dr. Edgar Figueiredo da Cruz e Silva (Centro de Biologia Celular, University of Aveiro, Portugal). 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 SacI and KpnI, and the 1.7-kb fragment was gel-purified (Figure 18). 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 121 twenty-one day-old progeny were analyzed by PCR for the presence of the transgene using the Pgk2 forward primer, 5′-GCGCACACCTCA GGACTATT-3′, and the SV40 reverse primer 5′-CTCGGTACCACCATGATTACGCCAAG-3′.
Figure 17 The PP1γ2-rescue construct.

Figure 18. PP1γ2 rescue construct cut with SacI and Kpn1.
2. Generation of the PP1γ1, PP1γ2 and PP1γ1+PP1γ2 rescue mice

The transgenic positive B6/SJL founder mice were mated either with Ppp1cc-null CD1 females or Ppp1cc +/- males, (Table 2) (Ppp1cc-null mice were obtained from Dr. Susan Varmuza, University of Toronto, Toronto, Canada). PP1γ2 transgene-positive-Ppp1cc +/- males were obtained by crossing PP1γ2 transgene positive-Ppp1cc +/- males to Ppp1cc +/- females (Table 2).

The double transgenic mice expressing PP1γ1 and PP1γ2 were obtained crossing the PP1γ2 transgene-positive-Ppp1cc +/- males with PP1γ1 transgene-positive-Ppp1cc +/- females as indicated in the mating scheme (Table 3).

Table 2. Generating the Pp1γ1 or PP1γ2 rescue mice
Table 3. Generating the double PP1γ1 and PP1γ2 rescue mice

![Diagram showing the generation of PP1γ1 and PP1γ2 rescue mice](image)

Figure 19. Null background assessment. PCR using a combination of primers IV-VI and IV-MA was used to detect the null background in the rescue mice. The roman numbers correspond to the primers targeting the exons present in the PP1 gamma gene. The combination of primers IV and VI target exons IV and VI respectively in the endogenous PP1 gamma gene. MA stands for
Mutant Allele, and primers directed against its sequence were used to assess the presence of the KO construct disrupting the endogenous PP1 gamma gene.

3. Expression of the transgene

Reverse transcription-PCR (RT-PCR) was used to detect expression of the PP1γ1 and PP1γ2 transgenes 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’-CGAACAACACTCCAGCATGAGA-3’. The 1040 bp product was detected by ethidium bromide staining following electrophoresis through 1% agarose. PCR was also performed to detect the transgenic Ppp1cc1 gene using the forward primer, 5’-TGATTCCTTCCTAAAGCCAAAGT-3’ and reverse, 5’-CGAAGAACTCCAGCATGAGA-3’. The 500bp product was detected by ethidium bromide staining following electrophoresis through 1% agarose.

4. 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 (Chakrabarti et al., 2007). Epididymal sperm extruded (as described in Sperm extrusion methods) in PBS was centrifuged for 3 min at 300xg and the sperm pellet resuspended in 1% SDS, boiled for 5 min followed by
centrifugation at 16000xg 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-PP1γ2 antibody directed against the PP1γ2 unique C-terminal region, followed by a peroxidase conjugated goat anti-rabbit secondary antibody (Chakrabarti et al., 2007). Blots were developed by enhanced chemiluminiscence.

5. Sperm extrusion methods from testis, caput, and cauda epididymis

Testicular sperm from the rescue animals were isolated using the methodology described by Chakrabarti et al., 2007. 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.

6. 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.

7. Sperm count and motility assessment

Caudae epididymides were lightly minced and incubated in KSOM medium (Summers, McGinnis, Lawitts, Raffin, & Biggers, 2000) for 15 min at 37°C in 5% CO2 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 (Pilder, Olds-Clarke, Orth, Jester, & Dugan, 1997).

8. 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.
9. **Histology and immunohistochemistry**

Tissues were embedded in paraffin and sectioned, then stained with hematoxylin-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-pp1γ2 antibody (1:100 dilution) for five hours at room temperature for PP1γ2-rescue testes and using a double-specificity anti-PP1γ1 and PP1γ2 for staining the PP1γ1-rescue testes (1:100 dilution). After washing, sections were incubated with a Cy3-conjugated secondary antibody (dilution 1:250) for 1h at room temperature. After washing, the sections were mounted, viewed, and photographed using an Olympus Fluorview 500 fluorescence microscope (Olympus, PA).

10. **Ultrastructure analysis of epididymal spermatoza 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% OsO4 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 (Pilder et al., 1997) in the laboratory of Dr. Stephen Pilder in the Department of Anatomy and Cell Biology from Temple University, PA.
RESULTS

1 Transgenic Expression of PP1γ2 in the Ppp1cc -/- Mouse

Ppp1cc2 transgenic mice carrying the construct shown in (Figure 17) were produced as described in Materials and Methods. The 500bp promoter of the testis-specific Pgk2 gene was used to drive transcription of mice Ppp1cc1 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 (Kessler et al., 2002); second, the Pgk2 promoter would drive transcription of Ppp1cc1 transgene in an appropriate fashion to allow for elevated translation of transgenic Ppp1cc1 in cells in which the level of wild-type PP1γ1 protein are normally high (late spermatocytes and spermatids); and third, because the Ppp1cc endogenous promoter expresses transcripts in most somatic tissues, its use would run the risk of expressing PP1γ1 everywhere, possibly producing undesired phenotypes.
Figure 20. Detection of PP1γ2-rescue transgene. PCR was used to detect the 1.7kb transgene in the genomic DNA of PP1γ2-rescue mice.

