RNF216 IS ESSENTIAL FOR SPERMATOGENESIS AND MALE FERTILITY

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ABSTRACT

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Spermatogenesis is a specialized differentiation process in which male germ cells undergo meiosis to form motile sperm. Interruptions in spermatogenesis, resulting in immobile, irregular, or lower concentrations of sperm, lead to male infertility. Mutations in genes involved in spermatogenesis have been shown to cause male infertility. RNF216 is a member of the RING finger domain family of E3 ubiquitin ligases that are involved in protein degradation. The physiological function of RNF216 in mammals in unknown. Here we show that RNF216 is highly expressed in the mouse testes. Targeted deletion of RNF216 in mice reveal that RNF216 is required for spermatogenesis and male fertility. However, RNF216 is not essential for animal viability or fertility in females. We further demonstrate that RNF216 exhibits E3 ubiquitin ligase activity *in vitro.* These findings underscore a potential function of RNF216 mediated protein degradation in mammalian germ cell development and male fertility.

I dedicate this thesis to Mom, Dad, Jenna, Austin, and the many people who have guided me during my educational journey.

"If I have seen further it is by standing on the shoulders of giants"-Sir Isaac Newton

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

KEY TO ABBREVIATIONS

APC/C	anaphase-promoting complex/cyclosome
BTB	Blood-Testes-Barrier
CDC20	cycle homologues 20
CDK	cyclin-dependent kinase
CDK1	cyclin-dependent kinase 1
cDNA	complementary DNA
Cre	Cre recombinase
DAPI	4’ 6-diamidino-2-phenylindole
DNA	deoxyribonucleic acid
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EMI1	Early mitotic inhibitor
FSH	Follicle Stimulating Hormone
FSHR	Follicle Stimulating Hormone Receptor
HECT	homologous to E6-AP C terminus
HRP	horseradish peroxidase
IAP	inhibitor apoptosis protein
IP	immunoprecipitation
kDa	kilodalton
LH	luteinizing hormone

NGS	normal goat serum
PBS	phosphate buffered saline
PCR	polymerase chain reaction
RING	really interesting new gene
RT	reverse transcriptase
SCF	S-phase kinase associated protein 1, cullin, and F-box
SKP2	S-phase kinase-associated protein 2
SSC	Spermatagonial Stem Cell
UPS	ubiquitin-proteasome system

Chapter 1: INTRODUCTION

1.1 MAMMALIAN SPERMATOGENESIS

Spermatogenesis is a cellular process by which spermatozoa, the male gamete, are produced from spermatagonial stem cells (SSC's) following meiotic division and differentiation (Mruk & Cheng, 2015). Spermatozoa provide two major contributions during conception: (1) to deliver the paternal genome and (2) to activate the developmental program. Disruptions in spermatogenesis may cause a multitude of problems in male fertility, ultimately leading to reproductive dysfunction (Cattanach, Iddon, Charlton, Chiappa, & Fink, 1997; Robertson et al., 1999). The following sections will give a comprehensive overview of the mammalian testes and male reproduction.

1.1.1 Organization of the Testes

The seminiferous tubules and interstitial tissue together, make the testis (L. D. Russell, Ettlin, Hikim, & Clegg, 1993). The interstitial tissue houses Leydig cells and is also where blood is circulated through the testes (Utiger, 1999). The Leydig cell is the primary source of circulating testosterone in males and secretes testosterone in response to Luteinizing Hormone (LH), produced by the pituitary gland (Cooke & Saunders, 2002). Testosterone diffuses into the seminiferous tubules to drive spermatogenesis (Ge, Chen, & Hardy, 2008).

The seminiferous tubule is the functional unit of the testis to produce spermatozoa. They are long convoluted tubules comprised of many concentric layers of different germ cells (Nakata et al., 2015). The seminiferous tubule is composed of seminiferous epithelium and tunica propria (L. D. Russell et al., 1993). The tunica propria is an underlying collagen network

with multiple myoid cell layers which work closely with the lymphatic network (Siu & Cheng, 2008).

