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Functional Studies of PTCHD3 During Spermatogenesis

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FUNCTIONAL STUDIES OF PTCHD3 DURING SPERMATOGENESIS

A thesis submitted to
the Graduate College of
Marshall University
In partial fulfillment of
the requirements for the degree of
Master of Science
in
Biological Sciences
by
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LIST OF ABBREVIATIONS

AMH	Anti-Müllerian Hormone
A-P	Anterior-Posterior
Azi1	5-Azacytidine Induced Gene 1
BBS	Bardet-Biedl Syndrome
CDS	CHORI-Sanger-UCDavis
CI	Cubitus Interruptus
CNV	Copy Number Variant
Cre	Cyclization Recombination (recombinase)
Dhh	Desert Hedgehog
Dpc	Days Post Coitum
Dpp	Days Post-Partum
E	Embryo's Stage
ECM	Extracellular Matrix
Emx2	Empty Spiracle Homologue Gene
En2	Homeobox Protein Engrailed-2
ES	Embryonic Stem Cells

ESHyb	Embryonic Stem Cells (hybrid)
FGF9	Fibroblast Growth Factor 9
FLC	Fetal Leydig Cells
Flp	Flippase Recombinase
FRT	Flippase Recognition Target
FSH	Follicle Stimulating Hormone
Fu	Fused
GLI	Zinc Finger Transcriptional Factor, mediating the Hh pathway
Hh	Hedgehog
IFT	Intraflagellar Transport
INSL3	Insulin-like Factor 3
Lhx9	Lens Intrinsic Membrane Homeo-box 9
Lim1	Lens Intrinsic Membrane 1
Loxp	Locus of X-over P1
L-R	Left-Right
MEF	Mouse Embryonic Fibroblast Cells
Pax	Paired Box Gene
PCR	Polymerase Chain Reaction

PDGF	Platelet-derived Growth Factor
PGD2	Prostaglandin D2
PMC	Peritubular Myoid Cells
PTCH	Protein Patched Homolog
RT-PCR	Reverse Transcriptase-Polymerase Chain Reaction
RND	Resistance –nodulation Division
Rpgr	Retinitis Pigmentosa GTPase Regulator Gene
Scc	P450 Side Chain Cleavage
Sf1	Steroidogenic Factor 1
Shh	Sonic Hedgehog
SMO	Smoothened
SOX9	Sry-box 9
SSD	Sterol Sensing Domain
SuFu	Suppressor of Fused
Sry	Sex determining Region Y Chromosome (Testis Determining Factor)
Wt1	Wilms' Tumor Suppressor Gene 1

ABSTRACT

Paracrine factor Desert hedgehog (Dhh) is essential for mouse spermatogenesis. However, the specific receptor of Dhh during spermatogenesis is unknown. This study aims to test the hypothesis that Ptchd3, a male germ cell-specific gene acts as a receptor for Dhh in spermatogenesis. In this study, a transgenic mouse model with Ptchd3 gene deletion was first successfully established. Then, in vivo fertility assay and in vitro analysis were performed on Ptchd3 null mutant male mice. The data obtained from the in vivo fertility experiments indicates that there is no statistical significance in offspring litter number (p -value 0.7973) and litter size (p -value 0.3648) among mutant, heterozygote and wild-type male mice. The data of in vitro sperm assay reveals that the abnormality /normality ratio of sperm morphology in Ptchd3 null mice demonstrates no statistical difference with that in wild-type mice (Tukey test interval ± 4.7 to ± 12.8). Taken together, these findings clearly attest that Ptchd3 is not essential for mouse spermatogenesis and fertility. However, whether Ptchd3 functions as a Dhh receptor remains undetermined. The knowledge gained from this research into the function of Ptchd3 on spermatogenesis could give us a better understanding of the Dhh signaling pathway in testis.

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

The Development of Male Reproductive System

Stem cell differentiation occurs during embryonic development. During this process, pluripotent stem cells turn into multipotent stem cells in different tissues (Eckfeldt et al. 2005). However, it is the cellular environments that allow them to become an organ-specific cell lineage (Rossant, 2001). At 9.5 days post coitum (dpc) in the mouse embryo, the bipotential primordia still have the ability to differentiate into testes or ovaries (Yao et al. 2003). These gonadal primordia, containing relatively undifferentiated cells that express transcriptional factors, are going to respond to signaling cues mediating the differentiation. One of these transcriptional factors is Steroidogenic factor 1(Sf1), whose expression regulates the adrenal and gonadal development and is localized in the urogenital ridge (Val at al. 2003) (Ikeda et al.1996) (Park et al.2007).

The crucial transcription factor that leads to testis development is the expression of testis determining factor of the Y chromosome, *Sry*. This factor starts the arrangement of testis cords and differentiation of Sertoli cells by interaction with the transcription factors, *Sry- containing gene 9* (SOX9), Sf1, Wilms' tumor (Wt1), and the GATA4 transcriptional factor (Fig. 1) (Koopman et al. 1991) (Huang et al. 1999).

Sertoli cells provide metabolic and structural support later on during the spermatogenesis process and are localized in the seminiferous tubule. They will become activated by the follicle stimulating hormone (FSH). Sertoli cells retain the gonadal primordial cells inside testis cords at 12.5dpc in mice. This retention is going to maintain the germ cells in G1 of mitosis, and prevent

them from entering meiosis, which will occur later on after birth (McLaren, 1988). During this process, Sertoli cells mediate the testis tissue differentiation by releasing anti-Müllerian hormone (AMH) (Fig. 1). This hormone makes the female ducts (mesonephric ducts) retract (Haider, 2004). As a result, Sertoli cells promote the presence of fetal Leydig cells. The Leydig cells, another type of somatic cells inside the testis and different from the Sertoli cells, does not express *Sox9* or *Sry*. Their differentiation is regulated in a paracrine way from an indirect signaling of the Sertoli cells that express *Sox9*, *Sry*, desert hedgehog (DHH) and platelet-derived growth factors (PDGFs). Null mutants of *Dhh* or *PDGFs* had produced mice with the phenotypes that showed an incomplete differentiation of Leydig cells (Fig. 1) (Barsoum and Yao, 2006). Nevertheless, when the differentiation of Leydig cells occurs in a proper way, they will secrete insulin-like factor 3 (INSL3), which makes the testis descend, providing an adequate temperature for stem cells to undergo male germ cell differentiation and development inside the testis (Fig. 1).

Later on Leydig cells regulate the expression of transcriptional factors such as *paired box gene 2 (Pax2)*, *Pax8*, *lens intrinsic membrane 1 (Lim1)* and *empty spiracle homologue gene (Emx2)* to differentiate the Wolffian ducts. This process begins with the degeneration of the pronephros and the transformation of the mesonephros to the Wolffian ducts at E 9-10 in mice. The Wolffian ducts originate from the mesonephric mesenchyme, creating the epithelial tubes of the duct (Barsoum and Yao, 2006). The Wolffian ducts near the testis develop into the epididymis.

The middle and posterior parts of the Wolffian ducts become the vas deferens and the seminal vesicle respectively (Lipschutz et al. 1999). After that, Leydig cells secrete testosterone at approximately E 15 to help the development of secondary sexual characteristics and male brain differentiation (Haider, 2004).

This process and regulation of the development of the male reproductive system has to occur fast since the embryo at early stages has the ability to become either female or male (Palmer and Burgoyne, 1991) (Eicher and Washburn, 1986) (Yao et al. 2003). In mice, this time frame is of 10.5 dpc-12.5dpc (Hacker et al. 1995). An interruption of *Sry* expression for 24 hours may cause a reversal from male to female since the cells may enter meiosis and produce ovaries instead (Eicher et al. 1995) (Nagamine et al. 1998) (Washburn et al. 2001).

For humans, *Sry* is a critical gene that is required to mediate male sex determination. However, this is not the case with mice, *M33*, *Emx2* and *Lhx9* (*lens intrinsic membrane homeobox 9 gene*) are also required. If one of these genes is absent, it can make a reversal from male to female by degenerating the Wolffian ducts at E13.5 (Brennan and Capel, 2004).

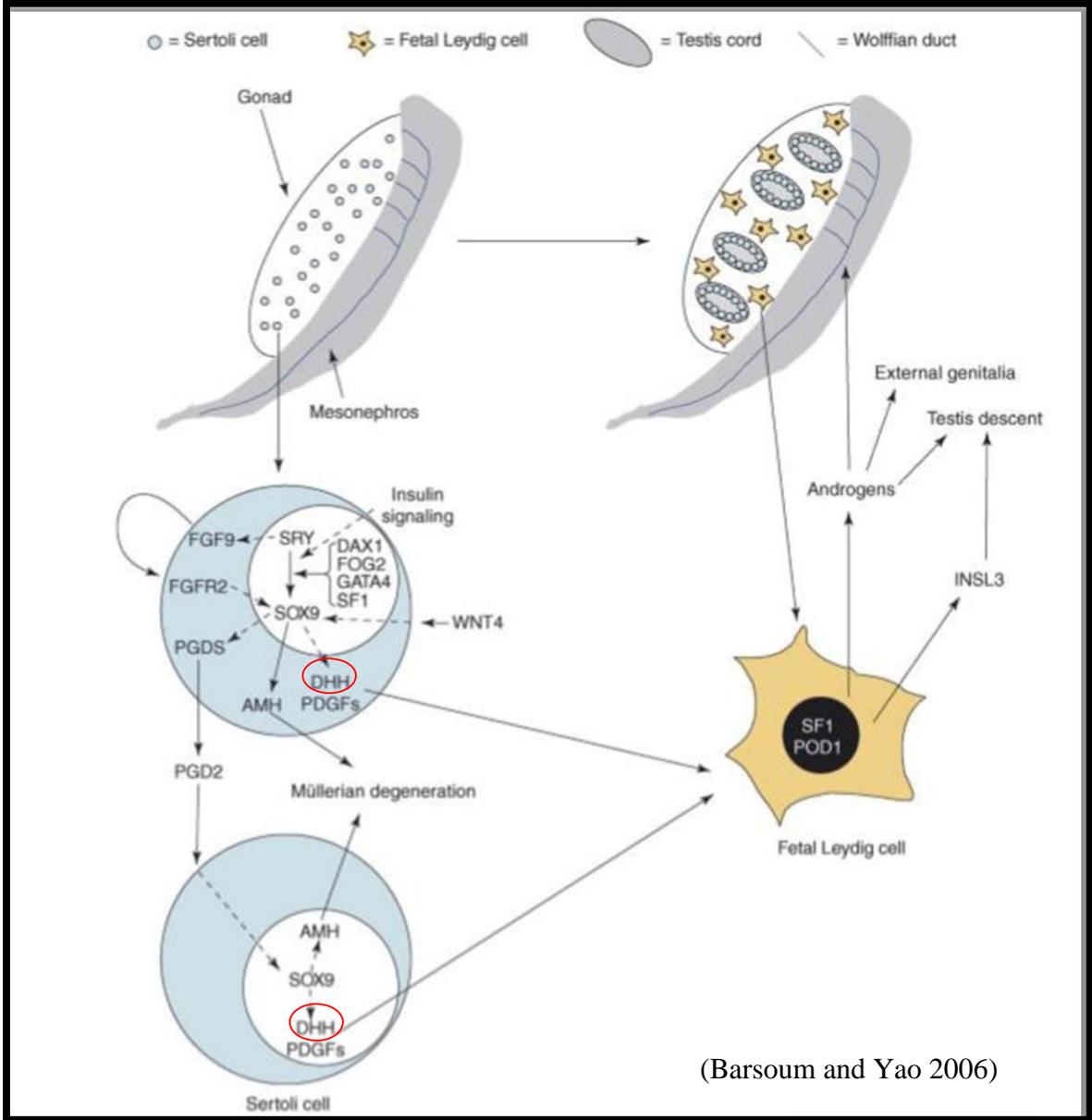


Fig 1. The major components in the development of the male reproductive system. For the purpose of this study, we will focus on how *Dhh* and its hypothetical receptor *Ptchd3* act during spermatogenesis.

Testis Anatomy

The testis is composed of several cell types. This section will discuss how all of them are involved in the testis development. The testis will start to develop when the germ cells migrate to the genital ridge then to the gonads (Harikae et al. 2013). The cells will become encased in the testis cord (Svingen and Koopman, 2013). When the germ cells are localized in the gonads the process of differentiation will start. The testis starts to develop after the expression of *Sry* in Sertoli cells. Before this step both male and female reproductive systems are basically the same. As mentioned previously, after the *Sry* expression, Sertoli cells differentiate and originate a cascade of events. *Sry* will need to be expressed in a gradient for a specific time to activate the expression of *Sox9*. The expression of *Sox9* will be localized in the primitive gonad, which will become testis tissue. However, the number of gonad cells expressing *Sox9* should be high otherwise the testis development will not continue and a sex reversal will occur. There are three mechanisms to prevent sex reversal, first by *Sox9* expression, second by fibroblast growth factor 9 (FGF9) expression and third by cell proliferation.

Therefore, for the first mechanism, cells in the gonad that are expressing *Sox9* will start recruiting adjacent cells and change their cell fate (from a gonad to a testicular fate) (Svingen and Koopman, 2013) (Palmer and Burgoyne, 1991). Prostaglandin D2 (PGD2) is also involved in this process, by inducing *Sox9* expression (Wilhelm et al. 2005). Second, FGF9 expression is needed to maintain the proper number of cells. Some studies believe that the function of FGF9 might be to mediate the expression of *Sox9* like PGD2. However, others believe that FGF9 acts as a repressor of the pre-ovary genes (Kim et al. 2006) (Jameson et al. 2012). Third, *Sry* will make the cells that express *Sox9* start dividing at a faster rate than the ones that do not express *Sox9*. As a result, the number of cells that express *Sox9* is increased (Schmahl et al. 2000). The

cells that come from this lineage will activate Sox9, which will allow them to change the cell fate this time to become pre-Sertoli cells (Sekido and Lovell-Badge 2008).

The pre-Sertoli cells will then organize themselves as the epithelium of the testis cords, retaining the germ cells in the lumen, where they will become mature Sertoli cells (Svingen and Koopman, 2013). The testis cord is composed of the Sertoli cells, which surround the germ cells, an exterior layer of peritubular myoid cells (PMCs) and an extracellular matrix (ECM) for establishing structural support (Svingen and Koopman, 2013). Sertoli cells are important in the establishment of testis vascularization and differentiation of peritubular myoid cells (Brennan and Capel, 2004). Sertoli cells and the PMCs create the basal lamina to divide the testis cord and the interstitial compartments (Skinner et al. 1985). Later on the testis cord will create loops that will be separated by the interstitial cells and will be elongated to give rise to the seminiferous epithelium (Archambeault and Yao, 2010) (Clermont and Huckins, 1961). The interstitium is composed of mesenchymal tissue, fetal Leydig cells (FLCs), and a blood vasculature (Svingen and Koopman, 2013).

The fetal Leydig cells (FLCs) on the other hand develop by paracrine trigger Dhh (Barsoum et al. 2009) (Huang and Yao, 2010) (Barsoum and Yao, 2010). Some Leydig cells form from the same precursor cells as the Sertoli cells, while the remaining are from perivascular progenitor cells, which are found in the mesonephric–gonadal junction (DeFalco et al. 2011) (DeFalco et al. 2013). The progenitor cells express Notch and androgens. Notch signaling is going to trigger the release of testosterone, which is necessary to maintain the proper number of progenitor cells (Barsoum and Yao, 2010) (Tang et al. 2008).

To originate the vasculature of the testis, endothelial cells separate from the arteries of the mesonephric plexus, acquire motility, and move through the testis to the anti-mesonephric region

(Coveney et al. 2008). It is in the anti-mesonephric region where they will be organized to form the coelomic vessel, the primary artery in the testis. The arterial network within the testis arises from the coelomic vessel and will branch to the tunica albuginea and the testis interstitium, and finally connect the rete of the testis (Brennan et al. 2003) (Bott et al. 2006) (Barsoum and Yao, 2006). The testis network venous on the other hand originates from the mesonephros, localized in the rete testis. The rete testis is localized in the top part of the seminiferous tubules and helps in moving the sperm from the tubules to the efferent tubules to mediate the ejaculation process.

Testis is covered by a protective tissue, which is composed of the tunica albuginea, smooth muscles and contractile cells (Middendorff et al. 2002) (Setchell et al. 1994). This protective tissue not only guards the testis but also is important for the blood flow and sperm movement (Setchell et al. 1994).

The nerves, nevertheless, so far have not been found to be essential during fetal testis development. But in adult testis they are involved in the regulation of hormones and are known to play a role during spermatogenesis (Chow et al. 2000) (Frankel and Ryan, 1981).

Spermatogenesis

Spermatogenesis. Spermatogenesis is the production of sperm from stem cells. This process takes about 34 days in mice and 74 days in humans. It occurs in the testes within the seminiferous tubules, where developing male germ cells and Sertoli cells are present (Gilbert, 2000) (Fan et al.2006).

Sertoli cells provide sufficient structural support to prevent the blood from contacting with developing male germ cells. They also provide nutrients necessary for male germ cells during spermatogenesis. The process of male germ cell development starts in the embryonic stage where primordial germ cells proliferate while migrating to the testis (Gilbert, 2000) (Wolpert et

al. 2002). These cells do not complete mitosis prior to the birth of the organism; therefore they are retained in the G1 phase of the cell cycle (Wolpert et al. 2002). Once the male mouse is born, the cell cycle is resumed and germ cells go on mitosis to produce more stem cells or spermatogonia (diploid 2n) (Fig. 2). Mitosis will follow, producing primary spermatocytes (diploid 4n) (Fig. 2) (Gilbert, 2000) (Wolpert et al. 2002). In order to get haploid cells, primary spermatocytes enter meiosis I to produce secondary spermatocytes (haploid 2n), which subsequently undergo meiosis II to produce spermatids (haploid n) (Fig. 2) (Gilbert, 2000) (Wolpert et al. 2002). When spermatids are formed, they need to pass through a process of cellular differentiation from being round spermatids to elongating spermatids and then to mature sperm (fig.2) (Gilbert, 2000) (Wolpert et al. 2002). Sperm continue maturation in the epididymis before ejaculation (Gilbert, 2000).

