THE ROLE OF SERINE/THREONINE PHOSPHATASES IN SPERM FUNCTION

A dissertation submitted
to Kent State University in partial fulfillment
of the requirements for the degree of Doctor of Philosophy

By
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May 2017

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<th>Definition</th>
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<tbody>
<tr>
<td>AKAP</td>
<td>A-kinase-anchoring protein</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>bp</td>
<td>Base pair</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine phosphate</td>
</tr>
<tr>
<td>CASA</td>
<td>Computer assisted sperm analyzer</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>CDS</td>
<td>Coding sequence</td>
</tr>
<tr>
<td>DIC</td>
<td>Differential interface contrast</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DOG</td>
<td>2-deoxy-D-glucose</td>
</tr>
<tr>
<td>ECL</td>
<td>Enhanced chemiluminescence</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>GSK3</td>
<td>Glycogen synthase kinase 3</td>
</tr>
<tr>
<td>HTF</td>
<td>Human tubal fluid</td>
</tr>
<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropyl-β-D-thio-galactopyranoside</td>
</tr>
<tr>
<td>IVF</td>
<td><em>In vitro</em> fertilization</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>OCC</td>
<td>Oocyte cumulus complex</td>
</tr>
</tbody>
</table>
PAS Periodic acid-schiff stain
PBS Phosphate buffered saline
PCR Polymerase chain reaction
PFA Paraformaldehyde
PGK2 Phosphoglycerate kinase 2
PIPs Phosphatase interacting proteins
PKA cAMP-dependent protein kinase
PMSF Phenylmethylsulfonyl fluoride
PPM Protein phosphatase Mg2+ or Mn2+ dependent
PPP Phosphoprotein phosphatase
PPP1C PPP1 catalytic subunit
PPP1R PPP1 regulatory subunit
PVDF Polyvinylidene difluoride membrane
RO Reverse osmosis
RIPA Radio-immunoprecipitation assay
RNA Ribonucleic acid
RT-PCR Reverse transcriptase PCR
SDS Sodium dodecyl sulphate
SDS-PAGE SDS polyacrylamide gel electrophoresis
Ser Serine
Spz1 Spermatogenic zip protein 1
SV-40 Simian virus 40
TBS Tris buffered saline
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>TCA</td>
<td>Trichloro acetic acid</td>
</tr>
<tr>
<td>Thr</td>
<td>Threonine</td>
</tr>
<tr>
<td>TPCK</td>
<td>Tosyl phenylalanyl chloromethyl ketone</td>
</tr>
<tr>
<td>TTBS</td>
<td>Tween tris buffered saline</td>
</tr>
<tr>
<td>ZP</td>
<td>Zona pellucida</td>
</tr>
</tbody>
</table>
This Thesis is dedicated to

“My journey companion: my beloved Husband”

“The people who made me who I am today: my Parents”
ACKNOWLEDGEMENTS

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Chapter I

INTRODUCTION

1.1 Mammalian Spermatogenesis

Gametogenesis in mammals requires that diploid cells undergo the process of reduction division known as meiosis. However, this process differs between males and females both in the timing and the endpoints. Oogenesis begins early in the fetal life with the goal of forming a limited number of gametes that are used over a defined reproductive lifetime. On the other hand spermatogenesis is not initiated until puberty and the formation of millions of gametes continues throughout the male adult life [1]. The nature of spermatogenesis requires a continuous stem cell population (Spermatogonial Stem Cells SSCs) to perform both self-renewal and differentiation. The fate of SSCs is determined by complex interactions between all types of testicular cells and is tightly regulated by growth factors and hormones.

1.1.1 The spermatogenic cycle

The first round of spermatogenesis in mice develops from a unique neurogenin 3 (NGN3) negative pool of prospermatogonia that transition directly into A₁ cells while the NGN3 positive spermatogonia remain undifferentiated and form the pool of stem cells [2]. Three different types of NGN3 positive cells have been identified based on cell and nuclear morphology A_{single} (A_{s}), A_{paired} (A_{p}), and A_{aligned} (A_{a}). Theses cells exist as either single cells or as a chain of interconnected
cells that arise as a result of incomplete cytokinesis. One of the most recently discovered markers expressed in these cells is ID4 that is expressed in the adult testis on a small subset of A cells [3]. Type A₄ spermatogonia sequentially divide to produce type A₂, A₃, and A₄ spermatogonia. Type A₄ spermatogonia differentiate into intermediate cells that undergo one mitotic division to give rise to type B spermatogonia. These type B spermatogonia undergo one last mitotic division to produce primary spermatocytes that eventually enter meiosis.

**Figure 1. Stages of spermatogenesis.**
Representation of a cross section through a seminiferous tubule showing germ cells at main stages of spermatogenesis, starting with spermatogonial stem cells at the top to differentiated testicular spermatozoa at the bottom.
Designed using Servier Medical Art
1.1.2 Developing germ cells

Primary spermatocytes situated midway in the epithelium of seminiferous tubules are the largest among the spermatogenic lineage. Division of each primary spermatocyte generates two secondary spermatocytes by meiosis I. The prophase of the first meiotic division is the longest step during which the DNA replicates in leptotene spermatocytes. Crossing over takes place shuffling the paternal genome in pachytene spermatocytes which then undergo a short diakinesis step where reduction division takes place to give rise to haploid secondary spermatocytes. Secondary spermatocytes then undergo meiosis II to produce four haploid round spermatids. The spermatids at this stage remain connected through cytoplasmic bridges allowing for diffusion of gene products between neighboring cells [4]. Spermatids then undergo a long process of morphological changes known as spermiogenesis to produce mature testicular spermatozoa. Spermatogenesis occurs in a synchronized cyclic pattern where the cellular associations of germ cells and testicular somatic cells including the hormone producing leydig cells and the supporting sertoli cells are maintained in a progressive fashion. The seminiferous epithelium can be categorized into discrete stages based on the cellular complement observed in a given segment of a seminiferous tubule. Twelve discrete stages have been identified in mice [2]. Figure 2 shows the cellular components of each stage.
Figure 2. The cycle of the seminiferous epithelium in the mouse testis.

A: Type A Spermatogonia, A$_{1-3}$: Type A$_{1-3}$ Spermatogonia, m: Mitotic Spermatogonia, In$_m$: Intermediate Spermatogonia, B: Type B Spermatogonia, B$_m$: Mitotic Type B Spermatogonia, Pl: Preleptotene Spermatocyte, L: Leptotene Spermatocyte, Z: Zygotene Spermatocyte, P: Pachytene Spermatocyte, D: Diakinetic Spermatocyte, m$_2$m: Early round Spermatid, 1-16: Spermatids 1-16.

Adapted from Griswold (2016), License Clearance ID: 4067930740973.
1.2 Spermiogenesis

During the process of spermiogenesis haploid spermatids undergo a complex morphological differentiation process that is vital for sperm function. These changes include condensation of the nucleus where DNA histones are replaced with protamines, formation of the acrosome, assembly of the flagellum, and arrangement of the mitochondria around the midpiece. Apart from these visibly unique structures, sperm also increase the level of gene transcription however; many of the produced mRNAs are stored for later translation [5].

1.3 Regulation of spermatogenesis

Spermatogenesis is an extremely well coordinated process that is regulated at two levels: extrinsic, and intrinsic. Extrinsic regulation involves the hormonal regulation of gene expression in somatic cells of the seminiferous tubules, which in turn modulates the activity of germ cells. LH produced from the pituitary gland acts on Leydig cells to produce testosterone and other androgens, while FSH acts on Sertoli cells along with testosterone to support germ cell maturation. Intrinsic regulation involves gene expression during spermatogenesis that is controlled at transcription, translation, and post-translational levels.

1.3.1 Transcriptional regulation

Regulation of stringent stage-specific gene expression during spermatogenesis is necessary for completion of both meiosis and male germ cell differentiation, and starts at the level of transcription regulators. Controlled levels of general transcription factors, and the expression of unique testis isoforms of transcription factors follows a carefully regulated program corresponding to the series of differentiation events occurring in the testis starting from
spermatogonia to round spermatids [6]. Some of the general transcription factors that are up-regulated during spermatogenesis are CREM (cAMP response element modulator), TBP (TATA binding protein), HSF2 (Heat shock factor 2), and OVOL1 (Ovo like transcriptional repressor 1) [7]. The most significant of them is CREM, named as the “master controller gene of spermatogenesis”. CREM is a cAMP responsive element binding protein that binds to CRE (cAMP response element) on promoters of target genes. CREM knockout mice are sterile due to arrest of spermatogenesis at the round spermatid stage [8, 9]. Infertility in these mice is caused by the lack of expression of a number of post-meiotic genes that are required for differentiation and contain CRE in their promoters, such as protamines 1 and 2, transition proteins 1 and 2, proacrosin, and calsermin, among others [10, 11]. A small number of transcription factors involved in spermatogenesis are testis specific and haven’t been reported to be present in somatic tissues. Examples are Spz1 [12] and Tisp40 [13]. In response to the up-regulation of many transcription factors during spermatogenesis, their target genes’ expression increases. Three major peaks of gene expression in the mouse testis have been identified: (a) during the mitotic phase, from 0 to 8 days postnatal (b) at the onset of meiosis, around 14 days postnatal (c) and during spermiogenesis, around 20 days postnatal. Some of those up-regulated genes are ubiquitously expressed in all tissues, while the majority are testis-specific transcripts. Transcriptional regulation of spermatogenesis is achieved in one of two ways:

(1) Activation of testis specific genes that have somatic homologs. Pgk2 (Phospho-glycerate kinase 2) is an example that has been studied for many years. The Pgk2 gene expressed only in male germ cells is located on chromosome 17. However, its somatic isoform is a homologous gene located on the X chromosome and expressed only in somatic cells [14].
The expression of testis specific genes that have no somatic homologs and are solely expressed in developing male germ cells. AKAP3 is a good example of this category [15]. AKAPs (A-kinase anchoring proteins) are proteins that anchor PKA to specific locations. In the sperm flagellum many AKAPs are present in the fibrous sheath and are tyrosine phosphorylated during capacitation [15].

Table 1. A list of testis specific genes common in mouse and human. Adapted from reference [16]

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Gene description</th>
</tr>
</thead>
<tbody>
<tr>
<td>GK2</td>
<td>Glycerol kinase 2</td>
</tr>
<tr>
<td>PDHA2</td>
<td>Pyruvate dehydrogenase alpha 2</td>
</tr>
<tr>
<td>SPERT</td>
<td>Spermatid associated</td>
</tr>
<tr>
<td>PGK2</td>
<td>Phosphoglycerate kinase 2</td>
</tr>
<tr>
<td>AKAP4</td>
<td>A kinase anchor protein 4</td>
</tr>
<tr>
<td>NUP210L</td>
<td>Nucleoporin 210 kDa-like</td>
</tr>
<tr>
<td>LDHC</td>
<td>Lactate dehydrogenase C</td>
</tr>
<tr>
<td>OXCT2</td>
<td>3-Oxoacid CoA transferase 2</td>
</tr>
<tr>
<td>SPATA3</td>
<td>Spermatogenesis associated 3</td>
</tr>
<tr>
<td>TEKT3</td>
<td>Tektin 3</td>
</tr>
<tr>
<td>PIWIL1</td>
<td>Piwi-like 1</td>
</tr>
<tr>
<td>MDH1B</td>
<td>Malate dehydrogenase 1B, NAD</td>
</tr>
<tr>
<td>SPATA16</td>
<td>Spermatogenesis associated 16</td>
</tr>
<tr>
<td>PPP3R2</td>
<td>Protein phosphatase 3, regulatory subunit B, beta isoform</td>
</tr>
<tr>
<td>SPAM1</td>
<td>Sperm adhesion molecule 1</td>
</tr>
<tr>
<td>ACRV1</td>
<td>Acrosomal vesicle protein 1</td>
</tr>
<tr>
<td>PLCZ1</td>
<td>Phospholipase C, zeta 1</td>
</tr>
<tr>
<td>SEPT12</td>
<td>Septin 12</td>
</tr>
<tr>
<td>ZPBP</td>
<td>Zona pellucida binding protein</td>
</tr>
<tr>
<td>Adcy10</td>
<td>Adenylate cyclase 10</td>
</tr>
<tr>
<td>TSGA10IP</td>
<td>Testis-specific, 10 interacting protein</td>
</tr>
<tr>
<td>TCP11</td>
<td>T-complex 11 homolog</td>
</tr>
<tr>
<td>ADAM2</td>
<td>ADAM metallopeptidase domain 2</td>
</tr>
<tr>
<td>ADAM3A</td>
<td>ADAM metallopeptidase domain 3A</td>
</tr>
<tr>
<td>ZPBP2</td>
<td>Zona pellucida binding protein 2</td>
</tr>
</tbody>
</table>
### 1.3.2 Post-transcriptional regulation

Post-transcriptional regulation of gene expression occurs at the mRNA level in one of two ways: either by alternative splicing or by silencing the message with miRNAs.

#### 1.3.2.1 Alternative splicing

Alternative splicing results in the generation of two or more isoforms of proteins from the same gene. This mechanism occurs highly in the testis and brain [17]. Exon skipping or inclusion occurs during the expression of mHK1 gene in developing male germ cells. In the testis the gene encodes for an alternately spliced mRNA: mHK1-S. The somatic isoform contains a porin binding region (PBR) that is absent in the testis isoform [18]. The use of an alternate promoter start site is another way of producing an alternate transcript of a single gene. The α-catalytic subunit of Protein Kinase A (PKA) exists in the testis as two isoforms Cα-1 and Cα-2. Cα-1 is ubiquitous while Cα-2 is specific to the testis. Both transcripts are encoded by the same gene “PRKACA” but using alternate start sites in intron 1. That process results in two proteins with
different N-termini. In a similar manner, the production of two protein isoforms with different C-termini from the same gene is possible through utilization of alternate polyadenylation (polyA) sites.

**Figure 3. Regulation of spermatogenesis.**
Illustration of different levels of gene expression during spermatogenesis, (i) Transcriptional regulation: Occurs through the binding of transcriptional enhancers or inhibitors to the promoter. (ii) Post-transcriptional regulation: Occurs through alternate splicing of mRNA. (iii) Translational regulation: Occurs through RNA binding proteins or long poly(A) tails. (iv) Post-translational regulation: Occurs through protein modifications such as phosphorylation.
1.3.2.2 Micro-RNA mediated gene regulation

miRNAs are single stranded non-coding RNAs of ≈ 22 nucleotides in length. miRNAs are coded by miRNA genes present in introns and are transcribed as part of the genes in which they reside. miRNA genes are transcribed by PolymeraseII to produce primary miRNAs that fold into a stem loop hairpin structure. Drosha trims the hairpin to produce a stem loop intermediate, which is transported to the cytoplasm where Dicer cuts both ends to produce a duplex. The duplex is unwound by a helicase to produce a single strand that is loaded onto a protein complex RISC (RNA induced silencing complex). The miRNA-RISC then binds to its target complementary mRNA and induces either translation suppression, or mRNA degradation [19]. Studies from Dicer knockout in germ cells clearly illustrate the importance of miRNAs in spermatogenesis and male fertility. The null males are infertile due to severe abnormalities in spermiogenesis [20]. Two miRNA clusters: miR-449 and miR-34b/c have been shown to be highly expressed in the testis at the onset of meiosis. Knockout of the miR-449 cluster had no visible effect on spermatogenesis or male fertility. However, the null mice exhibited up-regulation of mir-34b/c [21]. When a double knockout of both miR-449 and miR-34b/c was generated mice become infertile due to impaired spermatogenesis [22].

1.3.3 Translational regulation

The final stages of spermiogenesis in the testis require proteins essential for the differentiation of the round spermatids. By the time this process begins, nuclei have already begun to condense and tighten up their DNA, which makes transcription a nearly impossible task. This requires early germ cells to either synthesize proteins required for differentiation ahead of nuclear condensation or to synthesize transcripts of these proteins and store them in an
inactive state until required at the later stages. This translational regulation of transcripts can occur in two ways: a) inactivation of transcripts by mRNA binding proteins, b) translational delays of transcripts by adding an extensively long poly (A) tail to them.

1.4 The Spermatozoon

The spermatozoon is one of the most highly differentiated mammalian cells. Its unique structure is designed for the purpose of transporting a haploid set of male chromosomes through the female reproductive tract to the egg. The cell is divided structurally into three main regions: (a) the head, (b) the connecting piece, (b) and the sperm flagellum.

1.4.1 The Sperm Head

The sperm head shape and size varies widely among species. Yet all sperm heads share the same basic architecture. This indicates that critical aspects of the function of the sperm head have been conserved throughout evolution, such as acrosome reaction and sperm-egg binding. The head of the sperm bears the most important component of the male gamete: the paternal DNA. At the round spermatid stage transcription of the sperm specific protamins begins.

Figure 4. Mature mouse spermatozoa.
DIC picture of mature spermatozoa (60x).
However their RNA remains untranslated until the elongated spermatid stage. At this point they replace nuclear histones by forming strong disulfide links with the DNA increasing its packaging by 10 fold. This aids in the formation of the compact sperm head while protecting the genome from physical and chemical shearing during the sperm's journey [23]. The plasma membrane surrounding the sperm head is divided into two regions: (a) the acrosomal region, which consists of the anterior acrosome and the equatorial acrosome. (b) The post-acrosomal region. The acrosome is a specialized secretory vesicle that caps the anterior part of the sperm head. The contents of the acrosome include proteases and other hydrolytic enzymes that enable the sperm to penetrate the egg Zona Pellucida [24].

![Figure 5. Mouse sperm head. A schematic showing different regions of the mouse sperm head.](image)

### 1.4.2 The Sperm Flagellum

The flagella comprise the motile apparatus necessary for the movement and penetration of sperm into the egg at fertilization. The tail is divided into the midpiece, principle piece and end piece. The main structural component of the flagella; the “axoneme” is an evolutionary conserved microtubule-based structure and is the same apparatus of cilia in other tissues as the
trachea, oviduct, and sensory organs. However, the sperm flagellum differs from cilia in a number of ways. First, the midpiece axoneme is surrounded by outer dense fibers (ODF) and a mitochondrial sheath (MS). Second, most of the principle piece is surrounded by (ODFs) and a fibrous sheath (FS). The axonemes (9 + 2) organization is well conserved among eukaryotic cilia and flagella. The doublet microtubules numbered from 1 to 9 composed of a complete A-tubule and an incomplete B-tubule, surround a central microtubule doublet: C1 and C2. Many other proteins are bound to these microtubules and form a highly organized protein network. One of the most important is Dynein. Dynein is a force generating ATPase observed as a pair of projecting arms on the doublet microtubules. The radial spoke (RS) connects the outer microtubule pair to the central pair in a dynamic manner that allows sliding of the dynein arms upon utilization of ATP [25]. In addition to the structural microtubules and dynein, the sperm flagellum harbors many enzymes bound to the fibrous sheath. Many of them are sperm specific isoforms of glycolytic enzymes such as lactate dehydrogenase (Ldhx) and glyceraldehyde-3-phosphate dehydrogenase (Gapdh-s) [26].
Figure 6. Spermatozoan structure.
Schematic diagram showing the ultrastructure of different regions of the sperm tail, the midpiece axoneme is surrounded by outer dense fibers and a mitochondrial sheath. The principle piece axoneme is surrounded by outer dense fibers and a fibrous sheath coat.
Designed using Servier Medical Art
1.5 Epididymal sperm maturation and motility initiation

Testicular sperm are unable to fertilize an egg. After being released into the seminiferous tubules they are required to undergo a new stage of maturation through their passage into a long coiled tubule known as the epididymis. Epididymal sperm maturation is a phase that occurs specifically in mammals. Mammals have evolved that unique organ coincident with the evolution of internal fertilization. However, by the time testicular sperm reach the epididymis, their chromatin is already condensed and the cells are incapable of gene transcription and protein synthesis. From that moment onwards all structural and functional modifications become
possible only through post-translational modifications of preexisting proteins mainly protein phosphorylation.

The epididymis is divided into three main functional compartments from anterior to posterior: the caput, the corpus, and the cauda and serves four main roles in the spermatozoas journey: (1) Storage and protection of sperm after being released from the seminiferous tubules. (2) Transport of sperm from the site of production to the vas deferens. (3) The development of sperm motility. (4) The development of sperm fertilizing ability. Male germ cells and spermatozoa are separated from the rest of the male body by the blood-testis barrier and the blood-epididymis barrier. This creates a unique environment solely controlled by the sertoli cells and the epididymal epithelium. The epididymal fluid consists of water, inorganic ions, small organic molecules, and proteins. Sperm undergo both morphological and biochemical changes in the epididymis.

1.5.1 Morphological changes during epididymal maturation

During spermiogenesis in the testis, spermatozoa shed most of their excess cytoplasm into a cytoplasmic droplet surrounding the connecting piece. The cytoplasmic droplet remains attached to the sperm until they pass from the caput to the cauda epididymis then migrates to the midpiece. Cytoplasmic drops are believed to be involved in osmolality regulation [27].