The blood-testes-barrier (BTB) is an immunological barrier that divides the seminiferous epithelium into basal and adluminal compartments (Stanton, 2016). Its function is to keep post meiotic germ cell development separated from systemic circulation and regulate transitory molecules into the different compartments (Lie, Mruk, Lee, & Cheng, 2009). The BTB is an essential part of spermatogenesis because of its unique nature to open or close during certain stages of spermatogenesis (Mital, HInton, & Dufour, 2011). While open, the barrier fundamentally disassembles the apical portion of migrating preleptotene and leptotene spermatocytes, and then closes to reassemble the basal portion of the cell. This facilitates migrations while still maintaining the authenticity of the barrier (Siu & Cheng, 2008).

The seminiferous epithelium contains somatic cells called the Sertoli cells (Figure 1). This cell has many important roles including providing nutrients and physical support to the germ cells, dividing the tubule up by tight junctions to provide a protected environment for the germ cells, controlling spermiation, secreting proteins, fluids, and growth factors, and phagocytizing degenerating germ cells and excess cytoplasm (Griswold, 1995). Sertoli cells possess follicle stimulating hormone receptors (FSHR) which become stimulated by follicle stimulating hormone (FSH) produced by the pituitary gland. Inhibin and activin are proteins produced by the Sertoli cells and act as paracrine regulators of spermatogenesis (Sharpe, 1987). Sertoli cells are essential for germ cell survival and regulate the amount of sperm a male will produce (Johnson, Thompson, & Varner, 2007). Sertoli cells support a specific number of germ cells per cell, consequently increased numbers of Sertoli cells can support a larger population of germ

cells and generate more sperm after spermatogenesis occurs (Hess & Franca, 2008). The Sertoli cell population is established at the perinatal and prepubertal stages of development to dictate germ cell numbers and testes size at puberty (Hai et al., 2014).

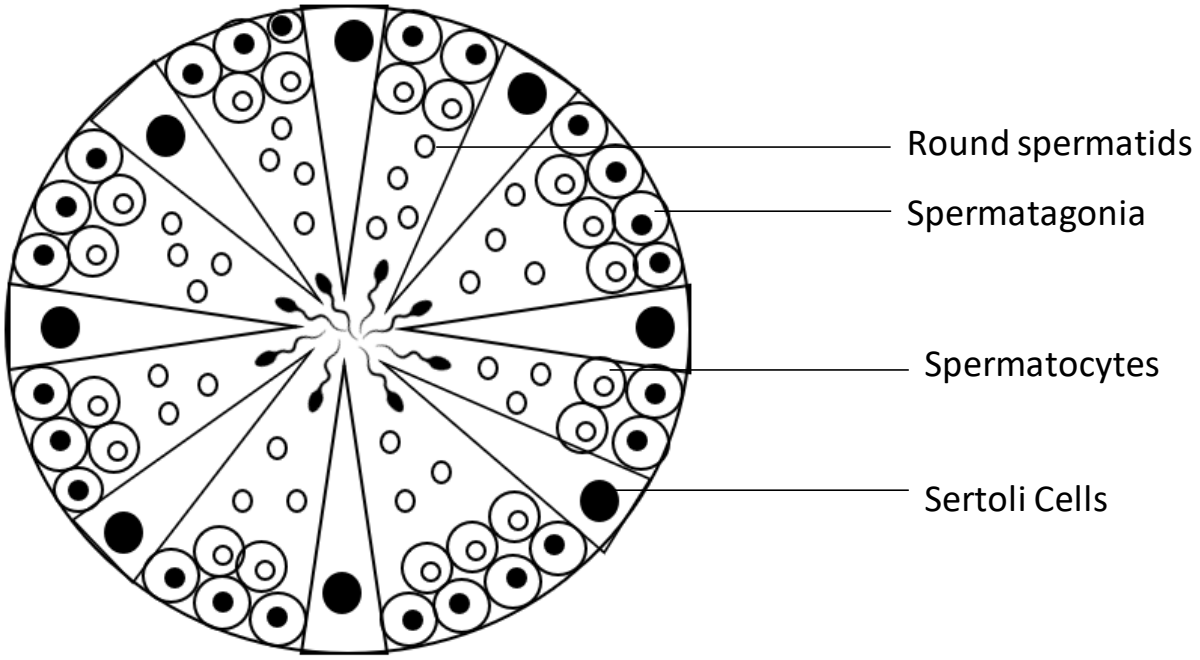

Figure 1: The Seminiferous Tubule Diagram showing the layout of cells that make up the seminiferous tubules. From the basement membrane working inwards towards the lumen, there are spermatogonia, spermatocytes, spermatids, and spermatozoa. Sertoli cells locate on the basement membrane but extend inward to support the germ cells.