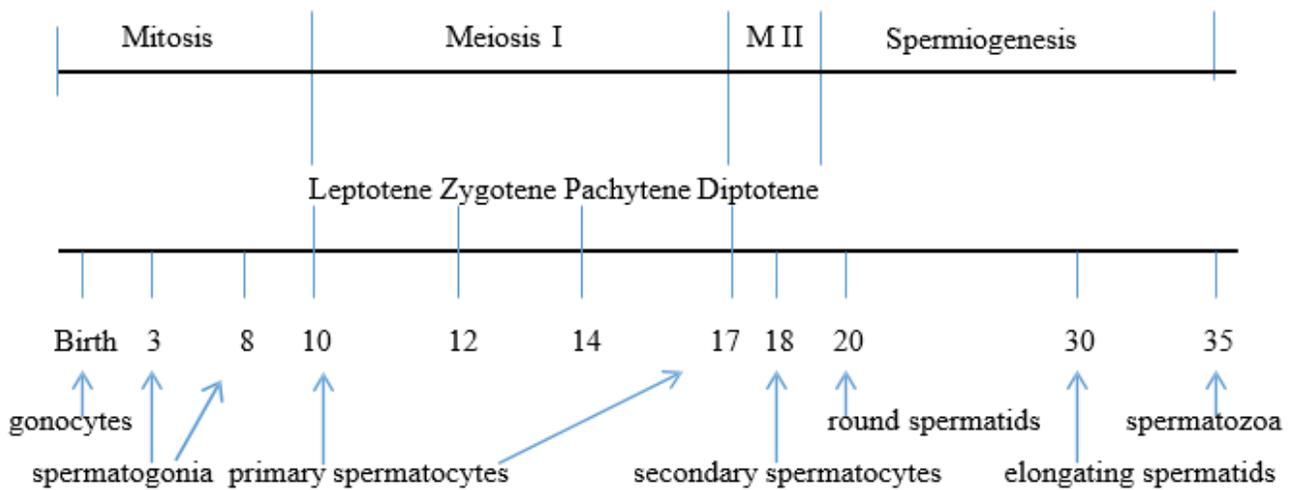


Fig 2. First wave of mouse spermatogenesis: Mouse male germ cell differentiation and development.

Sperm Modification in the Epididymis. The sperm need one to two week transition in the epididymis before acquiring all the modifications that are needed to reach full maturation (Dacheux and Dacheux, 2014). The correct microenvironment in the epididymis is essential for sperm motility. This environment is usually controlled by β -defensin, a polar molecule that is divided by hydrophobic and charged regions (Yeung et al. 1992). As its name suggests, β -defensin act as an antimicrobial defense (Zasloff, 2002). One of the membrane ion channels that β -defensin has been associated is Ca^{2+} channel.

Ca^{2+} is an important ion for the capacitation of the sperm. The process of sperm capacitation occurs when the sperm travels along the epididymis, where Ca^{2+} concentration increases by twofold higher from the caput to the cauda (Hoskins et al. 1983). Bin1b, a rat defensin, was found to produce different binding sites on the immature sperm, which seems to help sperm acquire Ca^{2+} , to facilitate the development of sperm motility (Zhou et al. 2004).

The first modification occurs when the sperm has to travel through the anterior epididymis, where water reabsorption is found. The water movement is controlled by the aquaporin channels that dictate the ionic composition (Dacheux and Dacheux, 2014). The contact of the sperm flagellum with this ionic composition generates the migration of the remaining cytoplasmic germ-cell and thus the first beating of the sperm (Dacheux and Dacheux, 2014). The cytoplasmic germ-cell forms a structure called cytoplasmic droplet (Dacheux and Dacheux, 2014). The specific function of this modification is unknown. However, it has been found that when the sperm travels from the anterior part through the epididymal caput, the cytoplasmic droplet moves from the beginning to the end of the middle piece of the flagellum (Dacheux and Dacheux, 2014). It has been found that abnormal migration of the cytoplasmic droplet leads to the reduction of sperm fertility (Cooper, 2005).

The following sperm modifications occur when the sperm continues to move through the epididymis. It is this period that changes in the lipid and protein composition of the sperm

membrane have been observed (Scott et al. 1967) (Dacheux and Voglmayr, 1983). The membrane proteins undergo proteolytic cleavages, which occur at the beginning of the epididymal transit (Dacheux and Dacheux, 2014). In this process, the electrostatic interaction will mediate the absorption of luminal epididymal proteins at the sperm surface and the integration of other proteins into the plasma membrane (Dacheux and Dacheux, 2014) found to produce different binding sites on the immature sperm, which seems to help sperm acquire Ca^{2+} , to facilitate the development of sperm motility (Zhou et al. 2004).

Male Fertility and Ciliopathies

A previous report showed that approximately 10-15% of human couples in European countries had conceiving problems. From this 10-15%, 60% of the cases were due to male fertility problems (Nieschlag and Behre, 1997). Previous studies revealed diverse causes of male infertility, for example, the spermatozoa number. A human ejaculation contains an average of 180 million spermatozoa (66 million/ml). However, usually only one spermatozoa fertilizes the egg, which means that the strategy is not cost-efficient.

Other studies investigated sperm morphology and motility (MacLeod and Gold, 1951). The motility of the sperm is important, since it allows the sperm to travel a long distance from the vaginal tract to the oviduct where the egg resides. Nevertheless, the morphology of the sperm is equally important since it allows the sperm to penetrate the corona radiata of the egg and fertilize it. Lindemann (2010) called this process the Mother Nature's Triathlon.

Nevertheless, the majority of infertile men show sperm with reduced or absent motility. Researchers have found that in most cases this motility problem arises from disruption in the formation of the flagellum (Escalier and David, 1984) (Chemes et al. 1998). The flagellum of the sperm is a type of modified cilium. Some researchers have discovered that the significance of

looking into the male sperm is not just to develop new techniques and medication for male fertility, but also to understand other diseases. Men who suffer from infertility also have problems with cilia elsewhere. Problems or disruption in cilium formation are called ciliopathies. The cilia are important for left to right side patterning that occurs during embryogenesis.

Kartagener's syndrome is a ciliopathy that often causes a shift of the organ from the left side to the right side, and causes patients to also suffer from chronic bronchitis and infertility. The defects during the cilium development result in the sperm missing motor proteins, which makes the sperm unable to move (Lindemann, 2010).

Brunner et al. (2008) and other researchers have looked at the function of retinitis pigmentosa GTPase regulator gene (*Rpgr*), a gene that is found in ciliated tissue. Some patients that suffered retinitis pigmentosa were also found to produce high abnormality in their sperm (Miendl et al. 1996) (Roepman et al. 1996). The retinitis pigmentosa is a condition that cause the loss of cells in the retina. Brunner hypothesized that *Rpgr* interacts with a microtubule protein that is in charge of mediating the intraflagellar transport (IFT) in the testis, resulting in defects of the spermiogenesis process (Kierszenbaum, 2002).

Another ciliopathy at which researchers have looked is the Bardet-Biedl syndrome (BBS). This condition displays different symptoms that vary from individuals even if they are related. Some of the symptoms can be light sensitivity, similar to the retinitis pigmentosa, polydactyly and infertility. Mouse studies have shown that blocking some BBS proteins results in mouse infertility, since sperm are unable to form the flagella. The sperm heads are present but there is no evidence of the flagella (Davis et al. 2007).

On the other hand, Hall et al. (2013) studied *5-azacytidine induced gene 1* (*Azil1*) null mice and found that this mutation led to absent or truncated sperm flagella. Similar to *Rpgr*, *Azil1* seems

to also interact with IFT during the formation of the flagellum. However, different from null BBS mice, this null mutant also exhibited some morphological problems in the head of the sperm.

***Mus musculus* as a Model**

Mus musculus, commonly known as the mouse, is currently one of the principal organism models to study developmental diseases. Scientists have used mice to understand numerous diseases due to gene similarities (99%) that mice share with humans (Waterston et al. 2002). Even though chimpanzee and other primates are more closely related to humans, the cost associated with mouse maintenance is relatively lower. Another benefit is that mice reproduce fast ($\approx 19-20$ days) and the litter size is higher than other mammalian models, allowing the analysis and comparison of multiple siblings. In addition, the mouse genome has already been sequenced which allows the comparison of diverse sequences to determine the relation among organisms.

Gordon and Ruddle (1981) were the first to generate a stable germ-line transmission of the mutant mice. This discovery allows companies to nowadays generate multiple commercial mutant traits, which make the biomedical research more accessible. Researchers can now make simple breeding events and produce their desired trait. Nevertheless, there are also companies and universities like University of California-Davis (UC-Davis) who have developed new strategies to produce a customized mutant mouse for their clients. Different technologies, such as the one from UC-Davis, give scientists the flexible tools to perform diverse genetic experiments, studying the function of the gene of interest. The common technology that is used in mice is the production of a knockout mouse line of the gene of interest. When producing a gene knockout in a mouse, the scientist beforehand needs to know or have an idea of the function of the gene of interest. If the expression of the gene is predicted to be essential for the mouse embryo to develop, the scientist or companies could use the Cre-LoxP technology to produce a tissue specific mutation (also called conditional knockout). Cre is a bacteriophage gene that allows scientists to generate a tissue specific

deletion by catalyzing the DNA recombination in loxP sites, leaving the essential genomic areas for the organism life intact. The loxP site is a small sequence (34bp) that is also derived from bacteriophage. It is composed of 8 bp in the middle and 13bp in both ends. The base pairs at the ends are repeatedly inverted. The Cre-LoxP technology, could also be used as an inducible Cre, which allows scientists to trace patterning during the embryonic development. This technology is also capable of being used to mediate insertions and translocations. Nevertheless, it is the deletion of this technology that is the most common application to be used to study a gene's function.

The Cre-LoxP works by having two loxP sites (34 bp) before and after the sequence of interest (Brocard et al. 1998). In order to mediate the deletion, both loxP sites should be oriented to the same direction. This will allow the deletion of part of the loxP sites by Cre recombinase, similar to an endonuclease. What occurs is that the loxP target locus will be cut into three pieces. The middle piece is going to include the sequence of interest (targeted gene). The ending two pieces will be united. Thus, after union, the middle piece is deleted from the genome.

The University of California-Davis has created the CHORI-Sanger-UCDavis (CSD) technology to generate a chimeric mouse, which can be used to produce either a conventional knockout (deletion in all tissues), wild-type, or conditional knockout (tissue specific deletion) mouse. The mutational result will depend on the different breeding strategies.

Hedgehog Signaling Pathway

Hh Signaling in *Drosophila melanogaster*. Hedgehog (Hh) functions as a ligand in the hedgehog signaling pathway, which starts with the secreted Hh protein ligand binding to the membrane receptor Patched (PTCH). PTCH usually acts as a negative regulator of another membrane protein Smoothed (SMO) (Gilbert, 2000) (Wolpert et al. 2002) (Rahnama et al. 2004) (Cohen, 2003). The binding of Hh and PTCH releases the inhibitory effect of PTCH on SMO.

Consequently, SMO is activated and initiates a signal transduction cascade, CI (Cubitus Interruptus) will become activated by a cytoplasm complex (composed of Costal-2, Fu (Fused) and suppressor of fused (SuFu) which interacts with microtubules. Activated CI will translocate from the cytoplasm to the nucleus. In the nucleus, CI acts as a transcription factor to activate gene expression of target genes. (Gilbert, 2000) (Wolpert et al. 2002).

Like the majority of other proteins that play a role during cell proliferation and development, *Hh* was first discovered in *Drosophila melanogaster* by Christiane Nusslein- Volhard and Eric F. Weischaus in 1980 in their attempt to look for mutation that interrupted the *Drosophila* larva body plan (Gupta et al. 2010). Since then, three *Hh* homolog genes were discovered in vertebrates, Indian Hedgehog, Desert Hedgehog (*Dhh*) and Sonic Hedgehog (*Shh*) (Sahin et al.2014).

Shh Signaling Pathway. Sonic hedgehog signaling in vertebrates is similar to Hh signaling that occurs in fruit flies. The main difference is that there are multiple Patched homologues in vertebrates, including Ptch1 (Patched1), Ptch2 (Patched2), and the recently-discovered Ptchd3. Also, there are three CI homologs Gli1, Gli2 and Gli3. However, not all Gli work as an activator of gene expression. For example, Gli1 is an activator of gene expression, while Gli2 or Gli3 can act as either activator or repressor of gene expression. In mammals, the cytoplasmic complex is composed of Stk36 (homologue of Fu), SuFu, and Gli. Sufu interacts with the microtubules to activate Gli (Fig. 3). As mentioned previously, activated Gli enters the nucleus and mediates changes in gene expression (Fig. 3).

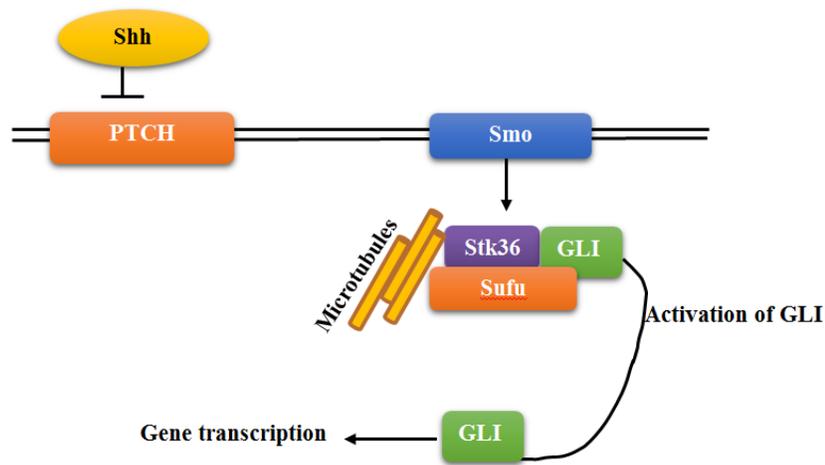


Fig 3. Sonic hedgehog signaling pathway

Models for Hh/Shh Signaling. Interestingly, Hh/Shh is important during left-right (L-R) patterning and is one of the pathways that are involved in ciliogenesis. In *Drosophila melanogaster*, the Hh gene is known to be a segment polarity gene, which is important in establishing the L-R axis and anterior-posterior (A-P) axis (in limb development). The Shh signaling has been shown to be either short-range or long-range signaling (Cohen, 2003). There are currently two published models that explain how Shh acts as a morphogen (autocrine) and an unpublished model that argues against the previously established ones. The first and more known model is called the Spatial model (or more known as the French's flag model) (Wolpert et al. 2002). In this model, signaling occurs in a concentration gradient, where different concentration will specify different cell fate. The second model is called the temporal model where the important part is not the concentration but the time of exposure of the ligand (Harfe et al. 2004). And the third model is proposed by Zhu, et al. (unpublished data), where Shh influences the early patterning of progenitor cells either by concentration or the time of exposure; however, the final cell fate is determined by the downstream signaling and cellular proliferation.

Shh in Organogenesis. Hh is involved in the development of fruit flies, playing a role in the

segmentation, wing, legs and brain development (McMahon, 2000). However, in vertebrates Shh (Hh homologue) is important in early embryonic patterning and morphogenesis of multiple organs. Sonic hedgehog determines digit number and digit patterning in the limb. Deletion of *Shh* expression leads to digit loss (Zhu et al. 2008). Also, *Shh* acts on the eye development. The eye development in the embryo starts to form as a single unit and sonic hedgehog allows it to split as two different fields. Mutation in Shh during the eye development does not allow eye splitting and result in a cyclops mutation (Gilbert, 2000). Sonic hedgehog has also been associated with the development of the patterning of the gut tube and its down-regulation is involved in the specification of the pancreas. When Shh is not down-regulated, the cell fate is inverted and begins to develop into the intestine (Gilbert, 2000).

Shh in Cancer. Sonic hedgehog not only is important during embryonic patterning but also regulates adult tissue homeostasis in both invertebrates and vertebrates (Ingham and McMahon, 2001). Mutation in the Shh pathway has been associated with cancer development and proliferation. Over 14 types of cancers have been found to have a mutation that leads to the activation of the Shh signaling pathway (Cohen, 2003). Some of these cancers include breast and liver cancer, esophageal carcinoma, basal cell carcinoma and medulloblastoma (Taipale and Beachy, 2001) (Rubin and de Sauvage, 2006). Type I cancers are usually associated with an independent activation of the pathway. Type II cancers are activated by an autocrine and ligand dependent signaling, where tumor cells and neighboring cells are expressing Shh and responding to the ligand.

Finally, type III cancers signal Shh in short-range, which promotes growth and proliferation of the cancer cells (Scales and de Sauvage, 2009) (Rubin and de Sauvage, 2006). Many scientists try to understand how Shh signaling behaves in a natural environment to predict the behavior of cancer cells. This research potentially leads to cancer treatments.

Shh in the Male Reproductive System. *Shh* signaling is involved in different adult tissues

that are important for reproduction. Sonic Hedgehog is involved in the development of prostate and external genitalia. Some studies have found its expression in the three regions of the adult mouse epididymis, cauda, corpus and caput (Fig. 4) (Turner et al. 2006) (Walterhouse et al. 2003). This finding is important, because a similar process occurs in the human epididymis. This tissue is divided into three intraregional segments. Many genes, including *Hh*, mediate the regulation of these segments (Turner et al. 2004). It seems that *Shh* recruits other proteins to create this patterning. Interestingly, when Turner et al. (2006) looked closer into the pattern, they found that Hh targets were not expressed during adult epididymis maturation; neither the receptors nor transcriptional factors of the pathway.