1.5.2 Biochemical Changes during epididymal maturation

Most of the biochemical changes that occur during sperm epididymal transit are related to protein exchanges with the epididymal lumen through epididymosomes, and post-translational modifications of sperm proteins [28]. Several epididymosomal proteins that are transferred to
spermatozoa have functions related to immunological protection, motility, or as decapacitation factors. CRISP1 and NCP2 are proteins that are believed to be acquired on the sperm surface from the epididymal fluid to regulate the cholesterol content of the spermatozoal plasma membrane and prevent premature capacitation [29]. Other proteins have been identified through large-scale proteomic analyses to differ among different regions of the epididymis.

### 1.5.3 Motility initiation

The most visible change in sperm during epididymal maturation is the activation of motility. Sperm isolated from the cauda epididymis show a symmetric and uniform flagellar waveform indicative of progressive motility. Caput sperm are non-motile, yet it is believed that at this stage they have mature flagellar machinery that is capable of activation. Studies on demembranated non-motile sperm have proved that they can become motile by changing the phosphorylation status of some proteins (addition of phosphatase inhibitors) or increasing the levels of intracellular cAMP [30], leading us to believe that the non-motile state of caput sperm is probably due to the inhibition of motility by the caput epididymal environment rather than a structural immaturity of the flagellum. The exact mechanism by which a number of factors initiate motility in the cauda epididymis is not fully known, but it is clear that they involve a change in protein phosphorylation of a wide range of sperm proteins. The main factors that lead to this altered protein phosphorylation state hence motility initiation are: pH, cAMP, and calcium.

#### 1.5.3.1 Intracellular pH
Motility of caput and quiescent sperm is inhibited by a low pH of the epididymal fluid and the intracellular environment of the spermatozoa. The high levels of lactic acid and proton ions in the epididymal lumen plays a major role in this process. In general Na⁺/H⁺ exchangers (NHEs) contribute to the basic homeostatic mechanisms that control pH, and are ubiquitously expresses in all tissues. Four different isoforms of NHEs are expressed in the testis, one of them has been reported as a sperm specific NHE (sNHE). The loss of this isoform causes infertility in males due to severe defects in sperm motility while intracellular alkalization of the null sperm partially restores the abnormal phenotype [31]. pHi regulates motility by affecting other downstream regulators such as the levels of cAMP through affecting the activity of sAC (soluble adenylyl cyclase) which has been shown to be pH dependent [32].

1.5.3.2 cAMP

Cyclic adenosine monophosphate (cAMP) is a key mediator of sperm motility initiation. In somatic cells cAMP is produced by a membrane bound adenylyl cyclase (mAC) through G-protein coupled machinery. However, sperm being a specialized cell has its unique form of a soluble adenylyl cyclase (sAC) that lacks a transmembrane domain, and is regulated by Ca²⁺ and HCO₃⁻. sAC once activated uses up ATP to produce cAMP. In turn cAMP acts as an activator of protein kinase A (PKA): a tyrosine kinase that phosphorylates a wide range of flagellar proteins. Transgenic mice null for sAC produce morphologically normal sperm, yet are infertile due to the lack of sperm progressive motility, indicating that PKA mediated tyrosine phosphorylation plays a key role in sperm motility initiation. The phenotype is rescued with the addition of cAMP to sperm in incubation media [33]. Interestingly, when comparing the phenotype of sAC deficient mice with the phenotype of PKA-Cα2 mutant mice, important differences can be noted. The loss
of sAC leads only to motility defects, while the absence of PKA leads to both morphological defects in the sperm head as well as motility defects. This suggests that PKA may have a role in spermiogenesis separate from its role in motility initiation [34, 35].

1.5.3.3 Calcium

Calcium ions are one of the main components of the epididymal fluid. Previous studies of the intracellular calcium content of sperm from different epididymal regions showed that non-motile caput sperm contain remarkably higher levels of calcium ions than motile caudal sperm. Since mature sperm do not contain the universal cellular calcium storage organelle the “endoplasmic reticulum”, it is believed that the sperm mitochondria serves as its calcium reservoir. The decline in \([\text{Ca}^{2+}]_i\) during sperm maturation may be due to the changes in mitochondrial influx and efflux characteristics and not only due to changes in the sperm plasma membrane permeability [36]. Male mice lacking the mitochondrial voltage-dependent anion channel type 3 (VDAC3) are infertile due to decreased motility of caudal sperm [37]. The plasma membrane calcium ATPase 4 (PMCA4) is another example of an intracellular calcium regulator present along the sperm flagellum. PMCA4 belongs to a group of calcium pumps that utilize ATP to pump out calcium from the cytosol through the plasma membrane in order to maintain homeostasis. Transgenic mice lacking PMCA4 develop normally to adulthood, yet males are infertile again due to a significant impairment of caudal sperm motility [38]. Catsper is a sperm specific voltage gated calcium channel present in the principle piece of the tail. Studies on Catsper Knockout mice show that males are infertile. Although the null sperm are motile, yet they are unable to undergo capacitation or hyperactivate when incubated in the designated media [39].
1.6 Capacitation and fertilization

Capacitation in the female reproductive tract is the final maturation process that sperm undergo before fertilization. During this process sperm undergo further morphological and biochemical changes, which include: (1) Changes in the plasma membrane of sperm due to the efflux of cholesterol, increasing the membranes fluidity. (2) Further increase in the levels of cAMP. (3) An increases in protein tyrosine phosphorylation levels. (4) An increase in intracellular pH and Ca\(^{2+}\) levels [40]. Capacitation is a series of events that start with a change in the motility pattern of the sperm known as “hyperactivation” where the flagellar beat movement changes from symmetrical to nonsymmetrical with increased amplitude. Two major factors that induce hyperactivation are bicarbonate ions (HCO\(_3\)-) and Ca\(^{2+}\). The bicarbonate transporter SLC4A2 is localized in the equatorial segment of the mammalian sperm head and four other SLC isoforms have been identified in the midpiece of mouse sperm. These transporters participate in the pH\(_i\) increase that takes place during capacitation and therefor leads to the activation of sAC [41]. It has been shown that the addition of HCO\(_3\)- to mature caudal sperm increases flagellar beat in a nonsymmetrical matter, which is a characteristic of hyperactivated motility. This vigorous movement aids the sperm in escaping from the oviduct epithelium and in penetrating the cumulus cells surrounding the unfertilized oocyte. The importance of Ca\(^{2+}\) has been illustrated in sperm lacking Catsper (Catsper1\(^{−/−}\)), where the null sperm are unable to detach from the oviduct epithelium due to their inability to undergo hyperactive motility [42].

Once the sperm comes in contact with the cumulus-oocyte complex (COC) a number of sperm surface proteins show hyluronidase activity such as sperm adhesion molecule 1 (SPAM1) and hyaluronoglucosaminidase 5 (Hyal5). Studies on mice knockout models of SPAM1 and Hyal5
show a compromised ability of sperm to penetrate the COC. The final stage of sperm capacitation: “acrosome reaction” is induced by the fusion of the sperm head with the Zona pellucida (ZP) where the enzymatic contents of the sperm acrosome are released to enable the fusion of the sperm with the oocyte oolemma. ZP3 has been identified as the only ZP isoform that induces elevation in sperm $[\text{Ca}^{2+}]_i$ and subsequent acrosome reaction. Many studies have been performed to identify the sperm surface protein that binds to ZP3. Five knockout mice models have been reported to have defective sperm-ZP binding: Clgn, Ace, Adam1a, Adam2, and Adam3 knockout mice. However, only ADAM3 has been shown to localize to the sperm surface and directly bind to ZP. It appears that the presence of the other four proteins is necessary for the optimum localization and function of ADAM3 [43]. Sp56 and proacrosin have also been identified as other candidate proteins that bind to ZP3 [44].

1.7 The role of protein phosphorylation in sperm function

At a certain point during spermatogenesis, round spermatids become transcriptionally inactive cells. Yet, they still have a long differentiation and maturation journey to undergo before they reach their target. Thus, regulation of protein function in post meiotic germ cells is achieved mainly through translational regulation or post-translational modifications of preexisting proteins. Spermatozoa are believed to be translationally silent to a large degree. Although minimal translation of stored mRNA occurs on mitochondrial ribosomes, post-translational modifications remain the main regulator of protein function [45].

Reversible phosphorylation is a major intracellular control mechanism in eukaryotes in general. It is involved in almost all cellular functions, from metabolism to signal transduction, cell division and memory. In spermatozoa, phosphorylation of proteins regulates events at every
maturation stage, from motility initiation in the epididymis to the acrosome reaction at the zona pellucida. The phosphorylation state of a protein is a dynamic process controlled by both protein kinases and protein phosphatases. Phosphorylation can occur on serine, threonine or tyrosine residues with serine phosphorylation being the most common. The human genome encodes 518 protein kinases and only 150 phosphatases, of which 107 are tyrosine phosphatases and fewer than 40, are serine/threonine phosphatases [46]. With the ratio of kinases to phosphatases being so high, the phosphatases are able to cover a wide range of cellular functions by interacting with a large number of regulators that localize the phosphatases to the required cellular compartment to ensure correct substrate specificity.

Serine/threonine phosphatases (PSPs) can be divided into three families as shown in figure 8. The PSPs of interest for this study are highlighted with red boxes.
1.7.1 Phospho-protein phosphatase 1 (PP1)

The PP1 protein phosphatases are present in various eukaryotic organisms ranging from budding yeast to mammals. They play key roles in metabolism, muscle contraction, and cell division. Four PP1 isoforms (PP1α, PP1β, PP1γ1, and PP1γ2) are expressed from three different genes in mammals (Ppplca, Ppplcb, and Ppplcc). PP1γ1, and PP1γ2 are differentially spliced products of the Ppplcc gene, an event that takes place only in mammals. While PP1α, PP1β, and PP1γ1 are expressed in a wide range of tissues, PP1γ2 is predominantly expressed in post-meiotic cells of the testis. All PP1 isoforms share a high degree of sequence similarity (90%) in their catalytic domain. Their differences reside mostly in their extreme C termini.

“PP1γ2” a mammalian specific isoform is essential for male fertility

PP1γ2 is produced by alternative splicing of the premature mRNA of the Ppplcc gene. This splicing event is specific to mammals and occurs highly in post meiotic germ cells of the testis. Non-mammalian species such as xenopus, turkey, and sea urchin bear PP1γ1, or PP1α in their testis and sperm [47]. Ppplcc contains eight exons and seven introns. The coding region of PP1γ1 contains exons 1-7 and 27 base pairs of intron 7, and the remaining sequence: the remainder of intron 7 and exon 8 form the PP1γ1 3’UTR. However, PP1γ2 is coded by all eight exons, while splicing out the entire intron 7, giving rise to a 325 amino acid protein with a unique 22 amino acid C-terminus tail. Mice lacking the Ppplcc gene deleting both PP1 isoforms PP1γ1 and PP1γ2 suffer from male infertility. Yet all other tissues are completely normal and females are fertile, indicating that the loss of PP1γ1 from somatic cells or female germ cells does not affect their function and other PP1 isoforms can probably substitute for its absence. Ppplcc knockout mice testes are devoid of spermatozoa due to germ cell arrest at the spermatocyte stage [48]. PP1γ2 in male germ cells is normally expressed post meiotically and remains to be the only
PP1 isoform present in mature spermatozoa. Other than the role of PP1γ2 in meiosis, it has been well established that PP1γ2 has a significant role in sperm motility initiation and regulation in the epididymis. Protein phosphorylation remains the most widely utilized mechanism for control of protein function in a wide range of cells and in spermatozoa in particular. Naturally the number of protein kinases in a cell exceeds the number of protein phosphatases. PP1γ2 being the only PP1 isoform present in sperm indicates that it controls a wide range of dephosphorylation events. Immotile caput epididymal sperm have high PP1γ2 activity whereas motile caudal epididymal sperm have low PP1γ2 activity. Furthermore inhibition of PP1 activity with okadaic acid (OA) or calyculin-A results in initiation of motility in immotile caput epididymal spermatozoa [49]. The PP1 catalytic subunit is always associated with one or more regulatory subunits forming a multimeric enzyme. These regulatory subunits control the enzymatic activity of PP1 and are involved in its localization to specific regions of the cell increasing its proximity to its substrates. Phosphatase interacting proteins (PIPs) are divided into three major categories: substrates, targeting subunits, or inhibitors [50]. PIPs are structurally unrelated, however most of them share an RVxF type motif that binds to a hydrophobic groove located behind the PP1 active site. The binding of this motif does not change the conformation of PP1s and serves only an anchoring purpose. Many PP1γ1 interacting proteins have been identified in somatic tissues most of which are general PP1 binding proteins. However, in the testis and spermatozoa a limited number have been studied. In the testis, endophilin B1t (a testis enriched isoform of endophilin B1a) was shown to bind to PP1γ2 but not to a mutant form of the enzyme lacking its specific C-terminus tail. Endophilin B1t also inhibits the phosphatase activity of PP1γ2 and is also absent in the testis of Ppp1cc null males [51]. PPP1R2 (I2) is capable of inhibiting the catalytic subunit of PP1 in many tissues. However, a novel isoform of PPP1R2 has been identified in spermatozoa, which
was previously thought to be an intronless pseudo-gene. This pseudo-gene appears to be specifically expressed in testis and the resulting protein contains a novel phosphorylation site at Ser127, which is lacking in the somatic isoform [52]. In sperm, GSK3 has been shown to phosphorylate I2 relieving its inhibitory effect of PP1γ2. This biochemical pathway is believed to be a major mechanism of controlling sperm motility initiation. GSK3 as well as PP1γ2 activity is higher in non-motile caput epididymal sperm and decreases as sperm acquire motility in the cauda epididymis. Inhibition of PP1γ2 activity initiates motility of sperm irrespective of calcium and cAMP levels indicating that PP1γ2 control of motility lies downstream of calcium and cAMP. Ca²⁺ and cAMP levels can affect PP1 activity through modulating the phosphorylation of I1 (PPP1R1) and DARP-32. However, since none of these proteins are expressed in spermatozoa, it is unlikely that this pathway plays a role in sperm motility [49]. PPP1R11 (I3) and PPP1R7 (Sds22) are other PP1 binding proteins expressed in testis and spermatozoa. They have been shown to form a complex with PP1γ2 and actin rendering the catalytic domain of the phosphatase inactive [53].

1.7.2 Phospho-protein phosphatase 2 B (PP2B or Calcineurin)

Calcineurin is a calcium and calmodulin-dependent serine/threonine phosphatase consisting of a catalytic subunit calcineurin A (CN-A) and a regulatory subunit, calcineurin B (CN-B). Three isoforms of the catalytic subunit have been identified: PPP3CA, PPP3CB, and PPP3CC, encoded by three separate genes, PPP3CC being exclusively expressed in the testis [54]. Two isoforms of the regulatory subunit exist: PPP3R1; which is ubiquitously expressed in all tissues, and PPP3R2 that is restricted to the male testis [55, 56]. CN-A shares some sequence and structural homology with both PP1 and PP2A, yet its activity is dependent on the binding of
calmodulin and CN-B. In the presence of high calcium, calmodulin binds to an autoinhibitory domain of CN-A preventing it from masking the catalytic site of the enzyme hence increasing its phosphatase activity. CN-B binds to CN-A in an irreversible manner. Its ability to activate the catalytic subunit depends on the availability of sufficient levels of calcium. CN-B has four Ca^{2+} binding sites. Two of those sites are constitutively bound to calcium and are essential for the structural integrity of the CN-A + CN-B heterodimer. The binding of Ca^{2+} to the remaining two sites increases the phosphatase activity of CN-A [57]. Calcium is a highly utilized signaling molecule in most cell types. Calcium dependent enzymes have been implicated in a wide variety of biological responses, from lymphocyte activation to neuronal and muscle development. Calcineurin was first extracted from bovine brains using calmodulin affinity chromatography and was believed to be an inhibitor subunit of phosphodiesterase [58]. It wasn’t until the 1980’s when calcineurin was identified as an independent phosphatase upon discovering its ability to dephosphorylate phosphorylase kinase [59]. In 1991 Schreiber and colleagues discovered that calcineurin is the target of two common immuno-suppressive drugs: Cyclosporin and FK-506 [60]. Since then many studies have been done on the effect of those drugs on calcium signaling.
Calcineurin was first reported to be expressed in testis as a unique isoform (PPP3CC) by Muramatsu et al. in 1992 [54]. PPP3CC mRNA was detected by northern blot in mouse testes as young as 20 days old. Similarly, PPP3R2 mRNA expression was reported to match PPP3CC [56]. The testis specific isoforms of both the catalytic and regulatory subunits differ than their somatic isoforms in the sequence of their N and C-termini. However the functional effects of those differences are not yet known. Although the role of calcium in sperm capacitation and
acrosome reaction has been well studied, a limited number of studies on the role of calcineurin in sperm function have been published. A recent study by Miyata et al. (2015) describes a unique role of PPP3CC in sperm epididymal maturation. The study reports that PPP3CC null mice are infertile due to abnormal sperm motility characterized by a rigid midpiece. Males produce normal numbers of sperm with no abnormalities in their testes architecture. Moreover, *in vitro* treatment of wild type sperm with the calcineurin inhibitors cyclosporine and FK-506 does not affect motility or fertilization ability, indicating that the role of calcineurin in sperm motility occurs during motility initiation in the epididymis and not after ejaculation or in the female reproductive tract. The study also shows that the phenotype of PPP3R2 knockout mice is similar to the phenotype of PPP3CC knockout mice indicating that the absence of either the catalytic or the regulatory subunit leads to a nonfunctional phosphatase. Interestingly, a number of studies published by Vijayaraghavan et al. (1989-1990) have reported that a dramatic change in the levels of intracellular calcium occurs as sperm progress from the caput to the cauda epididymis [61], and that calcium uptake from the epididymal fluid is regulated by the sperm mitochondria [36, 62]. These findings altogether point out a significant role of calcium and the calcium dependent phosphatase “calcineurin” in the epididymal maturation and motility initiation of spermatozoa.
The three aims of my dissertation are:

Aim I: To test the hypothesis that Pp1γ1 can replace the function of Pp1γ2 in testis and sperm.

Aim II: To identify the Ppp1cc gene’s minimal promoter sequence required for testis specific expression, and to identify the function of PP1γ2 C-terminus tail.

Aim III: To identify the role of the protein phosphatase PP2B (Calcineurin) in sperm motility.
Chapter II

MATERIALS AND METHODS

2.1 Mouse genomic DNA Isolation

Ear punches from mice were resuspended in 50μl of Alkali lysis buffer (25 mM NaOH and 2 mM EDTA, pH 12.0 in ddH₂O) and denatured at 95°C for 1 hr. Next, 50 μl of neutralizing buffer (40mM Tris-HCl, pH 5.0 in ddH₂O) was added. The samples were centrifuged at 1000xg and the supernatant was collected for genotyping PCR.

Table 2: List of primers used for genotyping PCR

<table>
<thead>
<tr>
<th>Primer reaction</th>
<th>Primer sequence</th>
<th>Genotype</th>
<th>Band size</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Ppp1cc KO &amp; PP1γ1 Rescue mice</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ppp1cc WT allele PCR</td>
<td>Intron IV forward</td>
<td>5’-CTCAGGCCAATGCTGTCTGC-3’</td>
<td>√</td>
</tr>
<tr>
<td></td>
<td>Intron VI reverse</td>
<td>5’-ACTCATAGCCCATCTTCAACCA-3’</td>
<td>√</td>
</tr>
<tr>
<td>Ppp1cc KO allele PCR</td>
<td>Intron IV forward</td>
<td>5’-CTCAGGCCAATGCTGTCTGC-3’</td>
<td>X</td>
</tr>
<tr>
<td></td>
<td>Neo Cassette (MA) reverse</td>
<td>5’-GGTGGATGTGAATGTAATGG-3’</td>
<td>√</td>
</tr>
<tr>
<td>Pp1γ1 transgene PCR</td>
<td>Exon VI forward</td>
<td>5’-GTGGTTGAAGATGGCTATGA-3’</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>SV40 reverse</td>
<td>5’-AAGCTGCAATAAACAAAGTTGG-3’</td>
<td>√</td>
</tr>
</tbody>
</table>
### 2.2 Generating and mating of transgenic mice

**Pp1γ1 rescue mice**

A gene construct designed by a former lab member: Nilam Sinha containing the *Pgk2* promoter – a modified Pp1γ1 cDNA sequence – SV40 polyA was injected into the pro-nuclei of fertilized B6SJL eggs. The injected eggs were implanted into the uteri of pseudo pregnant mothers. The procedure was carried out at the Transgenic Facility of Case Western Reserve University (Cleveland, OH). The produced offspring with the genotype *Ppp1cc(+/+)*, Pp1γ1tg(+/-) were shipped to Kent State Animal Facility. Pp1γ1 rescue mice were produced through breeding the received transgenic mice with *Ppp1cc* null female mice that were a gift from Dr. Susan Varmuza, Univerity of Toronto. (Subsequent breeding initiated by Tejasvi Dudiki).
Figure 10. A schematic comparing the PP1γ1 transgene construct design with both PP1γ2 and PP1γ1 mRNA.