1.1.2 Spermatogenesis in the Mouse Testes

Spermatogenesis is a specialized differentiation process by which male germ cells undergo meiosis to form motile sperm (Clermont, 1963). The three phases of spermatogenesis are spermatagonial mitotic division, meiosis, and spermiogenesis (Sharpe, 1987). Spermatogenesis occurs in waves (Oakberg, 1956). During spermatogenesis, each tubule progresses through these waves independently so that each tubule is at a different stage of spermatogenesis at a given time (Phillips, Gassei, & Orwig, 2010).

Spermatogenesis can be classified into mitosis, meiosis, and spermiogenesis (Griswold, 2016). Mitosis is the proliferative stage of spermatogenesis and starts in spermatagonia. Spermatagonial stem cells (SSC) are diploid (2n) precursor germ cells that locate on the basal lamina of the seminiferous tubules. They can be classified into undifferentiated type A, differentiated type A, intermediate, and type B SSC's (Clermont, 1963). Undifferentiated spermatagonia refers to spermatagonial stem cells that can colonize after germ cell transplantation. Differentiated spermatogonia are unable to colonize in the testes (D. DeRooij, 2001). A type and B type are dependent on gene expression pattern and appearance. Undifferentiated type A spermatagonia can have single, paired, or aligned appearances (D. G. DeRooij, VanDissel-Emiliani, & VanPelt, 1989). Differentiated type A spermatagonia have different forms called A1, A2, A3, and A4 (Hess & Franca, 2008). Stem cells are unique in their ability to differentiate or replicate. Some spermatagonia will keep their population by replicating and others will initiate spermatogenesis by differentiating into spermatozoa (Clermont, 1963).

Meiosis begins when spermatagonia divide to form preleptotene spermatocytes. Preleptotene spermatocytes sit on the basal lamia, and as spermatogenesis progresses to enter Meiotic prophase I, leptotene, zygotene, pachytene, and diplotene spermatocytes are consecutively formed (S.-R. Chen & Liu, 2015). Leptotene and zygotene spermatocytes move through the BTB and prepare for meiosis (L. Russell, 1976). Meiosis in the mouse occurs at stage XII of spermatogenesis, including meiosis I, secondary spermatocyte formation, and meiosis II, and is typically complete within one day (Hess, 1998). Meiosis I is where the division of 2n cells occur to give rise to 1n cells called secondary spermatocytes. During meiosis II, 2n

secondary spermatocytes divide, forming haploid (1n) cells called round spermatids (Hess & Franca, 2008). Spermatids then progress onto the next stages of spermatogenesis, spermiogenesis.

During spermiogenesis, haploid spermatids become elongated and condense their nuclei to form mature spermatozoa (Hess & Franca, 2008). These spermatozoa are released into the lumen of the seminiferous tubule for transport to the rete testes, where sperm is collected and transferred to the epididymis for sperm maturation and storage (Marchiani, Tamburrino, Muratori, & Baldi, 2017). During spermiogenesis, the acrosome, an enzymatic vesicle covering the head of the sperm, is formed. This structure is important for sperm to penetrate the egg (Berruti & Paiardi, 2011). The Golgi apparatus forms vesicles and produces granules containing the enzymatic components essential to cap the sperm nucleus (Susi, Leblond, & Clermont, 1971). The nucleus is capped when the acrosome granules touch the nuclear envelop of the sperm during early stages of spermiogenesis. The cap flattens out over one third of the nuclear surface and the spermatid begins to elongate and change shape (Anakwe & Gerton, 1990). As the sperm continues to mature, the nucleus continues to condense (L. D. Russell et al., 1993). Residual cytoplasm will become a cytoplasmic lobule to form a residual body, housing unused organelles such as ribosomes and mitochondria (Hess & Franca, 2008).