A total of 121 mice were genotyped by PCR for the presence of the PP1γ2-rescue transgene using the primers described in Materials and Methods. 19 out of 121 mice (~16%) turned to be positive for the rescue construct and after mating the positive mice accordingly (Table 2) to produce the Ppp1cc2 transgene-positive Ppp1cc -/- (rescue) males, only seven founders turned to be germline transmitters: lines A, B, D, E, F, G and Q. From these, only lines A, D, E and G were found to be high expressers of transgenic PP1γ2 (Figure 23A).

Transgene-positive Ppp1cc +/- male and female mice for the PP1γ2-transgene exhibited a wild-type phenotype as did PP1γ2-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 the rescue mice were comparable to those of
wild-type and *Ppp1cc*-/- mice.

**1.1 PP1γ2 mRNA detection**

Transgenic *Pp1cc2* mRNA expression was detected in the PP1γ2-rescue testis (Figure 21, R).

Detection was performed on the A-line, a high PP1γ2 expressor line.
Figure 21. The mRNA of transgenic PP1γ2 mice. (A) RT-PCR was performed on whole testes of PP1γ2-rescue mice (R) and the transgenic PP1γ2 mRNA was detected. (M) indicates the marker and (-) indicates the negative control. (B) Northern blot of steady state testicular RNA levels from wild-type homozygote (+/+), wild-type/KO heterozygote (+/-), wild-type homozygote plus transgene (+/+R), wild-type/KO heterozygote plus transgene (+/-R), Ppp1cc -/- rescue (-/-R), and KO (-/-). The probe used is specific for PP1γ2 RNA. It is noteworthy that the Ppp1cc -/- rescue testis expresses about as much PP1γ2 RNA as does the Ppp1cc +/- testis. Note that transgenic PP1γ2 RNA is smaller than endogenous RNA, and truncated Ppp1cc -/- RNA is even smaller.

1.2 PP1γ2 Protein Detection

Significant transgenic PP1γ2 protein expression was evident in rescue testes in which endogenous expression was absent (Figure 19). These findings suggested that an upper boundary of PP1γ2 protein expression exists and is tightly controlled. Moreover, as expected, transgenic PP1γ2 expression was restricted to the testis (Figure 22), due to the use of the spermatocyte-specific Pgk2 promoter to drive transcription of the transgene.
Figure 22. Presence of transgenic PP1γ2 in the PP1γ2-rescue mice. Western blot analysis shows transgenic PP1γ2 is restricted to the testis and is not expressed in the brain of PP1γ2-rescue mice following the testis-specific expression of the PGK2 promoter. Actin antibodies were used as controls to check for the integrity of the loaded samples.

Western blot analysis of testis extracts from rescue males showed that four transgenic lines expressed significant amounts of PP1γ2 in the testis (Figure 23A), with the E-line expressing the greatest amount, approximately one-third of the steady state level of wild-type (Figure 23B).
Figure 23. Degrees of PP1γ2 expression in the PP1γ2-rescue lines. The amount of PP1γ2 expressed in each rescue line testis was estimated by western blot analysis. (A) Line E expresses ~ 1/3 of wild-type PP1γ2 expression, line D, ~ 1/6, line A, ~ 1/7, and line G, ~ 1/15. (B) Detail of Line E transgenic PP1γ2 expression compared to WT.

1.3 Testes architecture in the PP1γ2-rescue mice

Previous studies have demonstrated that spermiogenesis is clearly impaired in Ppp1cc -/- mice (Varmuza et al., 1999) 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 PP1γ2-rescue testis sections (Figure 24) 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. DIC and DAPI staining also revealed presence of elongating
spermatids in the lumen of the PP1γ2-rescue seminiferous tubules at 20X and 60X respectively (Figure 25).

Figure 24. Hematoxylin-eosin staining of PP1γ2-rescue mouse testes. Testes cross sections from E-line rescue mice (A&B), null (C) and wild-type (D) were stained with hematoxylin-eosin. The stained sections were viewed with an Olympus IX70 microscope at 20X (A) and 40X magnifications (B-C). Note the seemingly wild-type architecture of the rescue testis at their corresponding magnifications. Arrows point at the presence of elongating spermatids in the lumen of the PP1γ2-rescue testes (A&B) and in the WT (D).
Figure 25. DAPI staining of PP1γ2-rescue mouse testes. Testes cross sections from the E-line rescue mice were observed using DIC microscopy (A) and stained with DAPI stain (B). Bracket (C) shows the composite picture. The stained sections were viewed with an Olympus IX70 microscope at 40X. The white arrow points at the presence of elongating spermatids (B) as seen in Figure 23.

1.4 Epididymides of PP1γ2-rescue mice

Many spermatozoa were evident in the lumina of the caudae epididymides stained with hematoxylin-eosin (Figure 26A) and Hoetsch (Figure 26B) from all lines of PP1γ2-rescue
mice, as well. Thus the presence of PP1γ2 in the testis of rescue mice restores spermiogenesis and testicular/epididymal sperm numbers to qualitatively normal levels.

