2015

Epigenetic Role of PTIP in Mouse Spermatogenesis

Chengjing Liu
liu27@marshall.edu

EPIGENETIC ROLE OF PTIP IN MOUSE SPERMATOGENESIS

A thesis submitted to
the Graduate College of
Marshall University
In partial fulfillment of
the requirement for the degree of
Master of Science
in
Biological Sciences
by
Chengjing Liu
Approved by
Dr. Guo-Zhang Zhu, Committee Chairperson
Dr. Gary Schultz
Dr. Marcia Harrison-Pitaniello

Marshall University
May 2015

ACKNOWLEDGMENTS

I would like to thank Dr. Guo-Zhang Zhu, Dr. Gary Schultz, Dr. Marcia Harrison-Pitaniello, and Miss Shaimar R. Gonzalez. First, I would like to thank my advisor Dr. Guo-Zhang Zhu for accepting me into his laboratory to pursue my Master's degree. Dr. Zhu has tirelessly supported and inspired me all the time during my study at Marshall University. I also feel thankful to Dr. Gary Schultz, who gave me the Microbial Genetics class and introduced me the field of genetics. Dr. Schultz also provided me first experience to do seminar presentation, encouraged me to improve the presentation skills, and gave me helpful suggestions for my thesis. I would also like to thank Dr. Marcia Harrison-Pitaniello, who vigorously helped me develop my professional skills and offered constructive suggestions for my thesis. I would like to thank the student Shaimar R. Gonzalez, who worked with me in the same laboratory, helped me learn experimental skills, and shared happiness and frustration with me.

Most importantly, I would like to thank my parents, Fangzheng Liu and Ping Li, for their financial sponsorship, emotional support and encouragement.

I appreciate everyone for providing me such a delightful experience of abroad studying and a wonderful environment to complete my thesis and Master's degree at Marshall University.

CONTENTS

LIST OF TABLES

LIST OF FIGURES

SYMBOLS AND NOMENCLATURES

PTIP……Pax2 Transactivation domain Interaction Protein

H3K4……..Histone 3 Lysine 4

RT-PCR ……... Reverse Transcriptional – Polymerase Chain Reaction

ATM…….....……. Ataxia Telangiectasia-Mutated

ATR……..... Ataxia Telangiectasia- and RAD3-related

MLL……... Mixed Lineage Leukemia

HMT…….……Histone MethylTransfera

dNTP…….……..deoxyribonuleotide Triphosphate

PBS…….....…….Phosphate Buffered Saline

EDTA……...……Ethylenediamineteraacetic Acid

APS……...……. Ammonium Persulfate

TBST……Tris-Buffered Saline and Tween 20

GAPDH……..……glyceraldehyde-3-phosphate dehydrogenase

ABSTRACT

In mammals, spermatogenesis is a biological process inside the testis to produce spermatozoa from spermatogonia. This process is governed by both genetic and epigenetic mechanisms and thus is a powerful system for epigenetic research. Methylation of histone 3 lysine 4 (H3K4) is an epigenetic mark, which has been found to be dynamically modulated in mouse male germ cells during spermatogenesis. Pax2 Transactivation domain Interaction Protein (PTIP) has been recently identified as part of a H3K4 methyltransferase complex. In this study, I hypothesize that PTIP is an essential epigenetic regulator in mouse spermatogenesis. To test this hypothesis, I first established a transgenic mouse model with conditional knockout of PTIP in postnatal male germ cells. Then I performed in vivo fertility assay, in vitro sperm assay, testis histology and Western blot. I found that PTIP conditional knockout males were as healthy as wild-type males, but they were infertile, had small testes, and did not produce sperm. In addition, our data demonstrated that H3K4 methylation was decreased in PTIP knockout testes. Taken together, our results validate my original hypothesis. Information collected from this study improves our understanding of male germ cell epigenome, offers novel insights into idiopathic infertility in men, and may provide new strategies for male contraception and assisted reproduction.

CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

Epigenetics

The term epigenetics describes the mitotic and meiotic modifications in genome function without a change in the DNA nucleotide sequence. These epigenetic changes may be heritable through cell division to next generations. In molecular terms, epigenetics represents a range of chromatin modifications including DNA methylation, histone modifications, remodeling of nucleosomes and higher order of chromatin reorganization.

In mammalian genomes, DNA methylation is considered to be primary context of the CpG dinucleotide. There are three main active enzymes involved in DNA methylation: DNA methyltransferase 1, DNA methyltransferase 3a and DNA methyltransferase 3b. They build and establish the DNA methylation patterns. DNA methylation is a repressive epigenetic modification and generally associated with gene silencing [1]. Histone modifications, on the other hand, are post-translational and significantly affect chromatin structure [2, 3]. Epigenetic regulators, such as histone acetyltransferase, methyltransferase, chromatin remodeling enzymes, fundamentally control gene expression through modifications of local chromatin.

Through controlling gene expression, epigenetic modifications define the phenotype of a cell without affecting the genotype, but the daughter cells have the same genotype as the parental cell [4]. This term epigenetics is also used to explain many parts of the transmission or alterations of phenotypic traits [5, 6]. All epigenetic mechanisms in a cell define its overall epigenetic state, which is called epigenome of the cell. Notably, the epigenome is dynamically altered upon changes of in vivo milieu (such as availability of growth factors) and/or in vitro environment (such as stress).

Hence, through epigenetic regulation, one single genome can result in the changes of cellular fate decision, cellular identity and tissue/body homeostasis. It is undoubtedly important to investigate how epigenetic regulators control cell differentiation and development through differential gene expression patterns. Thereby, doing research on novel epigenetic regulators, such as PTIP in this study, will provide new perspectives on human life and wellness.

Spermatogenesis

In mammals, spermatogenesis is a biological process in the testis to produce functional male gametes spermatozoa from stem cells spermatogonia. This process includes proliferation, differentiation and morphogenesis of male germ cells inside the seminiferous tubules, which also contain supporting somatic cells called Sertoli cells [7]. Inside a seminiferous tubule, spermatogonia reside nearby the basal lamina; spermatozoa locate in the lumen; and the Sertoli cell extends from the basal lamina to the lumen. Leydig cells, which belong to another somatic cell type in the testis and produce androgens, position in between the seminiferous tubules.

From the view of the cell cycle, each wave of spermatogenesis encompasses the consecutive mitosis, meiosis I, meiosis II and spermiogenesis. One wave of spermatogenesis takes about 74 days in human and 35 days in mouse. Mouse spermatogenesis starts shortly after birth, while human spermatogenesis does not commence until puberty. The first wave of mouse spermatogenesis is illustrated in Figure 1.

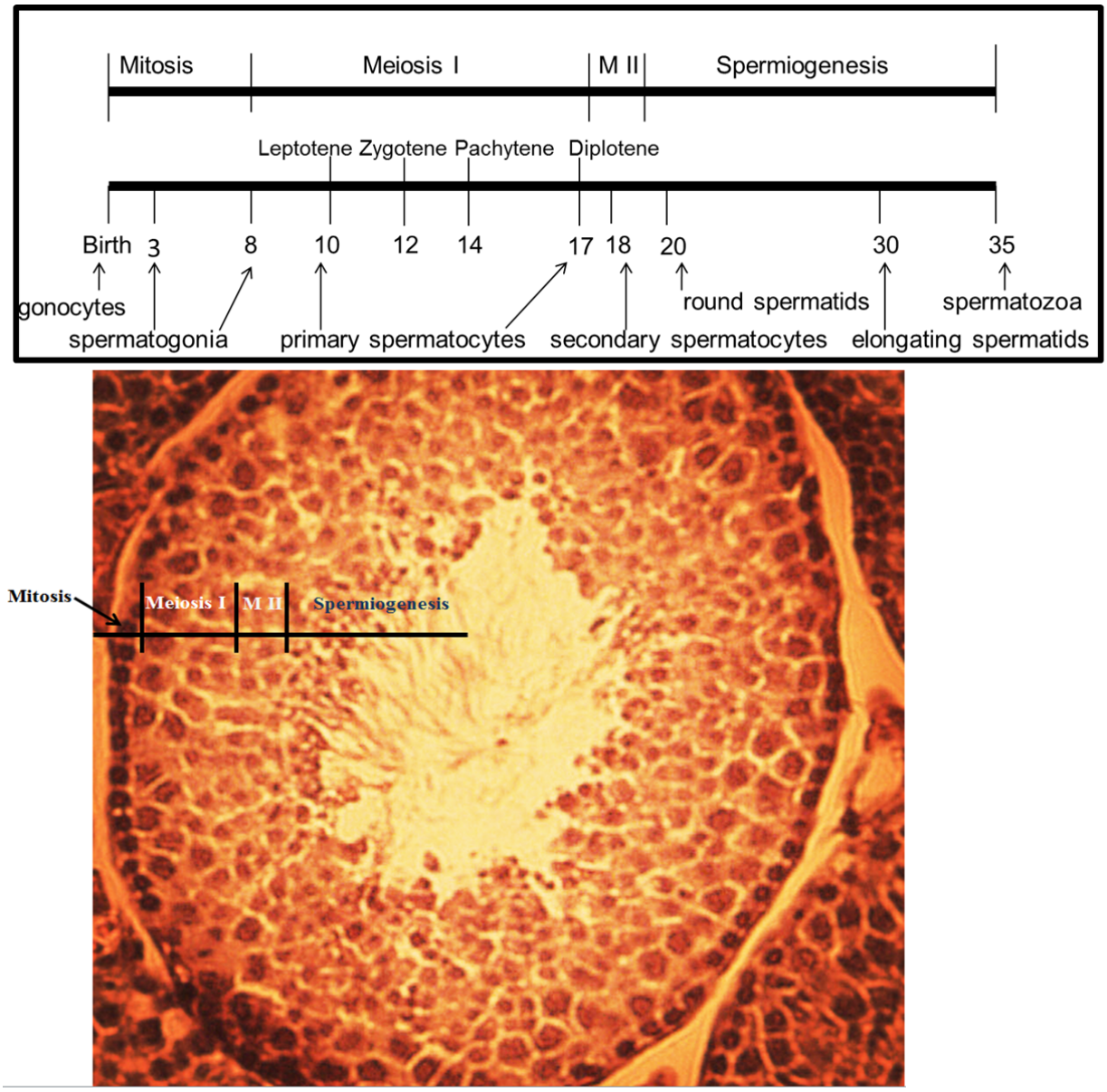

Figure 1. Upper panel: timeline of the first wave of mouse male germ cell differentiation and development (from birth to postnatal day 35). Lower panel: cellular events of spermatogenesis in one seminiferous tubule.

Stem cells spermatogonia (diploid, descendants of gonocytes) appear on postnatal day 3. They undergo a couple rounds of mitotic proliferation until postnatal day 8, and then enter meiosis I to produce primary spermatocytes (diploid) around postnatal day 10. During meiosis I, double-strand DNA break occurs at the leptotene stage; the homologous chromosomes are paired at the zygotene stage; the exchange of genetic materials between

maternal and paternal chromosomes happens at the pachytene stage; and the separation of homologous chromosomes take place at the diplotene stage. After meiosis I (around postnatal day 18), the secondary spermatocytes (haploid) undergo meiosis II to separate individual chromatid strands and generate round spermatid cells (haploid). Finally, round spermatids develop into spermatozoa (haploid) through the process of spermiogenesis, which is the differentiation program including enormous morphogenetic transformation [10].

It is now known that spermatogenesis is governed by both genetic and epigenetic mechanisms [8, 9]. Thus, this process (Figure 1) is a powerful system for epigenetic research because it involves the complicated transcriptional regulation, dynamic chromatin remodeling and remarkable genome reorganization. Indeed, several recent studies have shown the critical role of epigenetic mechanisms in genomic imprinting and meiotic recombination by investigating the spermatogenesis process [11]. However, little is known about the influence and interaction of epigenetic factors in male germ cells. Male germ cells are unique among the various cell types that constitute an animal body [12]. Therefore, my research about epigenetic regulation of male germ cells is interesting and significant. Completion of this study will offer new views on the germ cell epigenome and broadly define an epigenetic mechanism.

The PTIP gene

In my project, I focused on the gene--Pax2 Transactivation domain Interaction Protein (PTIP). PTIP is a ubiquitously expressed nuclear protein originally identified through a yeast two-hybrid screen. PTIP is a big protein (Figure 2), which contains six BRCT (BRCA1 C Terminus) domains (two on the amino-terminus and four on the carboxyl terminus) and one glutamine-rich region [13]. It has been shown to be essential for embryogenesis. PTIP-null in mice leads to embryonic death at E9.5 due to widespread cell death [14]. In addition, another predominant and important function of PTIP may be involved in the DNA damage repair by

the BRCT domains as phosphoprotein binding. Following ionizing radiation, the histone variant H2AX is phosphorylated by DNA-damage activated ataxia telangiectasia-mutated (ATM) and ataxia telangiectasia- and RAD3-related (ATR). Indeed, the PTIP has been shown to interact with 53BP1 in response to DNA damage [15, 16].

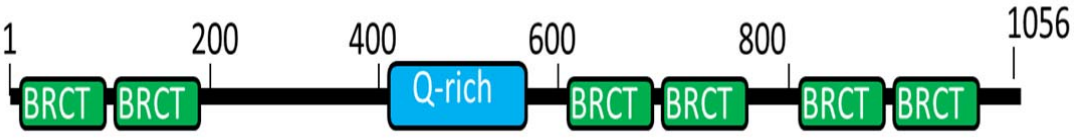

Figure 2. The protein domain structure of PTIP. BRCT=BRCA1 carboxyl-terminal domain; Q-rich=glutamine-rich region.

Subsequently, several studies have demonstrated that PTIP is part of a histone 3 lysine 4 (H3K4) methyltransferase complex [17, 18]. The histone methyltransferase complex contains histone H3K4 methyltransferase MLL3, MLL4, and histone H3K27 demethylase UTX (a JmjC domain-containing protein) [19, 20]. PTIP has been linked to gene activation through association with these two H3K4 methyltransferases [21]. However, the MLL3/4 complexes may not be involved in DNA damage repair. It is thought that PTIP responds to DNA damage independently with the MLL3/4 protein complexes. PTIP is also involved in immunoglobulin class switch DNA recombination in mature B cells [22] and DNA double-strand break repair in somatic cells [23, 24]. Furthermore, PTIP-null mutant mouse embryos showed reduced levels of methylated H3K4. However, there is no evidence that H3K4 methylation is the underlying mechanism for the function of PTIP in DNA double-strand break repair. Nonetheless, PTIP is capable of influencing chromatin architecture and regulating genome function. These studies clearly attest that PTIP is an epigenetic regulator in somatic cells. A role of PTIP in germ cells, however, has not been elucidated.