1.1.3 Seminiferous Epithelium and Staging in Mice

There are 16 stages of spermatogenesis in the mouse (Clermont, 1963). It is important to keep in mind that spermatogenesis is a continuum, where cross sections are viewed at “in-

between" stages, showing the translation from one stage to the next. However, in this thesis, I will refer to cross sections of the seminiferous tubules by the stages described in this section. The stages in the mouse occur sequentially until stage 16. The wave like pattern of spermatogenesis allows repetition to occur along the length of the tubule, moving distally from the rete testes in descending order towards the lumen of the seminiferous tubule. After the last stage is complete, the wave starts over from stage 1 (Davis, Snyder, Hogarth, Small, & Griswold,

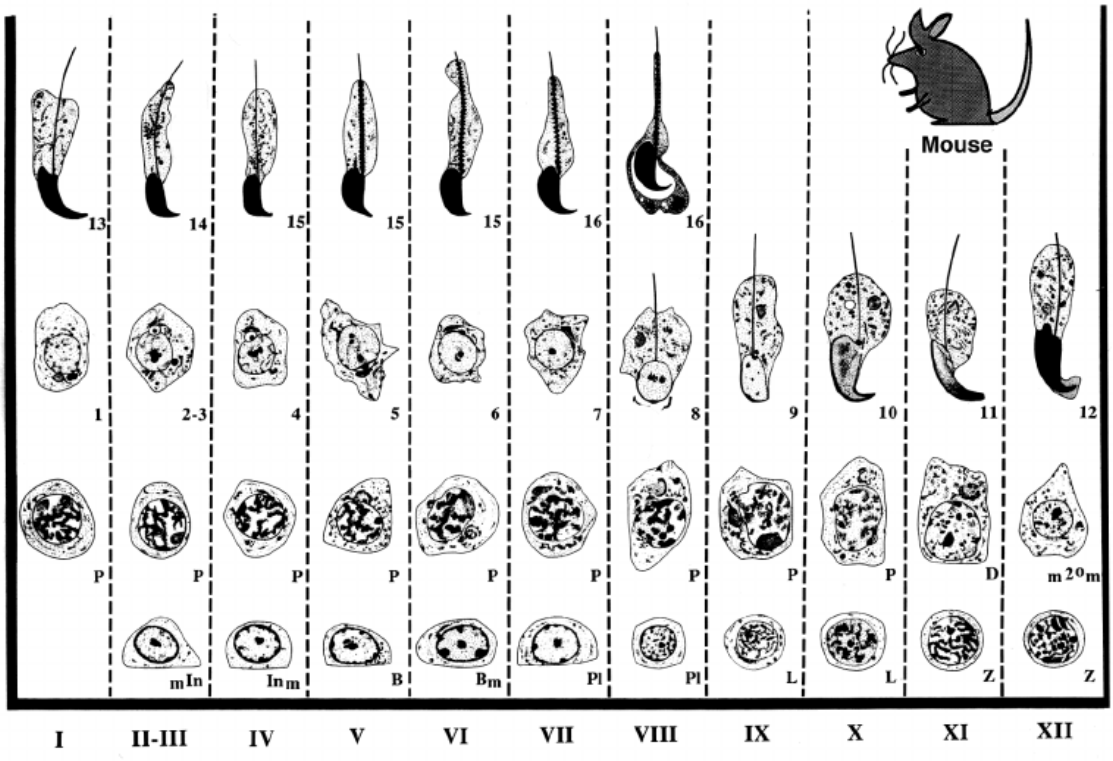

Figure 2: Stages of Spermatogenesis in Mice Diagram depicts the cell types present in the mouse at the 16 different spermatogenic stages. The vertical columns represent the different stages, all labeled by roman numerals 1 through 16. The bottom of the diagram is associated with cells closest to the basal lamina, with the cells towards the top of the diagram being associated with cells closest to the lumen of the seminiferous tubules. Spermatid development is labeled by Arabic numerals 1 through 19. Spermatagonia and spermatocytes are labeled as the following; In= intermediate spermatogonia, B= type B spermatagonia, Pl= pre-leptotene spermatocyte, L= leptotene spermatocyte, Z= zygotene spermatocyte, P= pachytene spermatocyte, D= diplotene spermatocyte. (Guan, 2009)

2013). Synchrony between the tubules only occurs during the first wave of spermatogenesis, however this is very temporary (Nebel, Amarose, & Hackett, 1961).