![Image of cauda epididymis sections]

**Figure 26. Caudae epididymides of PP1γ2-rescue mouse.** Hematoxylin-eosin stained cross sections through the lumina of the cauda epididymides from rescue E-line (A), KO (B) and wild-type (C) viewed with an Olympus IX70 microscope at 40X. Note the absence of sperm in the null epididymis. DAPI staining of the caudal epididymis at 40X shows the morphology of the sperm heads and absence of round cells in the lumen.

1.5 Protein localization in the PP1γ2-rescue testes

Immunohistochemical analysis of testis sections demonstrated that transgenic PP1γ2 under the control of the PGK2 promoter was mainly restricted to meiotic and post-meiotic germ cells, roughly comparable to the staining pattern in wild-type testis (Chakrabarti et al., 2007)
Figure 27. Presence of PP1γ2 in rescue mouse testis. Immunohistochemistry of PP1γ2-rescue (A), KO (B), WT(C) and Negative CTR (D) with secondary antibody alone. Sections were probed with rabbit anti-PP1γ2 antibody followed by goat anti-rabbit cy3-labeled secondary antibody. Slides were viewed with an Olympus Fluorview 500 microscope at 20X magnification.

1.6 PP1γ2-rescue sperm morphogenesis and motility

Absence of PP1γ1 and PP1γ2 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 (Figure 28F). In
contrast, testicular sperm heads appear to be normally hook-shaped in rescue mice (Figure 28A&B).

However, most mitochondrial sheaths appear to contain irregularities, including gaps especially at either their proximal, distal, or both ends (Figure 28A & B). In addition, most of these sperm contained residual cytoplasmic irregularities at the head/connecting piece junction (Figure 28B), suggesting some abnormality in the condensation process. Interestingly, when we examined caput (Figure 28C) and cauda epididymal rescue sperm (Figure 28D&E), 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 (Figure 28D). In many of these cases, the mitochondrial sheath appeared gapped or absent at what is normally its proximal end (Figure 28D). A smaller number of sperm also showed a similar hairpin phenotype at the midpiece/principal piece junction (Figure 28E). 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 4) 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.

Table 4. Sperm tail bend phenotype

<table>
<thead>
<tr>
<th>Sperm Type</th>
<th>Straight</th>
<th>Hairpin at Head/CP Junction</th>
<th>Hairpin at MP/PP Junction</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Testicular</td>
<td>218 (93.96%)</td>
<td>13 (5.6%)</td>
<td>1 (0.43%)</td>
<td>232</td>
</tr>
<tr>
<td>Caput</td>
<td>108 (50.9%)</td>
<td>94 (44.33%)</td>
<td>10 (4.7%)</td>
<td>212</td>
</tr>
<tr>
<td>Caudal</td>
<td>13 (8.8%)</td>
<td>109 (74.14%)</td>
<td>25 (17%)</td>
<td>147</td>
</tr>
</tbody>
</table>

*Significantly < Testicular (p<0.01)
*Significantly < Caput (p<0.01)
*Significantly > Testicular (p<0.01)
*Significantly > Caput (p<0.01)

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).
Figure 28. DIC imaging of testicular, caput, and cauda epididymal spermatozoa. (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 exhibiting hairpin bend at the midpiece/principal piece junction. (F) Caudal sperm from KO male mice. Note the round sperm head and mitochondrial sheath malformation. All sperm were viewed at 100X magnification.
1.7 Protein detection in the PP1γ2-rescue mice

Western blot analysis of caudal sperm protein extracts from PP1γ2-rescue mice showed that immuno-reactive PP1γ2 was indeed present, although, as expected, at lower levels than in extracts prepared from wild-type sperm (Figure 29).

![Western Blot Image]

Figure 29. PP1γ2 is present in the PP1γ2-rescue sperm.

1.8 Protein localization in the PP1γ2-rescue mice

We also tested to see if PP1γ2 protein expressed in testicular germ cells of rescue mice was properly incorporated into epididymal spermatozoa. Immuno-cytochemical analysis demonstrated that PP1γ2 localization was distributed along the tail and the head in a similar fashion to the pattern seen in wild-type caudal sperm (Figure 30).
Figure 30. Transgenic PP1γ2 detection in spermatozoa of PP1γ2-rescue mice.

Immunocytochemistry of PP1γ2 in caudal rescue sperm shows incorporation of PP1γ2 along the head and tail (A), similar to wild-type in (B). Slides were viewed using an Olympus Fluorview 500 at 60X magnification.

1.9 Ultrasctructure of PP1γ2-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 (Figure 31A-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 (Figure 31B). We also discerned that the heads of these sperm were elongated (Figure 31E), in contrast to the rounded heads of \textit{Ppp1cc-/-} testicular sperm (Figure 31A), and that they contained nuclei with completely condensed chromatin (Figure 31A-E). However, in some cases, concave deformities were observed in the proximal acrosome (Figure 31A & 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) (Figure 31A-C, & E). However, in contrast to \textit{Ppp1cc-/-} testicular sperm, where the number of ODFs in the midpiece was usually highly increased (Chakrabarti et al., 2007), the number of ODF and MT doublets internal to the 9 mitochondrial sheaths of rescue sperm were generally normal or occasionally decreased by one (Figure 31B, 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 (Figure 31F). In other cross sections through these more distal regions of the midpieces of rescue sperm, these and other abnormalities were evident (Figure 31H, J, & K). First, extruded ODFs/MT doublet complexes were present in unshed, asymmetrical cytoplasmic bulges peripheral to the mitochondrial sheath (Figure 31H). 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 (Figure 31G). 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 (Figure 31I). Tail disorganization appeared to be no less radical in the principal pieces of rescue sperm tails (Figure 31J & 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 (Figure 31 J). 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 (Figure 31K). 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 PP1γ2 activity in the wild-type caput epididymis are known to inhibit the initiation of sperm motility ((Vijayaraghavan et al., 1996)), 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 PP1γ2 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 (Figure 32). 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 PP1γ2 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.