The PP1γ2 coding sequence contains exons 1-7 and exon 8. The PP1γ1 coding sequence contains exons 1-7 and a retained fragment of intron 7. The remainder of Intron 7 is retained in the mature transcript along with exon 8 as a part of its 3’ UTR. The PP1γ1 rescue construct was designed to mimic PP1γ2 mRNA in the absence of intron 7, with the exception of the retained fragment encoding the last 8 amino acids of PP1γ1 protein.
PPp3r2 knockout out mice

The knockout mice were generated by electroporation of Embryonic Stem (ES) cells of B6SJL mice with the designed targeting vector. The targeting sequence contained LacZ and a
neo cassette replacing most of exon1, intron1, and 82 base pairs of exon2 with a 5’ end homologous to \textit{Ppp3r2} 5’UTR and a 3’ end homologous to \textit{Ppp3r2} exon2. Following homologous recombination the targeting vector replaced a single \textit{Ppp3r2} allele. The neo cassette (flanked with LoxP sites) was removed from the first generation of transgenic mice through breeding them with Cre’ mice. The final transgenic mice produced had one allele of LacZ replacing most of exon1, intron1, and 82bp of exon2. The mice were generated at KOMP Repository (UCDAVIS).

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure12.png}
\caption{Calcineurin regulatory subunit PPP3R2 knockout mice generation. A schematic showing the procedure followed by KOMP to generate the knockout mice. A) The targeting vector contained a LacZ reporter, a neo cassette surrounded by two LoxP sites, and homology regions overlapping parts of exon1 and exon2. B) The gene targeted by the vector is shown after homologous recombination, and after Cre deletion of the neo cassette from the targeted gene.}
\end{figure}

Heterozygous mice \textit{Ppp3r2}(+/−) were received from KOMP and the following breeding scheme was followed to produce \textit{Ppp3r2}(-/-) mice.
Figure 13. The breeding scheme followed to generate $Ppp3r2$ knockout mice. $Ppp3r2$ heterozygous mice $Ppp3r2(+/-)$ received from KOMP were bred with wild type mice $Ppp3r2(+/+)$ to generate the first generation of heterozygous mice derived from a single founder. Heterozygous mice resulting from the first breeding were bred together to generate the desired knockout $Ppp3r2(-/-)$. 
2.3 Fertility test

Transgenic male mice were mated with wild-type CD1 females over a period of 4 weeks, and the number of offspring in each litter was recorded. CD1 females that failed to become pregnant were subsequently tested for fertility by mating to wild-type CD1 males.

2.4 Mouse sperm isolation

Mice were euthanized in a carbon dioxide chamber then the cauda epididymis and vas deferens were isolated together. The artery of the deferens was carefully removed to avoid blood contamination. The tissue was then washed in PBS before being placed in a 60mm X 15mm petri dish containing fresh PBS warmed to 37° C. The cauda epididymis was punctured with a 26G (45mm) needle and the sperm were squeezed out from the cauda epididymis and the vas deferens using surgical tweezers. Sperm were allowed to disperse in the dish for 5-10 min at 37° C aided with occasional swirling. Upon complete dispersion, the sperm were transferred to a 1.5 ml centrifuge tube using a cut tip 1 ml pipette tip to avoid shearing of the sperm.

2.5 Sperm count

Following sperm isolation, 10 μl was diluted 20 times (mouse) or 100 times (bovine) in water. 10 μl of diluted sperm was loaded under the cover slip of a hemocytometer to fill up the chamber and allowed to sit for 5 minutes to settle before counting.

2.6 Quantitation of morphologically normal sperm

Following sperm isolation in PBS, the sperm suspension was centrifuged for 20 min at 700xg at 4°, the sperm pellet was resuspended in 4% PFA (paraformaldehyde) in 1X PBS and
incubated on ice for 30 min for fixation. Fixed spermatozoa were mounted on a clean slide and sealed under a coverslip. Sperm morphology was analyzed under 20X and 40X objectives. Randomly selected fields were observed and the proportion of defective sperm was counted out of a total of 100 sperm cells. Spermatozoa with the following characteristics were counted as defective: bent neck at the connecting piece, tail with an 180° bend at the midpiece-principle piece junction, malformed head. Method derived from Sinha et al. 2012 [63].

### 2.7 Sperm motility analysis

Sperm from the cauda epididymis and the vas deferens were isolated into PBS supplemented with 10mM glucose and warmed to 37°C. The sperm suspension was diluted to a concentration of approximately 2x10^7 sperm/ml. Motility was analyzed using 100μm deep chamber slides (SC-100-01-02-B, Leja) by CASA. Three to five random fields each of 60 frames at 60 frames per second were chosen for each sample and analyzed with the following settings: minimum contrast 35, minimum cell size 5 pixels, static cell size 7 pixels, static cell intensity 40, low size gate 0.17, high size gate 2.26, low intensity gate 0.35, high intensity gate 1.84, minimum static elongation gate 0, maximum static elongation gate 90, minimum VAP (velocity of average path) 50μ/s, minimum STR 50%, VAP cut off 10 μ/s and VSL (velocity of straight line) cut off 0 μ/s.

### 2.8 Sperm flagella tracings and analysis of flagella beat wave.

Sperm motility analysis was performed as described in the previous section. Video recordings were taken using a DVC camera (model: 340M-OO-CL) connected to a light microscope using a 10x magnification lens. Each recording was taken for 10 seconds with a
frame rate of approximately 100/Sec. Videos were converted into a sequence of images using Adobe Photoshop and flagella tracings were performed on 20 images. Flagella wave peak-to-peak amplitude was estimated from produced drawings.

2.9 Measurement of ATP levels

Isolated caudal sperm were washed in PBS and centrifuged for 10 minutes at 750xg. Sperm pellets were resuspended in HTF media (EmbryoMax, EMD Millipore) pre-equilibrated in 5% CO\textsubscript{2} at 37\textdegree C for at least 4 hours or in pre-equilibrated TYH medium with substrates (Glucose 10mM, Lactate 25mM, or Pyruvate 25mM) with or without the glycolysis inhibitor DOG (2-deoxy-D-glucose) or the mitochondrial respiration inhibitor Antimycin. 50\textmu l of sperm suspension was taken at different time points during the incubation and added to 450\textmu l of hot ATP buffer (100mM Tris-HCl and 4mM EDTA, pH 7.75) and boiled in a dry bath for 6 minutes. The lysed sperm suspension was snap frozen on dry ice, thawed then centrifuged at 15,000xg for 5 minutes at 4\textdegree C. Following centrifugation the supernatant was collected in a clean tube and used to perform the assay according to the manufactures instructions (ATP Bioluminescence Assay Kit HS II, Roche Life Science, IN, USA). ATP levels were calculated and adjusted according to sperm count and reported as nMoles/10\textsuperscript{7} sperm cells.

2.10 Hexokinase activity assay

Hexokinase was assayed spectrophotometrically in a system coupled with glucose-6-phosphate dehydrogenase G-6-PDH (protocol derived from Sigma Aldrich). The following equation represents the reaction performed:

D-Glucose + ATP $\xrightarrow{\text{Hexokinase}}$ D-Glucose-6-Phosphate + ADP
D-Glucose-6-Phosphate + βNADP $\xrightarrow{\text{G-6-PD}}$ 6-PG + βNADPH

One ml of the assay mixture contained: 20mM Triethanolamine pH 7.6, 200mM glucose, 7mM MgCl$_2$, 1mM βNADP, 1mM ATP, and 1 unit of G-6-PDH. The assay mixture was incubated for 10 minutes at 25°C before adding 50μl of sperm extract (in 1x RIPA), or 50μl of water for blank. Absorbance (340nm) was measured at both 0 minutes and 5 minutes after adding the sperm extract. The concentration of βNADPH was calculated after generating a standard curve for βNADPH absorbance at 340nm. One unit of hexokinase is defined as the amount of enzyme that catalyzes the phosphorylation of one micromole of substrate (thus produces 1 micromole of βNADPH) per minute at 25°C at pH 7.6.

2.11 Sperm cAMP measurement

Intracellular cAMP levels were measured using a colorimetric immunoassay (Direct cAMP ELISA kit, ADI-900-066, Enzo). Isolated caudal sperm were washed in PBS and centrifuged for 10 minutes at 750xg. Equal count sperm pellets were resuspended in 0.1M HCl for lysis, and lysates were processed according to manufacturers protocol. Absorbance was measured at 405 nm using a plate reader photospectrometer.

2.12 Sperm acrosome reaction assessment

Caudal epididymal sperm was squeezed out the vas deferens of three sacrificed mice into 1ml of HTF media pre-equilibrated in a CO$_2$ incubator overnight. Sperm were allowed to capacitate for 90 minutes in the presence of 5% CO$_2$ at 37°C before adding 10μM of Ionomycin to induce acrosome reaction. An equal volume of DMSO was added to the negative control tube. Following a 10-minute incubation period, sperm cells were fixed in 4% PFA for 20 minutes at
4°C, spread on a poly-L-lysine coated slide, and stained with Commassie blue G-250 for 10 minutes.Slides were then washed, mounted and observed with a 60X lens of an Olympus IX81 microscope. Acrosome reacted sperm appeared without a dark blue cap at the anterior of the spermatozoans head. The percentage of acrosome reacted sperm was obtained by counting a total of 100 cells per animal from random microscopic fields.

2.13 Filamentous actin staining

Freshly extracted caudal sperm were allowed to capacitate as described in the previous section. Acrosome reaction was induced by 10µM of Ionomycin incubated for 10 minutes. Both capacitated and acrosome reacted sperm were fixed in 4% PFA for 20 minutes at 4°C, spread on poly-L-lysine coated slides, washed with PBS then stained with Rhodamine-Phalloidin, 3µM in PBS (R415, Thermo Fisher Scientific) for one hour at room temperature. Slides were then washed with PBS and mounted with anti-fade medium (Prolong Live Antifade Reagent, P36975 Thermo Fisher Scientific), then examined using a 60X lens of a fluorescence Microscope (IX81, Olympus).

2.14 Sperm extract preparation

Counted sperm were pelleted down by centrifuging at 700xg for 20 min at 4°C. The PBS supernatant was carefully removed. The protein from pelleted sperm was extracted in the following ways according to each experimental requirement:

1) Whole sperm extract: The sperm pellet was resuspended in 1% SDS in water, heated to 95°C in a dry bath for 6 min then centrifuged at 16000xg at room temperature for 20 min. The supernatant was collected and boiled with Laemmli buffer (6%SDS, 25mM Tris-HCl pH 6.5,
50mM 31 DTT, 10% glycerol and Bromphenol blue) ready to be loaded for western blot analysis.

2) RIPA sperm extract: Following sperm counting, sperm pellets were resuspended in a volume of RIPA buffer (EMD, Millipore). The sperm suspension was incubated on ice for 30 minutes with repeated pipetting every 10 minutes. The suspension was then centrifuged at 16,000xg for 20 min. at 4°C. The supernatants were boiled with Laemmli buffer for 6 minutes then stored at -20°C. This portion was labeled (RIPA supernatant fraction). The pellet remained after centrifugation of the sonicated sperm suspension was resuspended in an equal volume of 1% SDS in water, heated to 95°C in a dry bath for 6 min then centrifuged at 16,000xg at room temperature for 20 min. The supernatant was collected and boiled with Laemmli buffer. This portion was labeled (RIPA pellet fraction)

2.15 Testis protein extract preparation

The mouse testes were isolated, weighed and placed into a test tube with 1ml of homogenization buffer (HB+) per mg of testis tissue then homogenized using a tissue tearor homogenizer on ice at level 4 for three 10 second bursts. The suspension was then centrifuged at 16,000xg for 20 min. at 4°C. The supernatants were collected and boiled with Laemmli buffer then stored at -20°C.

2.16 Protein estimation

Protein concentration in tissue extracts prepared in HB+ were estimated using DC protein assay kit II from Bio-Rad. Simultaneous to tissue extract preparation, bovine serum albumin (BSA) standards for protein estimation were prepared by dissolving 100mg of BSA in 1ml of HB+ and then serially diluting down to a concentration of 8mg/ml. BSA from the standards and
proteins from extracts were precipitated with equal volumes of 20% trichloroacetic acid (TCA) overnight. They were then centrifuged at 12,000xg for 15 min at 4°C and the protein pellets were air dried to be dissolved in 0.1 N NaOH. The BSA standards were serially diluted with 0.1 N NaOH to obtain a concentration ranging from 8 mg/ml to 0.0625 mg/ml. Reagent A’ was prepared by mixing 1ml of reagent A with 20μl of reagent S. 5μl of extract (unknown) and BSA standards (known) were loaded in triplicates for each sample in a 90 well plate and mixed with 25μl reagent A’ and 200μl reagent B. the samples were incubated at room temperature for 15 min with gentle rocking and their OD was measured using a spectrophotometer at 630nm. The OD of unknown was then converted to protein concentration in terms of mg/ml by plotting a standard graph with the standards.

2.17 Histology and immunohistochemistry

Testes were removed from sacrificed mice and immediately placed in bouin’s fixative solution for 12-16 hours. Fixed testes were then dehydrated by washing in a graded series of increasing ethanol concentrations: 70% ethanol for one hour, 95% ethanol for one hour, 80% ethanol for one hour, and 100% ethanol for 40 minutes twice. Then permeabilized in CitriSolv (Fisher Scientific) for one hour. Tissues were then waxed in warm paraffin for one hour (Tissue processing was performed using Shandon Citadel 2000 Tissue Processor, Thermo Fisher Scientific). Tissue were then removed from processor and embedded in paraffin wax warmed up to 56°C inside a plastic mold and allowed to solidify on a cold plate cover of a Shandon Histocentre 2 Embedding Center (Thermo Fisher Scientific). Then tissues were sliced into 5μm thick sections using a microtome (Leica Microsystems Inc.) and transferred to poly-L-Lysine coated slides. Testis sections were then de-paraffinized by sequential washing in Xylene for 5
minutes 2x then rehydrated by washing in a graded series of decreasing ethanol concentrations: 100% ethanol for 5 minutes 2x, 95% ethanol for 5 minutes 2x, 80% ethanol for 5 minutes 2x, 75% for 5 minutes 2x, and finally ddH₂O for 5 minutes 2x.

**Histology**

De-paraffinized, rehydrated sections were stained with PAS (Periodic Acid and Schiff, Leica Biosystems 38016SS4) following the manufacturers protocol. Stained sections were covered with a drop of Xylene based Histochoice Mounting Media (H157-475ML, amresco) followed by a coverslip and viewed using a 60X magnification lens (IX53, Olympus).

**Immunohistochemistry**

De-paraffinized, rehydrated sections were boiled in citrate buffer (10mM Citric acid, 0.05% Tween20, pH 6.0) for antigen retrieval for 1 minute followed by a resting time of 2 minutes then boiled once again for 1 minute. Slides were allowed to cool down while in citrate buffer for 30 minutes at room temperature. Then washed in ddH₂O for 5 minutes. Sections were blocked by incubation in a blocking solution (5% goat serum, 5% BSA in 1x PBS) for 1hr at room temperature in a humidified chamber. Sections were then incubated with primary antibodies at 1:20 dilutions (in blocking solution) at 4°C overnight in a humidified chamber followed by three 5 minute-washes in 1x PBS. Negative control sections were incubated in blocking solution instead of primary antibody. Sections were then incubated with goat anti-rabbit Cy3-conjugated secondary antibody (Jackson Immunoresearch) for 1hr at room temperature in a dark humidified chamber. Finally sections were washed three times for 5 min each in 1X PBS, counterstained with Hoechst or DAPI in 1X PBS for 10 minutes, washed a final wash in 1X PBS and mounted with anti-fade mounting media (Prolong Live Antifade Reagent, P36975 Thermo Fisher Scientific), then examined using a fluorescence Microscope (IX81, Olympus).
2.18 Staging of testis seminiferous tubules

PAS stained cross sections of fixed testis were imaged and subjected to staging analysis using the method described by Meistrich and Hess (2013). Sub-stages of germ cells were identified based on cell size, nuclei shape, and intensity of staining [64].

**Figure 14a. Diagram showing testicular germ cells at different developmental stages of the seminiferous epithelium.**
Figure 14b. A schematic guide for identification of seminiferous epithelium stages.
A binary key descision method for staging of seminiferous tubules derived from Meistrich & Hess (2013), License Clearance ID: 4067930740973.

2.19 Sperm Immunoprecipitation

200μl of sperm extract prepared in RIPA buffer were incubated with approximately 2-6μg of primary antibody. Another 200μl of sperm extract was incubated with diluted pre-immune serum of the antibody as a negative control. The tubes were kept on a rotator at 4°C overnight. The following day, Protein G-Sepharose 4 Fast Flow beads (GE Healthcare, Piscataway, NJ, USA) 30μl per tube, were washed with either RIPA twice. The extracts were then added to the beads and incubated on a rotator at 4°C for 2-4 hrs. The beads were centrifuged at 1000xg for 1 min at 4°C and the supernatant was collected and labeled as flow through (FT). The beads were then washed with TTBS four times and once with RIPA. The washed beads were then resuspended in 50μl of 1X Laemmli buffer, boiled for 6 minutes, centrifuged 16,000xg for 5 minutes, the supernatant was labeled Bound and later analyzed by western blot.

2.20 Western blot analysis

Protein samples were boiled in Laemmli sample buffer and separated by 12% SDS-PAGE (Mini-Protean II system, Bio-Rad Laboratories, Hercules, CA), then electrophoretically transferred to Immobilon-P, PVDF membranes (Millipore Corp., Billerica, MA, USA). After blocking non-specific binding sites with 5% nonfat dry milk in TTBS the blots were incubated overnight with primary antibody at 4°C. The primary antibodies used in this study are shown in table 2.1. After washing with TTBS the blots were incubated with the appropriate secondary antibody (Amersham, Piscataway, NJ) conjugated to horseradish peroxidase at 1:5000 dilution for 1 hr at room temperature. Blots were then washed with TTBS twice 5 min each. Blots were then developed with a homemade ECL chemiluminescence kit and imaged using a Fujifilm Darkbox LAS-3000 (Fuji).
2.21 Immunocytochemistry

Mouse sperm were isolated and washed with PBS by centrifugation at 700xg at 4°C for 10 min. The sperm pellet was resuspended in a calculated volume of 4% paraformaldehyde in PBS to reach an approximate concentration of 2x10^6 cells/ml and incubated at 4°C for 20 minutes. Following the fixation period, and equal volume of 0.2% Triton-X was added to the fixed sperm. 20-40μl of the sperm suspension was overlaid onto a poly-L-lysine (Sigma-Aldrich) coated slide and left to sit for 20 minutes at room temperature. Excess paraformaldehyde was rinsed off with PBS then the sperm were blocked with a solution of 5% goat serum in PBS for one hour at room temperature. The slides were overlaid with a solution containing the primary antibody of interest at a concentration of 1:200 to 1:100 in blocking buffer (5% goat serum in PBS) and incubated overnight at 4°C. The following day, the slides were washed in TTBS twice for 10 min each then incubated with Cy3-conjugated secondary antibody (111-165-144, Jackson ImmunoResearch) (1:200 in blocking buffer) for 1hr at room temperature in a dark chamber. Finally the slides were washed in TTBS three times for 10 min each, mounted with ProLong Diamond Antifade Mountant (ThermoFisher Scientific) and observed using an IX80 fluorescence microscope (Olympus, Melville, NY, USA).