1.1.4 Mouse Models of Male Infertility

Male infertility is largely idiopathic and difficult to understand, partly due to the lack of animal models to study it. The mouse (*Mus musculus)* shares 99% of its genes with humans and molecular pathways controlling spermatogenesis are highly conserved (Rosenthal & Brown, 2007). It has been estimated that genetics contributes about 60% to male infertility issues (Lilford, Jones, Bishop, & Mueller, 1994). We use the mouse as a model to study male infertility because of its genetic similarity with humans and accessibility. Mice are easy to maintain and manipulate, and share similar testes histology and developmental patterns as humans (Cooke & Saunders, 2002). Mice reach sexual maturity at six to eight weeks and have an average gestation of about three weeks (Silver, 1991).

Gene knockout technology has become a powerful tool to produce loss-of-function mutations in mice, and therefore a great tool to study mammalian spermatogenesis and male infertility. We can categorize genetic mutations affecting germ cell development into specialized categories to better understand the developmental dynamics of spermatogenesis.

Meiotic arrest is one of the main causes of male infertility (Cooke & Saunders, 2002). One example of meiotic arrest is the gene mutation of *Spo11* (sporulation protein meiosis-specific SPO11 homologue). *Spo11* cleaves DNA through a topoisomerase-like transesterase mechanism and mutation in this gene eliminates meiotic recombination (Diaz, Alcid, Berger, &

Keeney, 2002). Thus, *Spo11*-deficient spermatocytes are arrested before the pachytene stage of spermatogenesis and undergo apoptosis (Romanienko & Camerini-Otero, 2000).

Another example of meiotic arrest is *Dmc1h* (disrupted meiotic cDNA 1 homologue). *Dmc1h* encodes a RecA-like enzyme, involved in strand exchange, and is detected in leptotene to zygotene spermatocytes (Pittman et al., 1998). Disruptions in *Dmc1h* in the mouse cause germ cell arrest during the early zygotene stages and apoptosis to occur (Yoshida et al., 1998).

Ataxia telangiectasia (AT) is a disease caused by mutation in the *ATM* gene and patients with this mutation possess only spermatogonia (Y. Xu et al., 1996). Further studies show that Atr (ataxia telangiectasia- and rad3-related) protein accumulates in *ATM* knockout mice and does not allow germ cells to complete chromosome pairing during meiosis (Moens et al., 1999).

Dmrt1 (doublesex/Mab3-related gene) is a gene essential for male gonad development and postnatal testes differentiation (Raymond, Murphy, O'Sullivan, Bardwell, & Zarkower, 2000). Mice with *Dmrt1* mutations showed germ cell depletion during the first wave of meiosis, occurring only a few days after birth. This is the stage where pre-leptotene spermatocytes should start developing. Interestingly, mutations in this gene were shown to cause the same phenotype in human males as well (Ottolenghi & McElreavey, 2000).

A subgroup of genes has a crucial role during spermiogenesis and if mutated, can cause reproductive dysfunction and infertility. Tlf (homologue of the TATA-binding protein) is expressed highly in late pachytene spermatocytes, round spermatids, and elongated spermatids (Igor Martianov et al., 2001). It has been shown that Tlf is important for the formation of the acrosome and chromatin reorganization, a process that occurs during spermatid maturation (I

Martianov et al., 2002). If *Tlf* is mutated in the mouse, they exhibit multinucleated giant cells, corresponding to the degeneration of round spermatids, ultimately causing infertility (Igor Martianov et al., 2001).