CHAPTER TWO

HYPOTHESIS, SPECIFIC AIMS AND MOUSE MODEL

Hypothesis

In this study, I aim to test the hypothesis that PTIP is an essential epigenetic regulator in male germ cells during spermatogenesis. I used mouse spermatogenesis as the research system to examine this hypothesis. Spermatogenesis is a unique form of cellular differentiation and development involving remarkable genome reorganization and epigenome modifications in male germ cells. However, our understanding of the underlying molecular events and molecular players are still limited. Thus, this study is designed to offer novel insights into the epigenetic regulation of spermatogenesis. In addition, the understanding of male germ cell differentiation and development may lead to new strategies to improve human wellness.

Hypothetical working model

Our laboratory recently found that one epigenetic mark, methylation of histone 3 lysine 4 (H3K4), was dynamically modulated in mouse male germ cells during spermatogenesis. In immunohistochemistry assay, H3K4 di-methylation (H3K4me2) and H3K4 tri-methylation (H3K4me3) was clearly detected in spermatogonia, preleptotene spermatocytes, leptotene spermatocytes, zygotene spermatocytes, spermatids and to a less staining intensity, pachytene spermatocytes. H3K4 methylation has been linked to transcriptional activation [27] and meiotic recombination [28].

Our laboratory also found that PTIP and its associated H3K4 methyltransferases MLL3 and MLL4 were all expressed in spermatogonia, leptotene/zygotene spermatocytes, pachytene spermatocytes and round spermatids. Thus, I speculated that PTIP and its

associated histone-modifying enzymes might be involved in regulating epigenome remodeling during male germ cell development.

In this study, I used a hypothetical working model (Figure 3) to test my hypothesis: PTIP is an essential epigenetic regulator during spermatogenesis. In this working model (Figure 3A), PTIP interacts with an unknown DNA-binding protein X (likely a transcription factor) to promote the assembly of the histone methyltransferase complex (HMT complex) on the specific chromatin regions. Then the HMT complex can methylate histone 3 lysine 4 (H3K4). This methylated H3K4 subsequently remodels chromatin into an active state, which in turn promotes spermatogenesis (probably through activating gene expression). On the other hand, without PTIP (such as in the case of genetic mutation or artificial knockout) (Figure 3B), the HMT complex cannot methylate H3K4. So the chromatin is in a repressed state and blocks spermatogenesis (probably through repressing gene expression).

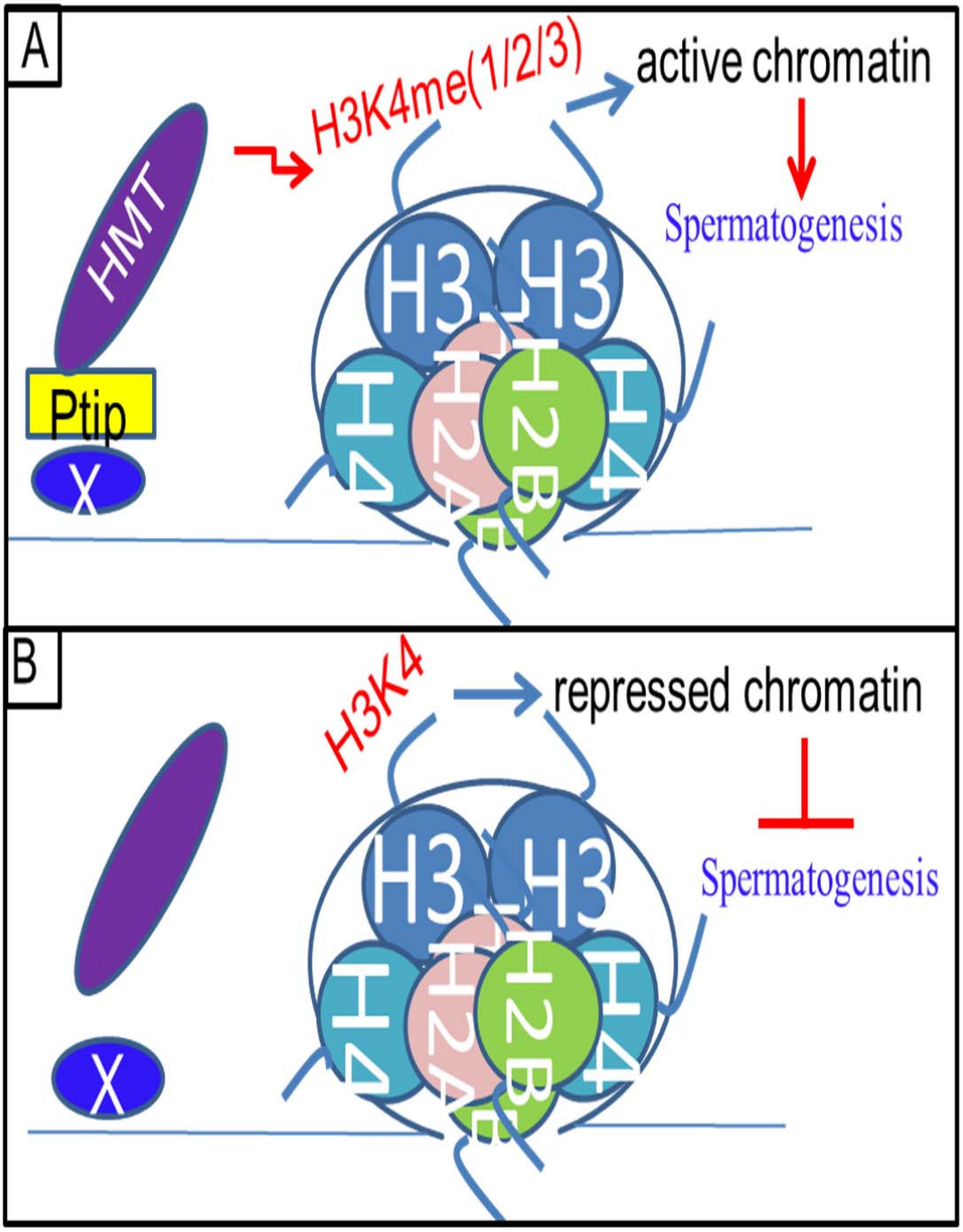

Figure 3. A hypothetical model of PTIP-mediated epigenetic regulation of spermatogenesis. HMT, Histone methyltransferase complex; H3k4me(1/2/3), histone 3 lysine 4 mono-, di-, and trimethylation; X, unknown PTIP-interacting protein.

Specific Aims

Specific Aim 1. Generate mutant mice with conditional deletion of PTIP in postnatal male germ cells

The transgenic mouse model with conditional knockout of PTIP in postnatal male germ cells can be generated through the stra8 Cre-*Lox*P recombination system. The genotyping is used to determine the mutant and wild type mice. Also, RT-PCR is used to test the efficiency of PTIP knockout.

Specific Aim 2. Examine male fertility of PTIP mutant mice

The in vivo fertility test is used to compare the fecundity between wild-type and PTIP mutant mice.

Specific Aim 3. Investigate spermatogenesis of PTIP mutant mice

Through in vitro assays of testis morphology, testis histology and sperm, the process of spermatogenesis in both wild-type and PTIP mutant mice are studied.

Specific Aim 4. Determine histone H3K4 methylation in PTIP mutant testis

Western blot is used to determine histone H3K4 methylation in PTIP mutant testis.

Conditional knockout of PTIP in postnatal male germ cells

Conventional knockout of PTIP in mice leads to embryonic death at E9.5 due to widespread cell death [14]. However, a new innovative technique to study PTIP's function in a tissue or cell specific matter is available. This new technique is conditional knockout of PTIP in postnatal male germ cells to carry out functional studies. In a conditional knockout mouse model, the critical exons of the target gene are flanked by the LoxP sequences, which can be specifically recognized by Cre recombinase and deleted from the genome. Thus, by

breeding the LoxP mice (target gene locus flanked by LoxP) with tissue/cell-specific Cre-expressing mice (Cre deleters), it is possible to specifically delete the flanked gene region and inactivate the gene in desired tissues/cells, while the target gene remains functional in all other tissues/cells.

In my study, I use the stra8 Cre-*Lox*P recombination system to excise out the target gene PTIP in postnatal male germ cells while PTIP is still expressed in all other cell types including somatic cells (Sertoli cells and Leydig cells) inside the testis. The mouse breeding strategy is shown in Figure 4. The mouse genotyping is verified by PCR on tail genomic DNA. In Stra8-Cre mice, the Cre recombinase only expresses in postnatal male germ cells [33]. In PTIP f/f mice, exon 1 of the PTIP gene is flanked by two *Lox*P sites [25, 26]. Thus, in PTIP f/f; Stra8-Cre mice, the Cre recombinase can specifically bind to the *Lox*P sites of PTIP and then excise out exon 1 of PTIP in postnatal male germ cells. Through this way, a transgenic mouse model with conditional knockout of PTIP in postnatal male germ cells is generated. This mouse model is extremely useful for studying PTIP's function in postnatal male germ cells during the biological process of spermatogenesis.

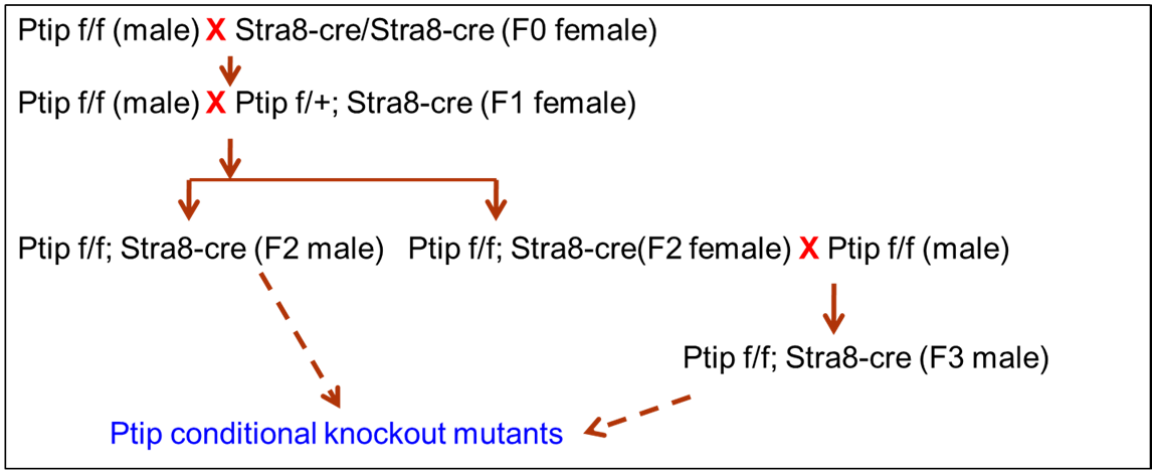

Figure 4. Generation of mutant mice with PTIP conditional knockout in male germ cells.

CHAPTER THREE

METHODS AND MATERIALS

Cell Culture

Human breast cancer cell line MCF7 (ATCC®, HTB-22™, Rockville, MD, USA) was cultured as described in the manufacture's instruction. Briefly, cells were cultured with the culture medium Eagle's Minimum Essential Medium (EMEM) (ATCC®, 30-2003™, Rockville, MD, USA) supplemented with 10% fetal bovine serum (FBS) (ATCC®, 30-2020™, Rockville, MD, USA) and penicillin-streptomycin (100 IU/ml) (Fisher Scientific, Pittsburg, PA, USA) at 37^0C in a humidified atmosphere with 5% CO_2 (Thermo Scientific Heracell 150i CO_2 Incubator, Waltham, MA, USA). To avoid contamination, cell culture was performed with a Ultra-Violet (UV) sterilizing hood (Thermo Scientific Cell Culture Hood 1300, Waltham, MA, USA).

Cells were passaged when the culture reached 70-80% confluence as described below. The culture medium was removed from the culture plate. Cells were rinsed once with 5-10 ml of phosphate buffered saline (PBS) (Fisher Scientific, Pittsburg, PA, USA). Then, 1.5 ml of Trypsin-EDTA solution (Fisher Scientific, Pittsburg, PA, USA) was added into the culture plate (Fisher Scientific, Pittsburg, PA, USA). The plate was incubated at 37^0C in a humidified atmosphere with 5% CO_2 for 5 minutes to allow the cells to detach. After incubation, 5 ml of the culture medium was added into the culture plate. The cells were aspirated by gently pipetting and transferred into a 15ml tube. The tube was centrifuged for 5 minutes at 1400rpm at room temperature. After centrifugation, the supernatant was discarded and the cell pellet was re-suspended in 5 ml of the culture medium by gently pipetting. An appropriate aliquot of the cell suspension was added into a new culture plate which contained 10 ml of the fresh culture medium. Lastly, the plate was incubated 37^0C in a humidified

atmosphere with 5% CO_2 (Thermo Scientific Heracell 150i CO_2 Incubator, Waltham, MA, USA).

To determine cell viability, 10 µl of the cell suspension and 10 µl of trypan blue (Fisher Scientific, Pittsburg, PA, USA) were added into a 1.5ml tube and gently mixed. After that, 10ul of the mixture was placed in each chamber of a hemocytometer (Fisher Scientific, Pittsburg, PA, USA). Cells were observed with a vistavision microscope (VWR, Radnor, PA, USA). Dead cells were stained in blue, while live cells appeared bright. Cells in the central square were counted. If cells were on the border outlining the central square, only the cells on the top and left borders of the square were counted. Cell concentration was then calculated by this equation: [number of (dead or live) cells] $\times 2 \times 10^4$ = (cells /ml).

Mouse Breeding

All animal care and use procedures described within were reviewed and approved by the Institutional Animal Care and Use Committee of Marshall University, and were performed in accordance with the Guiding Principles for the Care and Use of Laboratory Animals. Stra8-Cre mice [33] were originally from The Jackson Laboratory (Bar Harbor, Maine, USA). PTIP f/f mice [25, 26] were originally from the University of Michigan Ann Arbor (Ann Arbor, MI, USA). The mouse breeding strategy was illustrated previously (Figure 4).