![Figure 31. Ultrastructure of testicular PP1γ2-rescue sperm.](image)

Ultrastructure 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 mitochondrial 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.
Figure 32. Ultrasctructure of caudal PP1γ2-rescue sperm. Transmission electron micrographs of testicular rescue spermatids and sperm. A. Section of step 7 rescue spermatid showing normal development of acrosomal vesicle (arrow). B. Transverse section showing normally developed portion of midpiece of testicular rescue sperm. C. Transverse (upper arrow) and sagittal (lower arrows) sections through midpieces of testicular rescue sperm. Note the eccentric position of the outer dense fibers and axoneme within the encircling mitochondria in the transverse section, and the non-uniformity of mitochondrial gyre morphology and spacing as indicated by the arrows in the sagittal section. D. Another example of non-uniform mitochondrial gyre morphology and spacing, creating potential weak spots (arrows) in the testicular rescue sperm midpiece. E & F. Sagittal sections through the midpieces of testicular wild-type sperm showing uniform mitochondrial gyre morphology and spacing. G & H. Condensing chromatin within normally developing sperm heads of late elongating spermatids from rescue mice. I. Transverse section through principal piece of testicular rescue sperm showing malformed (gapped) fibrous
sheath rib (arrows). Note that ODFs 3 and 8 have not yet completely disappeared indicating that this section of the principal piece is near or at the midpiece-principal piece junction. J. Sagittal section through principal piece of testicular rescue sperm near the midpiece-principal piece junction. Arrow points to unusually large gap between fibrous sheath ribs, creating a potential weak spot through which ODFs and attached axonemal doublets can extrude. Note that in this case, the distal mitochondrial sheath abuts the annulus (arrow heads) and the principal piece, as in wild-type sperm.

2 Transgenic Expression of PP1γ1 in the Ppp1cc -/- Mouse

Ppp1cc1 transgenic mice carrying the construct shown in (Figure 15) were produced as described in Materials and Methods.

The founder mice were genotyped by PCR (Figure 33) using the primers described in Materials and Methods and mated accordingly to produce Ppp1cc1 transgene-positive Ppp1cc -/- (rescue) males (Table 2).
A total of 168 potential founder mice were genotyped by PCR for the presence of the PP1γ1-rescue transgene using the primers described in Materials and Methods. Only 13 out of 168 mice (~8%) turned to be positive for the rescue construct and after mating the positive mice accordingly (Table 2) to produce the Ppp1cc1 transgene-positive Ppp1cc -/- (rescue) males, only three founders turned to be germline transmitters: T, I and N lines.

PP1γ1 transgene-positive Ppp1cc +/- male and female mice exhibited a wild-type phenotype as did transgene positive Ppp1cc -/- females. In addition, the presence of the Ppp1cc1 transgene
did not appear to affect the development or life span of any male, and the general health characteristics of the rescue mice were comparable to those of wild-type and Ppp1cc -/- mice.

2.1 PP1γ1 mRNA detection

![Image of RT-PCR result]

**Figure 34. The mRNA of transgenic PP1γ1 mice.** RT-PCR was performed on whole testes of PP1γ1-rescue mice (R) to detect the Ppp1cc1 transgene mRNA expression of ~500bp long. (M) indicates the marker and (-) indicates the negative control.

2.2 PP1γ1 Protein Detection

Western blot analysis of testis extracts from mature rescue males showed that only one out of three PP1γ1-rescue mice lines had relatively high levels of testicular expression of transgenic PP1γ1 (Figure 35). Specifically, the T and I lines (data not shown) had very low expression or undetectable levels of PP1γ1, while only the N-line had relatively high levels of expression -approximately 1/4 of wild type (Figure 36).
Figure 35. Expression of PP1γ1 is detected in PP1γ1-rescue mice. Rescue and wild type testes extracts were subjected to SDS-PAGE followed by Western blot analysis with a monoclonal PP1γ1 antibody.

Figure 36. Degree of PP1γ1 expression in the PP1γ1-rescue N line. The amount of PP1γ1 expressed in each rescue line testis was estimated by western blot analysis. Lines T and I are not
shown due to the extremely low levels of PP1γ1 expression. Line N expressed ~1/4 of wild type PP1γ1 expression levels.