Ube2b (ubiquitin-conjugating enzyme E2 B) is a mammalian homologue of the *Saccharomyces cerevisiae RAD6* and encodes a ubiquitin-conjugating DNA-repair enzyme (Lawrence, 1994). It exhibits higher expression in post-meiotic male germ cells, when histones are replaced by transition proteins, helping with chromatin compaction (Koken et al., 1996). Males without *Ube2b* have developmental problems in post-meiotic germ cells when chromatin compaction starts and in consequence, most males have no mature germ cells or have reduced numbers of spermatozoa, showing signs of abnormality (Roest et al., 1996).

Many times, spermatozoa of mutated mice will exhibit abnormal looking sperm if they make it past meiotic arrest. *Theg* (testicular haploid-expressed gene) is a gene that encodes a protein specifically expressed in spermatids during early stages of germ cell development and is important for assembling cytoskeletal proteins (Yanaka et al., 2000). When *Theg* is knocked out of the mouse genome, any sperm that made it past the germ cell arrest appears abnormal in morphology (Yanaka et al., 2000).

Mutations in genes that regulate Sertoli cell interactions can also lead to male infertility. α-mannosidase-2a, is an enzyme encoded by a gene called *Mm*. If a carbohydrate N-glycan, which is synthesized by α-mannosidase-2a, is not properly synthesized, then germ cells do not adhere properly to the Sertoli cell, and are released prematurely (Akama et al., 2002). While there are several other genes and proteins that regulate spermatogenesis and are essential for

normal spermatogenic function in mice and humans, I have only described a few here to further prove why mice make great models to study male infertility.

1.1.5 Degrees of Male Infertility

Azoospermia is one form of male infertility, identified by complete absence of sperm, and can be classified as obstructive or non-obstructive (Chiba, Enatsu, & Fujisawa, 2016). Obstructive azoospermia exemplifies a blockage in the male reproductive tract (Wosnitzer & Goldstein, 2014). This type of infertility is sometimes hereditary, meaning a male could be born without vas deferens or have an unknown epidydimal obstruction (Meng et al., 2001). Non-hereditary obstructive azoospermia could be due to injuries to the male reproductive tract such as infections or vasectomies. These can usually be treated by surgery to remove and fix the obstruction. Non-obstructive azoospermia represents an inadequate production of sperm, perhaps caused by a problem in the spermatogenic cycle and can only be treated once the genetic cause is established (Schlegel, 2004).

Oligospermia is incomplete male infertility, where sperm are still being produced though there are problems with the sperm, including low sperm count, abnormal sperm, or low motility sperm (Turek, 2016). According to the Mayo Clinic, to be considered oligospermic, a man must have a semen sample with a concentration lower than 15 million sperm per milliliter.

1.2 RING FINGER 216

1.2.1 E3 Ubiquitin Ligase Families

E3 ligases can be classified as U-box domains, HECT (homologous to E6-AP C terminus) domains, and RING (really interesting new gene) domains. U-box domains mediate the interaction of proteins with ubiquitin conjugated targets (Ohi, VanderKooi, Rosenberg, Chazin, & Gould, 2003). HECT domain E3s primarily have roles in protein trafficking, immune response, and in cellular growth and proliferation signaling pathways (Kamadurai, 2013). RING fingers are Zinc coordinating domains that have specially spaced cysteine and histidine residues (Hedge, 2009). Their function at minimum, is to bring the E2 enzyme together with the E3 enzyme (Z. J. Chen & Sun, 2009).

1.2.2 RING Finger Domains

RING domains facilitate E3 ubiquitin ligase activity. RNF216 is a RING domain (Figure 3) that is 866 amino acids long. There are over 600 human genes that are RING based E3 ubiquitin ligases (Metzger, Hristova, & Weissman, 2012). RING domains bind to ubiquitin conjugating enzymes to help transfer ubiquitin to a target protein. They can function as monomers, dimers, or multi-subunit complexes (Metzger et al., 2012). For the cell cycle to progress normally, RING