Mouse Genotyping

Polymerase chain reaction (PCR) [34] on mouse tail genomic DNA was used to determine mouse genotypes, as described below. When the mouse was 17 days old, about 2-3mm mouse tail was snipped off and placed in a 1.5 ml micro-centrifuge tube. Then, 75ul of 50mM sodium hydroxide (NaOH) was added into the tube. The tube was incubated in a heating block (Fisher Scientific, Pittsburg, PA, USA) at 95 degrees for 30 minutes. After

incubation, the tube was taken out, spun, and cooled to room temperature. Then, 75ul of 50mM hydrochloric acid (HCl) was added into the tube to neutralize the solution, followed by addition of 15ul 1M Tris-HCl pH8 (Fisher Scientific, Pittsburg, PA, USA). The tube was mixed using a vortex mixer (Fisher Scientific, Pittsburg, PA, USA), and centrifuged at room temperature at 12000rpm for 10 minutes. The supernatant (containing the genomic DNA) was stored at 4 degrees.

Polymerase Chain Reaction (PCR)

One microliter of the genomic DNA solution (described above) was used as template in the PCR reaction with a total volume of 30ul. The other PCR components were 3ul of 10X reaction buffer (New England Biolabs, Ipswich, MA, USA), 0.6ul of deoxynucleotide (dNTP) mix (10mM) (Invitrogen, Grand Island, NY, USA), 0.6ul of Taq polymerase (New England Biolabs, Ipswich, MA, USA), 0.6ul of (Stra8 or PTIP) PCR primers (10uM) (Invitrogen, Grand Island, NY, USA), and 24.2ul of PCR-grade water (Invitrogen, Grand Island, NY, USA). The primers for Stra8 were ic202F 5'- GTG CAA GCT GAA CAA CAG GA-3' and ic381R 5'- AGG GAC ACA GCA TTG GAG TC-3'. The primers for PTIP were PTIP-B2(129) 5'-GGG AAC TGA TCT TCG ATG AGG-3' and PTIP-F1(795) 5'-GGT TCT CTT GCA GCA TCT CC-3'. The PCR parameters were 94℃ 3 min; 94 ℃ 30s, 58 ℃ 30s, 72 ℃ 1 min, 30 cycles; 72 ℃ 6 min; held at 4 ℃. The TGradient thermocycler was from Biometra (Göttingen, Germany).

Gel Electrophoresis

To make a gel, agarose powder (Fisher Scientific, Pittsburg, PA, USA) was mixed with the electrophoresis buffer 1X TAE (40mM Tris, 20mM acetic acid and 1mM EDTA, Fisher Scientific, Pittsburg, PA, USA) to 1.5% (W/V), then heated in a microwave oven until completely melted. After cooling the solution to about 50℃, ethidium bromide was added to

the solution (final concentration 0.5 μg/ml). The mixed solution was then poured into a casting tray containing a 10-well comb (Fisher Scientific, Pittsburg, PA, USA) in a chemical fume hood. After the solution was solidified to become a gel, the comb was pulled out. The gel was placed in a running apparatus (Fisher Scientific, Pittsburg, PA, USA) and covered with the running buffer 1X TAE. Ten microliter of the PCR products was loaded into the gel wells. The gel was run at 110 voltages for 30 minutes. After that, the gel image was captured by the Alpha Innotech gel documentation system (now the AlphaImager gel documentation system from ProteinSimple, San Jose, California, USA), according to the manufacture's instruction.

Testis Morphology and Histology

The mice (2-month-old) were terminated with carbon dioxide (CO_2) and the body weight was measured. The testes were isolated, immediately placed on ice, and weighed. Then the testes were fixed in 4% paraformaldehyde (Fisher Scientific, Pittsburg, PA, USA) for at least 24 hours at room temperature. After fixation, the testes were sent to School of Medicine, Marshall University for tissue sectioning. The testes were first embedded in paraffin, cut with a microtome (6 micron thickness) and stained with hematoxylin and eosin. The tissue slides were observed with the Leica DMI4000 microscope (Leica microsystems, Buffalo Grove, IL, USA). The histology images were captured with a digital camera (Leica microsystems, Buffalo Grove, IL, USA).

Sperm Analysis

The mice (2-month-old) were terminated with carbon dioxide (CO_2). The epididymides were isolated from the animal body, placed in a petri dish with 1.5ml of phosphate buffered saline (Fisher Scientific, Pittsburg, PA, USA), and cut 8 times with a dissecting scissor. The dish was sit at room temperature for 15 minutes so that the sperm

could be released from the epididymides. After that, the sperm solution was filtered through a nylon mesh to get rid of big tissue pieces. An aliquot of 15µl of the filtered sperm solution was added on a glass slide and covered with a glass slip. The sperm sample was observed with the Leica DMI4000 microscope (Leica microsystems, Buffalo Grove, IL, USA). The sperm images were captured with a digital camera (Leica microsystems, Buffalo Grove, IL, USA).

Sperm Immunofluorescence

The filtered sperm solution was prepared as described above and centrifuged at room temperature at 5,000rpm for 3 minutes. The supernatant was discarded and the sperm pellet was re-suspended in 500µl of 0.3% (W/V) bovine serum albumin (Sigma-Aldrich, St. Louis, MO, USA) in phosphate buffered saline (Fisher Scientific, Pittsburg, PA, USA). An aliquot of 200µl of the sperm suspension was added into a 1.5 ml micro-centrifuge tube, which contained either the primary antibody Ptchd3-Ab1 (10µg /ml) [35] or no primary antibody. The tube was sit at room temperature for one hour and then centrifuged at room temperature at 5,000rpm for 3 minutes. The supernatant was discarded and the sperm pellet was re-suspended in 200µl of 0.3% (W/V) bovine serum albumin (Sigma-Aldrich, St. Louis, MO, USA) in phosphate buffered saline (Fisher Scientific, Pittsburg, PA, USA). The tube was centrifuged at room temperature at 5,000rpm for 3 minutes. The supernatant was discarded and the sperm pellet was re-suspended in 200µl of 0.3% (W/V) bovine serum albumin in phosphate buffered saline. Then, 1.5µl of Alexa-488 goat anti-rabbit secondary antibody (Life Technologies, Grand Island, NY, USA) and 1.5µl of 4',6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich, St. Louis, MO, USA) were added into the tube. The tube was covered with aluminum foil to minimize light exposure and incubated at room temperature for 45 minutes by rotation. After incubation, the tube was centrifuged at room temperature at 5,000rpm for 3 minutes. The supernatant was discarded and the sperm pellet was re-

suspended in 100µl of 0.3% (W/V) bovine serum albumin in phosphate buffered saline. An aliquot of 15µl of the re-suspended sperm solution was added on a glass slide and covered with a glass slip. The sperm sample was observed with the Leica DMI4000 microscope (Leica microsystems, Buffalo Grove, IL, USA). The sperm images were captured with a digital camera (Leica microsystems, Buffalo Grove, IL, USA).

RNA Extraction from Mouse Testis

RNA was extracted from mouse testes using the method of Chomczynski and Sacchi [36]. Briefly, the mice (2-month-old) were terminated with carbon dioxide (CO_2). The testes were isolated and immediately placed on ice. The testis (wild-type: half testis; PTIP knockout: whole testis) was cut with a dissecting scissor to remove the outer tissue layer of tunica albuginea and placed in a 1.5 ml micro-centrifuge tube with 300 µl of the RNA extraction buffer Trizol (Ambion, Grand Island, NY, USA). The tube was vortexed vigorously to break off tissues and kept at room temperature for 30 minutes. After that, 60 µl of chloroform (Sigma-Aldrich, St. Louis, MO, USA) was added into the tube. The tube was vortexed for 15 seconds. The mixture was incubated for 10 minutes at room temperature. The tube was then centrifuged for 10 minutes at 12,000 rpm at 4℃. After centrifugation, the aqueous phase was transferred to a fresh tube carefully. After addition of 150 µl of 2-propanol (Sigma-Aldrich, St. Louis, MO, USA), the tube was vortexed for 10 seconds and incubated for 10 minutes at room temperature. Then, the tube was centrifuged for 10 minutes at 12,000rpm at 4℃. The supernatant was discarded carefully by pipetting and the RNA pellet was washed with 250 µl of 70% ethanol (Fisher Scientific, Pittsburg, PA, USA) twice. The RNA pellet was then air dried at room temperature for 7 minutes and then dissolved in 60 µl of nuclease-free water (Ambion, Grand Island, NY, USA) for 5 minutes at room temperature. The RNA concentration was determined by a Nanodrop Lite spectrophotometer (Thermo Scientific, Waltham, MA, USA).

Reverse Transcription –Polymerase Chain Reaction (RT-PCR)

Reverse transcription (RT) was performed using a kit from Clontech Laboratories (Mountain View, CA, USA). Two reaction tubes were set up. One tube contained 1 μg of total RNA from the wild-type mouse PS 257. The other tube contained 1 μg of total RNA from the PTIP mutant mouse PS 263. Two microliter of random primer (600 pmol/μl) was added into each tube. PCR-grade water was added to make a total volume of 13 μl. The reaction tubes were placed in the TGradient thermocycler (Biometra, Göttingen, Germany) and incubated at 65°C for 10 minutes. After incubation, the tubes were kept on ice. The 5X reaction buffer (4μl), dNTPs (10mM) (2μl) and reverse transcriptase (1μl) were added into each tube. The reagents in the tube were carefully mixed by flicking. The tubes were briefly centrifuged to collect the reagents to the bottom of the tube. Then, the tubes were placed in the TGradient thermocycler (Biometra, Göttingen, Germany) and incubated at 25°C for 10 minutes, then at 50°C for 60 minutes, followed by 5 minutes at 85°C to inactivate the reaction. After that, 80 μl of the elution buffer (EB) (QIAGEN, Valencia, CA, USA) was added into each tube. The resultant cDNA samples were stored at -20°C.

Polymerase Chain Reaction (PCR) was carried out as described in the previous section. Briefly, 1 μl of the above cDNA sample was used as template. The primers for β-actin were actin-forward 5'-GTG GGC CGC TCT AGG CAC CAA-3'and actin-reverse 5'-CTC TTT GAT GTC ACG CAC GAT TTC- 3'. The primers for PTIP were mPtip-1F 5'-AGT GAA GGT GAC TGC AGA GCT-3' and mPtip-1R 5'- TCA GGA CAA ACT CCG CAT TG-3'. The PCR parameters were as follows: 3 minutes at 94℃; 94℃ for 45 seconds, 58℃ for 45 seconds, 72℃ for 1 minute and 30 seconds, 30 cycles; and a final extension at 72℃ for 10 minutes.

Gel electrophoresis of the PCR products was carried out essentially as described in the previous section. The signal intensity of the PCR product bands were recorded by the Alpha Innotech gel documentation system (now the AlphaImager gel documentation system from ProteinSimple, San Jose, California, USA), according to the manufacture's instruction..

Protein Extraction from Mouse Testis

The mice (2-month-old) were terminated with carbon dioxide (CO_2). The testes were isolated and immediately placed on ice. The testis (wild-type: half testis; PTIP knockout: whole testis) was cut with a dissecting scissor to remove the outer tissue layer of tunica albuginea and placed in a 1.5 ml micro-centrifuge tube with 300 µl of the radioimmunoprecipitation assay buffer (RIPA) (Sigmal-Aldrich, St. Louis, MO, USA). The tube was vortexed and then kept on ice for 30 minutes. After that, the tube was centrifuged for 10 minutes at 12,000rpm at 4℃. The supernatant (containing proteins) was carefully transferred to a new 1.5 ml tube. The protein concentration was determined by a Nanodrop Lite spectrophotometer (Thermo Scientific, Waltham, MA, USA).

Western Blot: Protein Separation

Protein separation on a sodium dodecyl sulfate (SDS)-polyacrylamide gel (PAG) was carried out by the method reported previously [37]. Briefly, a SDS-PAG (4% stacking/10% resolving) was first made in the laboratory and placed into the electrophoresis apparatus (Bio-Rad, Hercules, CA, USA) filled with 1X SDS running buffer (25 mM Tris, 192 mM Glycine, 0.1% SDS, pH8.8.). The testis protein sample (100 µg) and 6X reducing buffer (2 µl) were added into a 1.5 ml tube (if needed, water was added to bring up to a total volume of 12 µl). The tube was heated at 100℃ for 3 minutes and then spun to collect the reagents to the bottom of the tube. The heated (denatured) protein sample was loaded into the gel well along

with the protein markers (New England Biolabs, Ipswich, MA, USA). The gel was run at 80 voltages for 10 minutes and then at 100 voltages for 1 hour.

Western Blot: Protein Transfer to the Membrane

After gel electrophoresis was done, the gel was removed from the running apparatus and placed in 1X transfer buffer (48 mM Tris, 39 mM Glycine, 1.3 mM SDS, pH9.0). Proteins were transferred from the gel to the nitrocellulose membrane (Bio-Rad, Hercules, CA, USA) by setting up a sandwich (blot paper, membrane, gel and blot paper) on the transfer machine (Bio-Rad, Hercules, CA, USA). Any air bubbles were carefully rolled out during the sandwich set-up. The transfer was run at 18 voltages for 5 minutes and then at 22 voltages for 40 minutes.

Western Blot: Protein Detection

After protein transfer, the membrane was placed in the blocking solution (3% (w/v) nonfat dry milk, 2.5% (w/v) bovine serum albumin in 1X TBST (10 mM Tris-HCl, 150 mM NaCl, 0.05% Tween, pH 7.5) in a plastic box and then incubated with gentle rotation for one hour at room temperature. After that, the primary antibody anti-GAPDH (Glyceraldehyde 3 phosphate dehydrogenase) (Ambion, Grand Island, NY, USA) or anti-H3K4me2 (Cell Signaling Technology, Beverly, MA, USA) was added into the blocking solution. The membrane was further incubated with gentle rotation for one hour at room temperature. Then, the membrane was washed four times for 5 minutes each with 8ml of 1X TBST. After washing, the membrane was incubated with the secondary antibody conjugated with alkaline phosphatase (1:8000 dilution in 1X TBST) (Promega, Madison, WI, USA) with gentle rotation for one hour at room temperature. Then, the membrane was washed four times for 5 minutes each with 8ml of 1X TBST. After washing, alkaline phosphatase activity on the

membrane was detected by color reaction with Western Blue stabilized substrate (Promega, Madison, WI, USA).