Table 3. List of Primary antibodies used for Western blot and immunocytochemistry

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Epitope</th>
<th>Host</th>
<th>Company</th>
<th>Conc.</th>
<th>Mol Wt</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-Ppγ2 (Rabbit 2 glycine), polyclonal</td>
<td>(C)SGLNPSIQKASNYRNNTVLYE&lt;sub&gt;COOH&lt;/sub&gt;</td>
<td>Rabbit</td>
<td>Yenzym</td>
<td>WB 1:5000</td>
<td>39kDa</td>
</tr>
<tr>
<td>Anti-Ppγ1 (K9 bleed 3), polyclonal</td>
<td>CTPPRGMITKQAKK&lt;sub&gt;COOH&lt;/sub&gt;</td>
<td>Rabbit</td>
<td>Yenzym</td>
<td>WB 1:4000</td>
<td>37kDa</td>
</tr>
<tr>
<td>Antibody Name</td>
<td>Description</td>
<td>Species</td>
<td>Supplier</td>
<td>Stain</td>
<td>Dilution</td>
</tr>
<tr>
<td>---------------------------------------------------</td>
<td>------------------------------------------------------------------------------</td>
<td>---------</td>
<td>------------------</td>
<td>----------------</td>
<td>------------</td>
</tr>
<tr>
<td>Anti-PPP1A (phospho T320) antibody (EP1512Y)</td>
<td>Residues surrounding Thr320 of human PP1α</td>
<td>Rabbit</td>
<td>Abcam</td>
<td>WB 1:100</td>
<td>37kDa</td>
</tr>
<tr>
<td>PP1 Antibody (E-9): sc-7482, monoclonal</td>
<td>Full length human PP1 Catalytic domain</td>
<td>Mouse</td>
<td>Santa Cruz</td>
<td>WB 1:1000</td>
<td>37kDa</td>
</tr>
<tr>
<td>Anti-SDS22 (peptide 2)</td>
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<td>Rabbit</td>
<td>Yenzym</td>
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<tr>
<td>Anti-I2 (C-terminus)</td>
<td>STTSDHLQHKSQSS_{COOH}</td>
<td>Rabbit</td>
<td>Yenzym</td>
<td>WB 1:2000</td>
<td>32kDa</td>
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<tr>
<td>Anti-I3</td>
<td>N-terminus</td>
<td>Rabbit</td>
<td>Yenzym</td>
<td>WB 1:2000</td>
<td>28kDa</td>
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<tr>
<td>Anti-TMEM225, polyclonal</td>
<td></td>
<td>Rabbit</td>
<td>Yenzym</td>
<td>WB 1:100</td>
<td>29kDa</td>
</tr>
<tr>
<td>Methyl-PP2A SC81063</td>
<td>302-309 of recombinant human PP2A</td>
<td>Mouse</td>
<td>Santa Cruz</td>
<td>WB 1:1000</td>
<td>36kDa</td>
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<tr>
<td>Anti-HK1 C35C4</td>
<td>Human Hexokinase I</td>
<td>Rabbit</td>
<td>Cell signalling</td>
<td>WB 1:2000</td>
<td>102kDa</td>
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<td>4G10</td>
<td></td>
<td>Mouse</td>
<td></td>
<td>WB 1:1000</td>
<td>N/A</td>
</tr>
<tr>
<td>Anti-Phospho-PKA substrates 100G7E</td>
<td>Synthetic Phospho-PKA substrate peptides</td>
<td>Rabbit</td>
<td>Cell signalling</td>
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<tr>
<td>Anti-β-Tubulin, polyclonal (ab6046)</td>
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<td>Rabbit</td>
<td>Abcam</td>
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<td>50kDa</td>
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<tr>
<td>Anti-PPP3R2, Polyclonal (14005-1-AP)</td>
<td>Human PPP3R2 full length – gst fusion protein</td>
<td>Rabbit</td>
<td>Proteintech</td>
<td>WB 1:1000</td>
<td>20kDa</td>
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<td>Anti-EGFP, #2956</td>
<td>N-terminus</td>
<td>Rabbit</td>
<td>Cell Signaling</td>
<td>WB 1:1000</td>
<td>30kDa</td>
</tr>
<tr>
<td>Anti-GSK3α, Monoclonal (D80E6)</td>
<td>Full length human GSK3α</td>
<td>Rabbit</td>
<td>Cell Signaling</td>
<td>WB 1:1000</td>
<td>55kDa</td>
</tr>
<tr>
<td>Anti-Phospho-GSK3α, mAb. (46H12)</td>
<td>Synthetic phosphopeptide corresponding to residues surrounding Ser21 of human GSK-3alpha.</td>
<td>Mouse</td>
<td>Cell Signaling</td>
<td>WB 1:1000</td>
<td>55kDa</td>
</tr>
<tr>
<td>Anti-Phospho-GSK-3α/β #9331</td>
<td>Ser21/9</td>
<td>Rabbit</td>
<td>Cell Signaling</td>
<td>WB 1:2000</td>
<td>55 and 47kDa</td>
</tr>
</tbody>
</table>
2.22 In Vitro Fertilization and Egg-Sperm Binding

Sperm was collected from two cauda epididymis and two vas deferens of 3–5 month old male mice in a 300μl drop of HTF media covered with mineral oil in a 35-mm dish. The caudal tissue was incised with the edge of a 30 G injection needle to allow the sperm to swim out and disperse for 10 min at 37°C under 5% CO₂ in a humidified incubator. To obtain mature eggs, 21–35 day old female mice were super ovulated by intraperitoneal injection (IP) of 5 IU of Gonadotropin from Pregnant Mare Serum (PMSG; Sigma). Forty-six to fifty hours after PMSG injection females were injected with 5 IU of Human Chorionic Gonadotropin (HCG; Sigma) intraperitoneally. Thirteen to fifteen hours following HCG administration, the female mice were euthanized with CO₂; their oviducts were aseptically removed and dissected free from surrounding tissues and placed in a culture dish containing 2ml of PBS medium warmed at 37°C. The cumulus oocytes complexes were removed from the ampullae using micro dissecting forceps. 15μl of the sperm suspension was added to a 250μl drop of HTF covered with mineral oil. The isolated cumulus masses were then transferred to the drop of HTF containing the sperm and incubated at 37°C in humidified atmosphere of 5% CO₂ in air for 4 hours. The eggs were then washed to eliminate excess sperm and cultured overnight in a 250μl drop of HTF medium 37°C in humidified atmosphere of 5% CO₂ in air. The following day the number of two-cell embryos was counted. The extent of fertilization was determined by the percentage of two cell stage embryos scored 24 h after insemination. For the egg-sperm binding assay following the four-hour incubation period, the eggs were moved into a fresh HTF drop, placed on ice to immobilize any sperm before capturing images using and inverted IX81 Olympus microscope. For IVF
2.23 Cloning, expression, and purification of recombinant TMEM225

2.23.1 Cloning

Total RNA was isolated from adult male testes as described in section 2.12 (Total RNA isolation). cDNA was prepared as described in section 2.13 (Reverse transcription PCR). The coding sequence of TMEM225 was amplified from testis cDNA using the forward primer: 5’-CTCTCGAGACAATGATGCACATCCCCAAAC-3’ (XhoI site introduced and shown in bold letters), and the reverse primer: 5’-GGAAGCTTCAGAGCCCAGGTTACCCGAC-3’ (HindIII site introduced and shown in bold letters). The PCR product was purified using QIAquick PCR Purification Kit (28104, QIAGEN) prior to digestion. The amplified and purified sequence was digested with XhoI (FD0694, FastDigest, ThermoFisher Scientific) and HindIII (FD0504, ThermoFisher Scientific) restriction enzymes following the manufacturers protocol. A “pRSET A” bacterial expression vector (V35120, Invitrogen) was digested with the same restriction enzymes in the presence of FastAP alkaline phosphatase (EF0654, ThermoFisher Scientific). The amplified, digested TMEM225 sequence and plasmid were run through a 1% agarose gel and the correct bands with the expected base pair sizes (TMEM225: 700 bp, pRSET plasmid: 4000bp) were excised and subjected to gel purification using QIAquick gel extraction kit (28704, QIAGEN). Ligation of TMEM225 cDNA into the vector backbone was performed using ANZA T4 DNA Ligase Master Mix (IVGN2104, Invitrogen). Ligated plasmid was stored in -80°C until bacterial transformation was performed.

2.23.2 DH-alpha cell Transformation

Deep frozen DH-alpha cell aliquots were removed from the -80°C freezer and allowed to thaw on ice for 20 minutes. Transformation was performed by adding 50µl of the ligated product
to the cells and allowed to incubate for 20 minutes on ice before heat shocking in a 42°C dry bath for 90 seconds followed by 10 minutes on ice. Transformed cells were cultured in 1 ml of SOC (20g/L Tryptone, 5g/L yeast extract, 0.5g/L NaCl, 0.186g/L KCl, 2.4g/L MgSO$_4$, 1% glucose w/v, pH 7.0) for 2 hours at 37°C with agitation of 250rpm. Following the two-hour incubation, the SOC was centrifuged at 12,000rcf for 1 min. the majority of the supernatant was discarded, and the pellet was resuspended in approximately 300µl of the remaining supernatant which was used to inoculate LB + ampicillin (75µg/ml) agar dishes. The dishes were incubated 14-16 hours at 37°C. The following day, individual colonies were chosen to inoculate tubes with 10 ml LB (10g/L Tryptone, 10g/L NaCl, 5g/L yeast extract, pH 7.0, ampicillin 75µg/ml). The inoculated LB was incubated overnight at 37°C with agitation 250rpm. The following day, 100µl of the culture was removed for DNA extraction and PCR analysis to identify the tubes with the transgene positive clones. Three clones were identified as positives from which 1ml was used to prepare glycerol stocks that were immediately stored in -80°C, and the remainder volume was used for plasmid purification using QIAprep Spin Miniprep Kit (27104, QIAGEN). The purified plasmid was stored in -80°C until transformation of BL21 cells was performed.

2.23.3 BL21 cell Transformation

Three tubes (one tube for each positive clone) of deep frozen BL21 cell aliquots were removed from the -80°C freezer and allowed to thaw on ice for 20 minutes. Transformation was performed by adding 1µg of the previously purified plasmid to the cells and allowed to incubate for 30 minutes on ice before heat shocking in a 42°C dry bath for 30 seconds followed by 10 minutes on ice. Transformed cells were cultured in 1 ml of SOC at 37°C with agitation for 2 hours. Following the two-hour incubation, the SOC was centrifuged at 12,000rcf for 1 min. The
majority of the supernatant was discarded, and the pellet was resuspended in approximately 300µl of the remaining supernatant, which was used to inoculate LB + ampicillin agar dishes. The dishes were incubated 14-16 hours at 37°C. The following day, individual colonies were chosen to inoculate tubes with 2 ml of LB + ampicillin. The 2ml cultures were incubated for 6 hours at 37°C with agitation 250rpm. Following the incubation period 750µl of each culture tube was added to 250µl of autoclaved glycerol and frozen immediately in a -80°C freezer for storage. The remaining culture was used to start a lager overnight 10 ml culture. The overnight 10 ml culture was used the following day to start a large-scale culture of 1 liter/clone. Large-scale cultures were incubated at 37°C with agitation for 2.5 hours until the OD of the LB measured using a spectrophotometer (600nm wavelength) reached 1.0. Induction was performed using a final concentration of 0.75M IPTG overnight at room temperature with agitation 250rpm. The following day cultures were centrifuged at 4000rpm for 15 minutes. The supernatant was discarded and the pellet was frozen in -200°C until cell lysis and protein purification was performed. One ml of each culture was removed before and after induction for analysis with SDS-PAGE and western blot for the presence of TMEM225.

2.23.4 TMEM225 extraction and purification

The cell pellet from the highest expressing clone was resuspended in 5ml of TBS (50mM Tris-Cl, 150mM NaCl, pH 8.0) containing protease inhibitors (10 mM Tris [pH 7.2], 1 mM EDTA, 1 mM EGTA, 10mM benzamidine-HCl, 1 mM PMSF, 0.1 mM TPCK, 0.1% (V/V) β-mercaptoethanol and 1 mM sodium orthovanadate) and sonicated on ice 10 second bursts, ten times with 20 second pauses. Following sonication the cell lysate was centrifuged for 20 minutes at 16,000rcf at 4°C. The supernatant was removed and subjected to immunoprecipitation with Nickel beads and FPLC fraction separation.
2.23.5 Nickel bead pulldown of His-tagged TMEM225

Nickel beads (88221, Thermo Fisher Scientific) were washed with TBS pH 8.0 then placed in a 10ml chromatography column (Econo-Pac Chromatography columns, 7321010, BIORAD) One ml of beads were used per 100ml of BL21 lysate. BL21 cell lysates were applied to the column and allowed to flow by gravity. Bound proteins were eluted using different concentrations of Imidazole: 100mM, 200mM, and 450mM. The three separate fractions were later subjected to western blot analysis.

2.23.6 Exchange chromatography

Anion exchange chromatography: BL21 cell lysate in TBS buffer pH 8.0 were passed through a HiTrap Q HP column (17-1153-01, GE Healthcare Life Sciences) using an FPLC system. Elution of fractions was performed using increasing concentrations of NaCl solutions: 50mM, 175mM, 300mM, 425mM, 625mM, and 1M sequentially.

Cation exchange chromatography: Prior to fraction separation, the input protein solution (in TBS pH 8.0) was exposed to dialysis for buffer exchange to a 50mM MES (M3671-250G, Sigma) buffer pH 5.8. The input was passed through a HiTrap SP HP column (17115101, GE Healthcare Life Sciences) using an FPLC system. Elution of fractions was performed using increasing concentrations of NaCl solutions: 125mM, 400mM, and 1M sequentially.

2.24 Testis immunoprecipitation with recombinant His-TMEM225.

One ml of testis lysate (100mg of testis tissue) was incubated with approximately 10ng of His-TMEM225 recombinant protein + 5µg of anti PP1γ2 antibody overnight at 4°C on a rotator. The following day, 1500µg of washed Protein G beads (10003D, ThermoFisher Scientific) were
incubated with the lysate mixture for 2 hours rotating at room temperature. The bound fraction was eluted by boiling the beads in 2x Laemmli sample buffer. Diluted pre-immune rabbit serum was used in exchange of PP1γ2 antibody for the negative control.

2.25 Transfection of LNCaP cells

LNCaP cells were cultured in RMPI-1640 media with serum and antibiotics (R8758 Sigma Aldrich) for 48 hours until 90% confluent in six well culture plates (9.5 cm²/well). Lipofectamine 2000 (Thermo Fisher Scientific) was diluted in serum free RMPI-1640 media (6µl Lipofectamine + 200µl media/well) and incubated for 5 minutes at room temperature. Four dilutions of plasmid DNA were prepared in serum free RMPI-1640 media: 1µg plasmid + 200µl media/well, 2µg plasmid + 200µl media/well, 5µg plasmid + 200µl/well, and 0µg plasmid + 200µl media/well (for the negative control). Diluted Lipofectamine and diluted plasmid were mixed together 1:1 and incubated for 20 minutes at room temperature. LNCaP cells were washed with warm PBS. 200µl of serum free, antibiotic free RMPI-1640 media was added to each well followed by 600µl of the Lipofectamine-plasmid mixture. The cells were cultured in a humidified CO₂ incubator at 37°C for five hours. Following the incubation period, Lipofectamine containing medium was washed off and replaced with RPMI-1640 media supplemented with serum and antibiotics. LNCaP cells were cultured for 48 hours then visualized using a fluorescent microscope (488nm excitation filter, Olympus IX81).
2.26 Total RNA isolation

100mg of fresh isolated mouse tissue was homogenized in 1ml cold Tri reagent (Sigma-Aldrich, St. Louis, MO, USA) 200μl of chloroform was added to the tissue homogenate and incubated for 15 minutes on ice. Followed by centrifugation at 12,000xg at 4°C for 15 minutes. The RNA enriched top layer was collected and 500μl of isopropanol was added before incubation at room temperature for 10 minutes prior to centrifugation at 10,000xg for 10 min at 4°C. The supernatant was collected and added to 1ml of 75% ethanol and vortexed gently. The mixture was centrifuged at 7,500xg for 7 minutes at 4°C and the supernatant was discarded. The pellet was semi dried and resuspended in approximately 50μl of Nuclease-Free Water (Promega P119C, Madison, WI). Finally the RNA concentration was measured using a Nanodrop spectrophotometer (ND-1000; Nanodrop technologies).

2.27 Reverse Transcription PCR

Extracted RNA concentration was adjusted to 200 ng/μl using Nuclease-Free Water (Promega P119C, Madison, WI). 4μl of RNA was used for each PCR reaction tube. QuantiTect Reverse Transcription kit was used (QIAGEN 205311) and the manufacturers protocol was followed. The genomic DNA was eliminated by incubating 800ng of RNA with RNase free DNase for 2 min at 42⁰C. It was then mixed with reverse transcriptase and random hexamers, and incubated at 42⁰C for 15 min. Finally the reverse transcriptase was inactivated by incubating the mixture at 95⁰C for 3 min. The cDNA concentration was measured and its purity was estimated by its 260/280 nm absorbance ratio using a Nanodrop spectrophotometer (ND-1000; Nanodrop technologies). 2μl of the cDNA was used for each PCR reaction tube.
2.28 Detection of the transgene (Endogenous PPP1CC promoter – EGFP - Pp1γ2 Cterminus tail) mRNA

Following the preparation of cDNA from various tissues, the transgene cDNA was amplified using the following primers: GFP-For 5’-CTAGGATCCATGGTGAGCAAG-3’ and GFP-Rev 5’-CTAGAATTCCTTGTACAGCTCGTC-3’ that detects a 710 bp band on a 1% agarose gel. The amplification of GAPDH cDNA was used as a control. The following primers were used: For: 5’-ACCACAGTCCATGCCATCAC-3’ Rev: 5’-TCCACCACCTGTTGCTGTA-3’.

2.29 EGFP fluorescence detection in testis tissue sections

Fresh testes were removed from euthanized mice, washed in PBS, coated and submerged in OCT (Optimum Cutting Temperature, Andwin Scientific 4583) then snap frozen in dry ice. Frozen testis were cut using a cryostat into 5µm thick sections and mounted on poly-L-lysine coated slides. Fixation was performed by exposing the mounted tissue to 3.7% formaldehyde vapor in a sealed container overnight at -20°C. Following fixation, slides were washed in PBS for 5 minutes, mounted with anti-fade mounting medium (Prolong Live Antifade Reagent, P36975 Thermo Fisher Scientific) and viewed using a fluorescent microscope (488nm excitation filter, Olympus IX81).

2.30 GFP pulldown

Tissue lysates were prepared in HB+ (supplemented with protease inhibitors) by sonication as described earlier. Before performing the pulldown 50µl of the tissue lysate
supernatant was separated, boiled with sample buffer and labeled as “Input”. Then one ml of the supernatant was incubated with 50µl of prewashed GFP-Trap_A beads (gta-10, chromotek) for one hour on a rotator at 4°C. Following incubation, the bead lysate suspension was centrifuged for 2 minutes at 5000rcf at 4°C. The supernatant was removed and boiled with Laemmli sample buffer and labeled as “Flow through”. The beads were then washed 3x with HB+ supplemented with 300mM of NaCl. Then boiled with 50µl 2x Sample buffer for 10 minutes at 95°C. Followed by a final centrifugation for 2 minutes at 5000rcf. The supernatant was collected and labeled as “GFP bound”. All fractions were then analyzed by western blot.

2.31 Measurement of total intracellular calcium

Isolated caput and caudal bovine sperm was washed three times in PBS and centrifuged at 4°C for 10 minutes at 750xg. Sperm pellets were resuspended in a calculated volume of 7% v/v TCA (trichloroacetic acid) to get a final sperm concentration of 10^8 sperm cells/µl. Sperm suspensions were sonicated at amplitude of 60% for 10 seconds twice while on ice. Following sonication the samples were centrifuged at 10,000xg for 15 minutes at 4°C. The supernatant was collected and diluted with RO filtered water 100 times. Standards were prepared by dissolving calcium chloride in 0.7% v/v TCA in RO filtered water. All samples and standards calcium levels were measured using Flame Photometry (Optima 8x00 ICP-OES, PerkinElmer, MA, USA). Calcium levels were reported as ng/10^8 sperm cells.

2.32 Statistical Analysis

All statistical analyses were performed by either student t-test (unpaired) or one-way ANOVA using Prism 6 Software. In all cases, significance was considered when p<0.05.
Chapter III

RESULTS

3.1 Aim I: To test the hypothesis that Pp1γ1 can replace the function of Pp1γ2 in testis and sperm.

Background

Protein Phosphatase 1 exists in mammals as four isoforms transcribed from three different genes: Ppplca encodes PP1α, Ppplcb encodes PP1β, and Ppplcc encode both PP1γ1 and PP1γ2 by alternative splicing of the primary mRNA. All PP1 isoforms share a high sequence homology in their catalytic domains. The differences that give rise to various isoforms lie in the extreme N- and C-termini of the proteins. In general, enzymes function in a multimeric complex associated with several regulatory subunits that target them to specific cellular compartments allowing them to be involved in multiple signaling pathways simultaneously. In vitro all PP1 isoforms are capable of dephosphorylating the same substrates. However, in vivo different isoforms serve different roles depending on their spatiotemporal expression. The three PP1 isoforms: PP1α, PP1β, and PP1γ1 are expressed in nearly all somatic cells. However, PP1γ2 is testis specific and restricted to post-meiotic germ cell. The loss of the Ppplcc gene encoding both PP1γ1 and PP1γ2, does not affect the function of somatic tissues where PP1γ1 is expressed. Indicating that PP1γ1 is replaceable by other PP1 isoforms. However, The loss of Ppplcc,
affects the male testis leading to male infertility due to spermatogenic arrest at the spermatid stage [48]. All PP1 isoforms PP1α, PP1β, PP1γ1, and PP1γ2 are expressed in the male testis. However their expression is restricted to somatic cells and pre-mieiotic germ cells. PP1γ2 expression in the testis begins at the post-mieiotic stage of developing germ cells and continues to be expressed in mature spermatozoa. Transgenic PP1γ2 expression using the testis specific promoter Pgk-2 on a Ppp1cc null background was able to rescue spermatogenesis and male fertility indicating that the PP1γ1 role in testis somatic cells and pre-mieotic germ cells is replaceable with other PP1 isoforms, similar to what occurs in somatic cells.

One can argue that the loss of PP1γ2 in the testis leads to a deleterious phenotype because PP1γ2 is the only PP1 isoform expressed at that specific developmental stage (post meiotic) in those specific cells (developing male germ cells) unlike somatic tissues and pre-mieiotic germ cells, where all three PP1 genes are usually expressed simultaneously in a single cell. But the question remains, why do germ cells in the testis shift from expressing all three PP1 isoforms: PP1α, PP1β, and PP1γ1, to the expression of a single unique isoform PP1γ2 after the onset of meiosis? Meiosis in the testis is associated with a wide change in the transcription pattern of many genes, silencing some, and up-regulating others, which is not surprising taking into account the uniqueness of the male gamete in both its structure and its mission.