Shh expression in the epididymis seems to be important in maintaining the luminal microenvironment conductivity, which is a determining factor for sperm maturation (Turner and Howards, 1978) (Yeung and Cooper, 2002). Disruption in the *Shh* pathway had resulted in reduced sperm motility, demonstrating that *Shh* indeed has a role in spermatogenesis (Turner et al. 2006).

Before the characterization of *Ptchd3*, *Ptch2* was known to be the receptor with highest expression in the testis, followed by a low expression of *Ptch1* (Mäkelä et al. 2011). *Ptch2* is expressed at the early stages of spermatogenesis (Fig. 5). However, previous studies conducted with *Ptch2* null mice had shown no significant difference in mouse fertility (Carpenter et al. 1998) (Nieuwenhuis et al. 2006). These findings suggest that *Ptchd3* could be the principal receptor for Hh during spermatogenesis since its expression is in the late stages of spermatogenesis (roundspermatids-sperm) (Fan et al. 2007). *Ptch1* had been found to be expressed in the interstitial cells, myoid cells and Leydig cells (Bitgood et al. 1996) (Clark et al. 2000). *Ptch1* had been shown to be necessary for Leydig cell differentiation at embryonic stages (Yao and Capel, 2002). *Gli1* is expressed in various germ cells from spermatogonia to round spermatids (Fig. 5) (Kroft et al. 2001)

(Mäkelä et al. 2011). Kroft et al. (2001) analyzed the ectopic Gli1 in spermatocytes and concluded that this transcriptional factor made the cells stop the meiotic cycle, indicating that Hh is required for proper spermatogenesis.

Ptch1, Ptch2, Smo and Fu, are expressed in both mitotic and meiotic murine germ cells. This is another piece of evidence that these cells have receptors required for Hh ligand response (Szczepny et al. 2006) (Morales et al. 2009).

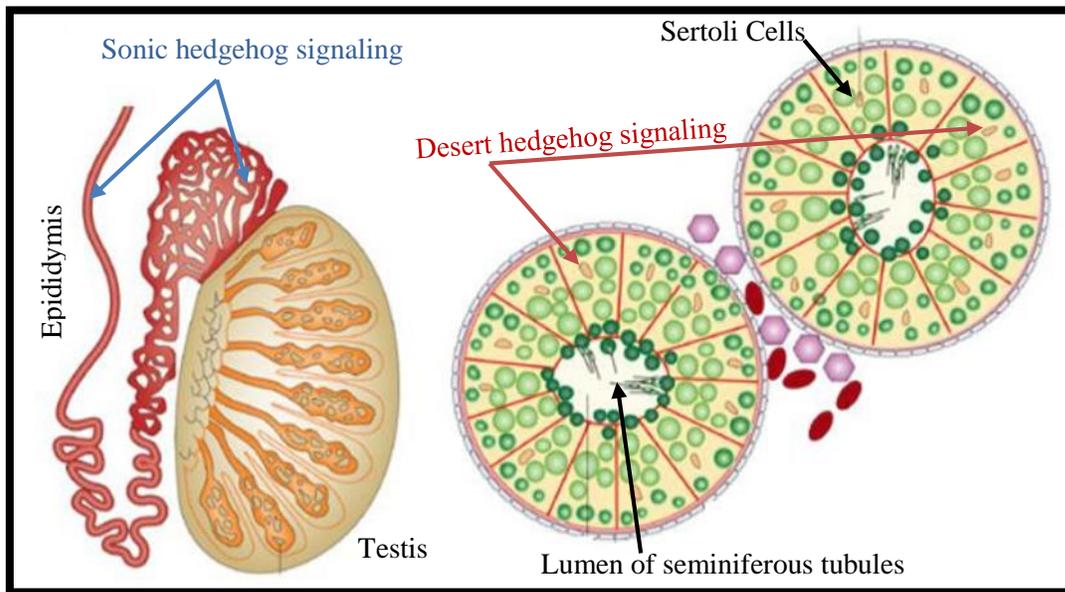


Fig 4. Hedgehog signaling in the testis and epididymis. Sonic hedgehog (blue) is expressed in all parts of the epididymis, while Desert hedgehog (red) is expressed in the testis and has especially been associated with Sertoli cells. This figure is modified from the report (Cooke and Saunders, 2002).

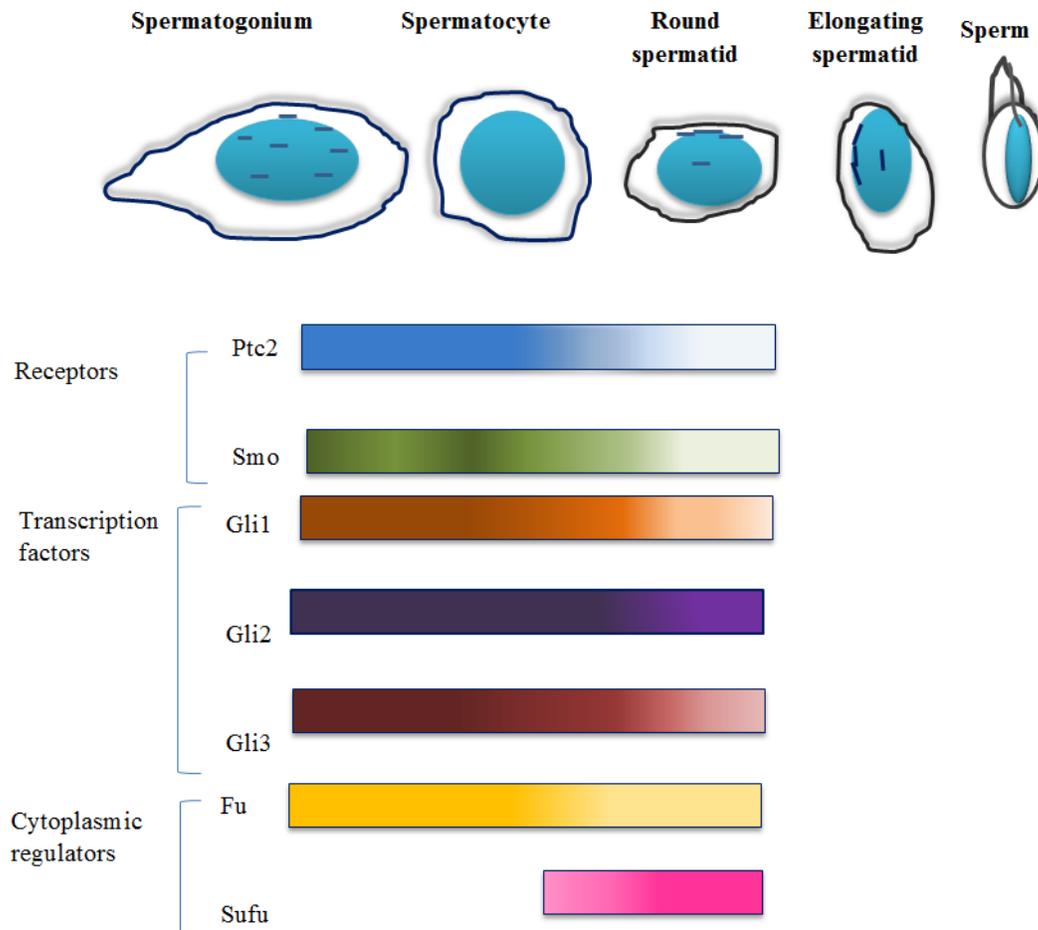


Fig 5. Hedgehog signaling components and their mRNA expression during spermatogenesis. This figure is modified from the report (Szczepny et al. 2006).

Desert Hedgehog during Spermatogenesis

Desert hedgehog (*Dhh*) plays a critical role in the spermatogenesis process, such as the development of the testis and the maturation of sperm (Szczepny et al. 2006). *Dhh* is the only hedgehog protein expressed in Sertoli cells during organogenesis (Fig. 4) (Szczepny et al. 2006).

Dhh helps differentiation of the fetal Leydig cells. At 11.5 dpc Sertoli cells begin to produce *Dhh* which allows the differentiation of Leydig cells to start. Leydig cells produce androgen, which is critical for developing male accessory organs. Yao and Capel (2002) simulated a *Dhh* null mutant using inhibitors of the pathway and observed that the number of Leydig cells was reduced albeit still present, which indicated that there was a genetic redundancy and that other *Hh* might play a role during this process. Genetic redundancy is not a surprise because Huang and Yao (2010) found that *Shh* mRNA was also expressed in the testis. Other studies have looked into the *Dhh*/*Ptch1* signaling and found that the specification or differentiation of Leydig cell lineage occurs because *Dhh* acts to upregulate the expression of steroidogenic factor 1 (*Sf1*) and *P450 side chain cleavage* (*Sccl*) expression in *Ptch1*-expressing precursor cells, which are found in the outer part of the testis cords (Yao et al. 2014).

Researchers had successfully produced a *Dhh* null mouse strain. The severity of the mutation depends on the genetic background of the mouse. Some mutants remain with normal testis but fail to produce mature sperm, while others end up with feminized external genitalia (Bitgood et al. 1996). On the other hand, other mice present problems in the testis patterning and produce irregular development of peritubular myoid cells, apolar Sertoli cells, lack of basal lamina, and anastomotic testis cords (Pierucci-Alves et al. 2001). Szczepny et al. (2006) confirmed these studies after generating a *Dhh* null mutant and found that males were infertile and did not produce mature sperm, indicating that the process of cellular differentiation was

affected at some point. Bitgood et al. (1996) also generated a mouse strain with a *Dhh* null allele. They found that the mutant mouse at 18.5 dpc exhibited small testis. These could be explained by the insufficiency of germ cells in the testis tubules and also the presence of residual Sertoli cells. After mutant males were born, the testis increased in size due to the continuation of germ cell mitotic cycle at 2-3 days post-partum (dpp). However, the mutant testis never reached the normal size afterward. At 10 dpp, meiosis begins. The haploid round spermatids form around 19- 21 dpp and the spermatozoa are released into the lumen of the seminiferous tubules at 5-6 weeks post-partum. *Dhh* seems to be important during this process of germ cell differentiation and development.

Overview of Ptchd3

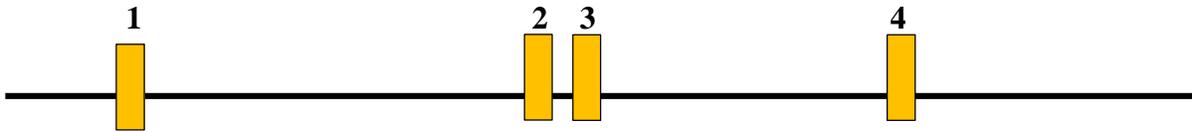
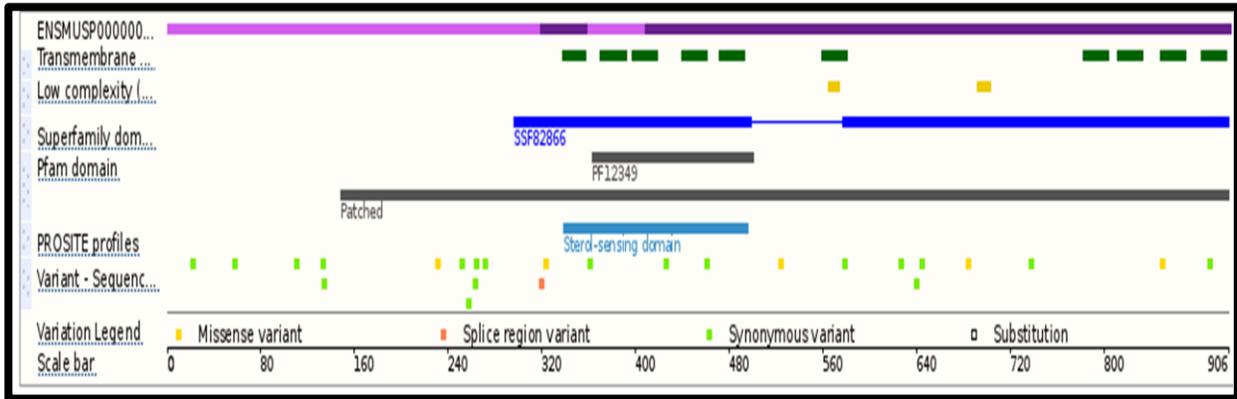
A previous study from our lab was the first to characterize a putative hedgehog receptor named Ptchd3, which is a male germ cell-specific gene. On sperm, the Ptchd3 protein was found in the mid-piece, and this location was conserved in humans, mice and rats (Fan et al. 2007).

Ptchd3 seems to fit well as the candidate receptor of *Dhh* during spermatogenesis. This proposed research tries to test if Ptchd3 is the essential receptor of *Dhh* in this process. During the characterization of this membrane protein Ptchd3, two transcript isoforms were found in mouse testis: Ptchd3a (the predicted protein product contains 410 amino acid residues) and Ptchd3b (the predicted protein product contains 906 amino acid residues) (Fan et al. 2007). Both isoforms were found to be transcribed on postnatal day 14. Ptchd3 gene is on chromosome eleven in mice and chromosome ten in humans. The Ptchd3b protein has a charge of -12.5, molecular weight of 101,813.36 g/mol and an isoelectric point of 5.1235. The presence of the 12 transmembrane domains in Ptchd3b (Fig. 6) is an indicator that this protein is a membrane protein. The presence of PTCH domain, sterol sensing domain (SSD) and resistance –nodulation-division (RND) super

family domains indicate that it might have multiple biological functions (Fig. 6), such as the regulation of embryonic stem cells (Fig. 26).

Gharamani Seno et al. (2011) studied *Ptchd3* in humans and found that this gene had copy number variant (CNV) in people. The CNV was found to be a product of a single ancestral event present in 0.6-1.6% of people with European ancestors. The expression of this gene was seen in different areas of the human body, but with highest expression in testis, lymph nodes and tongue. He concluded that *Ptchd3* was a non-essential gene at least in humans and its expression could increase fecundity but its absence did not cause infertility. Another study conducted by Smith et al. (2013) performed germ lines analysis to look at the genes that might be involved in colorectal tumorigenesis. He found that two patients of his study carried a truncated mutation in *Ptchd3*. After making a comparison of this mutation and somatic mutations in the wild-type allele that seen in the tumor patients, he concluded that *Ptchd3* was a tumor-suppressor and its mutation predisposed an individual to colorectal cancer.

Ptchd3 is present in different organisms (Fig. 27), for example in *C. elegans* (Soloviev et al. 2011). However, contrary to *Ptchd3* in mouse, in *C. elegans* it is expressed in multiple tissues. Soloviev et al. (2011) investigated the temporal and spatial pattern of *Ptchd3* expression in *C. elegans* during the embryonic development. He found that in *C. elegans* *Ptchd3* was essential to the survival of the embryo when it was transitioning to the larval stage.

A**B**

www.ensembl.org

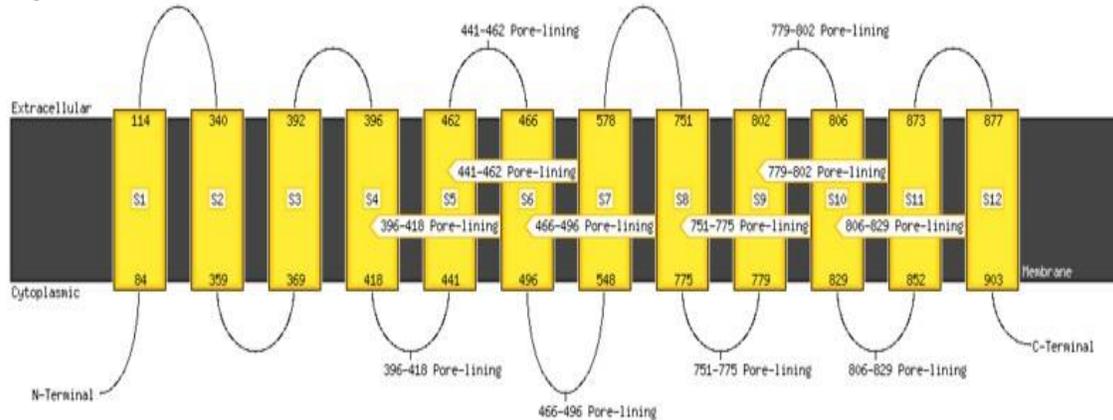
C

Fig 6. Ptchd3 gene and protein structure. (A) The Ptchd3 gene consists of 4 exons. (B) The conserved protein domains of Ptchd3 were analyzed at www.ensembl.org. Ptchd3a has two transmembrane domains and Ptchd3b has ten transmembrane domains. Ptchd3b also possesses a sterol sensing domain (SSD), Patched domain, two AcrB (Cation/multidrug efflux pump) domains and three RND superfamily domains (Fan et al. 2007). (C) Ptchd3b protein sequence was analyzed at the University of College London MEMSAT3 web server to obtain the protein topology, which consists of 12 transmembrane domains.

CHAPTER 2

SPECIFIC AIMS AND HYPOTHESIS

Objective and Specific Aims

The purpose of this research is to study if Ptchd3 has a function during spermatogenesis by observing the effects of Ptchd3 knockout in *Mus musculus*.

Specific Aim 1: Generation of Ptchd3 Knockout Mice. There were no Ptchd3 knockout mice commercially available when this project was initiated. Therefore, generation of a Ptchd3 knockout mouse line was greatly needed in order to study this gene's function in vivo.