Statistical Analysis

Data were analyzed by Student's t test and presented as mean ±S.D. (standard deviation). Statistical significant differences were defined as $p < 0.05$.

CHAPTER FOUR

RESULTS

Mouse genotyping was determined by PCR for the Stra8 gene

To generate Ptip mutant mice, Ptip f/f; Stra8-cre female mice were crossed with Ptip f/f male mice. The offspring should be Ptip f/f genotype. The offspring's Stra8 genotype was determined by PCR (Figure 5). The Stra8 PCR primers are 20 bp. The expected Stra8 PCR product is 179 bp. The genotype of the offspring with Stra8 positive should be Ptip f/f; stra8-cre and thus be mutant. The genotype of the offspring with Stra8 negative should be Ptip f/f and thus be wild-type.

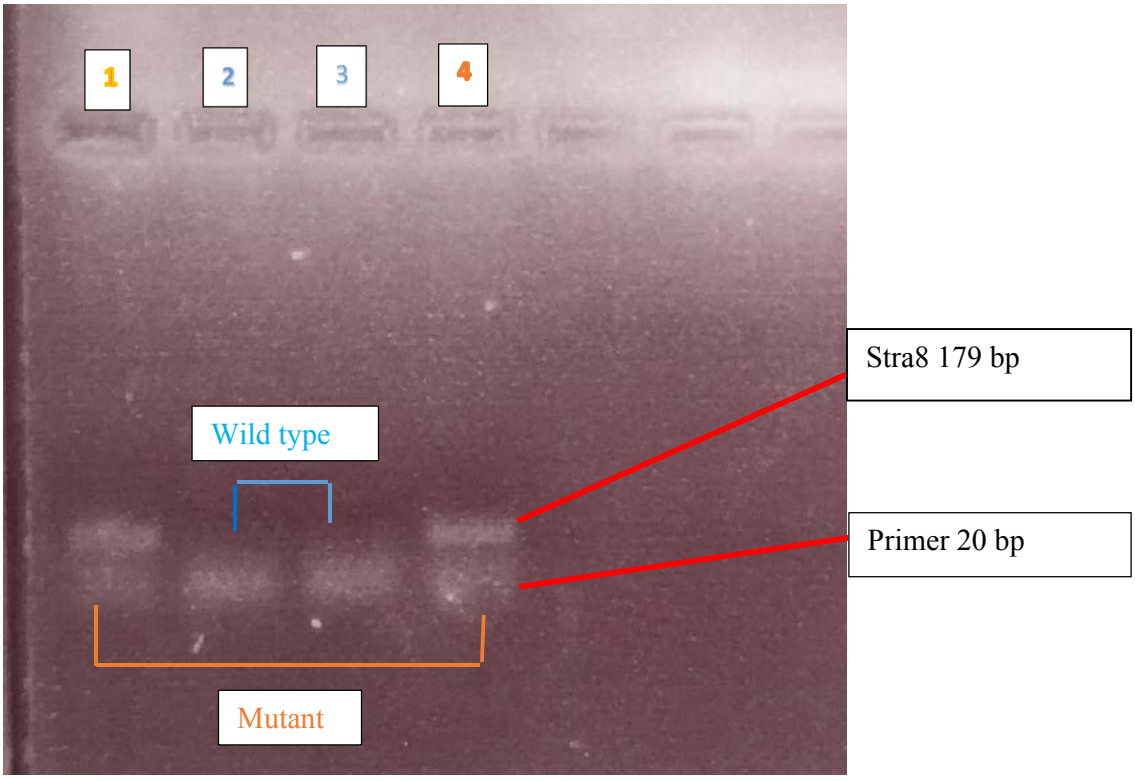

Figure 5. The genotype of the Stra8 gene is determined by PCR. Lane 1 and 4 represent two mutant mice (PTIP f/f; Stra8-Cre). Lane 2 and 3 exemplify two wild-type mice (PTIP f/f). All mice were from the F3 generation.

Therefore, as shown in Figure 5, the first mouse and the fourth mouse are mutant mice with conditional knocked out of PTIP in postnatal male germ cells. The second and third mice are wild-type. Through this genotyping, we could distinguish the wild type mice and mutant mice, and then use them in the following experiments.

Reverse Transcription –Polymerase Chain Reaction (RT-PCR)

After genotyping, I wanted to confirm the efficiency of PTIP conditional knockout in postnatal male germ cells. Hence, I performed RT-PCR to determine the expression levels of PTIP in mutant testes (10-weeks-old) (Figure 6). The expected beta-actin and PTIP PCR product size is 540 bp and 708 bp, respectively.

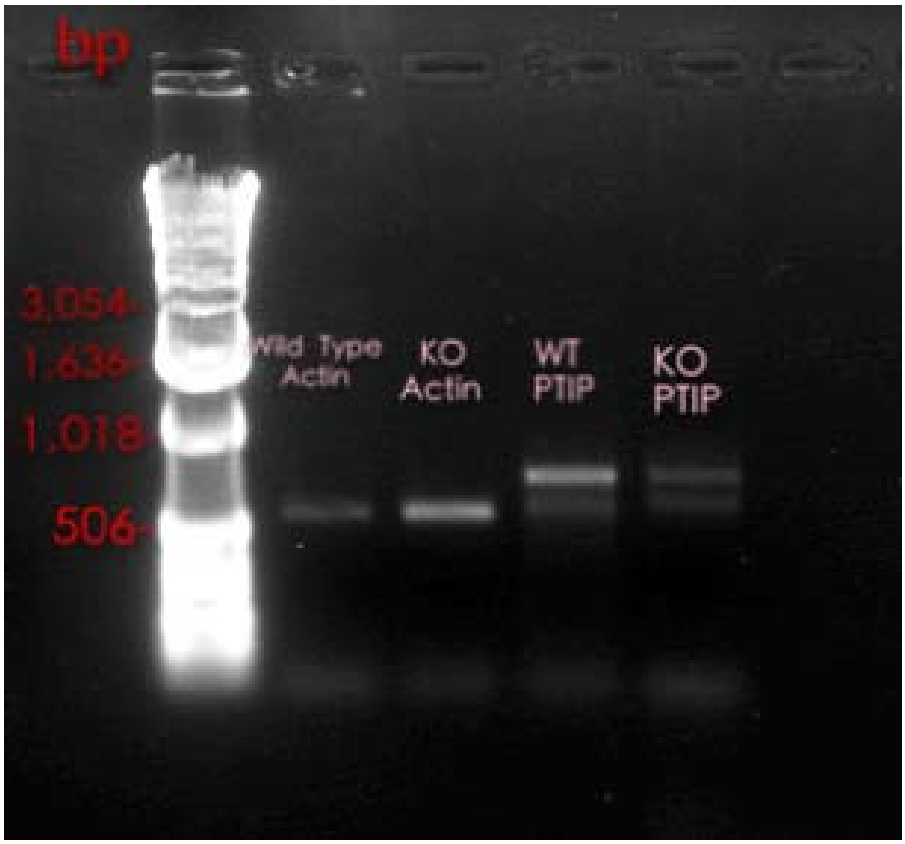

Figure 6. RT- PCR analysis of the PTIP knockout efficiency.

Then, I measured the signal intensity of beta-actin (housekeeping gene as internal control) and PTIP by the Alpha Innotech program (Table 1). As compared to beta-actin, the relative expression level of PTIP is 1.38 and 0.41 in wild-type and mutant testis, respectively. The conditional knockout efficiency is calculated as the percentage of (mutant PTIP level) / (wild-type PTIP level) and thus equals 29.7% (0.41/1.38 x100%). This number indicates that there is still some expression of PTIP in the mutant testis. This result is not surprising since our conditional knockout strategy only deletes PTIP in male germ cells but not in somatic cells (such as Sertoli cells and Leydig cells) in the testis. With this regard, the 29.7% expression level may match and reflect well the percentage of somatic cells in the testis.

mouse genotype	Signal intensity		PTIP/β-actin ratio
	PTIP	β-actin	
wild-Type Ptip f/f	1477.44	1070.82	1.379728
knockout Ptip f/f; Stra8-cre	674.54	1647.16	0.409517

Table 1: Analysis of PTIP expression level by using β-actin as internal control.

Thus, our results demonstrate that the conditional knockout efficiency is high enough (even though I cannot state 100% efficiency) for further phenotypic and functional studies.

Life of the PTIP conditional knockout mice

Unlike embryonic lethality of conventional PTIP knockout mice, the mutant mice (Figure 7, right one) with conditional knockout of PTIP in postnatal male germ cells were

born, grew, behaved, and lived just as normally as wild-type mice (Figure 7, left one). Hence, the mutant mice were suitable to test our hypothesis.

Figure 7. Images of wild-type (left) and mutant (right) mice.

Sterility of the PTIP conditional mutant mice

When the mice were two-months old, I did the in vivo fertility test by putting one male and two females in one cage for a period of two months. All females mated with the wild type (PTIP f/f) males produced offspring normally (Table 2). However, the females

mated with the mutant PTIP f/f; Stra8-cre males did not deliver any baby (Table 2). This result is significant ($p < 0.01$) and clearly attests that conditional knockout of PTIP in postnatal male germ cells leads to male sterility.

Sterility of the PTIP conditional mutant mice		
mouse genotype	litters (from 8 mated females)	averaged litter size (from 8 mated females)
Ptip f/f (n=4)	16	6.3 ± 1.3
Ptip f/f; Stra8-cre (n=4)	0	0

Table 2: In vivo fertility test of PTIP conditional mutant mice

Azoospermia of the Ptip conditional mutant mice

After I found that PTIP conditional mutant mice were infertile (Table 2), I wanted to determine if there was something wrong with sperm. Thus, I analyzed sperm from the epididymis, an accessory reproductive organ for sperm storage before ejaculation. As shown in Figure 8, even though I observed many sperm from the wild-type mouse (10-weeks-old), I barely saw any sperm from the mutant mouse (10-weeks-old). This result is significant ($p < 0.01$) (Table 3) and clearly shows that Ptip conditional mutant mice were azoospermia, suggesting that spermatogenesis might be defect in the mutant testis.

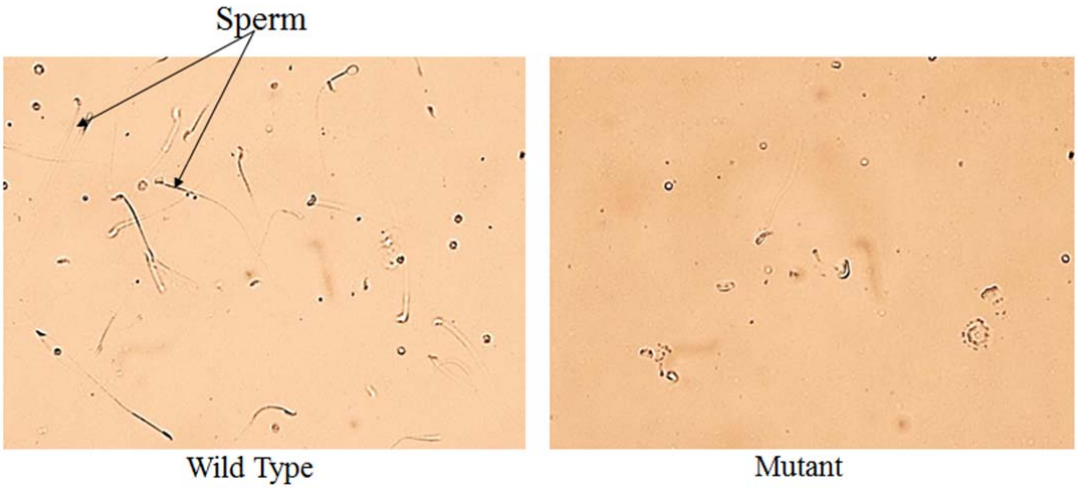

Figure 8. Analysis of epididymal sperm of wild-type and PTIP conditional mutant mice

mouse genotype	epididymal sperm ($x10^7$)
Ptip f/f (n=4)	2.4 ± 0.3
Ptip f/f; Stra8-cre (n=4)	0

Table 3: Sperm number analysis

Small Testis of the Ptip conditional mutant mice

The above results strongly suggest that spermatogenesis may be defect in the mutant testis. Thus, I wanted to analyze the morphology and weight of mutant testis. As shown in Figure 9, the upper four testes were from the wild-type mice (10-weeks-old), and the lower four testes were from the mutant mice (10-weeks-old). Although there was no difference in testis shape, the size of mutant testes was clearly smaller than that of wild-type testes. This result indicated that spermatogenesis was blocked in the mutant testes.

Figure 9. The morphology of wild-type (top fours) and mutant (bottom fours) testes.

Although I did not detect difference in body weight of the wild-type and mutant mice (Figure 10), the weight of the wild-type testes (0.1911 ± 0.0134g) was significantly heavier than that of the mutant testes (0.061 ± 0.0083g) ($p < 0.01$) (Figure 11). This result evidently showed that conditional knockout of PTIP in postnatal male germ cells leaded to small testes, indicating that spermatogenesis was blocked in the mutant testes.