Figure 3: RNF216 Domain Architecture

E3 multi subunits, like the anaphase-promoting complex/cyclosome (APC/C) and S-phase kinase associated protein 1, cullin, and F-box (SCF), are needed (Mocciaro & Rape, 2012). APC/C has 13 subunits, one of which is APC11, an E2 binding RING Finger protein (Metzger et al., 2012). To confer substrate specificity, two co-activators known as CDC20 (cell division cycle homologues 20) and CDH1 associate with the APC/C at specific stages of the cells cycle (Nakayama & Nakayama, 2006). The APC/C for CDC20 is activated during prometaphase through telophase. Securin is one of its ubiquitin-proteasome system (UPS) substrates, and ensures accurate separation of the sister chromatids (Peters, 2006). Cyclin B is another UPS substrate that must be degraded for proper activity of cyclin-dependent kinase 1 (CDK1) after mitosis is complete. Another set of targets, including CDC20 and S-phase kinase-associated protein 2 (SKP2), are tagged for degradation in the G1 phase of mitosis, when CDC20 gets replaced by CDH1. CDH1 gets inactivated by phosphorylation by CDK2, leading mitosis into the S phase. The APC/C targets CH1 for degradation. Early mitotic inhibitor (EMI1) deactivates the APC/C during late G1 to S phase and sometimes the G2 phase of mitosis (Frescas & Pagano, 2008).

1.2.3 RING Ubiquitin Pathway

Ubiquitin is a small protein, about 8.5 kDa in size or 76 amino acids long (Hegde, 2010). The ubiquitin pathway is a process that mediates protein degradation in a three-step enzymatic cascade (Roos-Mattjus & Sistonen, 2009). An E1 activating enzyme activates the ubiquitin by forming a thioester bond between itself and the cysteine active site on the E1 enzyme. It is transferred to the E2 conjugating enzyme where it forms a thioester linkage. The E3 ubiquitin ligase forms an isopeptide bond between the ubiquitin and the lysine residues on the target protein it is attached to (Kumar, Kao, & Howley, 1997). E3 ligases are essential for target

protein recognition (Chuang & Ulevitch, 2004). When the target protein is recognized and the E3 complex attaches, the target protein becomes poly-ubiquitinated and tags the protein for

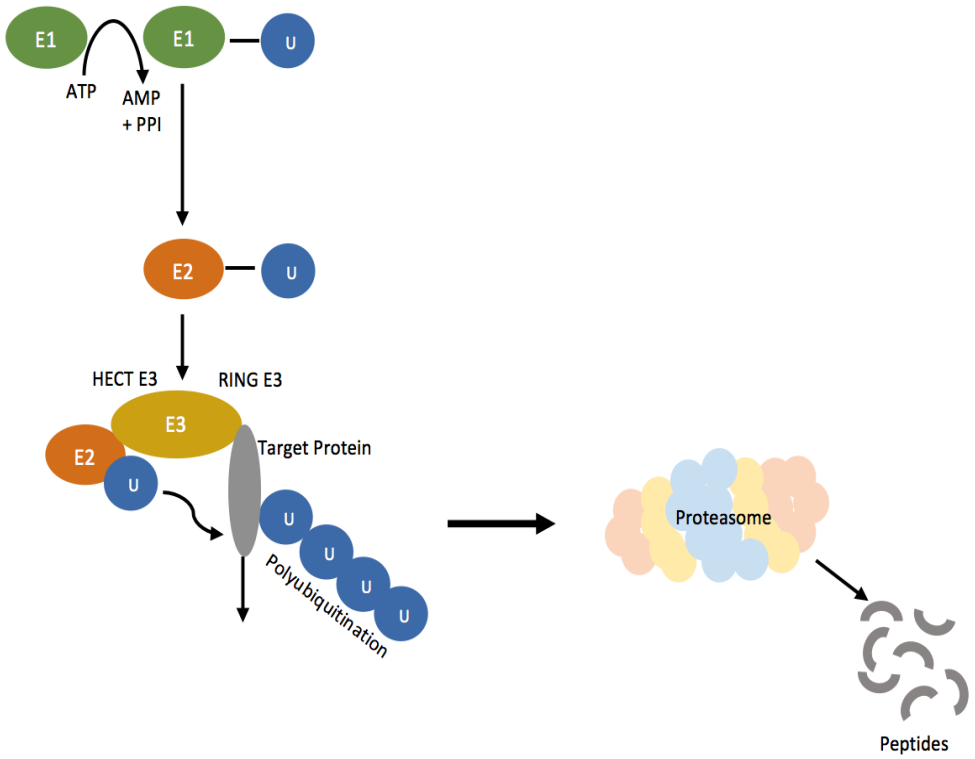

Figure 4: The E3 Ubiquitin Pathway Flow diagram showing the transfer of ubiquitin for targeted protein degradation via the proteasome. U= ubiquitin, E1= ubiquitin activating enzyme, E2= ubiquitin conjugating enzyme, E3= ubiquitin ligase.

degradation via the proteasome (Shang & Taylor, 2011) (Figure 4).