Specific Aim 2: In Vivo Fertilization Analysis. To assess if Ptchd3 plays a critical role during mouse spermatogenesis, we had to test the fertility of Ptchd3 knockout mice.

Specific Aim 3: In Vitro Sperm Analysis. There are many approaches to test male infertility, and one of them is to analyze sperm morphology (Lindemann, 2010) (MacLeod and Gold, 1951). It has been shown that Ptchd3 is located in the mid-piece of the sperm tail (Fan et al. 2007), where mitochondria are present and produce the required energy for sperm movement. Thus, we decided to in vitro assess sperm motility in Ptchd3 knockout mice.

Hypothesis

This study aims to test the hypothesis that Ptchd3 (Patched domain containing 3), a male germ cell-specific gene whose expression pattern is conserved in humans, mice and rats, acts as a receptor for *Dhh* in mouse spermatogenesis.

CHAPTER 3

MATERIALS AND METHODS

Generation of Mutant Mice and Genotypic Analysis

Mutant Generation. The *Ptchd3* knockout chimeric male mice were generated at University of California- Davis under the project CSD 24758 of Knockout Mouse Project.

The chimeric mice, BL3085-6, which contained an allele of *Ptchd3*^{tm1a} Wtsi were transferred to Marshall University animal facility and crossed with C57BL/6 female mice to produce F1 *Ptchd3*^{+/-} heterozygous mice (Fig. 7). The F1 mice were inter-crossed to obtain *Ptchd3*^{-/-} homozygous knockout mice.

The animal care and experiments 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 Care and Use of Laboratory Animals.

DNA Extraction. Once the offspring were approximately one month old, about 2-3mm of the tails was cut for DNA extraction. The tails were digested by using 75 μ L of 50mM NaOH and heating at 95°C for 30 min. Then 75 μ L of 50mM HCl and 15 μ L of 1M Tris HCl were added to complete the digestion process. The extracted DNA solution was then stored at 4°C.

PCR. The mouse genotype was determined by polymerase chain reaction (PCR) of the genomic DNA that was obtained from the tail. The primer pairs used to detect the knockout amplicon (389bp) were *Ptchd3-loxF* GAGATGGCGCAACGCAATTAATG and *Ptchd3-R* CAACTGTATCCCTCAAGAAACAAGCC (Fig. 8). The other set of primers, *Ptchd3-F* GCATGGCTGACTCACTTTCTTGACC and *Ptchd3-ttR*

GGGTTATATTTTGGGATTGCTGGCCC were used to detect the wild-type amplicon (543bp)

(Fig. 8). The PCR mix and PCR cycles are described in the appendix.

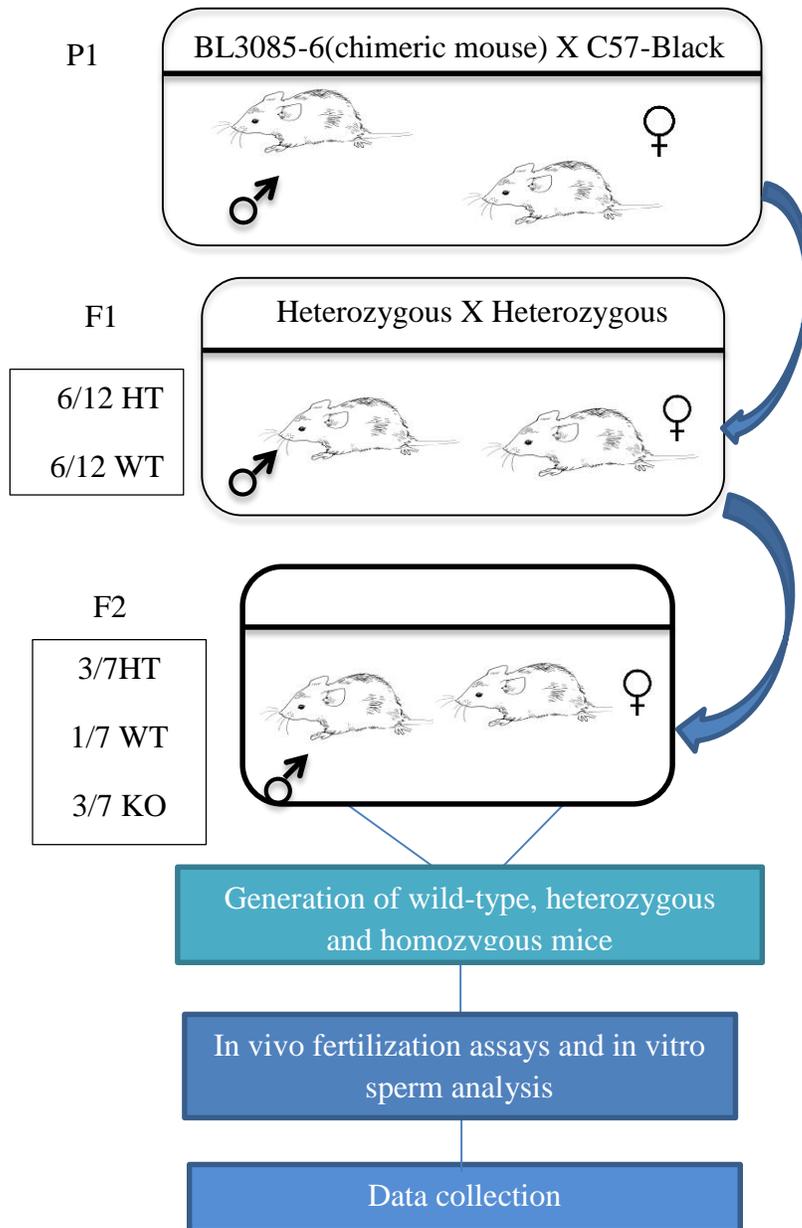


Fig 7. Generation of the Ptchd3 knockout mice and experimental design.

sample. Once the cut was made, the fat was pulled out, allowing the testes to be seen more clearly for their removal. The testes were collected and kept either at -20°C for RT-PCR analysis, or in 4% paraformaldehyde for histology. The testes could also be used as fresh samples for RT-PCR or Western blot analysis. In order to get the percentage of testis body weight, their weight was measured with an analytical balance and then the testis body weight was calculated using the formula below:

$$\text{Testis body weight} = \left(\frac{\text{Testis}}{\text{Body weight}} \right) \times 100.$$

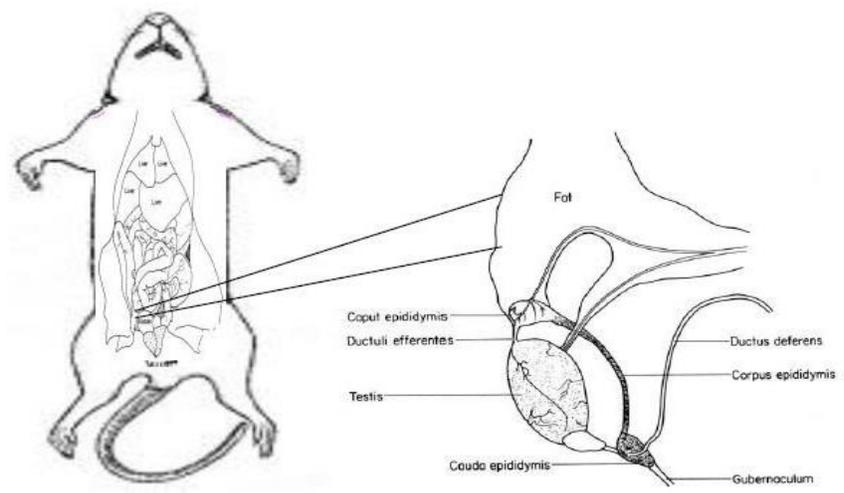


Fig 9. Dissection of mouse testis and epididymis. The illustration shows how the male mouse is dissected and the testis and sperm are collected.

Sperm Analysis and Abnormality

To perform sperm analysis, first the epididymis was cut and placed in 1.5 mL of 1X PBS. The sperm sample was filtered to avoid big pieces of tissues (for obtaining a pure sperm sample)

and spin down in the Eppendorf centrifuge 5415D for five min at 8000 rpm. In order to observe the sperm nucleus, DAPI was added and incubated at room temperature for 15 min using the rotator. The DAPI blue fluorescence stain would help to have a better visualization of the sperm head where the sperm nucleus is located. The DAPI staining serve as a confirmation to avoid bias when the abnormal/normal ratio of sperm morphology was performed (Fig. 16). The samples were analyzed on Leica DMI 4000B fluorescent microscope. The images (phase contrast and fluorescence) were captured with Leica DFC 400 digital camera.

$$\%Abnormality = \left(\frac{Abnormal\ Sperm}{Normal\ Sperm} \right) \times 100$$

Statistical Analysis

The data obtained from the experimental cages (litter size, litter number, testis body weight, sperm morphology and sperm motility) were analyzed using t-test (Microsoft Excel) or one-way ANOVA. The standard deviation and mean of each genotype was determined. The formulas used for standard deviation and mean are described below:

T-test statistical analysis

$$Average = \frac{\sum n_1 + n_2 + n_3 + \dots}{total\ mice\ analyze}$$

$$Standard\ Deviation = \frac{\sqrt{\sum (X - \bar{X})^2}}{n-1}$$

To corroborate the data, SAS and Prism statistical programs were used. An example of the commands used to run SAS program is indicated below.

SAS 9.4

Version: Example

```
PROC MEANS DATA=mice;  
Var abnormality;  
CLASS genotype;  
RUN;
```

```
PROC UNIVARIATE DATA=mice PLOT NORMAL;  
VAR abnormality;  
BY genotype;  
RUN;
```

```
PROC ANOVA  
DATA=mice;  
CLASS genotype;  
MODEL abnormality=genotype;  
MEANS genotype/Tukey;  
RUN;
```

RT-PCR

RNA Extraction. The RNA was extracted by the method of Chomczynski and Sacchi (1987). When the testes were removed, they were cut in halves and the epithelial layer was removed and discarded. The remaining tissue was placed in a 1.5 mL tube with 300 μ L of TRIzol reagent (from Life Technologies). The samples were mixed, homogenized and vortexed until completely dispersed. After that, 60 μ L of chloroform was added. The sample was vortexed for 15 sec, and incubated for ten min under RT. Later the sample was centrifuged for ten min at 12,000 g at 4°C. The aqueous phase was transferred to a new tube and the remaining was discarded. Then 150 μ L of 2-propanol was added and was vortexed for ten sec and incubated at RT for ten min. After that, the sample was centrifuged for ten min at 12,000 g at 4°C. The supernatant was discarded by pipetting and the RNA pellet was washed with 250 μ L of 70% ethanol in nuclease-free water.

Then the pellet sat at RT for about ten min for air dry. In order to finish the RNA extraction, 40 μ L of nuclease free water was added to the sample and the concentration was then measured by the Nanodrop spectrophotometer.

RT-PCR. Once the RNA concentration was determined by the Nanodrop, the Transcriptor First Strand cDNA Synthesis Kit (by Roche) was used to obtain the cDNA, which was used as a template for PCR. To make RNA-primer mix, 1 μ g of total RNA was added to a PCR tube with addition of 2 μ L of Random Hexamer Primer (from Roche) and 10 μ L of nuclease free water. The sample was then denatured at 65 $^{\circ}$ C in the thermo cycler for ten min. After denature, the sample was immediately placed on ice. Then the next components were added in the following order, first the Reverse Transcriptase Reaction Buffer, 5x (4 μ L), second Deoxynucleotide Mix, 10mM (2 μ L) and finally the Reverse Transcriptase (0.5 μ L). Then the sample was mixed by gently pipetting and spin down for 30s. After that, the sample was placed in the thermo cycler and incubated ten min at 25 $^{\circ}$ C, followed by 60 min at 50 $^{\circ}$ C and finally five min at 85 $^{\circ}$ C. At this point, the cDNA was generated and used for the next experiment.

The cDNA was then used as the PCR template. The primers for β -actin were (5'-GTG GGC CGC TCT AGG CAC CAA-3' and 5'-CTC TTT GAT GTC ACG CAC GAT TTC-3'). The primers FP1: 5'-CACCCAGCTCATCTACTTAGC-3' and RP1: 5'-CTACAAATTTAACACAGCCTCG-3' were used to produce an amplicon of 524bp to detect the short isoform, Ptchd3a (Fig. 10). The primers FP1: 5'-CACCCAGCTCATCTACTTAGC-3' and RP2: 5'-GAGCAGGGTTGTTTCCTGTATAG-3' were used to produce an amplicon of 700bp to identify the long isoform, Ptchd3b (Fig. 10) (Fan et al. 2007).

The primers for the short isoform Ptchd3a

Forward Primer-FP1: 5'-CACCCAGCTCATCTACTTAGC-3'

Reverse primer RP1: 5'-CTACAAATTTAACACAGCCTCG-3'

These primers can generate a PCR amplicon of 524 base pairs (bp).

The primers for the long isoform Ptchd3b

Forward Primer-FP1: 5'-CACCCAGCTCATCTACTTAGC-3'

Reverse primer RP2: 5'-GAGCAGGGTTGTTTCCTGTATAG-3

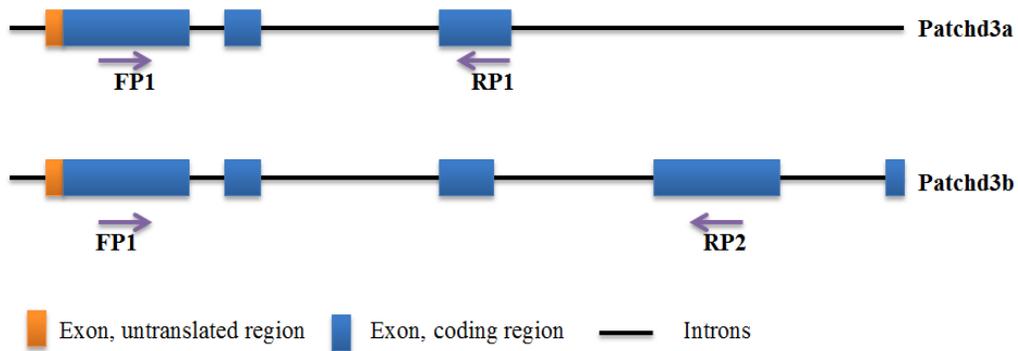


Fig 10. Position of the RT-PCR primers on the mouse Ptchd3 gene.

Sequencing

Sample Preparation. After obtaining the positive RT-PCR product we proceeded to make an additional round of PCR by adding 1 μ L of the sample to five different PCR tubes containing the PCR mix (making a total of 50 μ L reaction volume). Once the reaction was finished the volume of each PCR tube was combined for the PCR purification.

PCR Purification. Once the PCR samples were prepared, we used the QIAquick PCR Purification Kit to clean the samples. First PB was added and mixed, followed by the addition of 10 μ of 3 M sodium acetate. The samples were added to the QIAquick columns and centrifuged at 13,000 rpm for one min. After that, the flow-through was discarded and 750 μ of PE buffer was added to the columns, followed by a centrifugation at 13,000 rpm for one min. An additional centrifugation was performed to remove the excess of the PE buffer. The column was removed from the collection tube and placed in a new 1.5 mL microcentrifuge tube. After that, the DNA was eluted by adding 50 μ L of EB buffer and centrifuged at 13,000 rpm for one min.

Sequencing. The purified PCR products were sent to the genomic core facility at Marshall University for conventional sequencing. The DNA sequence and corresponding protein sequence were analyzed by CLC Main Workbench 6.0 and the MAMSAT3 web server provided by the University of College London.

Histology

Mouse testes were collected and preserved in 4 % paraformaldehyde. The fixed testes were then sent to John C. Edwards School of Medicine at Marshall University for slide preparation. The testes were embedded in paraffin and cut with a microtome (six micron thickness). Tissue slides were stained with hematoxylin and eosin. The slides were observed under Leica DMI 4000B

microscope and the images were captured with Leica DFC 400 digital camera.

Immunostaining

The dissection was performed as described previously in section 3.3 to obtain and isolate the epididymis in 1.5 mL of PBS. Then the epididymis was cut about ten times and left at RT for 15 min to allow the sperm to move out from the tissue. The sperm sample was filtered through a mesh to clear out big pieces of tissues, and then centrifuged at 5000 rpm for three min. After that, the supernatant was discarded and the sperm pellet was washed and suspended in 500 μ L of PBS with 0.3% bovine serum albumin in two different 1.5 mL tubes. 200 μ L of the sperm suspension was added. One tube contained 10 μ g/mL of primary antibody (Ptchd3-Ab1), and the other did not (control). Both tubes were incubated at RT for 45 min and centrifuged at 5000 rpm for four min. After centrifugation, the sperm sample was washed and suspended with 500 μ L of PBS with 0.3% bovine serum albumin. Then the secondary antibody (Alexa Fluor 488-conjugated goat anti-rabbit antibody) and DAPI (to observe the nucleus) were added (Fan et al. 2007). After that, the tubes were covered with aluminum foil to avoid light exposure and incubated at RT for 45 min by rotation. After incubation, the sample was centrifuged at 5000 rpm for 3 min to discard the supernatant. Finally, the sperm pellet was resuspended in 200 μ L of PBS. 10 μ L of the sperm suspension was placed on a glass slide, covered with a glass cover slip, and observed under Leica DMI 4000B fluorescent microscope. The images (phase contrast and fluorescence) were captured with Leica DFC 400 digital camera.