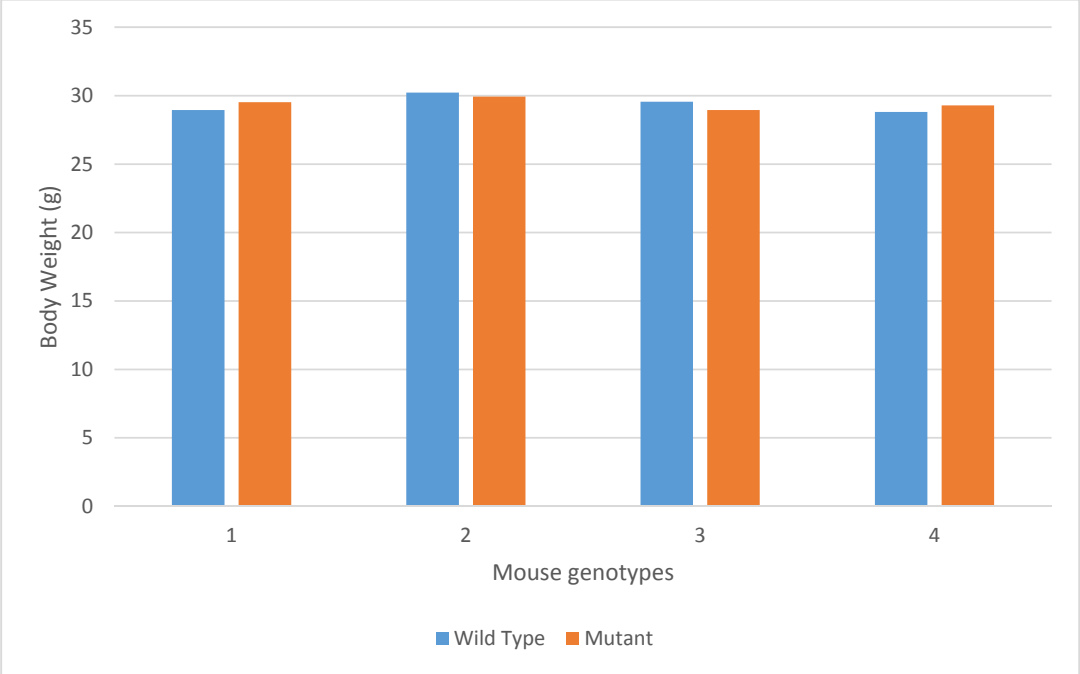

Figure 10. Body weight of the wild-type and mutant mice

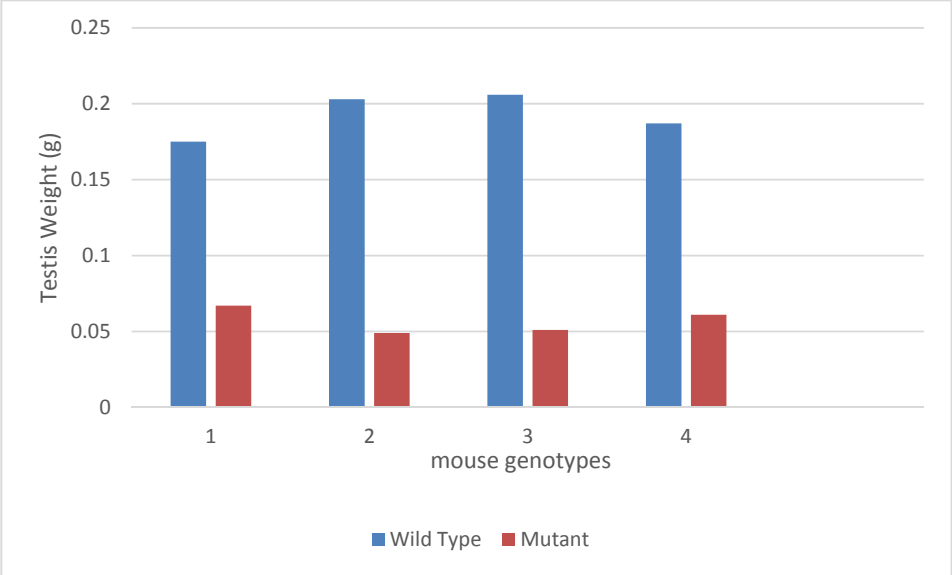

Figure 11. The analysis of testis weight.

Arrested spermatogenesis of the Ptip conditional mutant mice

I then performed testis histology to examine what happened inside the mutant testis (10-weeks-old). As shown in Figure 12, post-meiotic male germ cells (spermatids/spermatozoa) were completely absent in the mutant seminiferous tubules (right panel). Instead, degenerating cells (zygotene-like spermatocytes) were found in the adluminal compartment of the mutant seminiferous tubules (right panel). In contrast, all cell types of the spermatogenic lineage (from spermatogonia to spermatozoa) were present in the wild-type seminiferous tubules (left panel).

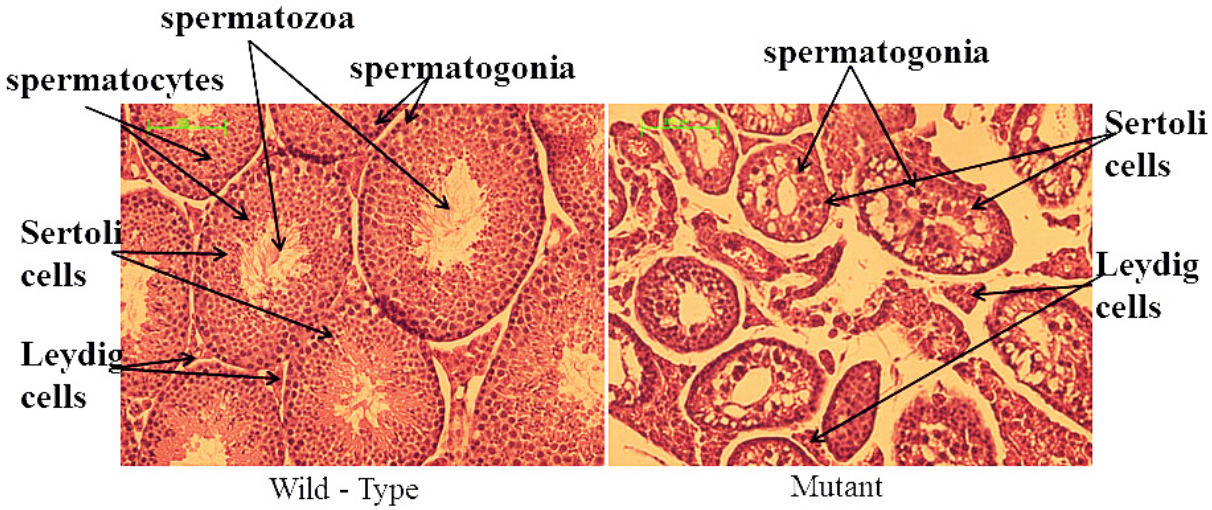

Figure 12. Histology of wild-type and mutant testes. (Scale Bar =100μm)

Spermatogonia (stem cells) lie in the periphery of the seminiferous tubule and appear dark dot (Figure 12, left and right panel). Many spermatozoa with long tail are seen in the lumen of the seminiferous tubule (Figure 12, left panel). In the middle part of the wild-type seminiferous tubule are various stages of developing male germ cells (Figure 12, left panel). Sertoli cells are seen in both wild-type and mutant seminiferous tubules. Leydig cells locate in between both wild-type and mutant seminiferous tubules.

This result is remarkable and confirms that the process of spermatogenesis is arrested in meiosis when PTIP in postnatal male germ cells is conditionally knocked out. This result supports our hypothesis and our working model (Figure 3).

Decreased H3K4 methylation in the Ptip conditional mutant testis

The above findings clearly demonstrated that PTIP conditional knockout in postnatal male germ cells resulted in spermatogenesis arrest, small testes, azoospermia and male sterility. Thus, I wanted to investigate the underlying molecular mechanism. Our working model (Figure 3) hypothesizes that PTIP functions through regulating H3K4 methylation. To address this, I carried out Western blotting on the protein samples prepared from the 10-weeks-old testes. I used GAPDH (glyceraldehyde-3-phosphate dehydrogenase) as the loading control (Figure 13, left panel). The level of H3K4 di-methylation in the mutant testis was noticeably decreased (Figure 13, right panel).

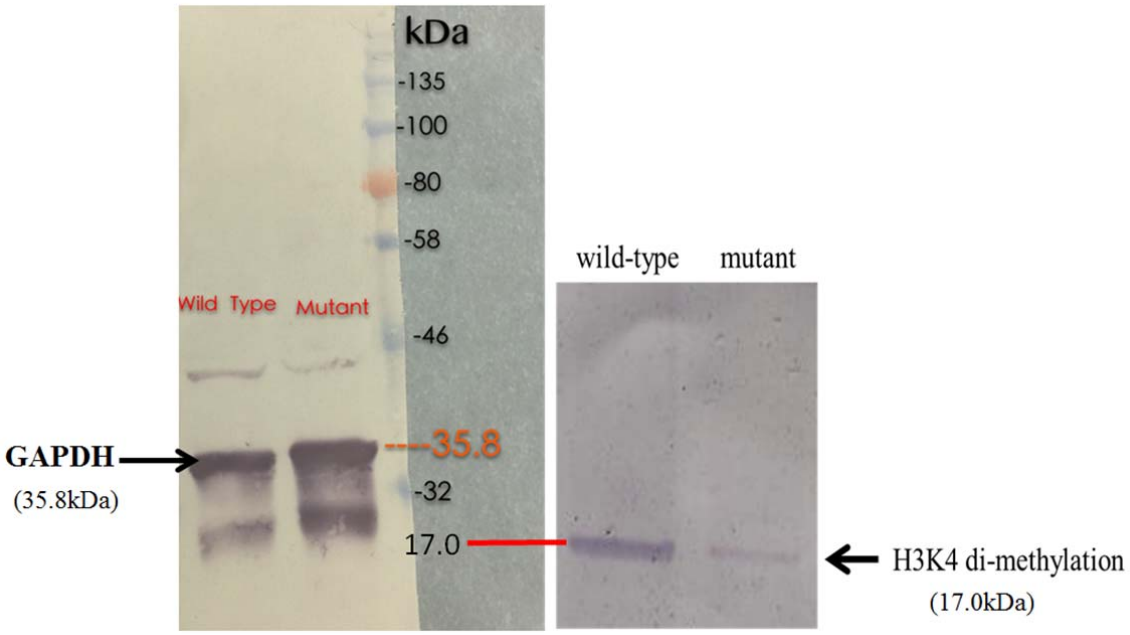

Figure 13. Western blot analysis of H3K4 methylation in the wild-type and mutant testes.

CHAPTER FIVE

CONCLUSIONS

This study was pursued to investigate PTIP's biological function in mouse postnatal male germ cells. In somatic cells, PTIP has been previously identified as a subunit of the histone methyltransferase complex, which includes methyltransferase MLL3/MLL4 and catalyzes methylation of histone H3 lysine 4 (H3K4). Methylation of H3K4 is an epigenetic mark and has been linked to gene activation. In this study, I aim to test the hypothesis that PTIP is an essential epigenetic regulator in male germ cells and is required for mouse spermatogenesis.

I used a range of approaches in this study, including genetics, cell biology, molecular biology, biochemistry, microscopy and bioinformatics. The collected data support the following conclusions.

Our genotyping (Figure 5) and RT-PCR (Figure 6 and Table 1) results indicate that a transgenic mouse line with conditional knockout of PTIP in postnatal male germ cells has been successfully generated. As expected, the PTIP conditional knockout mice lived and grew normally (Figure 7 and Figure 10). Our in vivo fertilization assay demonstrated that the knockout mice were infertile (Table 2). Our in vitro assays revealed that the knockout mice had small testes (Figure 9) and could not produce spermatozoa (Figure 8 and Table 2). The result of testis histology clearly showed that spermatogenesis was arrested in meiosis in the PTIP conditional knockout mice (Figure 12). Furthermore, Western blotting analysis disclosed that H3K4 methylation was decreased in the PTIP conditional knockout testes (Figure 13).

Taken together, these findings clearly validate our original hypothesis and working model. Knowledge gained from this study provides novel insights into epigenetic regulation

in male germ cells during spermatogenesis and may lead to new strategies for assisted reproduction and male contraception.

CHAPTER SIX

DISCUSSION AND FUTURE STUDIES

The protein PTIP is conserved among many species including humans. Several studies have shown that PTIP is an epigenetic regulator in somatic cells [30, 31]. Whether PTIP plays a similar role in postnatal germ cells is unclear. The data from this study evidently reveal that PTIP indeed is a critical epigenetic regulator in mouse spermatogenesis (Figures 8, 9 and 12).

Conventional knockout of PTIP in mice leads to embryonic lethality. In other words, no live mice can be obtained in conventional knockout of PTIP. Thus, I used the Cre-LoxP system to conditionally delete PTIP in mouse postnatal germ cells. This approach is innovative since it allows us to generate live animals to investigate spermatogenesis, a postnatal biological process. On the other hand, the efficiency of conditional knockout is dependent on the accessibility of the Cre recombinase to the target LoxP sites and hence can vary from 0% to 100%. Apparently, in our case, this efficiency is close to 100% (Figure 6 and Table 1), in consistence with the previous report in which the Stra8-Cre mouse line was originally created [33].

The Stra8-Cre mice express improved Cre recombinase only in postnatal spermatogonia from day 3 to day 8 [33]. Thus, it is likely possible that the PTIP gene (and the PTIP mRNA) is not completely deleted in spermatogonia. In line with this, I still observed spermatogonia in the PTIP conditional mutant seminiferous tubules (Figure 12). However, our results do not exclude the possibility that PTIP is critical in the renewal and differentiation of spermatogonia.

Our result indicates that spermatogenesis is arrested in meiosis I in the PTIP conditional mutant testis (Figure 12). This result will be confirmed in the future by analyzing

histology of 18-day-old testis. In addition, to determine the cause of meiotic arrest in PTIP-deficient spermatocytes, meiotic phenotypes, such as meiotic chromosomal synapsis will be further analyzed.

It should be noted that the cellular composition of the PTIP conditional mutant testis is significantly altered, such as the depletion of post-meiotic germ cells (Figure 12). Thus, the interpretation of Western blot result (Figure 13), which used the protein sample from the whole testis, should be cautioned. In the future, immunofluorescence should be carried out to examine H3K4 methylation specifically in PTIP-deficient male germ cells. Otherwise, a protocol for purifying male germ cells from the testis should be established and then Western blot analysis is followed.

In this study, only H3K4 di-methylation was analyzed in the mutant testis. Future studies should be carried out to detect H3K4 mono-methylation and tri-methylation in the mutant and wild-type testis.

H3K4 methylation has been linked to transcriptional activation. It will be interesting to see how the gene expression profile is altered in PTIP-deficient male germ cells. To address this, RNA profiling based on next-generation-sequencing technology should be performed.

PTIP has also been shown to function in the DNA damage repair. In meiosis I, DNA double-strand is programmatically broken and repaired in order for the success of meiotic recombination. Thus, in the future, we will determine whether PTIP plays a role in meiotic DNA double-strand break, repair and meiotic recombination.

Since post-meiotic male germ cells were absent in the mutant testis, whether PTIP plays a role in post-meiotic events, such as spermiogenesis, is unknown. Thus, utilizing a Cre

mouse line that expresses Cre at a later time point, such as postnatal day 16, will be helpful to explore the role of PTIP in post-meiotic germ cells.

REFERENCES

1. Bird A. (2002) DNA methylation patterns and epigenetic memory. Genes Dev 16: 6-21.

2. Allfrey VG, Faulkner R, Mirsky AE. (1964) Acetylation and methylation of histones and their possible role in the regulation of RNA synthesis. Proc Natl Acad Sci U S A. 51:786-94.