PP1γ2 is generated by alternative splicing of the primary PP1γ mRNA, Giving rise to a PP1 isoform with an extra 22-amino acid long C-terminus tail. This splicing event that starts at the secondary spermatocyte stage, is also accompanied by a dramatic increase in the levels of both PP1γ2 transcript and protein. Both the reason for the requirement of high levels of PP1γ2 and the function of its unique C-terminus tail are unknown. Studies performed using Ppp1cc null males show that the knockout male testes are devoid of mature spermatozoa in their seminiferous
tubule lumens. It has been believed that PP1γ2 has a testis specific role supporting spermatogenesis beyond the meiotic phase.

**Figure 15. Schematic of the generation of the two PP1γ isoforms.**
The *Ppplce* pre-mRNA has 8 exons and 7 introns. The Pp1γ1 mature mRNA is generated by splicing of introns 1 through 6. Intron 7 is retained in the mature transcript along with exon 8 as a part of its 3’ UTR. It encodes a protein containing 303 amino acids derived from the seven exons and 8 amino acids from the extended exon 7 (i.e. initial part of intron 7). In the male postmeiotic germ cells the introns 1-6 along with the 1.1kb long intron 7 are spliced out. Thus, producing a shorter Pp1γ2 transcript of approximately 1.7kb. The exon 8 gives Pp1γ2 its unique 22 amino acid C-terminus. Pp1γ1 and Pp1γ2 proteins are identical in structure and their catalytic domain except for the extra C-terminus tail in Pp1γ2.
The transgenic expression of PP1γ2 alone-without its somatic isoform PP1γ1-in the testes of the null males rescues spermatogenesis and male fertility in a dose dependent manner. The protein expression level threshold for the complete rescue of fertility is 75% of the PP1 protein levels expressed in Ppp1cc heterozygous males. Transgenic mice expressing levels of PP1γ2 less than 75% are infertile and show a partial rescue of spermatogenesis. They have low testis weights, reduced sperm number, severe sperm morphological abnormalities, and reduced sperm motility: both total and progressive. We can conclude two major findings from these studies: 1) The presence of PP1γ1 in the testis somatic cells and pre-meiotic germ cells is replaceable with other PP1 isoforms. 2) The levels of PP1γ2 in the testis positively correlate with normal sperm parameters hence, fertility.

The goal of this aim is to determine whether PP1γ1-when expressed in levels equal to or above the 75% threshold-is capable of replacing the role of PP1γ2 in post-meiotic germ cells and rescuing male fertility in Ppp1cc null mice. Through this aim we will determine whether the C-terminus tail unique to PP1γ2 is necessary for the enzymes role in spermatogenesis and sperm function.

After a number of failed attempts to express high levels of transgenic PP1γ1 in the testis using different construct designs, we reached a conclusion that the presence of intron 7 (the intron spliced out of PP1γ2 mRNA, yet retained in PP1γ1 mRNA as part of its 5’UTR) may lead to the instability of the transcribed transgenes. To support this hypothesis, bioinformatics analysis was performed on the sequence of intron 7. The analysis revealed multiple miRNA binding sites. Two specific miRNA sites were shown to be conserved among all mammals: miR-449 and miR-34. Interestingly both these miRNA’s have been reported to be expressed in testis at high levels in post-meiotic germ cells. Thus we designed a novel construct that resembles the
mRNA of PP1γ2, meaning that, the region normally spliced out of the PP1γ2 message: “Intron 7”, was removed from the transgenes 3’UTR. The final construct was designed and has successfully expressed levels of PP1γ1 similar to the levels of PP1γ2 in wild type mice testes. Analysis of these mice is the main subject of Aim I. (All constructs were designed by Nilam Sinha and initial rescue mice were analyzed by Tejasvi Dudiki)

Figure 16. Schematic of the generation of the PP1γ1 rescue transgene constructs. Rescues I to III constructs contain the entire sequence of intron 7 as part of their 3’UTR. The transgenic expression of PP1γ1 in the testes of the generated mice was very low. The Final Rescue construct lacks intron 7 (the region spliced out from PP1γ2 mRNA) resembling the PP1γ2 message. The region between the arrows is the extended exon 7 sequence, specific to Pp1γ1 mRNA. It is driven by the Pgk2 promoter for testis specific expression. The transgenic mice produced by the final construct, express high levels of transgenic PP1γ1 in their testes almost near wild type levels of PP1γ2.
3.1.1 Transgenic PP1γ1 protein amounts in testis and sperm of rescue males are comparable with those of endogenous PP1γ2 in heterozygous control.

To determine the amount of transgenic PP1γ1 protein expressed in testes and sperm of the rescue males, western blot was performed using testis and sperm extracts along with known amounts of recombinant His-PP1. Band intensity of the recombinant PP1 was compared with band intensity of PP1 from heterozygous control, PP1γ2 rescue, and PP1γ1 rescue testis and sperm extracts. Nanogram levels of PP1 protein were estimated as 7.7ng of PP1 in Ppp1cc heterozygous testes, 6ng of PP1 in PP1γ2 rescue testes, and 8.2ng PP1 in PP1γ1 rescue testes. Levels of PP1 in sperm extracts follow a similar pattern (Figure 17).
B. **Testis Pp1γ Protein**

![Bar chart showing PP1γ (ng/10μg testis) levels for different genotypes: Ppp1cc (+/+), Ppp1cc (+/-), Pp1γ2, and Pp1γ1.](image)

C. **Western Blot Analysis**

- **His-Pp1γ1/2 (5ng)**
- **Ppp1cc (+/-)**
- **Ppp1cc (+/-), Pp1γ2**
- **Ppp1cc (+/-), Pp1γ1**

- **Pp1γ1**
- **Pp1γ2**
- **Tubulin**
Figure 17. Transgenic protein expression in PP1γ1 rescue testis and sperm.
A) Western blot analysis of PP1γ1 rescue testes. 10µg of testis extracts from Heterozygous control “Ppp1cc (+/-)”, PP1γ2 rescue “Ppp1cc (-/-), PP1γ2”, and PP1γ1 rescue “Ppp1cc (-/-), PP1γ1” along with 10 and 5ng of His-His1γ1 and His-His1γ2 were analyzed by western blot and probed with anti-PP1γ1 (blot 1) and anti-PP1γ2 antibodies (blot 2). The blots were re-probed with antibodies against β-Actin to confirm equal protein loading. The blots are representative of three different experiments. B) Quantification of PP1 levels expressed per 10µg of testis extract of control and rescue mice were obtained by intensity analysis of immunoreactive bands of western blot. C) Western blot analysis of PP1γ1 rescue sperm extracts. 10⁶ sperm from “Ppp1cc (+/-)”, “Ppp1cc (-/-), PP1γ2”, and “Ppp1cc (-/-), PP1γ1” along with 5ng of His-His1γ1 and His-His1γ2 were analyzed by western blot and probed with anti-PP1γ1 (blot 1) and anti-PP1γ2 antibodies (blot 2). The blots were re-probed with antibodies against β-Tubulin to confirm equal protein loading. The blots are representative of three different experiments. D) Quantification of PP1 levels expressed per 10⁶ sperm of control and rescue mice were obtained by intensity analysis of immunoreactive bands of western blot.
3.1.2 Transgenic expression of Pgk2-PP1γ1 within cross sections of seminiferous tubules matches the expression of endogenous PP1γ2 in heterozygous control.

The developmental expression of PP1γ2 mRNA and protein have previously been shown by northern blot and western blot, to begin in the testes of 13 to 15-day-old mice coinciding with the emergence of the first wave of secondary spermatocytes and continue in high levels throughout the life of the adult mouse [65]. PP1γ2 shows a distinct cellular localization in wild type mouse testes sections appearing in post meiotic germ cells only: secondary spermatocytes, round spermatids, elongated spermatids, and testicular spermatozoa [66]. To examine the expression pattern of transgenic PP1γ1 in the testes of rescue males, paraffin embedded testis sections from both PPp1cc heterozygous control “Ppplcc(+/-)” and PP1γ1 rescue mice “Ppplcc(-/-).PP1γ1(+/-)” were deparaffinized, rehydrated, and subjected to immunohistochemical staining using specific anti-rabbit primary antibodies against PP1γ2 or PP1γ1 followed by Cyanine3 conjugated secondary antibodies. Figure 18 clearly demonstrates the presence of PP1γ2 expression in spermatocytes, spermatids, and testicular spermatozoa of heterozygous males. No PP1γ2 staining could be detected in spermatogonia aligned along the peripheral membrane of the seminiferous tubules, pachytene spermatocytes, or in peritubular interstitial cells. Figure 19 shows cellular localization of PP1γ1 in the testes of rescue males comparable to the localization of PP1γ2 in testes of heterozygous control.
Figure 18. Immunohistochemistry showing the expression and localization of PPP1CC2 in Ppp1cc heterozygous testis.
A) PPP1CC2 staining appears in the center of seminiferous tubules, and absent from germ cells near the basal membrane. B) Seminiferous tubules from image “A” counter-stained with Hoechst. C) A merged image of A and B. D) PPP1CC2 staining appears in secondary spermatocytes and absent from pre-miotic spermatogonial cells. E) Seminiferous tubules from image “D” counter-stained with Hoechst. F) A merged image of D and E.
Figure 19. Immunohistochemistry showing the expression and localization of PPP1CC1 in PP1γ rescue testis.

A) PPP1CC1 staining appears in the center of seminiferous tubules, and absent from germ cells near the basal membrane similar to the expression and localization of PPP1CC2 in heterozygous control testis. B) Seminiferous tubules from image “A” counter-stained with Hoechst. C) A merged image of A and B. D) PPP1CC1 staining appears in secondary spermatocytes and absent from pre-meiotic spermatogonial cells. E) Seminiferous tubules from image “D” counter-stained with Hoechst. F) A merged image of D and E. G) Seminiferous tubules stained with secondary antibody alone. H) Seminiferous tubules from image “G” counter-stained with Hoechst. I) A merged image of G and H.
3.1.3 Transgenic PP1γ1 expression in Ppp1cc knockout testes rescues both spermatogenesis and spermiogenesis.

Cross sections of the mouse testis show different groups of germ cells in different seminiferous tubules. Each defined group of germ cells indicates a particular phase of development. These phases are referred to as stages. In the mouse there are 12 stages that together constitute the cycle of the seminiferous epithelium. In order to examine the progress of spermatogenesis in the testes of rescue males, paraffin embedded testis sections were subjected to histological analysis following PAS stain. Different stages were identified according to the method described by Hess et al. [64]. Figure 20 shows the presence of all developmental stages I-XII in seminiferous tubules of PP1γ1 rescue testes. Each stage was defined by the presence of certain germ cell populations.

Stage I-IV: contains two generations of spermatids: round and elongated, with the elongated spermatids not aligning in the middle of the seminiferous tubule lumen, Type A spermatogonia that appear at a small distance from the membrane of the seminiferous tubule and are characterized by pale staining with an undefined nucleus, Pachytene spermatocytes: Larger cells that appear closer to the tubule membrane with very light cytoplasmic staining and a dark large lobular granule-like nucleus.

Stage V-VI: similar to the earlier stages. However, these stages contain type B spermatogonia that appear as small cells tightly bound to the membrane of the seminiferous tubule.

Stage VII-VIII: are characterized by the presence of elongated spermatids aligning in the middle of the seminiferous tubule lumen.
Stage IX-X: are characterized by the absence of spermatogonial cells, and the presence of a single generation of spermatids; spermatid stage 9 or 10, which appear with large oval shaped and faintly stained nuclei.

Stage X-XII: are characterized by the absence of spermatogonial cells, and the presence of a single generation of spermatids; spermatid stage 11 or 12, which appear with long thin densely stained nuclei.

Testis weight and sperm counts are additional indications of spermatogenesis progress. The average testis weight of PP1γ1 rescue males was comparable to that of heterozygous control (Figure 21A). Similarly the total number of sperm cells extracted from a single rescue male was comparable to the total number of sperm cells extracted from a single heterozygous male (Figure 21B). Sperm morphology was estimated by counting the number of morphologically abnormal sperm present among 100 sperm cells from a single male. Spermatozoa with the following characteristics were counted as morphologically abnormal: bent neck at the connecting piece, tail with an 180° bend at the mid piece-principle piece junction, malformed head. There was no significant difference observed between PP1γ1 rescue and heterozygous sperm regarding sperm morphology (Figure 21C).
Figure 20. PAS stained testis sections of PP1γ1 rescue mice showing various stages of seminiferous tubules at 60x magnification.
I-II) Stage I-II of the seminiferous epithelium, A-In: Type A-Intermediate spermatogonium, P: pachytene spermatocyte, Ser: sertoli cell, St1-2: spermatid stage 1-2, St13-14: spermatid stage 13-14. III-IV) Stage III-IV of the seminiferous epithelium, In: intermediate spermatocyte, P: pachytene spermatocyte, St3-4: spermatid stage 3-4, St15: spermatid stage 15. V-VI) Stage V-VI of the seminiferous epithelium, B: type B spermatogonium, P: pachytene spermatocyte, St5-6: spermatid stage 5-6, St15: spermatid stage 15. VII-VIII) pL: pre-leptotene spermatocyte, P: pachytene spermatocyte, St7-8: spermatid stage 7-8, St16: spermatid stage 16. IX-X) Stage IX-X of the seminiferous epithelium, L: leptotene spermatocyte, P: pachytene spermatocyte, Ser: sertoli cell, St10: spermatid stage 10. XI-XII) Stage XI-XII of the seminiferous epithelium, sSc: secondary spermatocyte, St11-12: spermatid stage 11-12, P: pachytene spermatocyte.
Figure 21. Phenotype of PP1γ1 rescue mice.
A) Comparison of testis weight expressed as the mean value ± standard error of mean (SEM) from seven males showing no significant difference between the groups (P > 0.05). B) Comparison of sperm number (the total number of sperm extracted from two cauda epididymis and two vas deferens of one male) values are expressed as the mean value ± standard error of mean (SEM) from seven males showing no significant difference between the groups (P > 0.05). C) Comparison of sperm morphology (the number of morphologically normal sperm out of 100 counted after fixation using a 40X microscope lens). Values are the mean value ± standard error of mean (SEM) from seven males showing no significant difference between the groups (P > 0.05).
3.1.4 Despite normal spermatogenesis, PP1γ1 rescue males are sub-fertile.

PP1γ1 rescue male mice were set up for breeding at the age of 8-9 weeks with CD1 wild type females aged between 8 to 16 weeks for a fixed time of 4 weeks. Only 31% of the tested males were fertile producing one litter over the 4-week period of mating. The size of the litter produced by the fertile males had an average of 6 pups. The infertile males were tested with a second CD1 wild type female for another 4-week period to confirm infertility (Figure 22).

Figure 22. Fertility of PP1γ1 rescue males compared to heterozygous controls. A) Fertility expressed as the percentage of fertile males, N= the number of males tested. All males were tested for four weeks. Their fertility was ascertained by testing with two wild type females. B) Average litter size produced by the fertile males. Bars show an average of 13 for heterozygous males vs. an average of 7 for the rescue males.
3.1.5 PP1γ1 rescue sperm have poor fertilization ability in vitro.

In some cases, male infertility can result from the inability of sperm to travel through the female reproductive track, which can be caused by a wide range of sperm abnormalities including motility defects. To test whether the cause of sub-fertility of PP1γ1 rescue males is limited to one of those defects, we performed in vitro fertilization using eggs from wild type females with sperm from PP1γ1 rescue or heterozygous control males. The average fertilization rate after IVF was calculated by dividing the number of zygotes that progressed to the 2-cell stage after overnight culture in HTF medium (37°C, and 5% CO₂ in a humidified incubator) by the total number of oocytes exposed to spermatozoa. There was a significant difference in fertilization rates between PP1γ1 rescue (5%) and heterozygous control males (52%). The fertilization rate of the heterozygous “Ppp1cc(–/-)” control mice was similar to that described in the literature for CD1 wild type males ≈ 43% [67] (Figure 23).
Figure 23. Fertility of PP1γ1 rescue males compared to heterozygous controls after IVF.
A) Fertilization rate was calculated as the percentage of 2-cell stage 24 hours after fertilization. Neggs are total number of eggs derived from three females. Error bars indicate the standard error of the mean of three different experiments. B) Bright field images of the 2-cell stage embryos resulting from the IVF experiments. Images are representative of three different experiments.
3.1.6 PP1γ1 rescue sperm show abnormal motility.

It is known from previous studies that the activity of PP1γ2 is high in non-motile caput sperm, and decreases as sperm mature and acquire motility in the cauda epididymis. To test whether PP1γ1 can replace the role of PP1γ2 in motility regulation, we measured sperm motility of rescue males using CASA (computer assisted sperm analyzer) immediately following sperm extraction from the vas deferens and cauda epididymis of males in PBS supplemented with 10mM glucose and warmed to 37°C. PP1γ1 rescue sperm showed a significantly lower percentage of total and progressively motile sperm (Figure 24A). The velocity parameters of the observed motile sperm were also low compared to heterozygous control sperm (Figure 24B). Interestingly PP1γ1 rescue sperm could not maintain their motility after 15 minutes of incubation in capacitating media HTF (37°C and 5% CO₂). After 15 minutes, the percent of total motile sperm decreased 50% and progressive motility disappeared (Figure 24C).

It was noticed from the motility video recordings of PP1γ1 rescue sperm, that those sperms that appeared motile, showed some rigidity in their flagella while swimming. Thus we performed a flagella beat wave analysis by tracing the movement of motile sperm flagella from high-speed (100frames/sec) video recordings. One video of a single sperm from a heterozygous male, and three videos of three sperm cells from a PP1γ1 rescue male were converted to a sequence of images and used to trace the moving flagellum. The peak-to-peak amplitude - identified as the distance between the highest and the lowest points of a wave- appears decreased in PP1γ1 rescue tracings compared to heterozygous (Figure 25).
A. Motility

B. Velocity

C. Pp1γ1 Rescue Motility in Capacitating Media
Figure 24. PP1γ1 rescue sperm motility parameters compared to heterozygous control.
A) Sperm motility analysis performed with CASA immediately upon sperm extraction in PBS supplemented with 10mM glucose at 37°C, shows a significant decrease in the percentage of total motile sperm (black bars) and progressively motile sperm (grey bars) in PP1γ1 rescue sperm compared to heterozygous control sperm. Sperm with a velocity greater than 50µm/sec were considered to be progressively motile. Motility is expressed as the mean value ± SEM from seven males. B) The velocity parameters: velocity average path (VAP), velocity curve line (VCL) and velocity straight line (VSL) are significantly lowered in Pp1γ1 rescue sperm compared to heterozygous control sperm. Bars are representative of the mean value ± SEM from seven males. C) Pp1γ1 rescue sperm motility after incubation in capacitating media “HTF”(pre-equilibrated in 5% CO2 overnight) at 37°C in a 5% CO2 incubator for 15 minutes. Total percent motility of Pp1γ1 rescue sperm significantly decreased, and progressive motility disappeared upon incubation. Bars are representative of the mean value ± SEM from 5 males.

Figure 25. Flagella beat wave schematic of PP1γ1 rescue sperm compared to heterozygous control.
A) Tracings of Ppp1cc heterozygous sperm flagella movement using image sequences imported from high speed video recordings (100 frames/sec) following extraction in PBS + 10mM glucose. 20 consecutive images were analyzed. The green flagellum represents the tracing of image no. 1; the red flagellum represents the tracing of image no. 20. B-D) Tracings of PP1γ1 rescue sperm flagella movement from three spermatozoa. PP1γ1 rescue sperm flagella wave show decreased peak-to-peak amplitude during motility compared to heterozygous control sperm.
3.1.7 PP1γ1 protein distribution in spermatozoa of rescue males does not match the distribution of PP1γ2 in heterozygous control.

The widely used RIPA lysis buffer (containing only 1% NP-40 as a detergent) extracts all cytosolic proteins and most membrane bound proteins, however nuclear proteins remain insoluble. The spermatozoa flagellum structures: the fibrous sheath, outer dense fibers, and axoneme are unique structures that bind proteins tightly. Flagella proteins such as beta-tubulin and a number of fibrous sheath bound glycolytic enzymes are not solubilized by RIPA. In wild type and Ppp1cc heterozygous sperm, PP1γ2 exists in both RIPA soluble and RIPA insoluble fractions almost equally. Figure 26 shows the distribution of PP1γ2 in sperm from two heterozygous males, and the distribution of PP1γ1 in sperm from two rescue males. It appears that PP1γ1 exists mostly in the insoluble fraction (pellet) of the sperm lysate, and to a less degree in the soluble (supernatant) fraction. The presence of beta tubulin in the insoluble fraction of the lysates and its absence from the soluble fraction confirms consistency of the lysis performed.
3.1.8 Sperm from PP1γ1 rescue males have lower levels of the PP1 binding protein PPP1R11 compared to heterozygous control sperm.