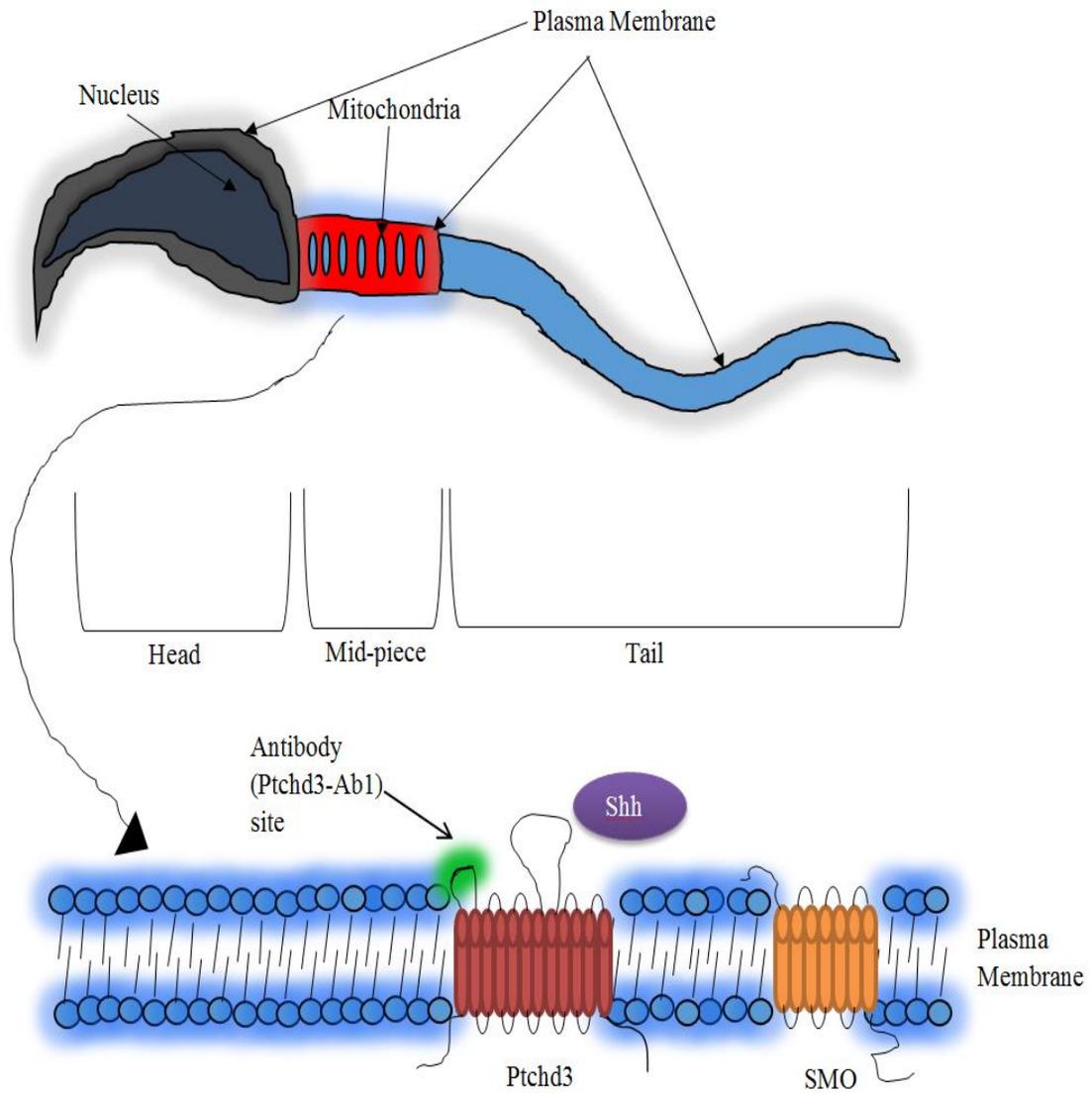


Fig 11. Antibody recognition of Ptch3. The antibody recognition site is located in the first extracellular loop.

Western Blot

Preparation of the Testis Protein Sample. The testes were isolated from the mice and one of them was cut in half with a blade. Half of the testis was placed in 1.5 ml microcentrifuge tube, and the other half was saved for RT-PCR. The epithelial layer was removed with a tweezer. The testis was spun down for several seconds. Then 5 μ L of SDS, 5 μ L of ABSF and 250 μ L of RIPA buffer were added (the final SDS concentration was 0.2%). Then the sample was placed on ice for ten min. After that, the sample was sonicated for 15 min. After sonication, the sample was centrifuged at 12,000 rpm, 4°C for 15 minutes. The supernatant was saved as the testis protein sample. The protein concentration was measured by Nanodrop.

Protein Separation. 0.1 mg of the testis protein sample was added to 2 μ L 6xR (DTT) and/or 6xNR protein loading buffer, and heated at 100°C for three minutes. After that the sample was spun down and kept on ice until being loaded to the gel. Afterwards 500ml of 1X SDS running buffer was added into the running tank so that it would fully cover the inner gel and half of the outer space of the running cassette. Then the protein samples (10 μ L of testis protein sample and 7.5 μ L of protein marker) were loaded into the gel wells. The gel was run at 80V for 10 min, followed by 100V for 1h. After running, the gel was removed from the cassette and the lowest part of the gel was discarded. The nitrocellulose membrane was placed in methanol and then washed 2x with distilled water and then everything (the sponges, filter papers, gel and membrane) was submerged in the wet transfer buffer for 15 minutes. Then the sandwich was built by placing the sponge, followed by filter paper (after every addition the assembled materials were rolled with a 2ml pipet to remove any bubbles) membrane, gel, filter paper and finally a sponge to close the cassette. The cassette was placed in the transfer box, which contained enough transfer buffer, and run at 100V for one hour to transfer the proteins to

the membrane.

Probing. The membrane was first blocked in 5% nonfat milk in TBST buffer (50mM Tris (pH 7.5), 150 mM NaCl, 0.1% Tween-20) for one hour at RT. After that the primary antibody Ptchd3-Ab1 (1:500 dilution, 10 μ L in 5 mL of TBST) was added. The membrane was incubated overnight at 4°C with rotation. After incubation, the membrane was rinsed twice with TBST, and then underwent one 15 min wash, followed by 3X wash (five min each) with TBST. All washing steps were done with shaking. After washing, the secondary antibody (mouse anti-rabbit, AP conjugated) was added (1:8000 dilution, 1 μ L in 8mL of TBST) and the membrane was incubated at RT for one hour with shaking. After incubation with the secondary antibody, the membrane was washed as described above. The membrane visualization was developed by adding 4mL of color solution (Promega).

Sperm Motility Analysis

To perform sperm motility assay, first the epididymis was cut and placed in 1.5 mL of 1X PBS. The 1.5 ml of sperm sample was filtered through a nylon mesh to avoid big pieces of tissue, transferred to a microcentrifuge tube, and then spun down in the Eppendorf centrifuge 5415D for one min at 8000 rpm. Later, the supernatant was taken out and discarded. 500 μ L of 1X PBS was added and the sperm pellet was re-suspended by pipetting. After that, the sample was incubated at 36°C for five min. 100 μ L of the sperm sample was taken out and diluted in 1mL of 1 X PBS. Later, 20 μ L of the sperm sample was mounted on a glass slide and observed under Leica DMI 4000B microscope. The videos were taken with a 3 MP resolution camera. The beating of the sperm was quantified for a minute for both wild-type and knockout sperm. The data was analyzed by t-test.

CHAPTER 4

RESULTS

To date, there are no published studies on Ptchd3 function in *Mus musculus*. Previous studies have just looked at the tissue expression of this transmembrane protein. In order to determine the function of an interested gene, researchers normally begin with an over-expression or knockout approach. Because this is the first study on Ptchd3, no Ptchd3 mutant mice are commercially available. In order to obtain Ptchd3 knockout mice in our laboratory, a custom-made transgenic mouse was first generated by UC Davis KOMP Repository Knockout Project. The resultant chimeric male mice were then transferred to Marshall University to produce Ptchd3 mutant mice.

Ptchd3 Knockout

The targeting vector Ptchd3-tm1a (KOMP) wtsi used to generate the chimeric males was illustrated below (Fig. 12). If successful, this targeting strategy would result in a mutant Ptchd3 mRNA (including endogenous exon1 and inserted EN2, IRES, LacZ and Poly A), which is expected to have reading frame shift and produce a truncated protein in the mutant mice.

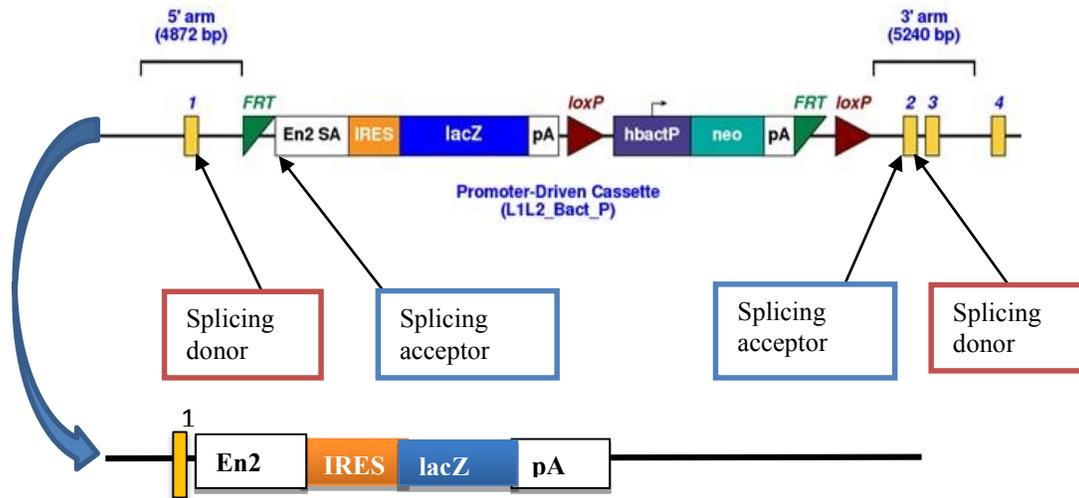


Fig 12. Mutation strategy used to generate Ptchd3 knockout mice

Genotyping

In a previous study (Fan et al. 2007), the expression of Ptchd3 was described and a particular pattern of expression was observed in the mouse testis. This expression suggests that Ptchd3 might be involved in spermatogenesis. To address Ptchd3's function, we adopted the gene targeting strategy. Two chimeric male mice were transferred from UC Davis to Marshall University, and subsequently mated with C57 Black females to produce the offspring. Only one chimeric male was able to transmit mutant Ptchd3 allele to its progeny (in other words, progeny with heterozygous genotype). The offspring genotypes were determined by PCR on the tail genomic DNA using the primers Ptchd3-loxF and Ptchd3-R, which identified the Ptchd3 wild-type mice with an amplicon of 543 bp and knockout mice with an amplicon of 389 bp (Fig. 13).

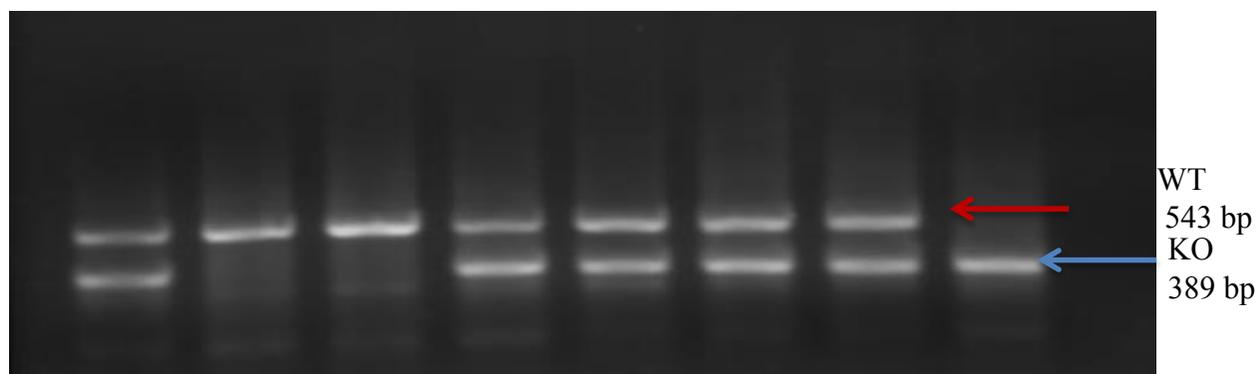


Fig 13. Genotyping of the *Pthcd3* transgenic mice. PCR and gel electrophoresis was performed to identify mutant mice.

In Vivo Fertilization Data

The in vivo fertilization experimental cages were established to monitor the offspring litter number and litter size. The one way-ANOVA test generated by Prism reveals no significant difference for litter number (p -value of 0.7973) (Fig. 14) and litter size (p -value 0.3648) (Fig. 15).

These in vivo fertilization results clearly demonstrate that *Ptchd3* is not essential for mouse fertility.

In Vivo Fertility Assay

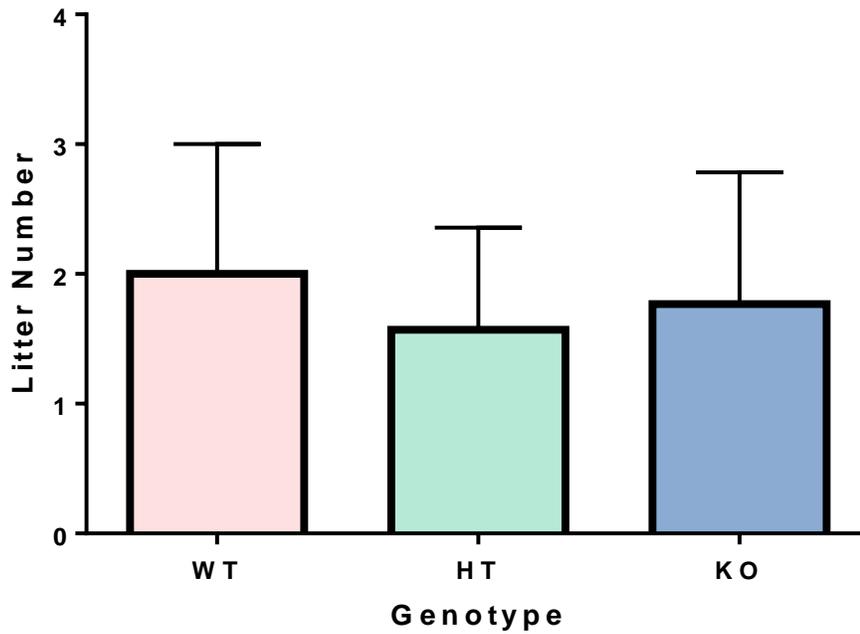


Fig 14. In vivo fertilization assay. Each experimental cage contained one male and two females. The data were collected from three wild-type, thirteen knockout and seven heterozygous male mice. A one way-ANOVA test generated by Prism software reveals a p-value of 0.7973.

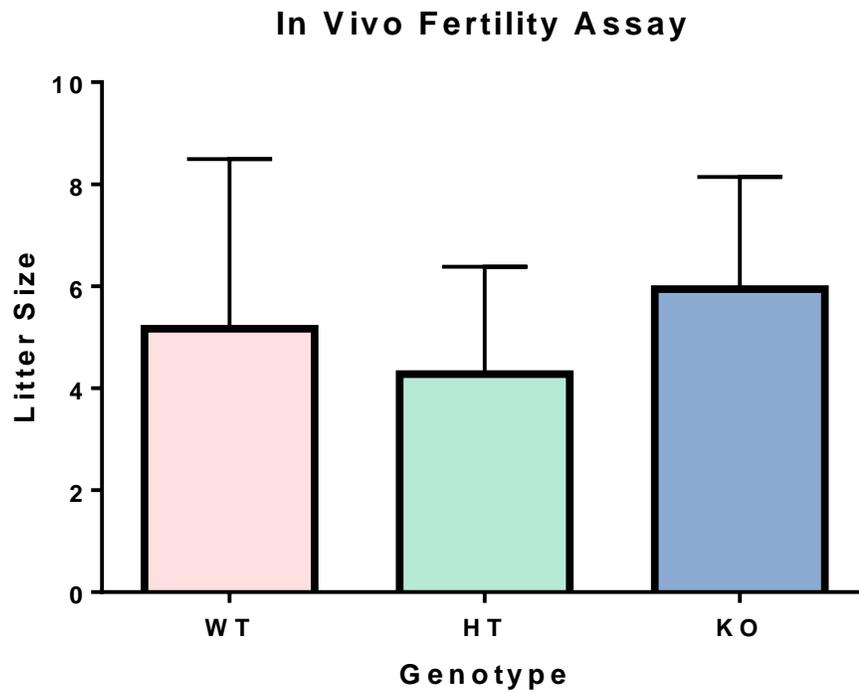


Fig 15. In vivo fertilization assay. Each experimental cage contained one male and two females. The data were collected from three wild-type, thirteen knockout and six heterozygous mice. A one way-ANOVA test generated by Prism software reveals a p-value of 0.3648.

In Vitro Sperm Morphology Analysis

The results obtained from the experimental cages for the in vivo fertilization somehow were not expected from a testis specific protein whose expression is conserved in the sperm of different mammals. Thus, we further analyzed sperm morphology in vitro. The abnormality/normality ratio was obtained by quantifying the sperm with abnormal morphology (Fig. 16) divided by the total sperm. The one way-ANOVA data reveals that there is some statistical significance (p -value 0.041*) (Fig. 17). Tukey test (performed on SAS 9.4) was then performed to determine which groups were statistically significant with one another. The comparison between KO and WT (interval ± 4.7 to ± 12.8) and HT and WT (interval ± 6.9 to ± 10.9) showed no statistical significance, since both comparisons were able to include zero. However, the comparison between KO and HT (interval ± 0.4 to ± 11.6) showed a statistical significance, since the values did not include zero. Nevertheless, this statistical significance might be explained by a bias (e.g. human factor) when we calculated the sperm abnormality or lower sample size.