2.3 Testes architecture of PP1γ1-rescue mice

The testes architecture in the PP1γ1-rescue mice appeared to be restored compared to that of PP1 gamma KO mice. Presence of vacuoles, a hallmark in the PP1 gamma KO mice described by Dr. Varmuza was significantly reduced and elongating spermatid heads (Figure 37) could be seen in the lumen of the seminiferous tubules.
Figure 37. Hematoxylin-eosin staining of PP1γ1-rescue mouse testes. Testes cross sections from N-line rescue mice (A), null (B) and wild type (C) were stained with hematoxylin-eosin. The stained sections were viewed with an Olympus IX70 microscope at 20X magnification. The testes architecture is partially restored in the PP1γ1-rescue mice testes (A) compared to KO testes (B) and WT testes (C). However, note the present of vacuoles (arrows) in the seminiferous tubules of the PP1γ1-rescue testes.
2.4 Protein localization in the PP1γ1-rescue testes

Immunohistochemical analysis of testis sections from these mice demonstrated that transgenic PP1γ1 was mainly restricted to meiotic and post-meiotic germ cells, as expected as a consequence of being under the control of the PGK2 promoter (Chakrabarti et al., 2007) (Figure 38A).

Figure 38. PP1γ1 distribution in the testes at 20X. The PGK2-driven expression of PP1γ1 is detected in the testes of PP1γ-rescue mice (A) compared to KO (B) and WT (C). Figure (D)
corresponds to the negative control. A double-specificity antibody against PP1γ1 and PP1γ2 was used for staining the N-line testes sections (A). Notice that in the WT testes (C), PP1γ1 expression is not detected in germ cells when using a specific PP1γ1 antibody.

2.5 Epididymides of PP1γ1-rescue mice

A mixture of spermatozoa and round cells were present in the lumina of the caudae epididymides stained with hematoxylin-eosin and Hoetsch (Figure 39) from the N-line of PP1γ1-rescue mice. The presence of PP1γ1 in the testis of rescue mice restores spermiogenesis, but at the same time, the epididymides of PP1γ1-rescue mice are filled with round cells, probably due to early release of round spermatids.

![Figure 39. Caudal epididymis of PP1γ1-rescue mice. PP1γ1-rescue epididymides (A) appear to be filled with sperm cells and prematurely released round cells compared to KO epididymides (B) and WT (C) using hematoxylin-eosin staining. Bracket (D) shows a DIC picture of the PP1γ1-](image)
rescue epididymides. DAPI staining was used to stain the nucleus of cells (E) in the caudal epididymides. Arrows point to the presence of round cells in the lumen.

2.6 Sperm morphogenesis

Absence of PP1γ1 and PP1γ2 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, displaying globular heads (Figure 28F). In contrast, when transgenic PP1γ1 is the sole isoform present, spermatozoa present a broader range of abnormalities compared to the PP1γ2-rescue spermatozoa (Figure 28).

Figure 40A and Figure 40K shows PP1γ1-rescue spermatozoa with heads bent backward similar to the ones present in the PP1γ2-rescue spermatozoa (Figure 28). In the PP1γ1-rescue spermatozoa there is presence of globular sperm heads as well, although they are much less prevalent (Figure 40D). Few numbers of sperm heads appear hooked shaped as seen in Figure 40B,E,H,K. Last but not least, a distinctive dorsally-compressed spermatozoa seem to be present only in the PP1γ1-rescue mice (Figure 40F).
Figure 40. PP1γ1 Rescue sperm morphogenesis. A) 10x DIC imagery of rescue sperm, arrows point at bent heads. At 60X morphological abnormalities are seen in the PP1γ1-rescue mice like thinning of the annulus (small arrows in B,H-J), headless sperm (arrow in C) and bent and globular heads (D,K-M). Panels (E) and (G) show mitochondrial sheath abnormalities reminiscent of PP1γ KO (Figure 28). The fibrous sheaths appear to be absent, panels (F) and (G).

Surprisingly, testes expression of transgenic PP1γ1 didn’t result in the incorporation of PP1γ1 into the sperm—not even in low levels- as seen in PP1γ2-rescue spermatozoa (Figure 29).
3 Transgenic Expression of PP1γ1 and PP1γ2 in the Ppp1cc -/- Mouse

Following the mating scheme outlined in Table 1, Ppp1cc1 transgenic female mice carrying the construct shown in Figure 33 were mated with transgenic males carrying the Ppp1cc2 construct shown in Figure 20, generating a double transgenic mouse expressing both constructs (Figure 41).

**Figure 41** Detection of both PP1γ1 and PP1γ2 constructs in null mice. PCR using the PGk2 forward primer and the SV40 reverse primer was used in order to detect mice carrying both transgenes. Subsequently, the double-positive mice were tested for presence of the null allele.
3.1 PP1γ1 and PP1γ2 mRNA detection

Transgenic *Ppp1cc1* and *Ppp1cc2* mRNA expression was detected in the double transgenic of PP1γ1 and PP1γ2-rescue testis (Figure 42, R). Only three animals were tested.

![Image of RT-PCR results showing PP1γ2 and PP1γ1 mRNA bands](image)

**Figure 42. The mRNA of transgenic PP1γ1 and PP1γ2.** RT-PCR was performed on whole tests of PP1γ1 and PP1γ2-rescue mice (R) and the transgenic PP1γ1 and PP1γ2 mRNA was detected at the expected sizes of 500bp and 100bp respectively. (-) indicates the negative control.