A number of PP1 interacting proteins have been identified in sperm. However, the exact molecular mechanism in which they regulate PP1γ2 activity is not completely clear for all of them, yet research on this topic is an ongoing project in our lab. The levels of PPP1R7 (known as Sds22), PPP1R2 (known as Inhibitor 2 or I2), and PPP1R11 (known as inhibitor 3 or I3) have been analyzed by western blot of sperm extracts. Figure 27 shows equal amounts of PPP1R7 and PPP1R2 in sperm extracts from both Ppp1cc heterozygous males and PP1γ1 rescue males. However, the amount of PPP1R11 appears to be lower in PP1γ1 rescue sperm extracts.

![Figure 26. Western blot analysis of soluble and insoluble fractions of RIPA sperm lysates from PP1γ1 rescue males compared to Ppp1cc heterozygous control.](image)

Left panel: the distribution of endogenous PP1γ2 protein within subcellular fractions of RIPA sperm lysates from $2 \times 10^6$ sperm cells of two heterozygous males. PP1γ2 is almost equally distributed between the soluble and insoluble fractions. Right panel: the distribution of transgenic PP1γ1 protein within subcellular fractions of RIPA sperm lysates from $2 \times 10^6$ sperm cells of two rescue males. Unlike PP1γ2, PP1γ1 protein appears more abundant in the pellet fraction. All lanes were loaded with $2 \times 10^6$ sperm. Blots were reprobed with anti-Tubulin as a control.
3.1.9 PP1γ1 does not bind to the PP1 inhibitor PPP1R2 in sperm of rescue males

Among the PP1 interacting proteins present in sperm, PPP1R2 is known for its inhibitory effect upon binding to PP1γ2. Threonine phosphorylation of PPP1R2 by GSK3 relieves this inhibition thus acts as an indirect activator of PP1γ2. Figure 28 shows the results of PP1γ2/1 immunoprecipitation performed on sperm lysates from both Ppp1cc heterozygous males and
PP1γ1 rescue males. PPP1R7, PPP1R11, and PPP1R2 were all pulled down with PP1γ2, however, only PPP1R7 and PPP1R11 were pulled down with PP1γ1 and PPP1R2 seemed not to bind to PP1γ1 in rescue sperm lysates.

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<th>A. PP1γ2 IP</th>
<th>B. PP1γ1 IP</th>
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<tr>
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<td><em>Ppp1cc(-/-), PP1γ1</em></td>
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**Figure 28. Western blot analysis of PP1 Immunoprecipitation performed with sperm lysates from Ppp1cc heterozygous males and PP1γ1 rescue males.**

PP1γ2 or PP1γ1 protein was immunoprecipitated from sperm extracts of heterozygous sperm and PP1γ1 rescue sperm respectively using specific antibodies. Protein-antibody complexes were pulled down with G-sepharose beads (Bound +), rabbit pre-immune serum was used as a negative control (Bound -), and 2x10⁶ sperm were loaded as input. Left panel: *Ppp1cc* heterozygous sperm immunoprecipitation. Separate blots were probed with anti PP1γ2, anti PPP1R7, anti PPP1R11, and anti PPP1R2 antibodies. Right panel: PP1γ1 rescue sperm immunoprecipitation. Separate blots were probed with anti PP1γ1, anti PPP1R7, anti PPP1R11, and anti PPP1R2 antibodies. PPP1R2 protein was not pulled down by PP1γ1 antibody in rescue sperm lysates. This figure represents a single experiment.
3.1.10 PP1γ1 rescue sperm show low levels of GSK3 alpha phosphorylation compared to heterozygous sperm.

Phosphorylation of GSK3α on Ser21 is known to correlate with the inhibition of the enzymes kinetic activity. In non-motile caput sperm GSK3 activity is high and decreases as sperm acquire motility in the cauda epididymis. The low motility parameters of PP1γ1 rescue sperm led us to investigate the activity of GSK3α through western blot. Figure 29 shows very low levels of GSK3α phosphorylation in caudal sperm of PP1γ1 rescue males compared to PP1γ2 rescue and Ppp1cc heterozygous sperm.

Figure 29. Comparative western blot analysis of sperm lysates from Ppp1cc heterozygous, PP1γ2 rescue, and PP1γ1 rescue males. Western blot gels loaded with equal sperm count; 2x10⁶, the first blot was probed with antibodies against Phospho-GSK3α, then probed with anti-GSK3α, GSK3α/β and PP1γ1. A second blot was probed with PP1γ2, followed by Tubulin. PP1γ1 rescue sperm showed low reactivity with Phospho-GSK3α antibody despite comparable reactivity with both GSK3α and GSK3α/β antibodies. This data is representative of three separate experiments.
3.1.11 Both cAMP levels and serine/threonine phosphorylation of PKA substrates in PP1γ1 rescue sperm, are comparable with those of heterozygous sperm.

Motility initiation is accompanied by an increase in cAMP levels and the activity of the cAMP dependent kinase PKA. cAMP levels were measured in Ppp1cc heterozygous, PP1γ2 rescue, and PP1γ1 rescue sperm. No significant difference was observed between the three groups (Figure 30).

The activity of PKA can be evaluated by western blot using antibodies that detect the phosphorylated residues of PKA substrates. Figure 31 shows a western blot of sperm extracts from Ppp1cc control, PP1γ2 rescue, and PP1γ1 rescue males, comparing the level of phosphorylation of PKA substrates. No difference was observed between the three lanes.

![cAMP levels comparison](attachment:image.png)

**Figure 30.** Comparison of sperm intracellular cAMP content among Ppp1cc heterozygous, PP1γ2 rescue, and PP1γ1 rescue males.

cAMP was measured in sperm lysates using a colorimetric immunoassay. Values are shown as picomoles of cAMP per 10^6 sperm cells. Bars represent the mean ± SEM from three separate experiments. No significant difference was observed between the three groups.
3.1.12 PP1γ1 rescue sperm have low levels of ATP.

ATP is the energy molecule utilized by the sperm flagella motor: the dynein ATPase. The amount of ATP generated by sperm correlates with motility parameters and fertilization capability. Figure 32A shows the ATP content of spermatozoa from both heterozygous control and PP1γ1 rescue males. Rescue sperm show low ATP content when sperm are incubated in the presence of both glycolytic and mitochondrial substrates. Figure 32B and 32C, show sperm ATP
levels in the presence of both mitochondrial and glycolytic substrates (Glucose + Lactate), mitochondrial substrates only (lactate), and glycolytic substrates only (glucose). The mitochondrial inhibitor “antimycin” was used along with glucose to prevent any ATP from being produced from the glycolysis end product “pyruvate”.

A. ATP

B. Ppp1cc (+/-) ATP
3.1.13 Hexokinase activity and phosphorylation in PP1γ1 rescue sperm are comparable to those of heterozygous control sperm.

The level of tyrosine phosphorylation of hexokinase increases its catalytic activity and enhances glycolysis and can be used as an indication of the enzymes activity. Figure 33 shows a
comparison of P-HK1 levels in *Ppp1cc* heterozygous control sperm and PP1γ1 rescue sperm. No difference in band intensity was observed between the two lanes. HK-1 antibody was used as a control to show equal levels of the enzyme in both mice lines.

Hexokinase activity was also measured by its ability to phosphorylate and trap the glucose competitor 2-Deoxy-D-glucose (DOG). DOG is phosphorylated by HK-1 in a similar matter to glucose. However, it does not undergo further glycolysis and remains trapped intracellularly. The decrease in ATP produced by a cell after replacing glucose in the surrounding media with DOG, is an indication that DOG has been phosphorylated by HK-1. In turn an indication of HK-1
activity. A comparison between the effect of DOG on ATP production of Ppp1cc heterozygous sperm and that of PP1γ1 rescue sperm showed that, despite the low overall levels of PP1γ1 rescue sperm ATP, yet the presence of DOG in the incubating media led to a decrease in ATP levels, which is an indication of some HK-1 activity (Figure 34A).

A third way of evaluating Hexokinase activity was performed by measuring the amounts of NADPH produced by oxidation of glucose-6-phosphate (G-6-P); an indirect method for measuring the amounts of G-6-P produced by hexokinase in sperm lysates. Figure 34B shows that there was no significant difference between the activity of hexokinase in sperm lysates of Ppp1cc heterozygous and PP1γ1 rescue males.
3.1.4 PP1γ1 shows altered subcellular localization.

We had previously observed an abnormal distribution of PP1γ1 in sperm supernatant and pellet fractions of rescue males. Thus we performed immunocytochemical staining of sperm from both Ppp1cc heterozygous, and PP1γ1 rescue males to see if there is any difference in localization of PP1γ2 and PP1γ1. Figure 35 shows the localization of PP1γ2 in heterozygous
sperm. It is present along the flagellum and in the equatorial and anterior acrosome. Figure 36 shows the localization of PP1γ1 in rescue sperm. PP1γ1 is also present along the flagellum. However, its localization in the head is different than PP1γ2. PP1γ1 is absent from the anterior or equatorial acrosome, and appears in the post-acrosomal region.

Figure 35. Subcellular localization of PP1γ2 in Ppp1cc heterozygous sperm. Fluorescence images of caudal sperm stained with anti PP1γ2 antibody. Top panel: Sperm head showing localization of PP1γ2 in the equatorial and anterior acrosome. Lower right panel: Sperm showing localization of PP1γ2 both in the head and tail. Lower left panel: Negative control sperm stained with secondary antibody only. All images are shown along with DAPI stain and bright field or merged with bright field.
Figure 36. Subcellular localization of PP1γ1 in PP1γ1 rescue sperm.
Fluorescence images of caudal sperm stained with anti PP1γ1 antibody. Top panel: Sperm head showing localization of PP1γ1 in the post-acrosomal region. Lower panel: Sperm showing localization of PP1γ1 both in the head and tail. All images are shown along with DAPI stain and merged with bright field.
3.1.15 PP1γ1 rescue sperm show a poor Zona Pellucida binding ability.

The ability of a spermatozoon to fertilize an oocyte, *in vivo* or *in vitro* depends on many factors. Once spermatozoa successfully reach the oocyte they are required to penetrate the oocyte cumulus complex (OCC), bind to the zona pellucida, undergo the acrosome reaction, and fuse with the oocyte oolemma. The ability of Pp1γ1 rescue males to bind to the ZP was tested following incubation of their sperm with oocytes obtained from CD1 wild type females for four hours. Rescue sperm show a compromised ability to bind to the ZP compared to *Ppp1cc* heterozygous sperm (Figure 37).

**Figure 37. Sperm-egg binding.**
A&B) Bright field images of wild type eggs after 4 hours of incubation with capacitated sperm of *Ppp1cc* heterozygous male. (C) Fluorescent image of fixed egg and bound sperm from heterozygous male, stained with Hoechst. D & E) Bright field images of wild type eggs after 4 hours of incubation with capacitated sperm of PP1γ1 rescue male. F) Fluorescent image of fixed egg and bound sperm from PP1γ1 rescue male, stained with Hoechst. Images are representative of 3 separate experiments.
3.1.16 PP1γ1 rescue males show a low percentage of acrosome reacted sperm upon addition of calcium ionomycin.

The presence of PP1γ2 in the anterior and equatorial acrosome, indicate that the phosphatase may have a role during acrosome reaction. The altered localization of PP1γ1 in the rescue sperm head lead us to test the ability of PP1γ1 rescue sperm to undergo the acrosome reaction in response to calcium ionomycin. Following sperm incubation in capacitating media for 60 minutes, acrosome reaction was induced in both Ppp1cc heterozygous sperm and PP1γ1 rescue sperm. Sperm were then stained with commassie blue and evaluated using a 40x magnification lens. Figure 40 shows the results summarized in a bar graph. A significant difference in the percentage of acrosome reacted sperm from both mice lines was observed. Figure 41 & 42 are microscopic images representing the data summarized in figure 40.

![Figure 38. Comparison of the percent of acrosome reacted sperm between Ppp1cc heterozygous and PP1γ1 rescue males.](image)

A bar graph representative of the number of acrosome reacted sperm evaluated under the microscope, after inducing AR by incubating capacitated sperm for 10 minutes with 10μM calcium ionomycin. A total number of 100 sperm cells fixed and stained with commassie blue were counted per sample. Values represent the mean ± SEM of three separate experiments. “*” indicates a significant difference ($P<0.05$).
Figure 39. Bright field microscopic images of comassie blue stained *Ppp1cc* heterozygous spermatozoa.

A-D) Sperm images before treatment with calcium ionomycin. The arrowheads point at stained intact acrosomes. E-H) Sperm images after treatment with calcium ionomycin. The arrows point at sperm heads lacking a stained acrosome.
Figure 40. Bright field microscopic images of commassie blue stained PP1γ1 rescue spermatozoa.
A-D) Sperm images before treatment with calcium ionomycin. The arrowheads point at stained intact acrosomes. E-H) Sperm images after treatment with calcium ionomycin. The arrows point at sperm heads lacking a stained acrosome.
3.1.17 PP1γ1 rescue sperm show low actin remodeling upon capacitation.

The increase in filamentous actin in the head of spermatozoa is a marker of capacitation. Actin remodeling is required prior to acrosome reaction, where it aids in the fusion of the outer acrosomal membrane and the inner plasma membrane before acrosomal exocytosis. Once the acrosome reaction is completed, actin filaments depolymerize. Staining of capacitated sperm with the filamentous actin stain “Phalloidin” allowed us to evaluate the degree to which F-actin formation occurs in both Ppp1cc heterozygous and PP1γ1 rescue sperm. Figure 38A-F shows images of Ppp1cc heterozygous sperm after 90-minute capacitation in HTF media (37°C, 5% CO₂). Figure 38G-j shows images of capacitated Ppp1cc heterozygous sperm after treatment with calcium ionomycin to induce acrosome reaction. Capacitated sperm show staining of F-actin in the head, while ionomycin treated sperm show no staining. Figure 39A-F shows images of PP1γ1 rescue sperm after 90-minute capacitation in HTF media (37°C, 5% CO₂). Figure 39G-j shows images of capacitated PP1γ1 rescue sperm after treatment with calcium ionomycin to induce acrosome reaction. A low number of capacitated sperm showed the presence of F-actin. The majority of sperm viewed lacked any visible staining. Similar to Ppp1cc heterozygous sperm, acrosome reacted sperm lacked any F-actin staining.
Figure 41. Actin remodeling after capacitation and acrosome reaction in *Ppp1cc* heterozygous sperm.
Figure 42. Actin remodeling after capacitation and acrosome reaction in PP1γ1 rescue sperm.
3.1.18 TMEM225 a sperm specific protein located in the equatorial acrosome binds to both PP1γ1 and PP1γ2.

**Background**

TMEM225 has been identified as a sperm specific protein in the rat and mouse. The amino acid sequence of TMEM225 suggests that it is a transmembrane protein with four transmembrane helices with its C and N terminus facing the cytoplasmic domain. The expression profile in the testis was first reported in rats by Yang et al. (2011) The study reported that TMEM225 expression appears to start at 13 months in the adult rat testis (the first wave of spermatogenesis in rats occurs around 40 days post birth) and is localized in spermatocytes and round spermatids, suggesting that TMEM225 might have a role in sperm apoptosis but not spermatogenesis [68]. A later study by Matsuura and Yogo (2015) reported TMEM225 expression in the mouse testis -by both RT PCR and western blot- to start at day 25pb coinciding with the first wave of spermatogenesis, with localization restricted to post-meiotic germ cells in the testis (by IHC). The same study reports the localization of TMEM225 in mature spermatozoa to be restricted to the equatorial region of the head and shows that it acts as a binding protein and inhibitor of PP1γ2 [69]. In this aim we investigate the ability of TMEM225 to bind to PP1γ1. Since TMEM225 contains a general PP1 binding motif RVxF in its C-terminus tail (amino acid 224-228) it is expected to bind both PP1γ2 and PP1γ1. However, its co-localization in the equatorial region of the spermatozoa head along with PP1γ2, and the fact that PP1γ1 in rescue sperm is absent from the equatorial region and present in the post-acrosomal region suggests that TMEM225- PP1γ1 binding might be missing in PP1γ1 rescue sperm.
An antibody against a peptide corresponding to 14 amino acids of the C-terminus tail (\(^{217}\text{NRPHTQARRVTWAL}^{230}\)) of TMEM225 was used to immunize rabbits for antibody production. Peptide synthesis and antibody purification were performed by Yenzym (Yenzyme antibodies, LLC). The antibody was tested against testis and sperm lysates from wild type mice. Immunoreactivity of the antibody was absent after western blot with 5\(\mu\)g of testis lysates and 2\(\times\)10\(^6\) sperm lysates. However, following western blow with 20 \(\mu\)g of testis lysate and 6\(\times\)10\(^6\) sperm lysates.
sperm lysate, a faint band shows at approximately 29kDa. (The calculated molecular weight: 26.5kDa)

3.1.18.1 TMEM225 antibody testing.

![Western blot image](image)

**Figure 44. Testing of the TMEM225 antibody.**  
Western blot of both testis and sperm extracts from a wild type male. 20μg of testis extract was loaded in the left lane and extract of 6x10⁶ sperm cells was loaded in the right lane. The antibody shows weak reactivity and a band with the size of approximately 29kDa.

3.1.18.2 TMEM225 is pulled down with either PP1γ1 antibody or PP1γ2 antibody in sperm lysates of both PP1γ1 rescue and Ppp1cc heterozygous control respectively.

In order to test the binding of TMEM225 to PP1γ2 and PP1γ1 in heterozygous and rescue sperm, we performed an Immuno-precipitation experiment on RIPA sperm lysates from both mice lines. PP1γ2/ PP1γ1 were pulled down from sperm extracts using specific antibodies
against each protein. Figure 45 shows western blot analysis following IP. TMEM225 antibody showed reactivity in the bound fractions of both PP1γ2 IP and PP1γ1 IP. However, no band was seen in the input lanes of either experiment, probably due to the low reactivity of the antibody and the relatively low number of sperm used for the input lysate extract.

**Figure 45. Western blot analysis of Immunoprecipitation of PP1γ2/PP1γ1.**

PP1γ2 or PP1γ1 protein was immunoprecipitated from sperm extracts of heterozygous males and PP1γ1 rescue males respectively using specific antibodies. Protein-antibody complexes were pulled down with G-sepharose beads (Bound +), rabbit pre-immune serum was used as a negative control (Bound -), and 2x10⁶ sperm was loaded as input. A) Ppp1cc heterozygous sperm immunoprecipitation. Blots were probed with anti PP1γ2 and anti TMEM225 antibodies. B) PP1γ1 rescue sperm immunoprecipitation. Blots were probed with anti PP1γ1 and anti TMEM225 antibodies.
3.18.3 PP1γ2 is not pulled down by TMEM225 antibody in sperm lysates of *Ppp1cc* heterozygous males.

The reverse of the previous experiment was performed on sperm extracts from *Ppp1cc* heterozygous males. TMEM225 was pulled down from sperm extracts using specific antibodies. Figure 46 shows that PP1γ2 protein was not pulled down along with TMEM225 as anticipated. The reasons for these results are unknown.

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**Figure 46. Western blot analysis of Immunoprecipitation of TMEM225.**

TMEM225 protein was immunoprecipitated from sperm extracts of heterozygous males using anti TMEM225 antibodies. Protein-antibody complexes were pulled down with G-sepharose beads (Bound +), rabbit pre-immune serum was used as a negative control (Bound -), and 2x10^6 sperm was loaded as input. Blots were probed with anti TMEM225 and anti PP1γ2 antibodies.
3.1.18.4 Recombinant TMEM225 expression.

The complete coding sequence of TMEM225 was amplified by PCR from testis cDNA using primers that introduced a XhoI restriction site at the 5’ end, and a HindIII restriction site at the 3’ end. The amplified sequence was inserted into a “pRSET A” bacterial expression vector, and used to express recombinant TMEM225 in BL21 E.coli. Five colonies growing in the presence of ampicillin were tested by PCR for the presence of the TMEM225 containing plasmid.

Figure 47. Schematic representation of the plasmid and insert used to make recombinant TMEM225.
A cDNA fragment containing the coding sequence of TMEM225 (CDS) was inserted into a pRSET A plasmid between the multiple cloning sites XhoI and HindIII. And used to transform BL21 E.coli cultures.
Figure 48 shows the results of BL21 transformation with the generated plasmid. Figure 48A represents PCR data from five plasmid positive colonies that grew on a culture plate containing ampicillin. Three of those colonies contained DNA for TMEM225 after testing with TMEM225 specific forward and reverse primers. Figure 48B represents western blot analysis of bacterial lysates after induction and expression of the inserted plasmid. Colony number 5 showed the highest band intensity for recombinant TMEM225 protein thus was selected for further protein purification. Figure 48C shows recombinant His tagged TMEM225 protein visible after probing bacterial lysates with antibodies against the attached His tag. The band was of similar size to the band obtained with anti-TMEM225 (approximately 34kDa). Figure 48C also shows the distribution of the recombinant protein between the supernatant and pellet fractions of bacterial lysates following sonication. The recombinant protein shows a reasonable amount present in the supernatant fraction.
Figure 48. Testing the expression of recombinant His-TMEM225 in BL21 lysates.
A) Results of PCR amplification of TMEM225 in five selected BL21 colonies growing in the presence of ampicillin. Forward and reverse primers amplifying the entire coding region of TMEM225 were used. B) Western blot analysis of TMEM225 positive BL21 cultures. Probed with anti-TMEM225 and showing a band with 34kDa of size. C) Right panel: Western blot analysis of BL21 culture lysate obtained from colony 5, before and after IPTG induction. Probed with anti-His antibody and showing a band of recombinant protein after IPTG induction at a similar size to the band obtained with anti-TMEM225; 34kDa. Left panel: Western blot analysis of BL21 culture lysate obtained from colony 5 showing the distribution of recombinant TMEM225 between the soluble (sup) and insoluble (pellet) fractions of the lysate following sonication.
3.1.18.5 Recombinant His-TMEM225 purification.