3. Luger K, Mäder AW, Richmond RK, Sargent DF, Richmond TJ. (1997) Crystal structure of the nucleosome core particle at 2.8 A resolution. Nature. 389(6648): 251-60.

4. Goldberg, AD, Allis, CD, Bernstein E. (2007) Epigenetics: a landscape takes shape. Cell 128, 635–638.

5. Heard E. (2004) Recent advances in X-chromosome inactivation. Curr Opin Cell Biol 16:247-55.

6. Jablonka E. (2004) The evolution of the peculiarities of mammalian sex chromosomes: an epigenetic view. Bioessays 26: 1327-32.

7. Hess RA, de Franca LR (2008) Spermatogenesis and cycle of the seminiferous epithelium. Adv Exp Med Biol 636:1–15.

8. Morgan, HD, Santos F, Green K, Dean W, Reik W. (2005) Epigenetic reprogramming in mammals. Hum. Mol. Genet. 14, R47–R58.

9. Allegrucci C, Thurston A, Lucas E, Young L. (2005) Epigenetics and the germline. Reproduction 129, 137–149.

10. Kimmins S, Kotaja N, Davidson I, Sanssone-Corsi P (2004) Testis-specific transcription mechanisms promoting male germ-cell differentiation. Reproduction 128(1): 5-12.

11. Ginsburg M, Snow MH, McLaren A. (1990) Primordial germ cells in the mouse embryo during gastrulation. Development 110, 521–528.

12. Surani MA, Hayashi K, Hajkova P. (2007) Genetic and epigenetic regulators of pluripotency. Cell 128, 747–762.

13. Lechner MS, Levitan I, Dressler GR. (2000) PTIP, a novel BRCT domain-containing protein interacts with Pax2 and is associated with active chromatin. Nucleic Acids Res. 28(14): 2741-51.

14. Cho EA, Prindle MJ, Dressler GR. (2003) BRCT domain-containing protein PTIP is essential for progression through mitosis. Mol Cell Biol. 23(5): 1666-73.

15. Manke IA, Lowery DM, Nguyen A, Yaffe MB (2003) BRCT repeats as phosphopeptide-binding modules involved in protein targeting. Science 302: 636-639.

16. Jowsey PA, Doherty AJ, Rouse J. (2004) Human PTIP facilitates ATM-mediated activation of p53 and promotes cellular resistance to ionizing radiation. J Biol Chem. 279: 55562–9.

17. Cho YW, Hong T, Hong S, Guo H, Yu H, Kim D, Guszczynski T, Dressler GR, Copeland TD, Kalkum M, Ge K. (2007) PTIP associates with MLL3- and MLL4-containing histone H3 lysine 4 methyltransferase complex. J Biol Chem. 282 (28): 20395-406.

18. Patel SR, Kim D, Levitan I, Dressler GR. (2007) The BRCT-domain containing protein PTIP links PAX2 to a histone H3, lysine 4 methyltransferase complex. Dev Cell. 13(4): 580-92.

19. Cho YW, Hong T, Hong S, Guo H, Yu H, Kim D, Guszczynski T, Dressler GR, Copeland TD, Kalkum M, Ge K. (2007) PTIP associates with MLL3- and MLL4-containing histone H3 lysine 4 methyltransferase complex. J Biol Chem. 282: 20395–406.

20. Issaeva I, Zonis Y, Rozovskaia T, Orlovsky K, Croce CM, Nakamura T, Mazo A, Eisenbach L, Canaani E. (2007) Knockdown of ALR (MLL2) reveals ALR target genes and leads to alterations in cell adhesion and growth. Mol. Cell. Biol. 27 1889-1903.

21. Swigut T, Wysocka J. (2007) H3K27 demethylases, at long last. Cell.131(1): 29-32.

22. Schwab KR, Patel SR, Dressler GR. (2011) The role of PTIP in class switch recombination and long range chromatin interactions at the immunoglobulin heavy chain locus. Mol Cell Biol. 31(7): 1503-11.

23. Jowsey PA, Doherty AJ, Rouse J. (2004) Human PTIP facilitates ATM-mediated activation of p53 and promotes cellular resistance to ionizing radiation. J Biol Chem. 279: 55562–9.

24. Manke IA, Lowery DM, Nguyen A, Yaffe MB (2003) BRCT repeats as phosphopeptide-binding modules involved in protein targeting. Science 302: 636-639.

25. Kim D, Patel SR, Xiao H, Dressler GR. The role of PTIP in maintaining embryonic stem cell pluripotency. Stem Cells. 2009;27:1516–23.

26. Schwab KR, Patel SR, Dressler GR. Role of PTIP in class switch recombination and long-range chromatin interactions at the immunoglobulin heavy chain locus. Mol Cell Biol. 2011;31:1503–11.

27. Jiang D, Kong NC, Gu X, Li Z, He Y. (2011) Arabidopsis COMPASS-like complexes mediate histone H3 lysine-4 trimethylation to control floral transition and plant development. PLoS Genet. 7(3): e1001330.

28. Yamada S, Ohta K, Yamada T. (2013) Acetylated Histone H3K9 is associated with meiotic recombination hotspots, and plays a role in recombination redundantly with other factors including the H3K4 methylase Set1 in fission yeast. Nucleic Acids Res. 41(6): 3504–3517.

29. Lee S, Lee DK, Dou Y, Lee J, Lee B, Kwak E, Kong YY, Lee SK, Roeder RG, Lee JW. (2006) Coactivator as a target gene specificity determinant for histone H3 lysine 4 methyltransferases. Proc. Natl. Acad. Sci. U. S. A. 103: 15392–15397.

30. Hayashi K, Yoshida K, Matsui Y. (2005) A histone H3 methyltransferase controls epigenetic events required for meiotic prophase. Nature. 438: 374–8.

31. Miller T, Krogan NJ, Dover J, Erdjument-Bromage H, Tempst P, Johnston M, Greenblatt JF, Shilatifard A. (2001) COMPASS: a complex of proteins associated with a trithorax-related SET domain protein. Proc. Natl. Acad. Sci. U. S. A. 98: 12902–12907

32. Ivashkevich AN, Martin OA, Smith AJ, Redon CE, Bonner WM, Martin RF, Lobachevsky PN. (2011) γH2AX foci as a measure of DNA damage: a computational approach to automatic analysis. Mutation Research. 711(1-2): 49–60.

33. Sadate-Ngatchou PI, Payne CJ, Dearth AT, Braun RE. (2008) Cre recombinase activity specific to postnatal, premeiotic male germ cells in transgenic mice. Genesis. 46(12): 738-42.

34. Bartlett JMS, Stirling D. (2003) "A Short History of the Polymerase Chain Reaction". PCR Protocols. Methods in Molecular Biology 226 (2nd ed.). pp. 3–6.

35. Fan J, Akabane H, Zheng X, Zhou X, Zhang L, Liu Q, Zhang YL, Yang J, Zhu GZ. (2007) Male germ cell-specific expression of a novel Patched-domain containing gene Ptchd3. Biochem Biophys Res Commun. 363(3): 757-61.

36. Chomczynski P, Sacchi N. (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem. 162: 156-159.

37. Laemmli UK (1970). "Cleavage of structural proteins during the assembly of the head of bacteriophage T4". Nature 227 (5259): 680–685.

APPENDIX 1

DATA FIGURES AND TABLES

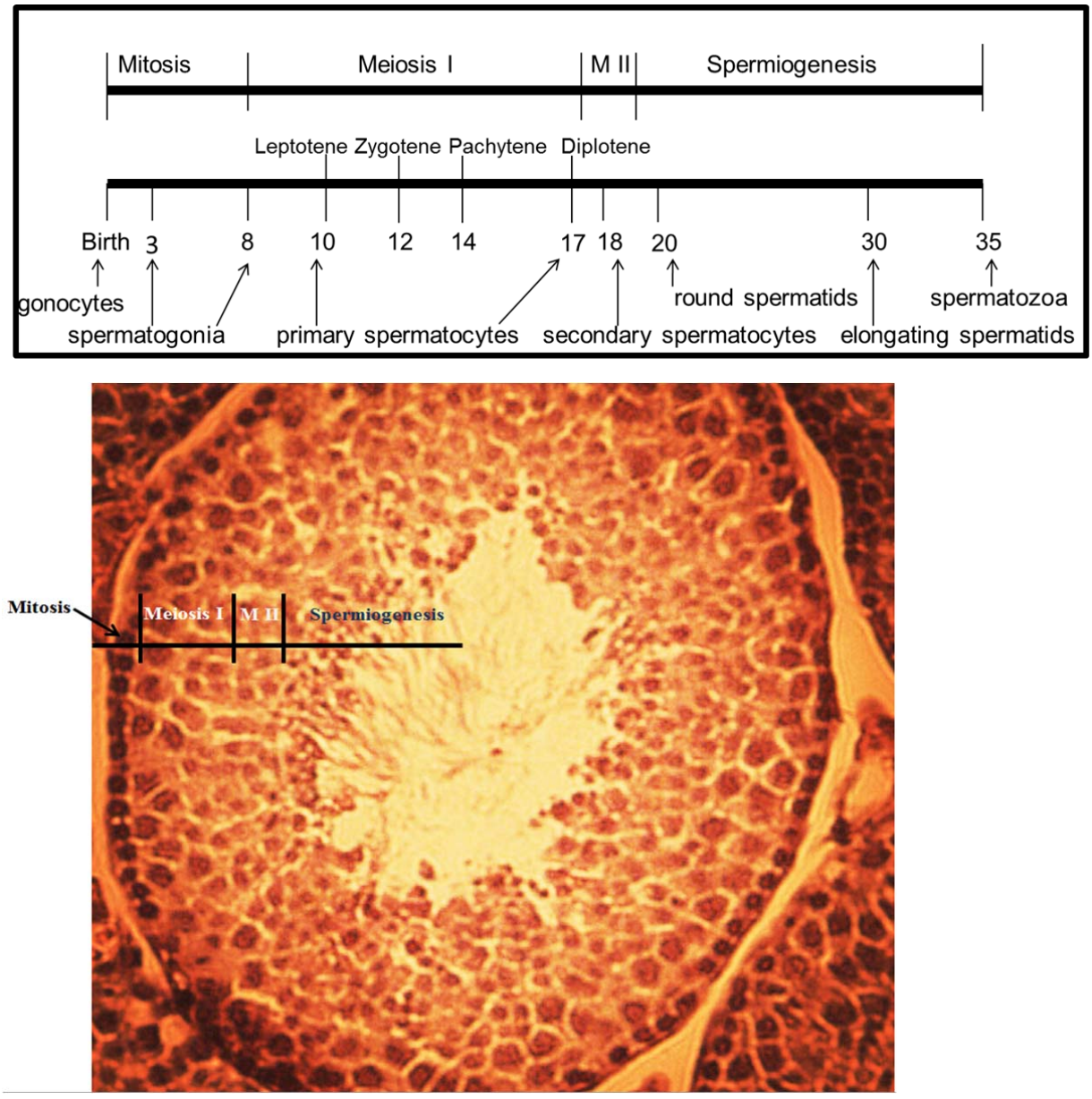

Figure 1. Upper panel: timeline of the first wave of mouse male germ cell differentiation and development (from birth to postnatal day 35). Lower panel: cellular events of spermatogenesis in one seminiferous tubule.

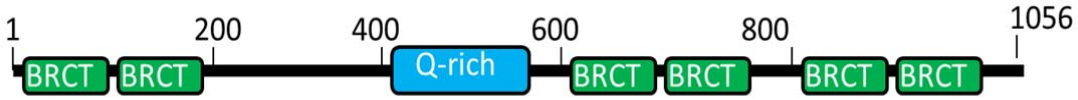

Figure 2. The protein domain structure of PTIP. BRCT=BRCA1 carboxyl-terminal domain; Q-rich=glutamine-rich region.

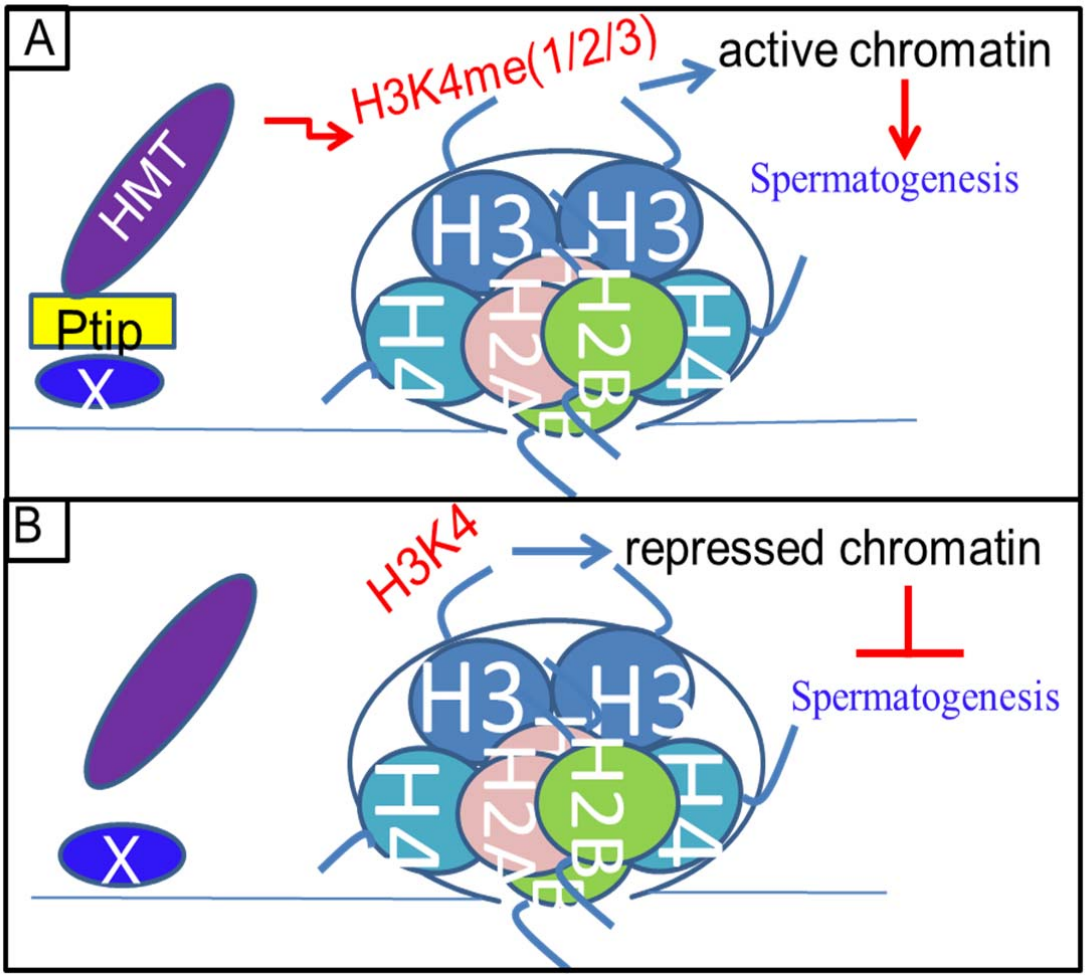

Figure 3. A hypothetical model of PTIP-mediated epigenetic regulation of spermatogenesis. HMT, Histone methyltransferase complex; H3k4me(1/2/3), histone 3 lysine 4 mono-, di-, and trimethylation; X, unknown PTIP-interacting protein.