3.2 Protein Detection of PP1γ1 and PP1γ2

Western blot analysis of testis extracts from the double PP1γ1 and PP1γ2 rescue males showed testicular expression of PP1γ1 and PP1γ2 close to their respective lines A and N (Figure 43A). Again, only the PP1γ2 isoform was incorporated in sperm (Figure 43B).
Figure 43. Presence of PP1 gamma isoforms in the double transgenic mice. (A) Western blot analysis shows transgenic PP1γ1 and PP1γ2 are expressed in the testes of the double transgenic
mice. (B) The sperm of the double transgenic mice shows incorporation of transgenic PP1γ2 but not PP1γ1 compared to WT sperm.

3.3 Testes architecture of PP1γ1 and PP1γ2-rescue mice

The testes architecture of the double transgenic mice seemed virtually indistinguishable from the PP1γ2-rescue testes (Figure 24). However it seemed to differ from the PP1γ1-rescue testes (Figure 37) for their absence of vacuoles, a hallmark of the PP1 gamma KO mice (Figure 24). Presence of vacuoles, a hallmark in the PP1 gamma KO mice described by Dr. Varmuza was significantly reduced and elongating spermatid heads (Figure 37) could be seen in the lumen of the seminiferous tubules.
Figure 44. Hematoxylin-eosin staining of PP1γ1 and PP1γ2-rescue mouse testes. Testes cross sections from A and T-line double-rescue mice (A), null (B) and wild type (C) were stained with hematoxylin-eosin. The stained sections were viewed with an Olympus IX70 microscope at 20X magnification. The testes architecture is also restored in the PP1γ1 and PP1γ2-rescue mice testes (A) compared to KO testes (B) and WT testes (C) and is virtually indistinguishable from the PP1γ2-rescue mice testes.
3.4 Epididymides of PP1γ1 and PP1γ2-rescue mice

Caudae epididymides stained with hematoxylin-eosin (Figure 45) from the double transgenic mice were virtually indistinguishable from the caudae epididymides of PP1γ2-rescue mice.

Figure 45. Caudal epididymides, of PP1γ1 and PP1γ2-rescue mice. PP1γ1 and PP1γ2-rescue epididymides, (A) are filled with sperm cells compared to KO epididymides (B) and WT (C) using hematoxylin-eosin staining. The PP1γ1 and PP1γ2-rescue epididymides are virtually undistinguishable from the PP1γ2-rescue epididymides.

3.5 Sperm morphogenesis and motility

Spermatozoa from double rescue mice seemed to show a slight amelioration of their abnormalities observed with PP1γ1 and PP1γ2-rescue mice. Numerous spermatozoa appeared to be pristine and normal (Figure 46A). However, the morphological abnormalities described in the PP1γ1 and PP1γ2-rescue spermatozoa were still present in the spermatozoa for PP1γ1 and Pp1γ2-rescue mice (Figure 46B-F). Sperm presented the PP1γ2-rescue hallmark phenotype with heads jack-knifed (Figure 46D), thinning of the annulus (Figure 46B), mitochondria abnormalities (Figure 46F) while numerous spermatozoa presented a globular-like head morphology (Figure
E). Also reminiscent of the abnormalities seen in PP1γ1-rescue, sperm heads appeared compressed in the axial plane (Figure 46C).

In contrast with the total absence of motility described in the PP1γ1-rescue sperm, the double transgenic PP1γ1 and PP1γ2-rescue sperm showed some degree of motility albeit below recordable levels. Sustained levels of motility were not attained and all spermatozoa became immotile minutes after their extrusion from caudal epididymides.

**Figure 46. Caudal sperm of PP1γ1 and PP1γ2 mice.** DIC imagery at 60X of PP1γ1 and PP1γ2-rescue sperm shows the slight improvement in the sperm morphology (A). However, PP1γ1-rescue and PP1γ2-rescue morphological abnormalities are still seen: thinning of the annulus (B), sperm heads jack-knifed (D) and compressed (C), globular heads (E) reminiscent of PP1γ1-rescue spermatozoa and KO and abnormal formation of the mitochondrial sheath (F).
DISCUSSION

Targeted disruption of the gene ultimately responsible for expression of both PP1 gamma protein isoforms, PP1γ1 and PP1γ2, results in male infertility stemming from impaired spermatogenesis, particularly in the spermiogeneic phase, where, presumably, spermatid apoptosis is widespread (Varmuza et al., 1999). Even though the other protein phosphatase 1 isoforms, PP1α and PP1β, are substantially upregulated in the Ppp1cc −/− testis (Chakrabarti et al., 2007), neither is able to substitute for the absent PP1 gamma 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 PP1γ2 subunits can complement the single PP1γ isoform in yeast (Gibbons, Kozubowski, Tatchell, & Shenolikar, 2007). It is also interesting that although PP1γ1 is expressed in nearly all tissues and the PP1γ2 protein is testis-restricted (being the only PP1 gamma isoform present in mammalian spermatozoa), the absence of PP1γ1 has no apparent detrimental effect on any process other than (possibly) testicular function in male mice. Because PP1γ2 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 PP1γ1 and PP1γ2 from the spermatocyte-specific promoter of Pgk2 (Figure 14, Figure 15, Figure 17) so that the independent PP1γ1 and PP1γ2 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 (Kessler et al., 2002).