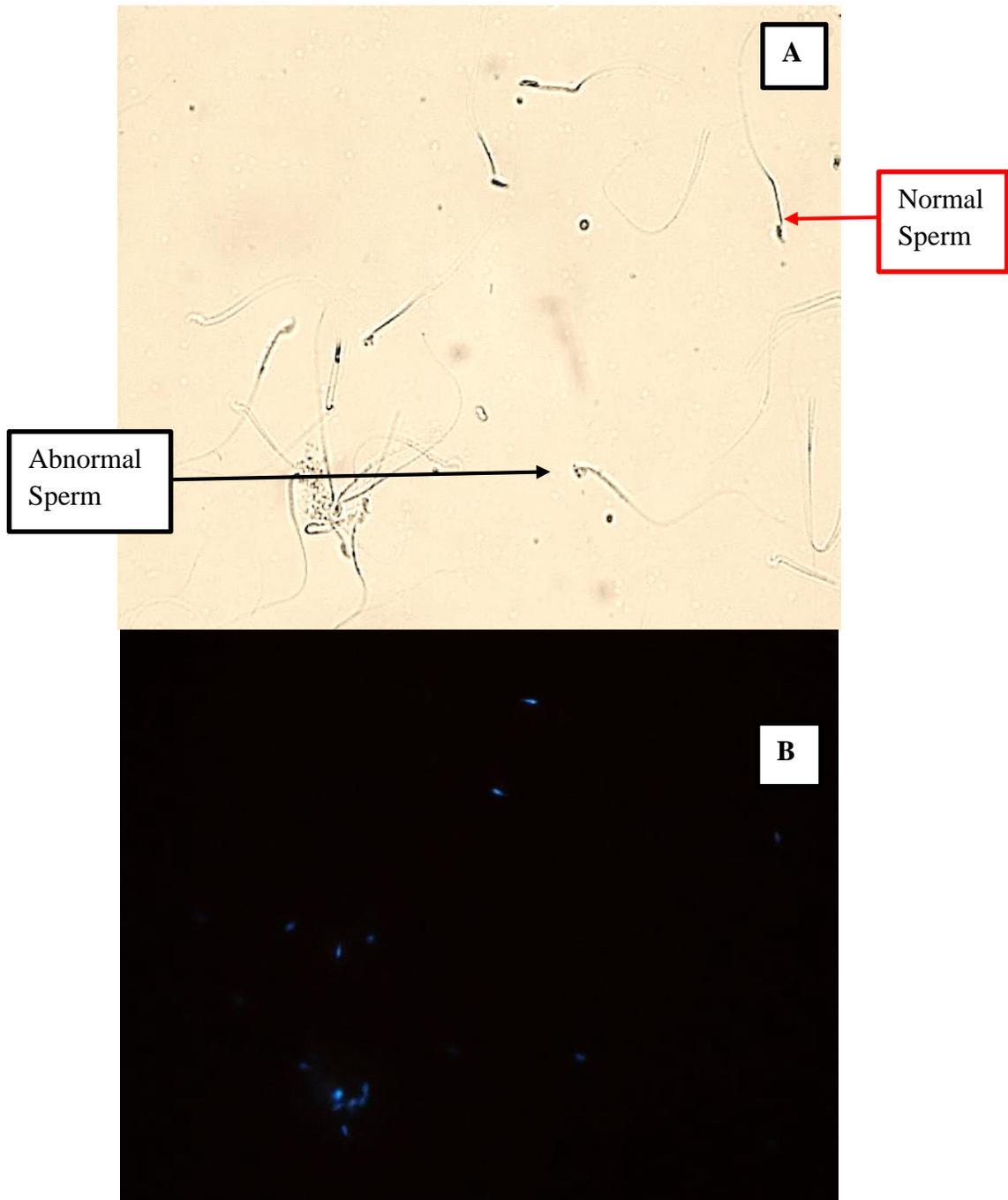


Fig 16. Sperm morphology of *Ptchd3* knockout mice. A) Black arrow shows an abnormal sperm while red arrow represents a normal one. B) DAPI staining of sperm nucleus. The samples were analyzed by Leica DMI 4000B fluorescent microscope. The images were captured with Leica DFC 400 digital camera.

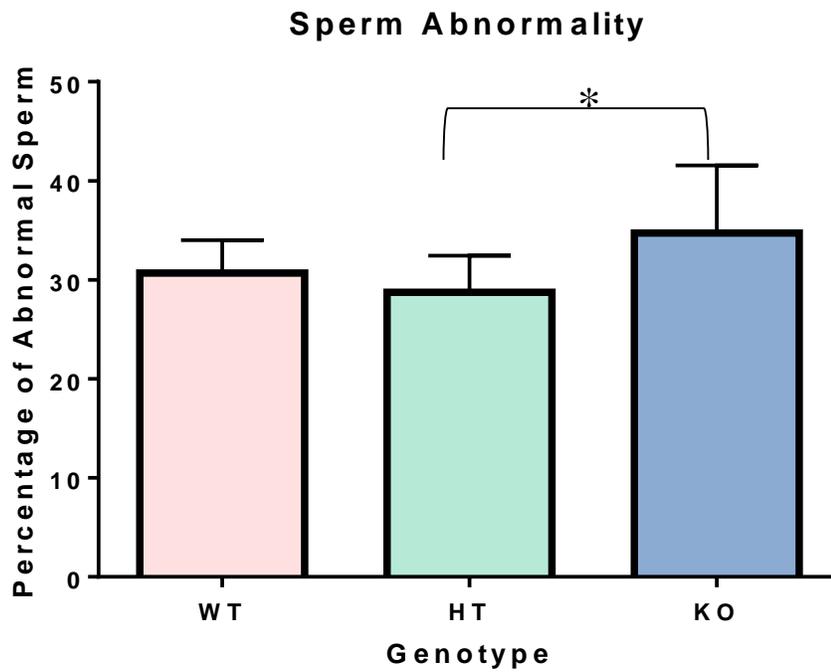


Fig 17. Sperm morphology analysis. The data were collected from three wild-type, thirteen knockout and eleven heterozygous mice. A one way-ANOVA test generated by Prism software reveals a statistical significant (p-value 0.041*).

Testis Body Weight

We then measured body and testis weight of wild-type, heterozygous and null mutant mice, and analyzed the ratios of testis to body weight. The one-way ANOVA indicates that there is no significant difference (p -value 0.0638) among the three tested groups (Fig. 18).

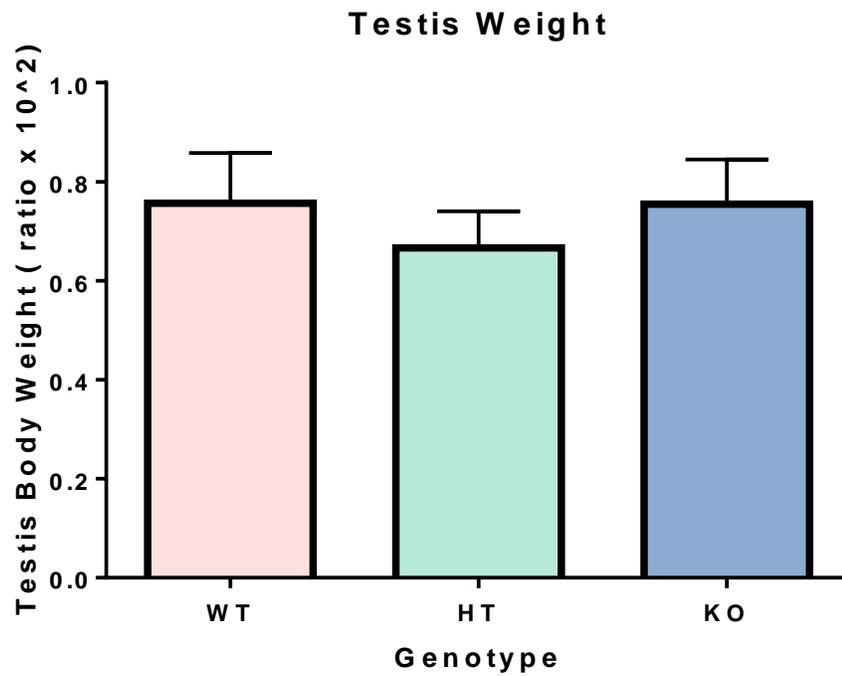


Fig 18. Analysis of testis body weight. The data were collected from three wild-type, fourteen knockout and nine heterozygous mice. A one way-ANOVA test generated by Prism software reveals a p-value of 0.0638.

RT-PCR

To determine the mRNA expression levels of Ptchd3 in mutant and wild-type mice, semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) was performed on cDNAs derived from the testis. After reverse transcription, one set of primers (Fp1/RP1) was used to amplify the short Ptchd3 isoform Ptchd3a and the other set of primers (FP1/RP2) to amplify the long Ptchd3 isoform Ptchd3b (Fan et al. 2007). The RT-PCR would reveal how the knockout strategy works at the transcriptional level. We run β -actin as a control since it is a housekeeping gene, and its expression should be the same among different samples. We observed a constant expression of β -actin in different samples, which was expected (Fig. 19a).

However, the RT-PCR for Ptchd3b as well as Ptchd3a (data not shown) shows a slightly higher amplicon (\approx approximate 80 bp) in the knockout mice, as compared with that in the wild-type mice (Fig. 19b). This result validates that the knockout strategy was successful at the mRNA level. However, the RT-PCR for Ptchd3b as well as Ptchd3a (not indicated below) shows a slightly higher amplicon (\approx approximate 80 bp) for the knockout than the wild-type (Fig. 19b).

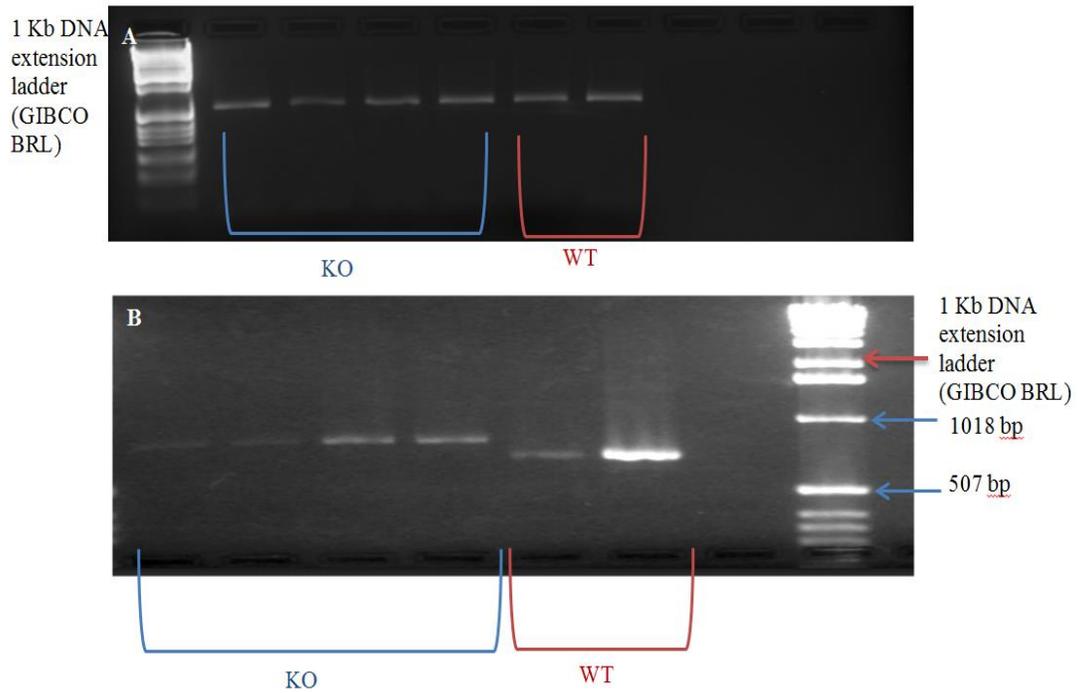


Fig 19. RT-PCR A) β -actin RT-PCR shows that all samples have similar expression. B) The result shows the expression of Ptchd3b isoform in both knockout (Approx. 780bp) and wild-type mice (700bp).

Immunofluorescence

The RT-PCR result indicated that we did not detect any wild-type mRNA in the knockout testis. To confirm that the Ptchd3 protein was indeed altered in the knockout mice, we conducted immunofluorescence on the sperm by using the antibody Ptchd3-Ab1, which recognizes the first extracellular loop of the Ptchd3 protein (Fig. 11). However, we observed the similar fluorescent pattern in the mid piece of both wild-type and knockout sperm (Fig. 20). This result indicates that the truncated Ptchd3 produced by the knockout strategy still contains the antigen site recognized by the antibody Ptchd3-Ab1, and is still able to get to the sperm plasma membrane.

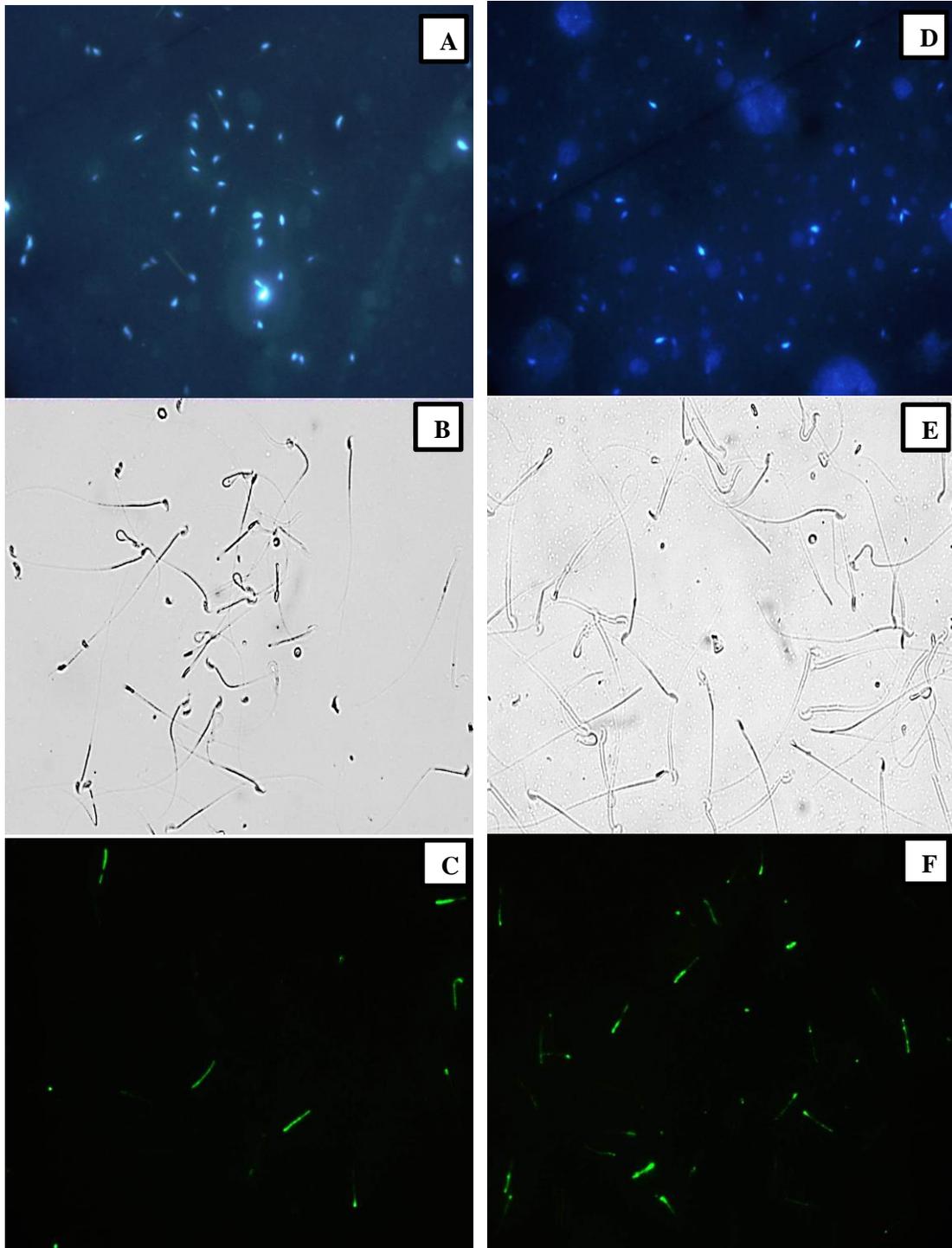


Fig 20. Immunofluorescent assay of mouse sperm. The antibody Ptchd3-Ab1 was used in the immunofluorescent assay on the wild-type (panels D-F) and Ptchd3 knockout sperm (panels A-C). Panel A and D: DAPI staining in the sperm nucleus. Panels B and E: phase contrast. Panel C and F: Alexa-488 green fluorescence. Green fluorescence was found in the mid piece of both wild-type and knockout sperm.

Sequencing

The RT-PCR analysis on the Ptchd3 knockout testis revealed an unexpected PCR amplicon (≈ 780 bp) (Fig. 19). In order to determine that this amplicon was not due to a result of non-specific amplification or contamination, the PCR product (≈ 780 bp) was purified and further analyzed by DNA sequencing.