Cultures derived from colony 5 were used for recombinant protein purification. Figure 49 summarizes the steps followed. For the first stage of purification Bacterial culture lysates (the soluble supernatant fraction) was incubated overnight with Nickel beads to pull down His-TMEM225 through its His tag. Western blot analysis of the different eluted fragments suggested that His-TMEM could not bind to the nickel beads efficiently. The majority of the recombinant protein was present in the flow through (not shown) and a significant amount was eluted with the weakly bound proteins with 100mM Imidazole. The second stage of purification was performed using an FPLC system with an anion column. Western blot from figure 49 shows that the majority of the recombinant protein did not bind to the anion column and passed along with the flow through. The third stage of purification was performed using an FPLC system with a cation column. It is clear from the western blot and commassie stained blot in figure 49, that a reasonably good amount and clean protein was obtained following elution of the protein from the cation column with 125mM of NaCl. This Final fraction was used for the pull down assay described later.
3.1.8.6 Recombinant TMEM225 protein binds to PP1γ2 in testis lysates of *Ppp1cc* heterozygous males.

Recombinant His-TMEM225 protein was incubated overnight with testis lysates of *Ppp1cc* heterozygous males. Protein complexes were pulled down with PP1γ2 antibody conjugated to G-Protein dynabeads. Nickel beads were not used since His-TMEM225 could not
bind to Nickel despite containing a His tag. Figure 50 shows the results of the pull down. Recombinant TMEM225 was detected by an anti-His antibody in the bound fraction pulled down along with PP1γ2 protein. No reactivity was observed in the negative control bound.

**Ppp1cc(+/−) Testis**

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**Figure 50. Recombinant His-TMEM225 pull down.**
Western blot analysis of *Ppp1cc* testis pull down experiment. Input: Testis lysate. Bound +: Testis lysate + His-TMEM225 pulled down with anti PP1γ2 antibody. Bound -: Testis lysate + His-TMEM225 pulled down with pre-immune rabbit serum.
Discussion of Aim I

PP1γ1 can replace PP1γ2 to support spermatogenesis in the male testis.

The first study describing the infertile phenotype of Ppp1cc null males was published in 1999 by Varmuza et al. [48] Since then, it has been widely believed that the testis specific isoform of Ppp1cc is essential for the progression of male spermatogenesis. The knockout males were infertile due to an arrest of germ cell development at the stage of round spermatids. Their testes contained a high number of apoptotic cells and were devoid of any mature spermatozoa. PP1γ2 being the only PP1 isoform expressed in post-meiotic germ cells could not be compensated by any other endogenous phosphatase, unlike somatic tissues where the loss of Ppp1cc leads to the up-regulation of other PP1 isoforms (mainly PP1α). Moreover, The levels of PP1γ2 protein expressed in the testis are very high compared to the levels of PP1γ1 expression in any other tissue. Females null for the Ppp1cc gene are normally fertile, and meiosis in the ovary is not affected by the loss of PP1γ. These findings altogether supported the hypothesis that PP1γ2, as a testis-enriched phosphatase is essential for the progression of meiosis in the male testis. For the first time our data falsifies the previously believed hypothesis. Previous studies from our lab have shown that the progression of spermatogenesis positively correlates with the amounts of PP1γ2 protein expressed in the testis. The fertility of Ppp1cc null males was rescued by the transgenic expression of PP1γ2 in the testis driven by the testis specific promoter Pgk2. A number of transgenic mice line were generated expressing different amounts of the PP1γ2 transgene. Through analysis of those mice it was concluded that testis weight, sperm number, and the percentage of normal sperm, increased with the increase in transgenic PP1γ2 expression. However, fertility was only rescued when the amount of PP1γ2 expressed reached a certain
threshold (75% of the protein levels expressed by Ppp1cc heterozygous males). Those rescue lines expressing transgenic PP1γ2 below the threshold value, showed partial rescue in spermatogenesis yet they were infertile. The PP1γ1 rescue males we generated were capable of expressing PP1γ1 in the testis of Ppp1cc null males at levels equivalent to those of endogenous wild type PP1γ2. The males showed comparable testis weights, total sperm counts, and normal sperm morphology compared to fertile Ppp1cc heterozygous males. Their testis architecture appeared normal upon analysis of PAS stained testis sections, with all stages of seminiferous tubule epithelium present containing all developmental stages of germ cells. This data suggests that irrespective of the PP1 isoform expressed in the testis, it is the levels of PP1 that determines the efficacy of spermatogenesis in the testis.

Non-mammalian species such as turkey and sea urchin express other PP1 isoforms: PP1α or PP1γ1 in their testes throughout spermatogenesis. However many differences in sperm morphology and mechanisms of fertilization exist between mammalian and non-mammalian species. Female mammals have a long reproductive tract that requires fertilization to occur internally. For that purpose mammalian sperm must have evolved in a way that allows them to overcome the various barriers along their journey to the egg. One characteristic of mammalian sperm that is believed to have evolved for that purpose is their ability to undergo hyperactive motility inside the female reproductive tract. Therefore we tested whether PP1γ1 could substitute for PP1γ2 in supporting sperm function and fertilization.

Despite Normal spermatogenesis PP1γ1 rescue males are sub-fertile.

Upon breeding with wild type females, approximately 70% of PP1γ1 rescue males failed to produce any litters. The 30% fertile males produced litters that were smaller in size than the
average litter produced by a *Ppp1cc* heterozygous male; 6 pups compared to 12 pups. This subfertility phenotype was hard to explain as no differences in PP1 protein amounts were detected between the fertile and the non-fertile rescues (not shown). Fertility of the rescue males was also tested following *in vitro* fertilization of wild type eggs. The percentage of two cell stage embryos produced using rescue sperm was 5% compared to 52% after using heterozygous sperm. The reason for the difference in fertility after *in vivo* vs. *in vitro* testing is unknown. Some factors involving the way sperm is affected by the female reproductive tract environment may be responsible for the observed difference.

“**PP1γ2** a mammal specific phosphatase is required for sperm epididymal maturation.”

Long before the generation and analysis of the *Ppp1cc* null males, a suggested role of PP1γ2 in sperm motility was described by Vijayaraghavan et al. (1996) [49]. The activity of the enzyme was reported to be high in non-motile caput sperm, and decrease as sperm pass through the cauda epididymis and acquire motility. Inhibition of PP1γ2 in non-motile caput sperm by calyculin A (CA) or okadaic acid (OA) induced motility to some degree. It become evident that PP1γ2 plays a role in motility initiation in the epididymis.

The epididymis is a ductal structure that forms during the embryonic development of mammals. Different regions of the epididymis arise from different cell lineages in the embryo, indicating a functional specificity of each region. By the time an embryo is born, its epididymal ducts are completely coiled. However, further post-natal development continues up to the adult stage. Non-mammalian species lack an epididymis but have a more simple, uncoiled tract that only serves to deliver gametes [70]. Interestingly PP1γ2 is only expressed in testes and sperm of mammalian species. Non-mammalian species such as turkey bear PP1α in their sperm.
The effect of replacing PP1γ2 with PP1γ1 in sperm, lead to abnormal motility. All motility parameters were significantly lower than those of heterozygous control, including progressive motility, velocity, and flagella beat pattern. PP1γ1 rescue sperm were not able to maintain their motility in capacitating media, nor undergo any capacitation dependent activity, such as acrosome reaction or filamentous actin formation.

Sperm motility is directly dependent on the ability of the motor apparatus of the flagellum to utilize ATP through the axoneme bound dynein ATPase. Among sperm of different mammalian species, those that generate more ATP have higher velocity parameters. It is evident that the levels of ATP correlate positively with sperm motility. Upon measuring the ATP levels of PP1γ1 rescue sperm, it appeared that they produce a significantly lower amounts of ATP compared to heterozygous control sperm. Our data indicates that energy production through both glycolysis and oxidative phosphorylation is compromised.

Interestingly PP1γ1 has previously been shown to bind to Sds22, I2, and I3 in the testis of PP1γ1 rescue males (unpublished). However, our data show that PP1γ1 could not bind I2 in caudal sperm. Moreover, GSK3α Serine phosphorylation in caudal sperm of the rescue males was dramatically decreased. The absence of GSK3α phosphorylation is an indication of its high kinase activity. GSK3 is a known regulator of PP1γ2 activity. When GSK3 is active it phosphorylates I2 and dissociates it from PP1γ2, activating the phosphatase. However, low kinase activity of GSK3 is associated with less I2 phosphorylation and its increased binding to and inhibition of PP1γ2. PP1γ1 rescue caudal sperm appear to maintain a state similar to that of wild type caput sperm. Caput sperm are normally non-motile and have high levels of both GSK3 and PP1 activity. Both enzymes are crucial regulators of motility development in the epididymis.
An important factor that regulates protein phosphatases in general, is their ability to bind to specific anchor proteins that bring them to a close proximity to their substrates. The subcellular localization of PP1γ1 protein within the rescue spermatozoa reveals that there is a disruption in the distribution of the protein inside the cell. Sperm lysates of rescue males prepared using RIPA buffer, reveal that PP1γ1 exists mainly in the insoluble fraction of the lysate, while PP1γ2 in heterozygous sperm is normally distributed almost equally between the soluble and insoluble fractions of the lysate. The significance of this data comes from the fact that most insoluble sperm proteins are those that are tightly bound to the flagellum structures such as the fibrous sheath. The abundance of PP1γ1 in the insoluble fraction of the rescue sperm lysate may have a role in the abnormal flagella beat wave and low motility parameters.

Results from our ICC experiments also reveal altered PP1γ1 localization in the heads of rescue sperm. Normally PP1γ2 is present in the anterior acrosome and the equatorial region of the head, which indicates its possible involvement in acrosome reaction or sperm-egg binding. The presence of PP1γ1 in the post-acrosomal region of the head may have a role in the poor egg binding and fertilization ability of rescue sperm. A recently discovered PP1γ2 binding protein “TMEM225” was reported to be present in the equatorial region of the sperm head [69]. Our initial hypothesis was that TMEM225 acts as a specific anchor for PP1γ2 and not PP1γ1. However, our data revealed that TMEM225 binds both PP1γ2 and PP1γ1 in sperm of heterozygous males and rescue males respectively.
3.2 Aim II: To identify the Ppp1cc gene’s minimal promoter sequence required for testis specific expression, and to identify the function of PP1γ2 C-terminus tail.

Background

Meiosis during spermatogenesis is accompanied by a dramatic change in the transcriptome of testicular germ cells. The transition from a diploid cell to a haploid cell that needs to undergo a strict morphological differentiation process is such a delicate transition. It includes activation of some genes, silencing of others, and most important of all it includes differential isoform expression of some ubiquitously expressed genes. Hundreds of somatic expressed genes have testis specific isoforms, and are produced in many different ways, ranging from alternative splicing of premature mRNA, to the use of alternate start and end sites, to many others. Testis specific transcription factors play a major role in this transition. They can act as either activators or silencers upon binding to specific motifs in a genes promoter.

The expression pattern of Ppp1cc in the testis is pretty unique. In early diploid germ cells the gene expresses PP1γ1 at basal levels, similar to the levels of PP1γ1 expressed in most somatic tissues. During meiosis in primary pachytene spermatocytes Ppp1cc starts shifting from the expression of PP1γ1 to the expression of PP1γ2. This process occurs due to the alternative splicing of Ppp1cc premature RNA through removal of intron 7 –which includes PP1γ1s stop site-, retaining exon 8 as part of PP1γ2s coding sequence, and introducing a new stop site. It is unclear why at this specific stage the splicing occurs. It is believed though, that in pachytene spermatocytes a number of miRNAs become expressed (including miR449 and miR34) which have targets in the spliced out intron 7, leading to the instability of PP1γ1 mRNA. In post-
meiotic secondary spermatocytes a dramatic increase in the levels of both PP1γ2 mRNA and protein are observed, indicating the introduction of an additional activator of Ppp1cc promoter at that stage. The requirement of the testis for high levels of the phosphatase in post-meiotic germ cells is expected and understandable. However, the mechanism in which this sudden increase in promoter activity is unknown. We had analyzed the Ppp1cc promoter for the presence of any testis specific transcription factor binding sites. An analysis of an 800bp sequence (-400 to +400) of the Ppp1cc promoter was performed using the Jaspar database online tool. It revealed a binding site for the testis specific transcription factor Spz1 between positions +257 and +272. Interestingly the 16 base-long sequence is conserved among a number of mammalian species.

For the first part of this aim we tested the ability of a 0.752kb region of the Ppp1cc promoter to drive testis specific expression.

The mature spermatozoon contains a large number of protein kinases. This is expected, as protein phosphorylation is one of the most utilized protein regulators in a cell that is devoid of transcription and translation. However the number of protein phosphatases present in sperm is surprisingly low compared to the number of protein kinases. In general, protein phosphatases do have a wider range of substrates, and are usually regulated by their PIPs (Phosphatase interacting proteins). PP1γ2; the testis specific isoform of the Ppp1cc gene is the only PP1 isoform present in post-meiotic male germ cells and spermatozoa. PP1γ2 along with PP2A and PP2B are the three most studied Ser/Thr phosphatases in sperm. PP1γ2 differs from its somatic isoform PP1γ1 in its unique 22 amino acid long C-terminus tail. Up to this moment the specific function of this C-terminus tail is unknown. It has been speculated that it may have a role in anchoring the phosphatase to specific structures in the spermatozoa. Such structures are unique to the
composition of the sperm cell and are not present in somatic cells, such as the flagellum outer
dense fibers (ODFs), the fibrous sheath surrounding the tail, or even the axoneme.

For the second part of this aim we used a transgenic mouse model expressing a
transgenic peptide corresponding to PP1γ2’s 22 amino acid C-terminus tail. The peptide is linked
to a reporter “EGFP protein” and is driven by a 0.7kb piece of Ppp1cc’s endogenous promoter.
This transgenic model served both part one and part two of this aim. 1) The identification of
Ppp1cc’s minimal promoter sequence required for testis specific expression. And 2) The
identification of the function of PP1γ2’s C-terminus tail.
3.2.1 “Endogenous promoter-EGFP-C-terminus tail” transgenic construct design.

![Diagram showing the design of the transgenic construct with 0.75 kb Ppp1cc Promoter and PP1γ2-C-terminus regions, and a region marked as EGFP and SV40.](image)

**Figure 51.** Design of the construct for the “Endogenous promoter – EGFP – Cterminus” transgenic protein.
The construct was designed and cloned by a former lab member: Nilam Sinha.

3.2.2 Expression of the transgene in LNCaP cells.

The ability of the 0.752 kb *Ppp1cc* promoter to drive expression of the transgene was tested in cell culture prior to generation of the transgenic mice. Prostate carcinoma cells (LNCaP) were chosen for this experiment based on the fact that a number of testis specific genes have been reported in the literature to be expressed in a number of prostate carcinomas [71]. Figure 52 shows FITC fluorescent microscopic images of transfected LNCaP cells (A-C) at 40x magnification. Comparison of those images with the negative control (image D) indicates successful expression of the EGFP-C-terminus tail plasmid.
3.2.3 Transgenic mice generation.

The genetic construct was injected into the pro-nuclei of fertilized B6SJL eggs. The injected eggs were implanted into the uteri of pseudo pregnant mothers. The procedure was carried out at the Transgenic Facility of Case Western Reserve University CWRU (Cleveland, OH) and the produced offspring were shipped to Kent State Animal Facility.
3.2.3.1 Genomic DNA spiking for PCR genotyping sensitivity

Prior to receiving the transgenic animals from CWRU, mouse genomic DNA spiking with the generated plasmid was performed for the purpose of testing the sensitivity of the PCR system that was designed to genotype the transgenic mice. The nano-gram value of the transgene that is equivalent to one copy was calculated using an online tool provided by “Case Transgenic and Targeting Facility”. One copy of the transgene per diploid mouse genome was equivalent to 0.8pg of plasmid per 1µg of mouse genomic DNA. Figure 53 shows the results of the spiking experiments. The primers used for PCR were able to amplify a sufficient amount of DNA from 1 copy of the plasmid in the presence of genomic DNA.

Figure 53. Genomic DNA spiking.
Images of gel electrophoresis following PCR of plasmid DNA in the presence of genomic DNA (top panel) and in the absence of genomic DNA (lower panel). Lane 1: 100 copies of plasmid/ diploid genome. Lane 2: 10 copies, Lane 3: 1 copy, Lane 4: 0.1 copy, Lane 5: 0.01 copy, Lane 6: 0.001 copy.
3.2.3.2 Identification and genotyping of founder mice.

A total of 110 animals were received from CWRU. After genotyping only 6 founder mice were positive for the EGFP transgene. Out of the six founders only 3 mice had passed on the transgene to their first generation of offspring (Table 4).

Table 4. EGFP transgene positive founder mice.

<table>
<thead>
<tr>
<th>Tg+ Founder</th>
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<tbody>
<tr>
<td>WT, GFP*</td>
</tr>
<tr>
<td>Line Name</td>
</tr>
<tr>
<td>Number of pups with Tg+ (first generation)</td>
</tr>
<tr>
<td>Number of male pups with Tg+ WT, GFP*</td>
</tr>
<tr>
<td>Cage1F2</td>
</tr>
<tr>
<td>GFP4</td>
</tr>
<tr>
<td>12</td>
</tr>
<tr>
<td>0</td>
</tr>
<tr>
<td>0</td>
</tr>
<tr>
<td>Cage6F2</td>
</tr>
<tr>
<td>GFP5</td>
</tr>
<tr>
<td>11</td>
</tr>
<tr>
<td>4</td>
</tr>
<tr>
<td>2</td>
</tr>
<tr>
<td>Cage6F5</td>
</tr>
<tr>
<td>GFP6</td>
</tr>
<tr>
<td>7</td>
</tr>
<tr>
<td>0</td>
</tr>
<tr>
<td>0</td>
</tr>
<tr>
<td>Cage16M5</td>
</tr>
<tr>
<td>GFP1</td>
</tr>
<tr>
<td>7</td>
</tr>
<tr>
<td>1</td>
</tr>
<tr>
<td>1</td>
</tr>
<tr>
<td>Cage21F4</td>
</tr>
<tr>
<td>GFP3</td>
</tr>
<tr>
<td>8</td>
</tr>
<tr>
<td>1</td>
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<tr>
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</tr>
<tr>
<td>Cage23M4</td>
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<td>GFP2</td>
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<tr>
<td>8</td>
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3.2.3.3 Transgenic protein expression in testes of transgene positive founder males.

Testis lysates of Tg+ males derived from three positive founders were subjected to western blot analysis and probed with both anti-EGFP antibody, and anti-PP1γ2 antibody to compare their levels of transgene expression. PP1γ2 antibody was raised against a peptide sequence corresponding to the 22 amino acid C-terminus tail of the protein, which allows it to detect the EGFP-C-terminus transgene at approximately 32kDa. It also detects the endogenous PP1γ2 protein at 39kDa. Thus Tg+ testis lysates show a double band when probed with anti-PP1γ2 antibody (Figure 54). The male belonging to the line “GFP5” had the highest transgenic
protein expression. It was bred with a wild type female and the Tg\(^+\) male pups were used for further experiments.

**Figure 54. Comparative western blot of testis lysate among EGFP transgene positive founder males.**

10µg of testis lysate from EGFP transgene positive founder males were loaded in each lane and probed with anti-GFP antibody (upper panel), anti PP1\(\gamma\)2 C-terminus antibody (middle panel), and anti beta-tubulin as control (lower panel). anti PP1\(\gamma\)2 C-terminus antibody detects both endogenous PP1\(\gamma\)2 at 39kDa and the transgenic protein at 32kDa.

Testes of Tg\(^+\) males were snap frozen, sectioned, then fixed with formaldehyde vapor according to the method described by Jockusch et al. (2003) [72]. Sections were observed using an FITC 488nm excitation filter. Figure 55A taken with a 20x magnification lens, shows green fluorescence in one seminiferous tubule out of 5 shown in the picture. Figure 55B taken with a
40x magnification lens shows green fluorescence in two adjacent seminiferous tubules. However, the fluorescence is absent from the center of the tubule where testicular spermatozoa accumulate. The same sections were viewed with a TRITC 550nm excitation filter as a negative control.