As a consequence of the independent expression of Ppp1cc2 transgene in the Ppp1cc-/- mouse, PP1γ2 spatial expression approximates wild-type levels in both the testis (Figure 22) (Soler et al., 2009), however, transgenic expression of Ppp1cc1 reached relatively low levels in comparison (Figure 33). Interestingly, only transgenic PP1γ2 and not PP1γ1 is detected in caudal sperm (Figure 29). This is a particularly intriguing minor outcome, since even both proteins are under the same controlled conditions, PP1γ2 but not PP1γ1 is the only one being incorporated in sperm. This outcome suggests that although highly similar, intrinsic differences between both proteins might still affect the distribution of both isoforms as seen in wild type male mice.

Normal testis architecture within the seminiferous tubules of PP1γ1 and PP1γ2-rescue mice is restored (Figure 24 and Figure 37) (Soler et al., 2009). In addition, spermatozoa derived by swim-out from the cauda epididymis of the PP1γ1 and Pp1γ2 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 PP1γ1 and PP1γ2-rescue rescue mouse appeared to have substantial numbers of spermatozoa (Figure 26, Figure 39). However, while the epididymides in the PP1γ2-rescue mice was composed of spermatozoa, the epididymides of PP1γ1-rescue mice presented a mixture of spermatozoa and round cells, possibly corresponding to early-released spermatids (Figure 39E white arrows). Double expression of transgenic PP1γ1 and PP1γ2 in mice with the null background produced a virtually indistinguishable PP1γ2-rescue phenotype.
Contrary to our initial expectations, transgenic expression of either PP1γ1 and PP1γ2 or expression of both in the Ppp1cc -/- testis failed to fully restore fertility in rescue males (Soler et al., 2009). Motility analysis showed that the great majority of caudal spermatozoa derived from PP1γ2-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.

Interestingly, PP1γ1-rescue spermatozoa presented a total absence of motility while transgenic expression of both PP1γ1 and PP1γ2 isoforms seemed to improve the above mentioned phenotypes (Figure 46). Not surprisingly, expression of PP1γ1 and PP1γ2 in the null background produced spermatozoa with a recollection of morphological abnormalities seen in the PP1γ1 and the PP1γ2-rescue mice (Figure 28 and Figure 40 respectively).

High resolution light and electron microscopy of caudal PP1γ2-rescue sperm provided a variety of structural causes for the lack of sperm motility (and thus, infertility) (Soler et al., 2009). Testicular PP1γ2-rescue sperm have normal sickle-shaped heads, as opposed to deformed, rounded heads in Ppp1cc -/- testicular sperm (Figure 28F), strongly suggesting that PP1γ2 activity regulates sperm head morphogenesis. However, the flagellar aberration seen in testicular sperm from Ppp1cc -/- mice, a malformed, abbreviated or gapped mitochondrial sheath, is only partially ameliorated in testicular PP1γ2-rescue sperm, while irregularly shaped, residual cytoplasmic bulges are frequently observed at the head/connecting piece junction. (Figure 28A,B,C). These findings have provided obvious reasons for the loss of movement in the PP1γ2-rescue sperm, and suggest that either PP1γ1 plays an additional role in flagellar morphogenesis or PP1γ2 activity is not sufficient in the rescue testis, where the level of
transgenic PP1γ2 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 (Soler et al., 2009). 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 (Figure 28, Table 4). 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 (Zheng et al., 2007). 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, in the PP1γ2-rescue sperm 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 4). More importantly, caput and caudal PP1γ2-rescue sperm are also significantly different from each other (p<0.01) for both hairpin phenotypes (Soler et al., 2009). 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 PP1γ2 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 PP1γ2 activity is further suppressed (Vijayaraghavan et al., 1996). Electron micrographs of rescue PP1γ2-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 ultrastructure of testicular rescue germ cells is relatively improved compared to that of caudal rescue sperm (Figure 32). 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 (Olson, Winfrey, Nagdas, Hill, & Burk, 2005), (Andersen et al., 2003) (Olson, Winfrey, Hill, & Burk, 2004), 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 (Soler et al., 2009). It is possible that expression of the putative 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 (Figure 28A-D). 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 (Olson et al., 2005), is substantially reduced in both Ppp1cc -/- and PP1γ2-rescue testis (S. Vijayaraghavan, unpublished observations).

Comparatively, the structural abnormalities presented by the PP1γ1-rescue sperm constituted a more heterogeneous cadre (Figure 40). The bent-head phenotype of PP1γ2-rescue mice appeared to be less prevalent (Figure 40A) while sperm heads appeared globular (Figure 40D) and flattened (Figure 40F). Nevertheless, PP1γ1-rescue sperm shared several abnormalities also present in the PP1γ2, like thinning of the annulus (Figure 40B,H-J), a feature reminiscent from the selenium-deficiency phenotype described in mice lacking the seleno-protein P1 or the putative seleno-protein receptor, apolipoprotein E receptor 2 (Andersen et al., 2003; Olson et al., 2004; Olson et al., 2005). In addition, PP1γ1-rescue sperm also presented an abnormal and incomplete mitochondrial sheath (Figure 40G,H), an abnormal whip-like end piece, (Figure 40F) and several head-less sperm (Figure 40C).