The result obtained from DNA sequencing indicated that appearance of the unexpected PCR product (≈ 780 bp) likely stems from the methodology implemented in the generation of Ptchd3 transgenic mice. The mutant mice were generated via conventional insertion, predictably resulting in a frameshift mutation and the production of truncated/non-functional Ptchd3a and Ptchd3b proteins. We found that the inserted DNA, causing the truncated protein, was from partial exon 2 of mouse En2 (homeobox protein engrailed-2) (Fig. 21). The sequence data also showed that the antibody (Ptchd3b-Ab1) recognition site was not changed in the truncated protein (Fig. 21), which was the reason that we still could observe immunofluorescent staining on the knockout sperm (Fig. 20). The truncated protein sequence was analyzed at the MAMSAT3 web server provided by the University of College London. The result shows that truncated Ptchd3 only has two transmembrane domains (Fig. 22), as compared with the wild-type protein which has twelve transmembrane domains (Fig. 6).

```

Ptchd3b-En2-KOMP ATGATCTCCTCCAAGGTGGCGCCCGGAGAGGAGGAGCAAGGGGAGTACCGTCCAAGGTGGAGCTCGGAGAGGCG
Translation +1 M I S S K V A P G E E E Q G E S P S K V E L G E A
80
Ptchd3b-En2-KOMP GAGGAGCAGCGGGAGGCACCGTTGGAAGGCCAACTGGAAGCCGTGCCACTTGATGAGGCGGGCCCTAGCTGGACC
Translation +1 E E Q R E A P L E G Q L E A V P L D E A G P S W T
100
Ptchd3b-En2-KOMP ACCGGGCGGCCACTGGAGCGCTTGCCACCGCTGGGTCAGGAGGCACCTCCGCCGCGTCCGTCGCCACACCAACTGC
Translation +1 T G P P L E R L P P L G Q E A P P P R R C H T N C
120
Ptchd3b-En2-KOMP TTGGAAGCACCGCTGTCGCGGGGCTTCCAACCGCTTTGGCGCCACAGTGGGCGCGAACCCCTGGCTGTTTTTGCTC
Translation +1 L E A P L S R G F Q R F G A T V G A N P W L F L L
140
Ptchd3b-En2-KOMP GGGCCTGCACTGCTGACGGCCTCCCTGGGCACCGGTCTCATTTTTCTACCCAAGGAAAAGGAGAAGCTGGAGGAG
Translation +1 G P A L L T A S L G T G L I F L P K E K E N L E E
160
Ptchd3b-En2-KOMP CAGTACACACCGATTGGCAGCCAGCCAAAGGCAGAGCGGCGTTTTGTGCAGGGTCATTTTCAGTACCAATGACACT
Translation +1 Q Y T P I G S P A K A E R R F V Q G H F S T N D T
180
Ptchd3b-En2-KOMP TACCGCTTCTCTGCCTCCAGGACCAGCTCCGAGACCAACTTTGCATCCATCTTGGTGGTGTCCCTCGCCAAGTCA
Translation +1 Y R F S A S R T S S E T N F A S I L V V S L A N S
200
Ptchd3b-En2-KOMP CTGCTGGAGCCAGAAAATATTCAAAGAGGTGAGCAAGCTGGACCAGGCGGTGCAGGCGCTGAAAAGTTGTCCAGGAA
Translation +1 L L E P E I F K E V S K L D Q A V Q A L K V V Q E
220
Ptchd3b-En2-KOMP AATGGAACGCAGATTCTATACCAAGAGGTGTGTGCCAAGTACAAGACGCTGTGCGTGCCGCCCAACCCCTCTGCTG
Translation +1 N G T Q I L Y Q E V C A K Y K T L C V P P N P L L
240
Ptchd3b-En2-KOMP TTTTCTGGCAGCATAACTCGTCTGTTGAACTGTGCGACCTCACCTTCCCTATCCACAACAGCCCAACCCAGCTC
Translation +1 F S W Q H N S S L N L S D L T F P I H N T P T Q L
260
Ptchd3b-En2-KOMP ATCTACTTAGCGGGCTTCTTTGGGGGAAATGTTTTGGGTCAAATGACTGGAAAAAGTCAACGACTCGTGGAGTCC
Translation +1 I Y L A G F F G G N V L G Q M T G K S Q R L V E S
280
Ptchd3b-En2-KOMP CGAGCAATGAGACTACTGTAACCTCAAGACGGAGGACCCCGAAGACAGTGAACGTAGCCAGGCATGGCTGACT
Translation +1 R A M R L L Y Y L K T E D P E D S E R S Q A W L T
300
Ptchd3b-En2-KOMP CACTTTCTTGACCATTTTAATGACATGAAGAGCAGTCTGACCCCTGGAAGAGATAGAGGTCCAGGTCCTCCGAAAAC
Translation +1 H F L D H F N D M K S S L T L E E I E V P G P E N
320
Ptchd3b-En2-KOMP CAAAAGAAGAAGAACCCCTAACAAAGAGGACAAGCGGGCTCGCACAGCCTTCACTGCTGAGCAGCTCCAGAGGCTCA
Translation +1 Q R R R T L T K R T S G L A Q P S L L S S S R G S
340
Ptchd3b-En2-KOMP AAGGCTGAGTTTCAGACCAACAGGTGGTCTACTTTTCATCCCTTCCAGGCAGCTGGAGTTTGAAGGCAACCTCCAA
Translation +1 R L S F R P T G G L L F I P F Q A A G V * G N L Q
360
Ptchd3b-En2-KOMP GACCGTGATCCCCCTGTTTCACTTGGCATAACATTTAATCATACTCTTTGCAGTTGATCATGCTCCAGGTTGGA
Translation +1 D R D P P V S L G I H F N H T L C S C I M L Q V G
380

```

Fig 21. Sequencing analysis of mutant Ptchd3 (Ptchd3-EN2-KOMP). The sequence reveals that 115 bp of exon 2 of mouse engrailed-2 (highlighted in green) was inserted between exon 1 and exon 2 of the authentic Ptchd3 mRNA, resulting in truncated and partially mis-translated protein 370 amino acid residues (from a reading frame of 1113 bp), (stop codon TGA was highlighted in red). The antigen site recognized by the antibody Ptchd3-Ab1 was highlighted in yellow.

TM Helix Map

Feature predictions are colour coded onto the sequence according to the sequence feature key shown below.

```

1  M I S S K V A P G E E E Q G E S P S K V E L G E A E E Q R E A P L E G Q L E A V P L D E A G P S W T 50
51  T G P P L E R L P P L G Q E A P P P R R C H T N C L E A P L S R G F Q R F G A T V G A N P W L F L L 100
101 G P A L L T A S L G T G L I F L P K E K E N L E E Q Y T P I G S P A K A E R R F V Q G H F S T N D T 150
151 Y R F S A S R T S S E T N F A S I L V V S L A N S L L E P E I F K E V S K L D Q A V Q A L K V V Q E 200
201 N G T Q I L Y Q E V C A K Y K T L C V P P N P L L F S W Q H N S S L N L S D L T F P I H N T P T Q L 250
251 I Y L A G F F G G N V L G Q M T G K S Q R L V E S R A M R L L Y Y L K T E D P E D S E R S Q A W L T 300
301 H F L D H F N D M K S S L T L E E I E V P G P E N Q R R R T L T K R T S G L A Q P S L L S S S R G S 350
351 R L S F R P T G G L L F I P F Q A A G V X
  
```

KEY	Transmembrane Helix	Pore lining Helix	Extracellular Region	Cytoplasmic Region	Disordered	Disordered protein binding	Domprcd Boundary	DomSSEA Boundary
Annotations	M	N	L	E	E	E	A	B

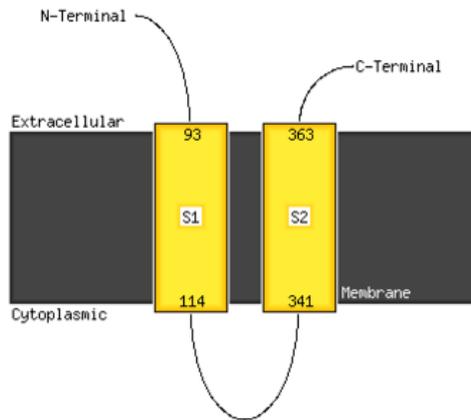


Fig 22. Protein topology of mutant Ptchd3. The protein topology was analyzed on the MAMSAT3 web server provided by the University of College London. The truncated and partially mis-translated mutant Ptchd3 protein (from a reading frame of 1113 bp) was predicted to possess two transmembrane domains instead of twelve transmembrane domains of the wild-type protein.

Testis Histology

Testis histology was carried out to determine whether Ptchd3 knockout affected spermatogenesis inside the testis. As shown in Fig. 23, there were not any noticeable changes in the histology between wild-type and knockout testis. The seminiferous tubules in the knockout testis appear normal and contain male germ cells at different developmental stages (Fig. 23). Spermatozoa are seen in both wild-type and knockout lumens (Fig. 23). This result indicates that spermatogenesis was not compromised without Ptchd3. In other words, Ptchd3 is not required for germ cells to complete the differentiation and development from spermatogonia to spermatozoa.

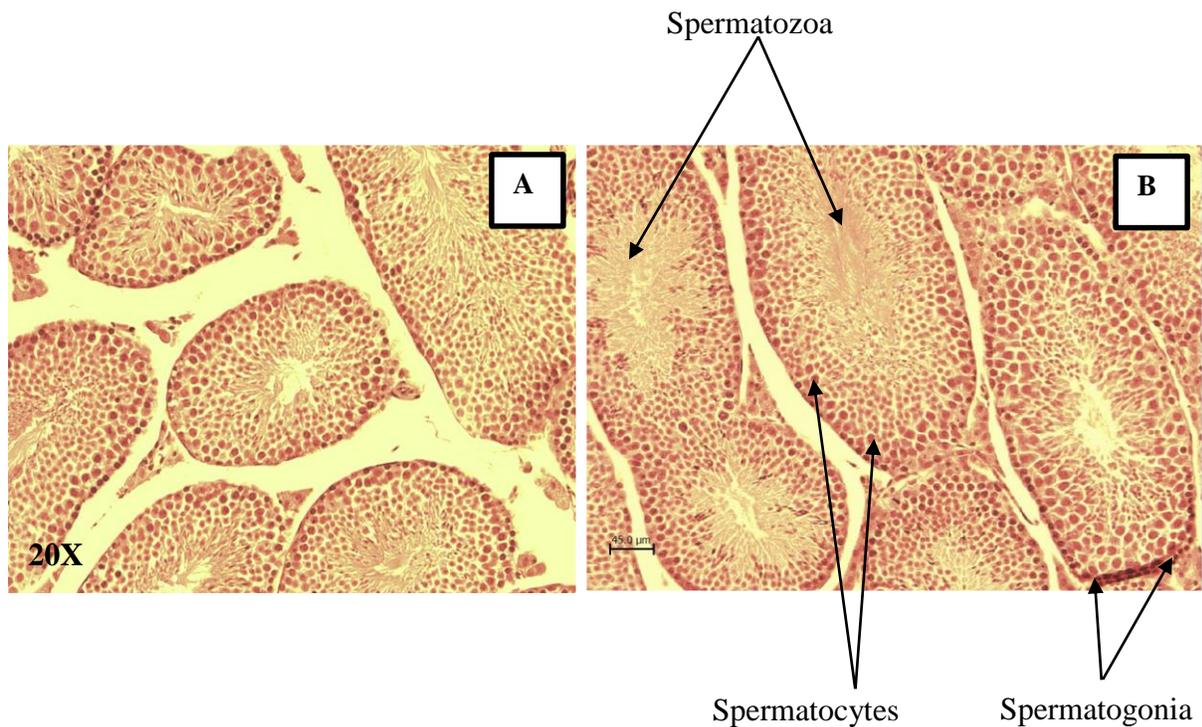


Fig 23. Histology analysis. Tissue slide (H&E staining) from (A) WT and (B) KO testis was observed with Leica DMI 4000B microscope. Normal spermatogenesis was seen in the KO testis.

Western Blot

To further confirm Ptchd3 knockout at the protein level, we performed Western blot. The protein samples were prepared from wild-type and knockout testis. The antibody Ptchd3-Ab1 was used as the primary antibody. Even with multiple attempts, we could not obtain a clean blot (Fig. 24), suggesting that the antibody Ptchd3-Ab1 might not be suitable for western blotting.

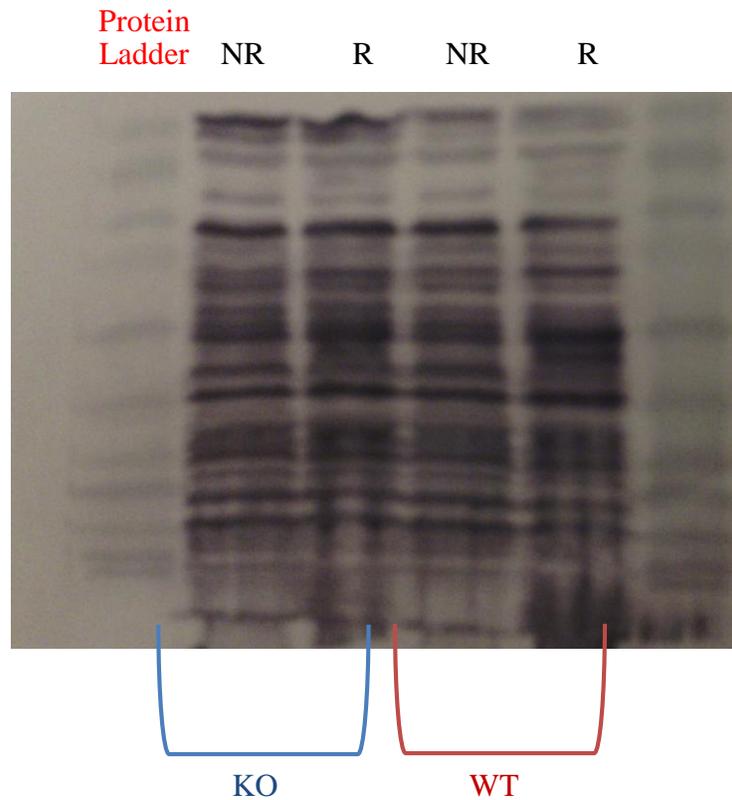


Fig 24. Western blot on testis protein samples. The primary antibody was Ptchd3-Ab1 (2.5 μ g/mL) and the secondary antibody was mouse anti-rabbit, AP conjugated (0.1 μ g/mL). The first two lanes show the knockout samples (non-reducing (NR) and reducing (R)). The last two lanes show the wild-type samples (non-reducing (NR) and reducing (R)). No specific band was detected in the samples.

Sperm Motility Analysis

There are many studies that can be performed to assess male fertility, such as in vivo fertilization, sperm morphology, sperm shape, sperm quantity, and sperm motility. Our result of in vivo fertilization and sperm morphology clearly demonstrate that there is no significant difference between wild-type and Ptchd3 knockout mice. However, the Ptchd3 protein is known to be localized in the sperm mid-piece, an area that is rich in mitochondria. Mitochondria in the sperm serve as an energy generator providing the ATP necessary for the sperm movement. As Gharamani Seno et al. (2011) pointed out, Ptchd3 could play a role in proper sperm motility. To test this, we examined sperm movement on wild-type and knockout sperm. The number of sperm movement (sperm beating) in one minute was recorded. The t-test (performed on Microsoft excel) showed that there was no significant difference in sperm movement (p -value of 0.1452) (Fig. 25). Thus, Ptchd3 is not necessary for sperm motility.

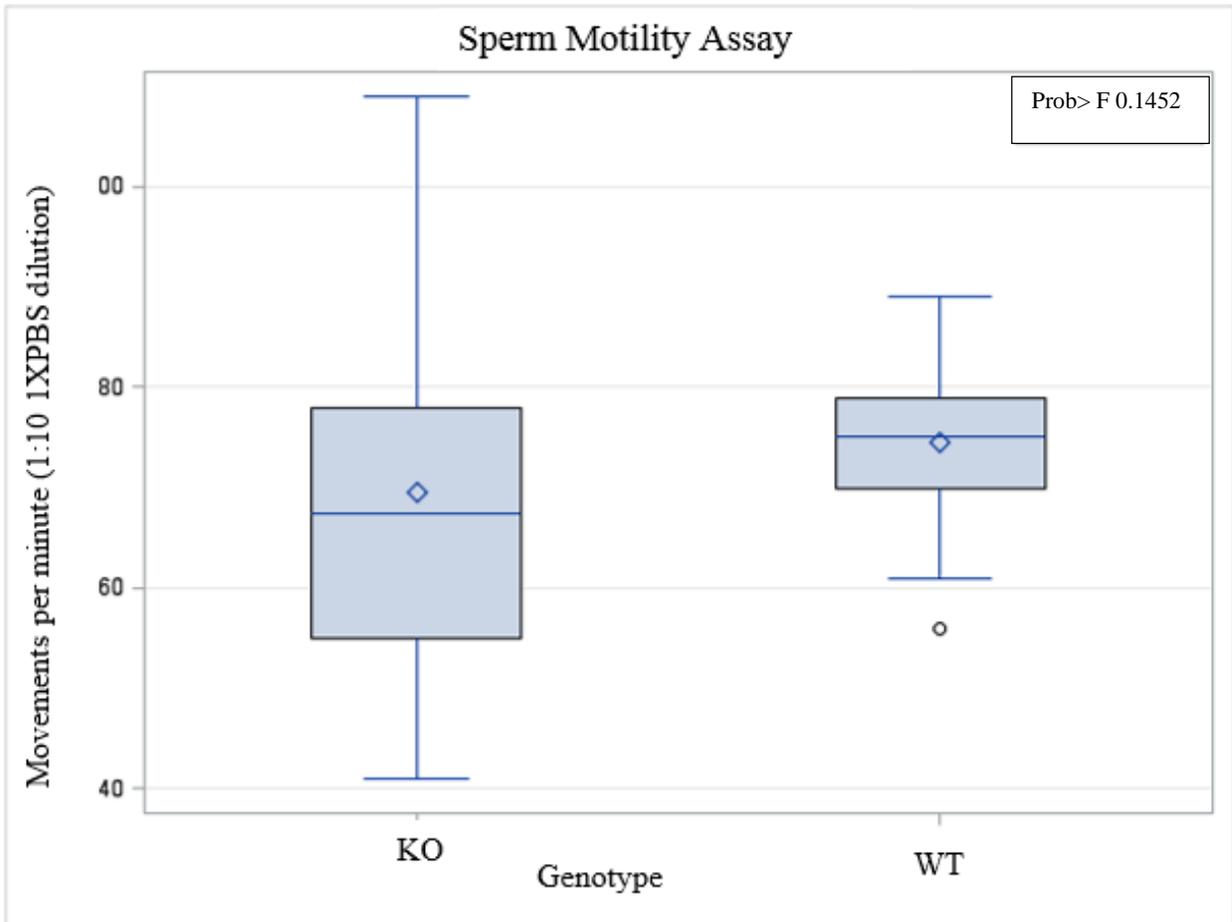


Fig 25. Sperm motility assay. The number of sperm movement (beating) was counted by eye under Leica DMI 4000 microscope. Twenty wild-type sperm and twenty knockout sperm were analyzed. A t-test statistical analysis shows no significant difference between KO & WT (p - value 0.1452).