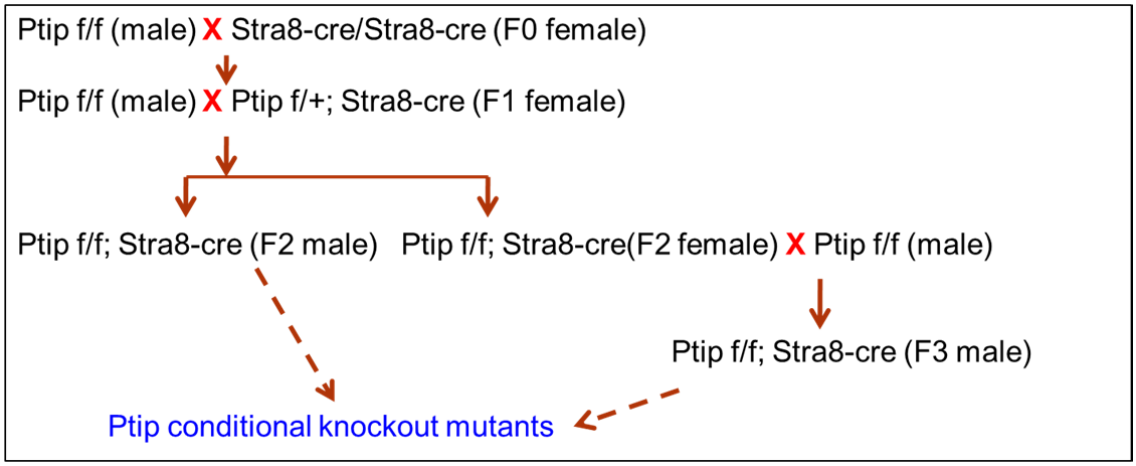

Figure 4. Generation of mutant mice with PTIP conditional knockout in male germ cells.

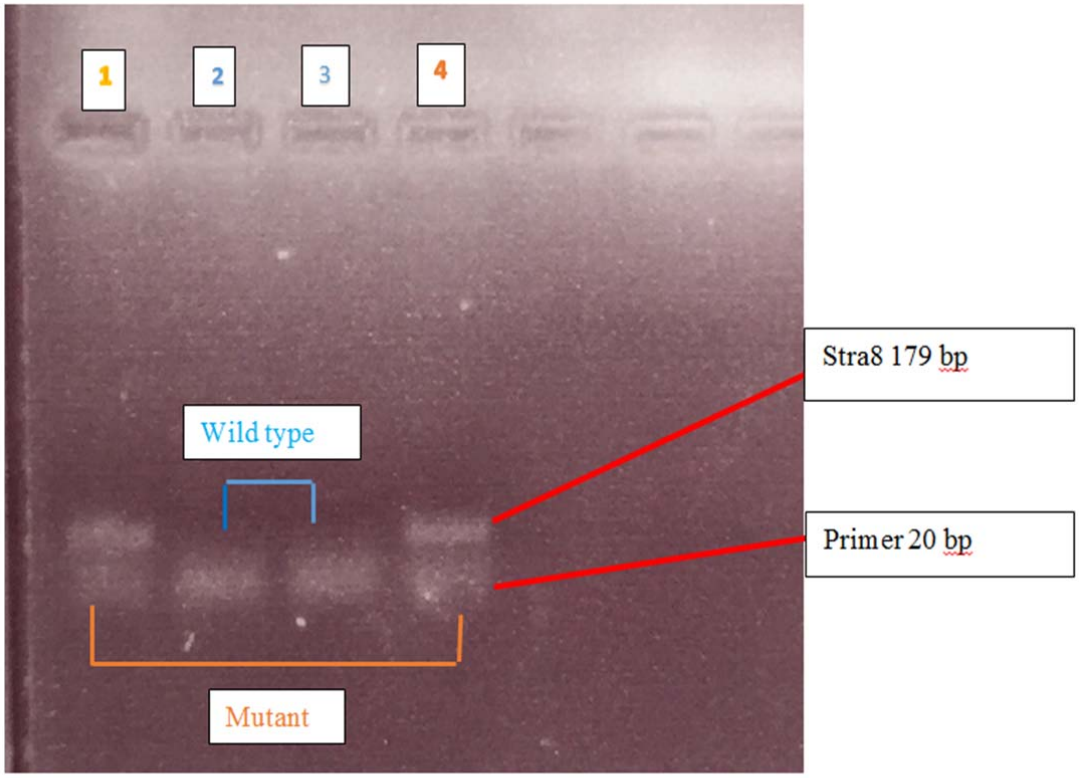

Figure 5. The genotype of the Stra8 gene is determined by PCR.

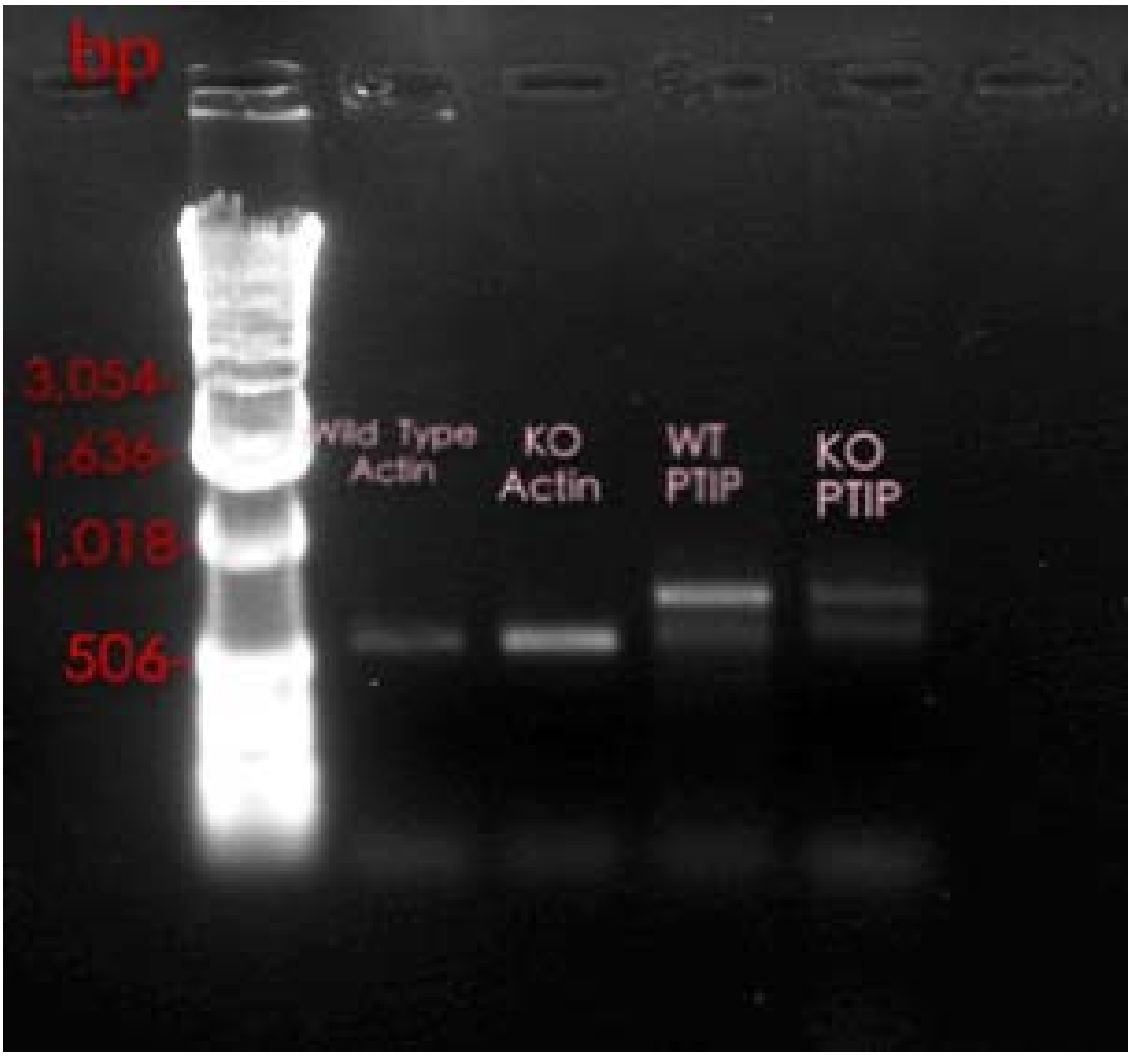

Figure 6. RT- PCR analysis of the PTIP knockout efficiency.

Figure 7. The life of wild-type (left) and mutant (right) mice.

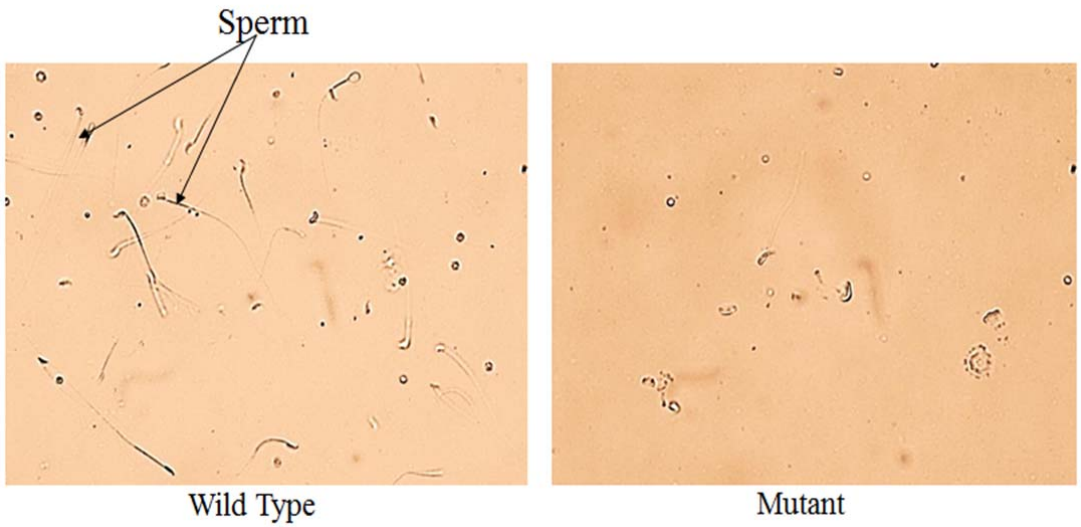

Figure 8. Analysis of epididymal sperm of wild-type and PTIP conditional mutant mice

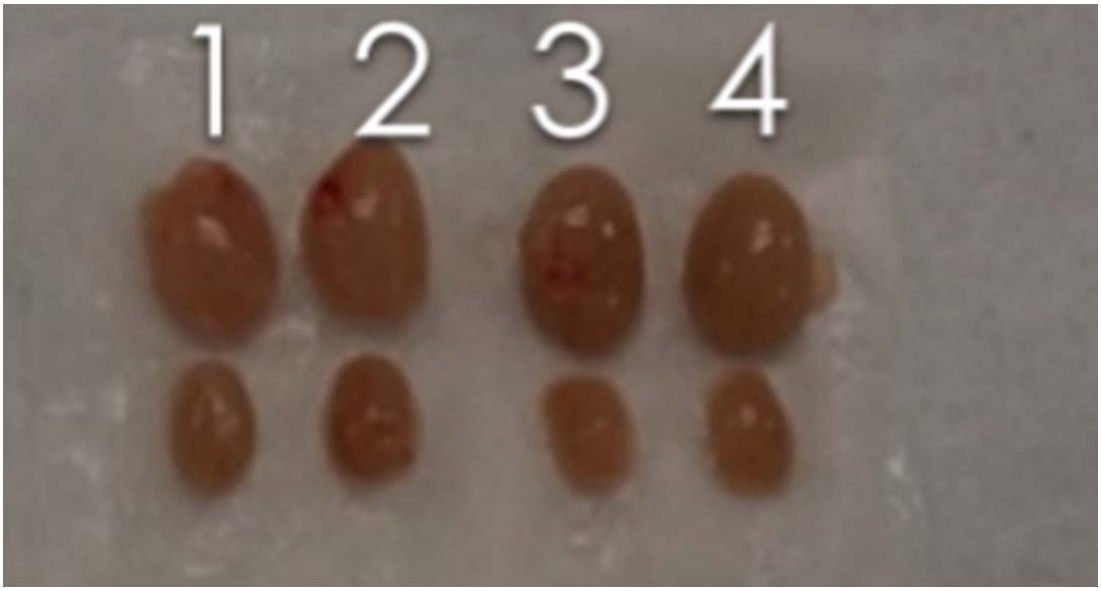

Figure 9. The morphology of wild-type (top) and mutant (bottom) testes.