Unless PP1γ1 also plays a major role in directing spermiogenesis, the exact reason for the lack of complete rescue of sperm structure and function in PP1γ2 mice is not entirely clear (Soler et al., 2009).
However, several testable possibilities exist. First it could be that relatively low levels of PP1γ2 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 high-expressor lines of transgenic mice. Surprisingly, the presence of the transgene fails to increase PP1γ2 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 PP1γ2 translation from its mRNA. Such a mechanism can be at the level of initiation of protein synthesis or in the breakdown 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 (Figure 23). 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 occurs from pre-existing mRNA (Iguchi, Tobias, & Hecht, 2006). Thus, the reduced stability of the mRNA from the *Ppp1cc2* transgene may compromise PP1γ2 protein levels in spermatozoa.

Another possibility for the lack of a complete rescue is that normal spermatogenesis may require expression of PP1γ2 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 (Zhang, Stroud, Eddy, Walter, & McCarrey, 1999). Immunohistochemical analysis of testis sections shows that a strong signal for PP1γ2 first appears in primary spermatocytes and remains strong through all further stages of germ cell development.
(Figure 27), although western blot analysis also has shown that a weak signal for PP1γ2 is
detected in the testis of 8-day old mice (corresponding to spermatogonial/somatic cell
expression) (Chakrabarti et al., 2007). Thus, PP1γ2 may be essential for gene expression and for
other biochemical processes in primary spermatocytes.

Therefore, it is conceivable that a lack of PP1γ2 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 (Hecht, 1990),(Iguchi et al., 2006). Among these are proteins of the outer dense
fibers which are unique features of the flagella of mammalian spermatozoa. It is possible that
PP1γ2, a PP1isoform 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, we cannot rule out the requirement of a function for PP1γ1 and PP1γ2 in Sertoli
cells and/or Leydig cells. That is, one or both PP1 gamma isoforms may be part of a signaling
system that must operate between Sertoli cells and developing germ cells to ensure normal
spermatogenesis.

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Thus, the initial hypothesis of this thesis proposing that PP1γ1 would have a function in
building the spermatozoa while PP1γ2 function would be specific in providing motility has been
partially disproved. The experiments provided by the transgenic expression of PPa1cc2 in the
null background mice suggests that PP1γ2 has a two-fold role instead: enabling spermatogenesis as well as providing limited non-forward motility as seen in the PP1γ2-rescue spermatozoa.

Forced expression of transgenic PP1γ1 alone in the null background of mice testes seemed to also enable spermatogenesis but up to a lesser degree compared to PP1γ2. In comparison to the PP1γ2-rescue mice, the complete lack of motility in the PP1γ1-rescue spermatozoa suggests that PP1γ1 role per se enabling spermatogenesis may be less effective than that of PP1γ2 alone.

While expression of both transgenes in the null background seemed to increase motility slightly, attempts to record it proved unsuccessful. However, the morphology of spermatozoa from PP1γ1 and PP1γ2 double rescue seemed ameliorated, while displaying a recollection of the structural abnormalities seen in either PP1γ1 or PP1γ2-resuce mice. Importantly, the double transgenic mice also proved to be infertile.

Conclusions

- Transgenic expression of PP1γ1 and PP1γ2 restores spermatogenesis
- Neither PP1γ1 or PP1γ2 expression under the PGK2 can restore fertility in male mice
- Sperm defects are severe in PP1γ2-rescue mice and spermatozoa present a low degree of motility
- Sperm defects are more heterogeneous in PP1γ1-rescue mice and spermatozoa are completely immotile.
- Concomitant expression of transgenic PP1γ1 and PP1γ2 under the PGK2 promoter doesn’t restore fertility in male mice.
Future Experiments and Directions

In order to address the remaining questions that this work has left unanswered, future experiments will include generation of rescue mice using the PP1 gamma endogenous promoter instead of PGK2 in order to re-create the bona fide expression patterns of PP1γ1 and PP1γ2. Conditional Knock Out (KO) mice will also be used in order to test whether Sertoli Cells and Leydig cells role are required to produce fertility-competent spermatozoa. Finally, creating an isoform-specific KO mouse will allow examination of how the spatio-temporal expression of PP1 gamma is essential in male mice testes.

Epilogue

Would PP1γ2 be a good anti-conceptive target?

Taking into consideration the results discussed in this work, a question arises of whether PP1γ2 would serve as a good target for developing a male-specific contraceptive drug. The relative fragility that seems to surrounds expression and action of PP1γ2 and its high mammalian and testes specificity make this protein phosphatase a good target for developing an anti-conceptive drug. Theoretically, and according to the studies discussed here just attaining a slight decrease in its expression or activity could yield the desired though reversible contraceptive effects.

However, such a drug should not target the hole PP1γ2 catalytic domain per se due to the protein isoform redundancy that exists in the male testes. Instead, in order to decrease its potential side effects, the hypothetical drug could make use of PP1γ2 unique C-terminal instead, and temporarily redirect the protein toward degradation via the proteosome.
Appendix

Three nucleotide mutations were found to be present in the sequence of the PP1γ2 cDNA used to create the PP1γ2-rescue mice. These mutations lead to three amino acid substitutions being L52R, I104V and L118F, generating a mutant version of PP1γ2. Subsequent pulldown and microcystein experiments showed these mutations didn’t alter PP1γ2 binding capabilities with its known regulators sds22 and i3 revealing that its functionality was not likely to be altered.

Further experiments are underway to generate transgenic mice with an unaltered version of PP1γ2.

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