CHAPTER 5

CONCLUSIONS

This study was pursued to investigate Ptchd3's biological function(s) in mouse. Ptchd3 was previously identified as a male germ-cell specific gene in mouse (Fan et al. 2007). The deduced Ptchd3 protein contains a Patched domain, which is known as a binding structure for Hedgehog (Hh) ligands (including sonic hedgehog Shh, Indian hedgehog Ihh and desert hedgehog Dhh) (Fan et al. 2007). Dhh has been previously shown to be essential for testis development and spermatogenesis (Szczepny et al. 2006). Thus, in this study, we aim to test the hypothesis that Ptchd3 functions as a Dhh receptor and is required for mouse spermatogenesis.

We 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, RT-PCR and sequencing results confirm that we were able to successfully generate a transgenic mouse line with conventional Ptchd3 knockout. This knockout approach resulted in the production of a mutant Ptchd3 protein that was truncated, partially mis-translated, and was still able to reach the sperm plasma membrane.

Similar to the findings in human study (Gharamani Seno et al. 2011), our study indicates that Ptchd3 is not essential for the mouse life. The knockout mice lived healthy without having any overt changes in body growth, body weight, and behavior.

We were able to observe male germ cells at all developmental stages in the knockout testis. We also found that, contrary to Dhh mutant mice (Bitgood et al. 1996), no Ptchd3 null

mice displayed feminized external genitalia (data not shown). Therefore, Ptchd3 is not required for mouse testis development and is dispensable for mouse spermatogenesis. These results point out that there might be other mechanisms to maintain cell proliferation and differentiation from spermatogonia to spermatozoa.

Our data indicate that Ptchd3 mutant mice had the capability to reproduce without any problem, which was like a previous study on null Ptch2 mice (Carpenter et al. 1998). Thus, like Ptch2, Ptchd3 is not vital for mouse fertility. Also, a recent study in humans reported that null PTCHD3 did not cause infertility (Gharamani Seno et al. 2011).

There was no significant difference of sperm movement between knockout and wild-type sperm, which indicates that Ptchd3 is not critical for mouse sperm motility.

Our in vitro sperm morphology assay indicated that there was no statistical difference between Ptchd3 knockout sperm and wild-type sperm. The lack of statistical significance between wild-type and knockout in the sperm morphology assay as well as in other assays (in vivo fertilization and testis body weight) might be due to low numbers of WT, which can create a bias and reduce the statistical power. However, the sperm morphology assay indicated that there was statistical difference between Ptchd3 knockout sperm and heterozygous sperm. One explanation might be that mutant Ptchd3 protein folds in a delicate way so that the net functional outcome is even worse. Otherwise, the complexity of mouse genetic background may be accounted for that observation.

Taken together, these findings clearly disprove our working hypothesis. However, our data cannot completely rule out the possibility that Ptchd3 might function as one of the Dhh receptors.

CHAPTER 6

DISCUSSION AND FUTURE STUDIES

Our results demonstrate that mouse *Ptchd3* is not an essential gene in life, testis development, spermatogenesis, and sperm physiology (morphology, motility and fertility). These findings are somewhat surprising, since *Ptchd3* gene is conserved in many organisms (Geer et al. 2010) and its protein is found on the mid-piece of mouse, rat and human sperm (Fan et al. 2007). On the other hand, based on protein domain structure, *Ptchd3* belongs to the patched family, which is the membrane receptor for hedgehog ligands (including sonic hedgehog, Indian hedgehog and desert hedgehog) and has six members identified so far (including *Ptch1*, *Ptch2*, *Ptchd1*, *Ptchd2*, *Ptchd3* and *Ptchd4*) (Geer et al. 2010). Hence, genetic redundancy may compensate the loss of one particular family member, such as *Ptchd3* in this study. With this regard, *Ptch1* and *Ptch2* indeed have been shown to be expressed in developing germ cells in testis (Mäkelä et al. 2011) and may functionally exchange with *Ptchd3*. Interestingly, previous studies also showed that *Ptch2* null mice were fertile (Carpenter et al. 1998) (Nieuwenhuis et al. 2006). Thus, it is possible that there are multiple Dhh receptors in testis and losing any one of them does not visibly compromise testis development, spermatogenesis and fertility.

Interestingly, genetic redundancy for the PTCH family members has been reported lately. Adolphe et al. (2014) studied null *Ptch1*, null *Ptch2* and double mutant mice, in order to assess the function of the PTCH family members in epidermal development. They found that null *Ptch1* alone produced some defects in epidermal development but the cells still were able to develop eventually. However, the loss of both *Ptch1* and *Ptch2* inhibited the epidermal lineage specification and differentiation (Adolphe et al. 2014).

Therefore, in order to study genetic redundancy that might occur during testis development and spermatogenesis, double null mutants of the PTCH family members need to be generated. In addition, future studies are needed to determine whether Ptchd1, Ptchd2 and Ptchd4 are also expressed in male germ cells.

Thus far, Ptchd3 is the only family member that has been shown on sperm (Fan et al. 2007). Since our data reveal that deletion of Ptchd3 does not affect sperm physiology, we predict that other patched family member(s) may be present on sperm. This interesting prediction needs to be addressed in the future.

This study did not directly examine whether Ptchd3 functions as one hedgehog receptor. In the future, Ptchd3 may be ectopically expressed in a suitable mammalian cell line, and then hedgehog-Ptchd3 binding assay should be performed.

The antibody Ptchd3-Ab1 used in this study recognizes an antigen between amino acid residues 131-150 of both Ptchd3 isoforms (Fan et al. 2007). Although this antibody works in immunofluorescent assay, apparently it is not suitable for Western blotting. In addition, this antibody also recognizes the mutant Ptchd3 protein from the mutant mice. In the future, an antibody targeted to the carboxyl-terminus and working in immunoblotting should be developed and then applied to differentiate the wild-type and mutant Ptchd3 protein.

The procedure used to count sperm movement in this study is subjected to human bias and thus is not ideal. In the future, a better protocol to determine sperm motility is greatly needed.

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APPENDIX A



Office of Research Integrity
Institutional Review Board

December 2, 2014

Gou-Zhang Zhu, PhD
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Huntington, WV 25755

Dear Dr. Zhu:

This letter is in response to the submitted thesis abstract for Shaimar R. Gonzalez Morales entitled "*Functional Studies of Ptchd3 during 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,

A handwritten signature in blue ink that reads 'Bruce F. Day'.

Bruce F. Day, ThD, CIP
Director

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APPENDIX B

REGULATION OF PTCHD3 EXPRESSION

The expression of Ptchd3 in different cell types was analyzed on Ensembl.



Fig 26. Regulation of Ptchd3 expression. This analysis shows that Ptchd3 has some promoters associated with embryonic stem cells (ES), embryonic stem cells hybrid (ESHyb), or mouse embryonic fibroblast cells (MEF).

APPENDIX C

PTCHD3 TAXONOMIC TREE

The FASTA sequence of the Ptchd3 protein was analyzed in both NCBI (Blast) and Ensembl to generate a taxonomic tree of its different homologs.

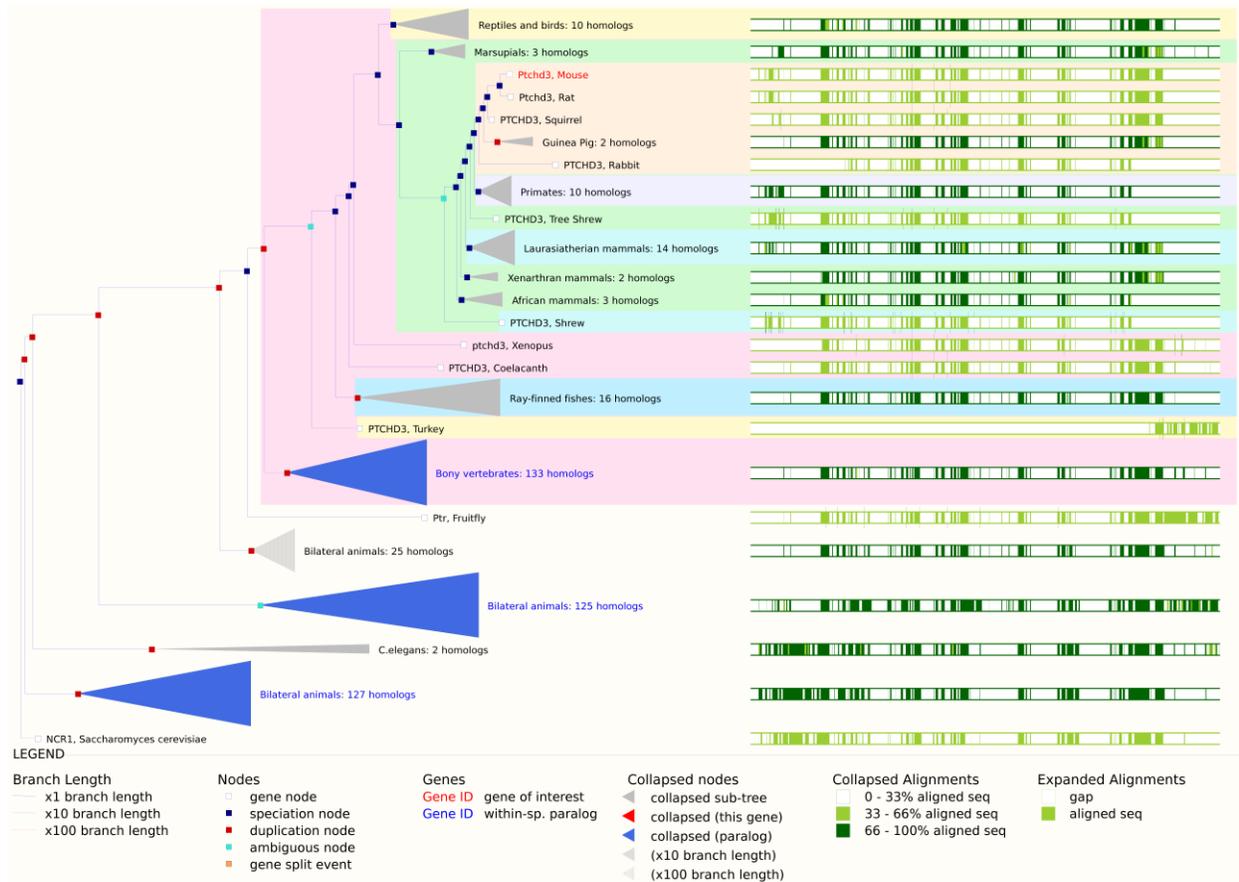


Fig 27. Ptchd3 homology tree. The taxonomic tree analysis was generated by Ensembl. It can be seen here that the Ptch3 protein homolog is found in multiple organisms, suggesting that Ptchd3 might have a conserved and important function during germ-cell differentiation.

APPENDIX D

GENOTYPING

Table 1. PCR mix

H2O	23.6 μ L
10X Buffer	3 μ L
Dntps	0.6 μ L
Primer Forward	0.6 μ L
Primer Reverse	0.6 μ L
Taq	0.6 μ L
Total	28.4 μ L

DNA: 1 μ L of tail genomic DNA

Table 2. PCR Cycle

1	94.0°C	0h 3 m 0s
2	94.0°C	0h 0 m 30s
3	60.0°C	0h 0 m 30s
4	72.0°C	0h 1m 20s-239
5	72.0°C	0h 6 m 0s
6	25. 0°C	24h 0 m 0s

Table 3. Gel (1.5%)

Agarose	0.6 grs
TAE	40 mL
Ethidium Bromide	3 μ L
<hr/>	
<i>*Running the Electrophoresis Gel</i>	
110 volts for 45 min-50 min	
10 to 15 μ L of DNA sample to a 1.5% gel	

APPENDIX E

RT-PCR

Table 4. RT-PCR mix

H ₂ O	23.2 μ L
10X Buffer	3 μ L
Dntps	0.6 μ L
Primer Forward	0.6 μ L
Primer Reverse	0.6 μ L
Taq	0.6 μ L
Total: 29.52 μ L DNA: 2 μ L	

*Follows the same steps of the genotyping in the previous appendix.

APPENDIX F

WESTERN BLOT

Table 5. Recipe for the Radioimmunoprecipitation assay (RIPA) lysis buffer

RIPA Lysis Buffer	For 1L
0.137 M NaCl	27.4 mL 5M NaCl (8.01g)
20mM Tris pH 8.0	20mL 1M Tris pH 8.0
10% glycerol	100mL glycerol
1% NP-40	10mL NP-40 or alternative
0.1% SDS	10mL 10% SDS
0.1% Na Deoxycholate	1 g Na Deoxycholate
	832.6 mL DDH ₂ O

* The buffer is autoclaved and stored at 4°C.

Table 6. Recipe for the SDS gel (resolving and stacking gel)

Resolving Gel 10%	Stacking Gel 5%
4.9 mL DH2O	3.6 mL LDDH2O
2.5 mL AA/BIS	0.63 mL AA/BIS
2.5 mL Tris pH 8.8	1.25 mL Tris pH 6.8
0.1 mL 10% SDS	50 μ L 10% SDS
50 μ L 10% APS	25 μ L 10% APS
5 μ L TEMED	5 μ L TEMED

Table 7. Recipe for the wet transfer buffer and the SDS running buffer

Wet Transfer Buffer	10 X SDS Running Buffer
Tris base 3.03g	Tris base 30.2g
Ciclyne 14.4g	Glycine 144g
Methanol 200 mL	10g SDS
800 mL DH2O	1000 mL DH2O

VITA

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EDUCATION

Marshall University, Huntington, West Virginia
M.S. in Biological Sciences
Graduate GPA: 3.86
Research Focus: Developmental Biology: May 2015

University of Puerto Rico- Mayagüez Campus, Mayagüez, Puerto Rico
B.S. in Biology Graduated: December 2011

Marshall University, Huntington, West Virginia
National Exchange Program: Fall 2011

- Took undergraduate courses and participated in research

EXPERIENCE

Marshall University, Huntington, West Virginia
Graduate Research Assistant Fall 2013-Present
Principal Investigator: Dr. Guo-Zhang Zhu

National Cancer Institute (NCI), Frederick, Maryland
Cancer Research Inter Summer 2014
Principal Investigator: Dr. Susan Mackem, M.D., Ph. D.

Marshall University, Huntington, West Virginia
Graduate Research Assistant Fall 2011-Spring 2013
Principal Investigator: Dr. Simon Collier, Ph. D.

University of Puerto Rico-Mayagüez Campus,
Mayagüez, PR
The Biocassava Plus Program
Research Assistant Spring 2009-Summer2011, Spring 2012
Principal Investigator: Dr. Dimuth Siritunga, Ph. D.

University of Puerto Rico-Mayagüez Campus,
PR UPRM Collection of Invertebrates
Undergraduate Research Assistant Spring 2008 - Fall 2009
Principal Investigator: Dr. Nico Franz, Ph. D.

Caribbean Biotechnologies Inc. Mayagüez, Puerto Rico
Research Assistant Summer 2008
Principal Investigator: Dr. Jorge E. González Cruz, Ph. D.

PUBLICATIONS

Abstract

Gonzalez-Morales S, Zhu G. Functional Studies of Ptchd3 during Spermatogenesis. In Vitro Cell.Dev.Biol.-Animal. Vol. 50. Issue Abstract. Spring 2014

Posters

Gonzalez-Morales S, Zhu G. (2014) Functional Studies of Ptchd3 during Spermatogenesis. **Presented at:** Sigma Xi- Marshall University, Huntington, West Virginia and 2014 World Biology Forum, Savannah, Georgia

Gonzalez-Morales S, Zhu J, Mackem S. (2014) Do the same cells that express sonic hedgehog also respond to it? Implications for morphogen function in the limb. **Presented at:** Student Poster Day, National Cancer Institute, Frederick, Maryland and Summer Poster Day 2014, National Institutes of Health, Bethesda, Maryland

PROFESSIONAL ACTIVITIES

2015 In Vitro Biology Meeting, Tucson, Arizona/ May 30- June 3, 2015
Student Program Chair and Convener for the Medicinal Plants, Propagation and Nutraceuticals Session

HONORS AND AWARDS

- Summer Intramural Training Award (Summer IRTA), 2015
- SACNAS National Conference Travel Award, 2014
- Cancer Research Training Award (CRTA), 2014
- Marshall University Department of Biological Sciences Travel Award, 2014
- Marshall University-Dr. Leonard J. Deutsch Graduate College Professional Development Fund, 2014
- Who's who Among Students in American Universities and Colleges, 2013
- Legislative Scholarship, 2010
- Academic Competitiveness, 2006