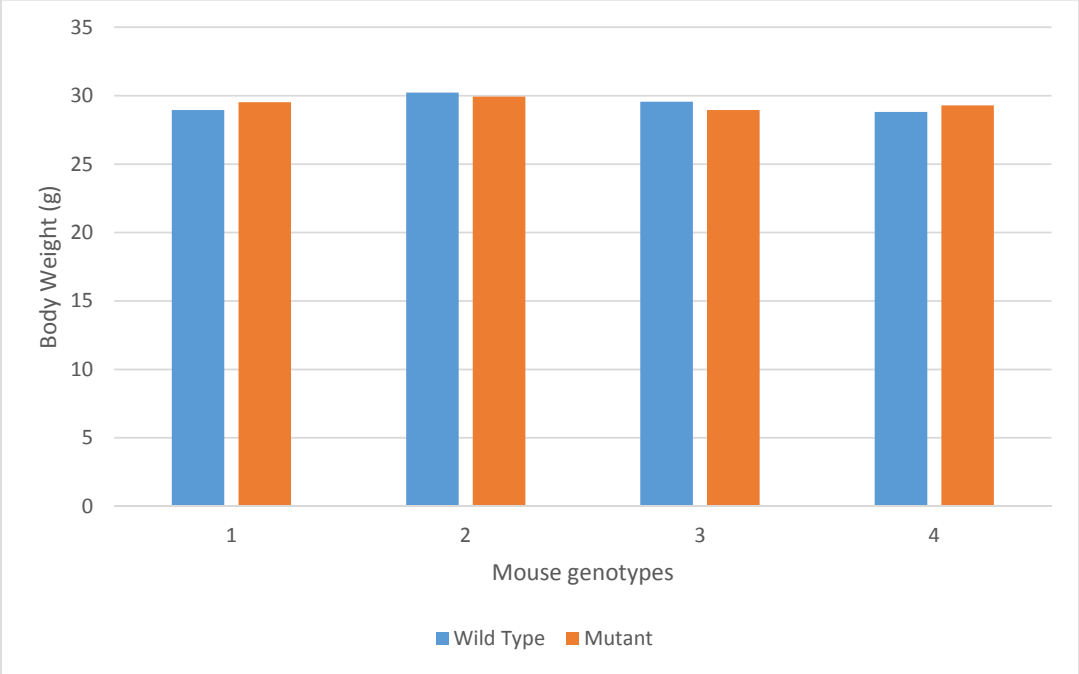

Figure 10. Body weight of the wild-type and mutant mice

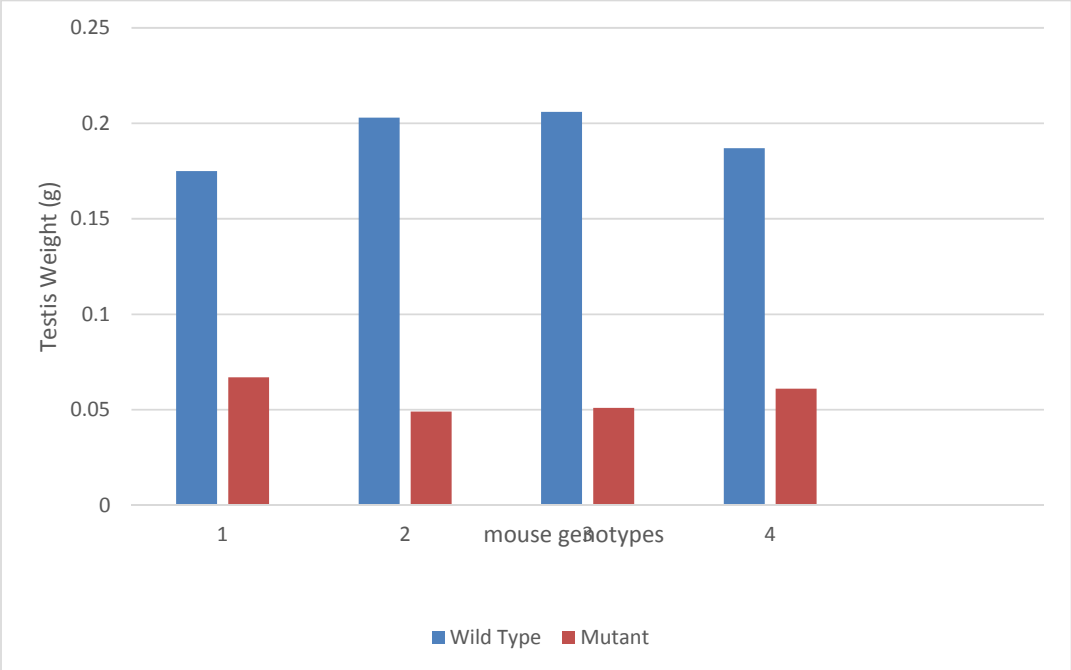

Figure 11. The analysis of testis weight.

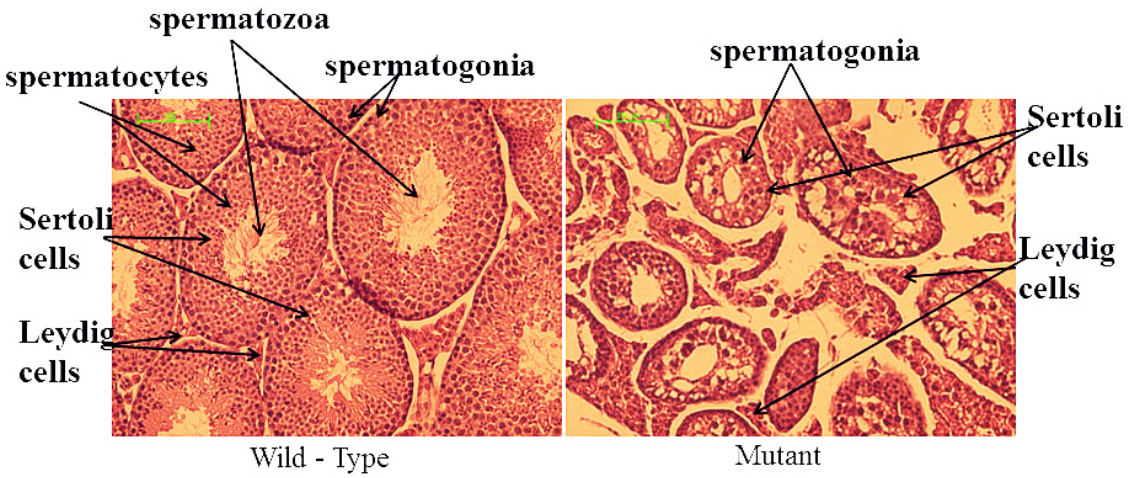

Figure 12. Histology of wild-type and mutant testes. (Scale Bar=100µm)

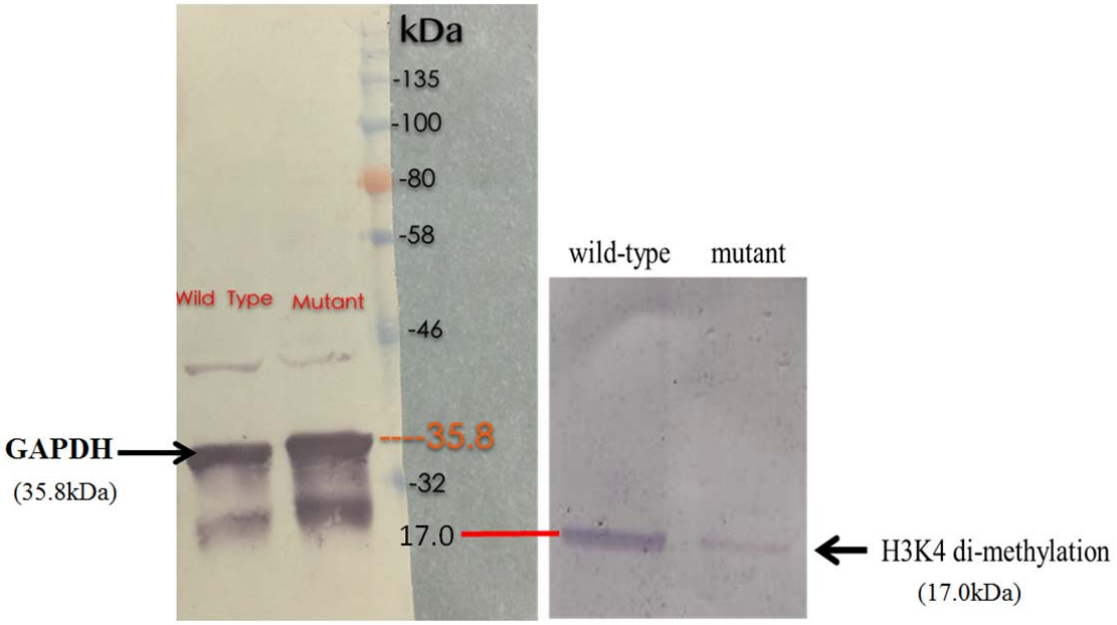

Figure 13. Western blot analysis of H3K4 methylation in the wild-type and mutant testes.

mouse genotype	Signal intensity		PTIP/β-actin ratio
	PTIP	β-actin	
wild-Type Ptip f/f	1477.44	1070.82	1.379728
knockout Ptip f/f; Stra8-cre	674.54	1647.16	0.409517

Table 1: Analysis of PTIP expression level by using β-actin as internal control.

Sterility of the PTIP conditional mutant mice		
mouse genotype	litters (from 8 mated females)	averaged litter size (from 8 mated females)
Ptip f/f (n=4)	16	6.3 ± 1.3
Ptip f/f; Stra8-cre (n=4)	0	0

Table 2: In vivo fertility test of PTIP conditional mutant mice

mouse genotype	epididymal sperm ($x10^7$)
Ptip f/f (n=4)	2.4 ± 0.3
Ptip f/f; Stra8-cre (n=4)	0

Table 3: Sperm number analysis

APPENDIX 2

LETTER FROM INSTITUTIONAL RESEARCH BOARD

MARSHALL UNIVERSITY
www.marshall.edu

Office of Research Integrity
Institutional Review Board

December 2, 2014

Gou-Zhang Zhu, PhD
Department of Biological Sciences
Marshall University
One John Marshall Drive
Huntington, WV 25755

Dear Dr. Zhu:

This letter is in response to the submitted thesis abstract for Chengjing Liu entitled *"Epigenetic Role of PTIP in Mouse Spermatogenesis."* After assessing the abstract it has been deemed not to be human subject research and therefore exempt from oversight of the Marshall University Institutional Review Board (IRB). The Institutional Animal Care and Use Committee (IACUC) has reviewed and approved the study under protocol #518 (IRBNet #367812). The applicable human and animal federal regulations have set forth the criteria utilized in making this determination. If there are any changes to the abstract you provided then you would need to resubmit that information to the Office of Research Integrity for review and a determination.

I appreciate your willingness to submit the abstract for determination. Please feel free to contact the Office of Research Integrity if you have any questions regarding future protocols that may require IRB review.

Sincerely,

Bruce F. Day, ThD, CIP
Director

Curriculum Vitae

Personal Information

Name: **Chengjing Liu**

TEL: (304)-840-7065 ***E-mail:***liu27@marshall.edu

Address: 20 Aspen Place Huntington, WV (25705)

Educational Background

Sep.2013-present ***Marshall University***

College of Science

Master graduate program

Department of Biological Sciences

GPA:3.50

Sep.2012-Jun.2013 ***Marshall University***

LEAP program

Sep.2010-Jun.2012 ***Liaocheng University***

College of life science

Undergraduate

Major in Biological Sciences

Sep.2007-Jun.2010 ***Qilu Normal University***

College of Biological Science

Undergraduate

Major in Biological Sciences and Technology

Reviewed Publications

2013 *Yang, Z., Wu, J., Liu, F., Mao, G., Liu, Y., Chen, Y., . . . Pang, Y. (2013). Detection of the pandemic H1N1/2009 influenza A virus by a highly sensitive quantitative real-time reverse-transcription polymerase chain reaction assay. Virologica Sinica, 28(1), 24-35. doi:10.1007/s12250-013-3290-0*

2011 *Chengjing Liu. (2011). Research on a health beverage of Coix seed and Chinese yam. Beverage Industry, issn.1007-7871.*

Research Experience

Sep.2013-present Research on biological functions of the PTIP gene in the process of spermatogenesis. Thesis research: determining the biological functions of PTIP (PAX transcription activation domain interacting protein) in spermatogenesis using a transgenic mouse model. PTIP has been lately identified as part of a histone H3 lysine 4 (H3K4) methyltransferase complex and is essential for mouse embryonic development. For my thesis research, I hypothesize that PTIP is an essential epigenetic regulator in male germ cell development and

differentiation. To test my hypothesis, I am generating mutant mice with conditional knockout of PTIP in postnatal male germ cells. Thus far, I have collected several remarkable results. A manuscript based on my research findings is now being written.

Lab Skills：

(1) gene amplification and gene cloning; (2) DNA detection and manipulation; (3) cell culture and maintenance; (4) usage of conventional and fluorescent microscopes; (5) morphological and histological analysis of testis; (6) mouse genotyping, Western blot, RT-PCR; (7) summarize and present research findings

Jun.2011-Aug.2011 Internship the Animal Laboratory in the College of Life Science, Shandong University Content and Achievement of internship: Managed the BALBc mouse colony. Established the raising environment with proper temperature, humidity, light, cage and pad. Detailed the fodder, water, sex, and preventive measures and so on.

Jan.2010-Sep.2010 Internship: Taizhou Institute of Viruses, Jiangsu Province. (Subordinate of Nanjing University) Content and Achievement of internship: Studied biological knowledge and did experiments on the Influenza A virus, 09 H1N1. Extracted the plasmids, ran DNA electrophoresis and published the article as a co-author.

Activity and Practice Experience

April/2014	During graduate period, I actively participated into varied activities related to biology, such as the Brain Expo held by our school, I was in charge of a volunteer docent. I had the opportunity to make an impact on the community through working with the Marshall University Brain Expo, where we brought kids from all levels and got them interested in neuroscience by doing activities that showed them how the brain works in a fun way.
Aug.2014-present	I worked as Teaching Assistant in Marshall University, in charge of the instruction of a laboratory course "human physiology laboratory" to undergraduate students. This experience not only consolidated my basic knowledge, but also improved my spoken English so much.
March.2011	I obtained national teacher certification in China. So I actively participated in supporting education as a science teacher.
Sep.2007-2010	Student committee: the Secretary of the Youth League Branch Committee, Director of the Discipline Department of Student Union. Main responsibilities: Inspected discipline on night lessons from Monday to Thursday. Led classmates to study the party constitution and party discipline. Organized the New Year's party.

Honors

2011	College Students Match of Technology and Academic Star, Liaocheng University.
2010	Excellent Graduation Paper, Qilu Normal University.
2009-2010	Model Cadre of the CCYL, Qilu Normal University.