3.2.3.4 The selected 0.752 kb region of the \textit{Ppp1cc} promoter is sufficient to drive testis specific expression.

To test the tissue expression pattern of the transgene, seven tissues in addition to testis were extracted from scarified Tg$^+$ males. Based on the initial western blots performed using the founder male testes, it was noticed that the transgenic expression of the EGFP-C-terminus tail protein was relatively low. Thus prior to western blot, all tissues were subjected to EGFP enrichment using EGFP immunoprecipitation. Figure 56 shows western blot of analyzed tissues probed with anti- PP1$\gamma 2$ antibody: Liver, stomach, kidney, lung, spleen, heart, brain, and testis. Low levels of PP1$\gamma 2$ expression was noted in stomach, lung, spleen, and heart. Brain tissue shows a relatively higher amount of PP1$\gamma 2$ compared to the other tissues with the exception of

![Figure 55. Fluorescent images of frozen testis sections from both EGFP$^+$ males and wild type males.](image)
A-B) Fluorescence images of Tg$^+$ testis sections taken with a 485nm excitation filter. C) Fluorescence image of WT testis sections taken with a 485nm excitation filter.
testis. However, none of these tissues show any band of 32kDa in size in the “GFP Bound lane”. Indicating that the transgene “EGFP-C-terminus tail” driven by the 0.7kb \textit{Ppp1cc} endogenous promoter was specifically expressed in the male testis and not any other somatic tissues.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{western_blot.png}
\caption{Comparative western blot of tissue lysates from GFP\textsuperscript{*} males following EGFP protein enrichment.}
\end{figure}

10µg of tissue lysates were loaded in each lane following EGFP enrichment by immunoprecipitation. Input: unprocessed tissue lysate, Flow through: unbound fraction of lysate, GFP Bound: lysate fraction containing proteins immunoprecipitated by anti EGFP antibody conjugated to agarose beads.

To support the previous western blot data, reverse transcriptase RT-PCR was performed on mRNA extracted from eight different tissues in addition to testis: liver, stomach, kidney, lung, spleen, heart, muscle, and tongue. The transgene message was amplified from the generated cDNA using primers that flank the coding region of EGFP. The PCR performed generated a
band of 700bp in size, which is equal to the length of EGFP CDS. The results shown in figure 57 confirm the testis specific expression of the transgene. Primers amplifying the CDS of the housekeeping gene GAPDH were used as an internal positive control.

![Figure 57. Gel electrophoresis following RT-PCR of cDNA from multiple tissues of an EGFP+ male.](image)

mRNA was extracted from multiple tissues of a Tg+ male. The coding sequence of the GFP transgene was amplified using primers spanning the complete CDS, resulting in a 700bp band (upper panel). Primers for amplification of the housekeeping gene GAPDH were used as an internal control (lower panel). DNA: genomic DNA of the Tg+ male, GFP Plasmid: 1ng of GFP plasmid, H2O: PCR negative control (no DNA).

3.2.3.5 The transgenic EGFP-Pp1γ2-C-terminus tail protein expression is restricted to testicular germ cells and absent in mature spermatozoa.

Following western blot analysis of sperm extracts from Tg+ males, no band for the transgenic protein was observed after probing the blots with anti- Pp1γ2 antibody. Similarly, after EGFP enrichment of the sperm lysates; no band was detected (Figure 58). We concluded
that the transgenic protein was excluded from mature spermatozoa for an unknown reason. This western blot data matches the fluorescence images of frozen testis sections, where the green signal was absent from the center of the seminiferous tubules where testicular sperm accumulate.

**Figure 58. Western blot analysis of testis and sperm lysates of GFP⁺ male before and after GFP enrichment.**

10 µg of testis lysate and sperm lysates from 2x10⁶ sperm cells from two Tg⁺ males: line GFP1, and line GFP5 probed with anti PP1γ2 C-terminus antibody to detect the transgene. Anti-tubulin was used as a loading control (upper panel). Testis and sperm lysates from the highest expressing line GFP5 after EGFP enrichment by immunoprecipitation, probed with anti PP1γ2 C-terminus antibody (lower panel).
3.2.3.6 Transgenically expressed Pp1γ2-C-terminus tail in the testis does not disrupt the function of endogenous Pp1γ2, nor does it bind to PP1γ2s known regulators; Sds22, PPP1R2, and PPP1R11.

One of our hypotheses during the design of this experiment was that over expression of Pp1γ2’s C-terminus tail in the testes of wild type males would compete with the proteins that would normally bind to endogenous Pp1γ2’s C-terminus tail, and probably lead to a disruption in the function of Pp1γ2. That disruption would hypothetically result in a phenotype similar to that of Ppp1cc null males. Based on the breeding data obtained from the mating of Tg+ males with wild type females, it become clear that the transgenic males are fully fertile similar to their wild type littermates. Thus we analyzed the testis architecture, sperm count and sperm morphology of Tg+ males. Figure 59 shows that the seminiferous tubules of transgenic male testes appear comparable to wild type following microscopic examination of PAS stained testis sections. Total sperm numbers were also comparable to wild type sperm numbers. The lower panel of figure 59 shows normal sperm morphology of the Tg+ males.
Up to this date not a single protein has been shown to bind to the Pp1γ2 specific C-terminus tail. To test whether any of the known regulators of Pp1γ2 in sperm binds to its C-terminus tail, we performed an EGFP pull down experiment using testis lysates of Tg⁺ males. Following western blot analysis of the pull down fractions it appears that none of the following proteins: Sds22 (PPP1R7), PPP1R2 (I2), nor PPP1R11 (I3) bind to the transgenically expressed C-terminus tail (Figure 60).
Figure 60. Western blot of GFP immunoprecipitation from GFP+ male testis lysates. EGFP-C-terminus tail transgenic protein was immunoprecipitated from testis lysates of a Tg+ male. Western blots were probed with anti-PP1γ2, anti-Sds22, anti-PPP1R2, and anti-PPP1R11. Input: unprocessed testis lysate, GFP Bound: lysate fraction containing proteins immunoprecipitated by anti EGFP antibody conjugated to agarose beads. Flow through: unbound fraction of lysate.
Discussion of Aim II

0.752kb of the *Ppp1cc* promoter is sufficient to drive testis specific expression, yet insufficient in obtaining high levels of expression.

The minimal *Ppp1cc* promoter region required for testis specific expression was based on bioinformatics analysis to identify the main evolutionary conserved regions in the genes upstream sequence. Based on the analysis a region 517bp upstream the transcription start site (TSS) and 235bp downstream the TSS was selected. That region contained an evolutionary conserved transcription factor-binding site for Spz1; a testis specific transcription factor expressed in post-meiotic germ cells. Upon generation and analysis of the transgenic mice with the specified promoter, we observed low levels and uneven expression of the GFP transgene among the seminiferous tubules of fixed testis sections. After western blot analysis of a number of tissue lysates subjected to GFP pull-down, none of the somatic tissues tested, showed any expression of the transgenic protein. RT-PCR of mRNA from the same tissues was tested for transgenic expression, results supported western blots indicating that testis specific expression driven by the 0.752kb promoter was successfully achieved.

Previously published data from our lab describes a transgenic mouse line expressing PP1γ2 on a *Ppp1cc* null background driven by a 2.6kb *Ppp1cc* promoter region [63]. The transgenic mice achieved expression levels equal to 40% of the endogenous PP1γ2 expressed by a wild type male, which is higher than what was achieved using the 0.752kb fragment. It is possible that the 0.752kb promoter fragment contains regions that regulate testis specific expression only, while the larger 2.6kb region contains general transcription factor binding sites that boost the genes expression.
The transgenic expression levels of PP1γ2-C-terminus tail were too low to affect or disrupt the function of the endogenous PP1γ2.

High levels of the expression of the transgene were not successively achieved. That is the most likely reason for a lack of phenotype of the transgenic males. Moreover, the transgenic protein was not incorporated into spermatozoa. The reason for that is unknown. It is possible that sperm get rid of unnecessary proteins along with excess cytoplasm through the shedding of the cytoplasmic droplet. The presence of the EGFP tag attached to the C-terminus tail may as well alter the final 3D shape of the peptide affecting its original ability to bind to any proteins.

Following EGFP immunoprecipitation from testis lysates of transgenic males, western blots were performed to test the binding of the EGFP-C-terminus tail to the known PP1 interacting proteins in testis: SDS22, I2 and I3. Although it is widely known that those PIPs bind PP1γ2 and all PP1 isoforms through an RvXF domain, it is still possible that additional binding sites may exist. However, none of the tested PIPs appeared to bind to the transgenic protein. These results cannot rule out the involvement of PP1γ2s C-terminus tail in this binding, as the 22 amino-acid sequence of the tail may affect the overall conformational structure of PP1γ2, yet when present as an isolated peptide becomes functionless.
3.3 Aim III: To identify the role of the protein phosphatase PP2B (Calcineurin) in sperm motility

Background:

Calcineurin or PP2B is a ubiquitous Ser/Thr phosphatase that is controlled by the levels of intracellular calcium. In response to increased levels of calcium in the cell, the calcium binding protein “calmodulin” becomes saturated with Ca$^{2+}$ ions and binds to a calmodulin-binding region of the calcineurin catalytic subunit. That binding causes a conformational change in the structure of the catalytic subunit moving away an auto-inhibitory arm from the enzymes catalytic site, thus exposing it to its substrates. Another regulatory subunit that is tightly bound to the catalytic subunit, PPP3R, also becomes saturated with calcium further activating the enzyme. This mechanism has been well studied in many cell types mainly due to the high importance of calcium signaling in all tissues.

Calcineurin is expressed in testis and spermatozoa as a testis specific isoform “PPP3CC”. Its regulatory subunit is also expressed as a testis specific isoform “PPP3R2”. Prior to the publication of a recent study by Miyata et al. (2015) [73], little was known about the role of calcineurin in sperm function. Although the role of calcium and calcium channels in sperm has been an area of research for many years, published studies on this topic had focused on the role of calcium signaling during sperm hyperactivation and acrosome reaction. The study by Miyata et al. described the phenotype of a mouse model lacking a functional calcineurin enzyme in the testis. They studied two different mice models, one lacking the catalytic subunit PPP3CC and the other lacking the regulatory subunit PPP3R2. Both mice lines showed a similar phenotype (as disruption of one subunit lead to the degradation of the other). The resulting males were infertile
despite normal testicular weights, sperm numbers, and sperm morphology. The only abnormality the sperm showed was abnormal motility due to a rigid midpiece. The authors suggested that the inability of the calcineurin lacking sperm to fertilize was due to abnormality in their epididymal maturation process, as inhibiting calcineurin in vitro did not affect the fertilization ability of spermatozoa.

Based on these findings, and the fact that the phenotype of the calcineurin knockout sperm resembles the phenotype of GSK3α knockout sperm—which is currently being studied in our lab—we decided to investigate the role of calcineurin in epididymal sperm maturation and its relationship to other enzymes that have a predominant role in motility initiation in the epididymis.

### 3.3.1 Intracellular calcium levels of sperm decrease dramatically during epididymal maturation.

Since calcineurin is a Ca$^{2+}$ dependent phosphatase, we measured the levels of intracellular calcium in spermatozoa from both caput and cauda epididymis. Due to the limited number of sperm that could be extracted from mice and the high cell number requirements of this method, we performed this experiment using bovine sperm extracts. Calcium signaling is a universal regulatory mechanism utilized by all living cells, and we suspect that the role of calcium during epididymal maturation of spermatozoa will be similar among most mammalian species. Sperm cells were lysed using trichloroacetic acid TCA and intracellular calcium levels were measured using flame photometry. Figure 61 shows the content of intracellular calcium of caput sperm almost 50% higher than that of caudal sperm.
3.3.2 PPP3R2 KO sperm show an increase in phosphorylation of GSK3α.

The phenotype of PPP3R2 KO males resembles the phenotype of GSK3α KO males. Both are infertile despite normal spermatogenesis, and show abnormal motility characterized by a rigid midpiece. GSK3α is an important regulator of sperm motility during epididymal maturation. The activity of GSK3α has been shown to decrease as sperm move from the caput to the cauda epididymis and acquire motility. GSK3α is also known to phosphorylate PPP1R2 (I2) and inhibit its binding to PP1γ2 in caput sperm, thus acting as an indirect activator of PP1. The decreased GSK3α activity in caudal sperm leads to a decrease in PP1γ2 activity as well. Both of these enzymes roles in motility initiation are central and essential to sperm function. One of the markers of GSK3α activity is the degree of phosphorylation of its Ser21 residue. Unlike most kinases, GSK3α is constitutively active, and is inhibited upon its phosphorylation. PP2A; a Ser/Thr phosphatase expressed in sperm is thought to modulate the activity of GSK3α through 

![Figure 61. Intracellular calcium levels of bovine caput vs. caudal epididymal sperm.](image)

Sperm total calcium measured by flow cytometry is expressed as ng. of calcium per 10^8 sperm cells. The bars represent mean values ± SEM obtained from 5 separate experiments. “*” indicates a significant difference between the two groups (P<0.05).
dephosphorylating its Ser\(^{21}\) residue. PP2A has been shown to be highly active in caput sperm, as sperm move to the cauda epididymis PP2A becomes methylated and inactivated.

Western blot analysis was performed on sperm extracts from PPP3R2 KO males. Figure 62 shows an increase in phospho-Ser\(^{21}\) of GSK3\(\alpha\) in KO sperm extracts compared to WT. The levels of GSK3\(\alpha\) protein were unchanged. PP1\(\gamma\)2 protein levels and phosphorylation levels were comparable with WT. Hexokinase tyrosine phosphorylation was also unchanged. A slight increase in the phosphorylation of PKA substrates was observed in two bands around 20, and 40kDa.

**Figure 62. Comparative western blot of sperm lysates from WT vs. PPP3R2 KO males.**

Left panel: Sperm lysates of 2x10\(^6\) and 3x10\(^6\) sperm cells from both WT and PPP3R2 KO males were subjected to western blot and probed with anti-Phospho GSK3\(\alpha\), anti- GSK3\(\alpha\), anti-4G10, anti Phospho PP1, anti-PP1\(\gamma\)2, and anti-tubulin antibodies. Right panel: Sperm lysates of 2x10\(^6\) and 3x10\(^6\) sperm cells from both WT and PPP3R2 KO males were subjected to western blot and probed with anti-phospho PKA substrates, and anti-tubulin antibodies. The arrows point at bands that belong to proteins that are hyperphosphorylated.
The activity of PP2A has been reported to affect the phosphorylation of GSK3α [74], thus we investigated the methylation state of PP2A in sperm of the KO mice. PP2A methylation regulates its activity as sperm move from the caput to the cauda epididymis. Figure 63 shows no difference in the methylation of PP2A between WT and PPP3R2 KO sperm.

**Figure 63. Comparative western blot of testis and sperm lysates from WT vs. PPP3R2 KO males.**

10 µg of testis lysate and sperm lysates from 2x10⁶ sperm cells from both WT and PPP3R2 KO males were subjected to western blot and probed with anti-Phospho GSK3α/β, anti-GSK3α/β, anti-methyl PP2A, and anti-tubulin antibodies.
3.3.3 PPP3R2 KO sperm have high ATP levels compared to WT sperm.

The decreased motility of PPP3R2 KO sperm, and the abnormal beat of their flagellum led us to investigate their energy production status. Thus we measured the amount of sperm produced ATP in the presence of different substrates. Figure 64A shows a surprisingly high content of ATP in sperm of PPP3R2 KO males after incubation in a media that supports both glycolysis and mitochondrial oxidative phosphorylation OXOPH. When sperm were incubated in a media supplemented with glucose and in the presence of the OXOPH inhibitor antimycin, the ATP levels of PPP3R2 KO sperm were comparable to those of WT sperm. However, in the presence of lactate as the only energy source, ATP of KO sperm was more than 50% higher than that of WT sperm (Figure 64B).

![ATP graph]
Figure 64. ATP content of sperm from WT vs. PPP3R2 KO males.  
A) ATP levels of WT sperm and PPP3R2 KO sperm following 10 minute incubation in pre-equilibrated HTF media. B) ATP levels of WY sperm and PPP3R2 KO sperm following 10 minute incubation in pre-equilibrated TYH media supplemented with either lactate, or lactate + antimycin. Values are expressed as nmoles of ATP per 10^7 sperm cells. Bars are represent mean values ± SEM of three separate experiments. “*” indicates significant difference between the groups (P<0.05)
Discussion of Aim III

The high levels of intracellular calcium in caput sperm suggest high activity of calcineurin.

Calcineurin is an enzyme that relies on intracellular calcium levels to regulate its activity. An increase in intracellular sperm calcium levels occurs at different stages throughout the sperm journey, hyperactivation being the most studied stage. The fact that in vitro inhibition of calcineurin by incubating sperm with cyclosporine or FK-506 does not affect its motility or its fertilization ability, indicates that calcineurin does don’t act downstream of calcium during hyperactivation. Yet the abnormal phenotype of sperm when calcineurin is absent in vivo at the epididymal maturation stage “PPP3R2 KO” strongly suggests that the enzymes function is essential during that specific developmental stage.

Calcineurin and GSK3α play an interactive role in regulating epididymal sperm maturation.

The loss of a functional calcineurin enzyme from spermatozoa leads to abnormal motility and an increase in GSK3α phosphorylation, thus a decrease in its activity. The effect of the loss of GSK3α on sperm function and fertility has been well studied in our lab using GSK3α KO mice. Our data strongly suggest that GSK3α has a central role in motility initiation in the cauda epididymis and that calcineurin acts along with GSK3α during this developmental stage of the spermatozoa. GSK3α is a kinase involved in many cellular processes. There is evidence that the lack of GSK3α inhibits hexokinase 1 thus leading to impaired glycolysis in KO sperm. The
direct relationship between GSK3α and calcineurin has not yet been investigated. However, a possibility remains that a sub-pool of GSK3α may act as a substrate for calcineurin.

**Calcineurin may regulate mitochondrial activity.**

Previous studies have linked high levels of calcium in caput sperm to mitochondrial regulation. Unlike ejaculated sperm that contains active calcium channels along its flagellum—for example: CATSPER- which upon stimulation with progesterone present in the female reproductive tract, allow the influx of calcium into the sperm cell which leads to activation of the sperm soluble adenylyl cyclase sAC, and an increase in cAMP levels that cause an active PKA to phosphorylate a wide range of proteins leading to hyperactivated motility. Caput sperm cells plasma membrane is impermeable to calcium, and the intracellular regulation of calcium levels occurs through mitochondrial uptake and are affected by the redox state of the mitochondria [62]. Our data suggests the loss of a functional calcineurin in sperm affects mitochondrial activity leading to its hyperactivation. The strong link between the role of sperm mitochondria in calcium homeostasis and the altered mitochondrial activity in PPP3R2 KO sperm, suggests that calcineurin may act as a downstream effector of calcium to maintain high levels of oxidative phosphorylation in non motile caput sperm, whereas the decrease in calcium seen in caudal sperm leads to a shift from OXOPH to glycolysis which is the main ATP producing pathway utilized by the motile flagellum. We propose that the loss of calcineurin from sperm prevents that caput to cauda shift in energy production pathways. One study on a knockout mouse model that lack of calcineurin catalytic subunit in muscle tissue, reports that the affected cells form a long network of fused hyperactive mitochondria [75]. The study shows that Drp1 a protein involved in regulation mitochondrial fusion and fission is hyperphosphorylated in the KO mice muscle
tissues, and may be a possible substrate of calcineurin. Drp1 has been reported to be present in sperm. However, more studies need to be performed on the relationship between sperm Drp1 and sperm calcineurin.
Overall Conclusion

This thesis sheds light on the role of two main Ser/Thr phosphatases in sperm function: PP1γ2, and Calcineurin (PP2B). The results show for the first time that spermatogenesis in the testis can be supported by the somatic PP1 isoform PP1γ1, and that the unique male germ cell phosphatase “PP1γ2” has probably evolved in mammals along with the evolution of the mammalian epididymis for the sole purpose of supporting sperm epididymal maturation.

We suggest that calcineurin has high activity in caput sperm due to the high levels of intracellular calcium. And that the effect of high calcium levels along with high phosphatase activity in caput sperm, have an inhibitory effect on sperm motility, opposing the effect of calcium on motility during sperm hyperactivation in the female reproductive tract. We propose that calcium signaling in sperm function has a biphasic role depending on which calcium dependent proteins are selectively activated at each developmental stage of the sperm journey. Calcineurin may also have a role in regulating mitochondrial activity. A shift in energy production pathways and substrate utilization occurs during sperm transition from the caput to the cauda epididymis, and calcineurin is believed to play a role in this process.

With PP1γ2 shown to be irreplaceable by PP1γ1 during sperm epididymal maturation, the following step would be comparing the phosphoproteome of PP1γ2 bearing sperm with those of PP1γ1 bearing sperm. A wide phosphoproteome analysis will reveal the specific substrates of PP1γ2 that PP1γ1 are unable to dephosphorylate, highlighting proteins with an essential role in epididymal maturation and sperm motility regulation. There is a possibility that the phenotype of PP1γ1 rescue males is caused by the effect of the transgenes incorporation into a random region
of the mouse genome. The only way to test this hypothesis would be the generation of a new transgenic model using a gene knock-in technique, where the endogenous promoters function will be maintained, and there will be no random incorporation of genetic material into the genome. If the results of that new transgenic model support our data, then only we can confirm our conclusion.

There is strong evidence that calcineurin has a role in mitochondrial energy production and activity. The following step would be analyzing PPP3R2 KO males for additional mitochondrial function parameters, such as oxygen consumption or measurement of a number of mitochondrial enzyme activities. Scanning electron microscopy can be performed to look for any mitochondrial elongation or fusion.
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