1.2.4 Regulation of RING Finger Domains

Substrate modification can regulate ubiquitination (R. J. Deshaies & Joazeiro, 2009). The substrate, E2, or E3 enzymes can be influenced by phosphorylation. Substrate phosphorylation is most well understood and has been studied in the Sic1 (Substrate inhibitor of CDK1) protein (Verma et al., 1997). Sic1 is stable early in the G1 phase of mitosis and becomes phosphorylated at many sites when the G1 cyclin-Cdk is activated late during the G1 phase. These phosphorylated sites are important for ubiquitination. The phosphorylated Sic1 protein binds to

the Cdc4 subunit of the SCF for Cdc4. This binding sets up ubiquitination by its E2 enzyme Cdc34 (R. J. Deshaies & Joazeiro, 2009).

E2s and E3s can be regulated by phosphorylation (R. J. Deshaies & Joazeiro, 2009). APC/C subunits can be phosphorylated by mitotic cyclin dependent kinases (Cdk), enhancing ubiquitin ligase activity by recruiting an activator protein called Cdc20 (Rudner & Murray, 2000). Alternatively, the APC/C activator Hct1/Dch1 can be phosphorylated by mitotic Cdk, inhibiting its activity, and preventing it from binding to the APC/C (Zachariae, Schwab, Nasmyth, & Seufert, 1998).

RING E3s can be regulated by conjugation with ubiquitin family proteins (R. J. Deshaies & Joazeiro, 2009). RING E3s are often ubiquitinated by autocatalytic processes (Galan & Peter, 1999). In the multi-subunit SCF complex, E2 can bind to the RING subunit and catalyze ubiquitination of the F-box subunit, resulting in its degradation (Zhou & Howley, 1998). Autoubiquitination is a homeostatic mechanism, where a bound substrate protects the F-box from being ubiquitinated. This results in an accumulation of the F-box protein only when it's substrate is present (R. Deshaies, 1999). The RING domain of inhibitor apoptosis protein (IAP) can promote the turnover of caspase-3 and other theoretical proteins, and acts as a cis degron activated by proteins that promote cellular death (Yang, Fang, Jensen, Weissman, & Ashwell, 2000). Sometimes autoubiquitination can work in the opposite way where it can switch on ubiquitin ligase activity (Mallery, Vandenberg, & Hiom, 2002).

RING E3 activity can be controlled by binding partners or regulated by small molecule ligands (R. J. Deshaies & Joazeiro, 2009). Dipeptides can bind to the E3 ubiquitin ligase and

enhance its activity towards a transcriptional repressor and block mRNA expression that encode a specific dipeptide transporter (Turner, Du, & Varshavsky, 2000). This causes a positive feedback loop with dipeptides enhancing expression of the dipeptide transporter and increases the cells uptake of dipeptides.

Substrate competition could regulate E3 ligases (R. J. Deshaies & Joazeiro, 2009). The order in which substrates are degraded is crucial to the role they have in anaphase and therefore, the APC/C mediates the turnover of substrates in a very specific order (Rape, Reddy, & Kirschner, 2005). The most processive substrates are degraded first because they possess a high likelihood of acquiring degradation competent chains when they bind to the APC/C. Being the most processive, they can catalyze reactions without releasing their substrate better than those with a lower processivity. Substrates with a low processivity must gain a ubiquitin chain long enough to tag itself for degradation via the proteasome, therefore they must shuttle on and off the APC/C several times. By requiring this consecutive shuttle action to occur, they must wait for the more processive substrates to be degraded first (Rape et al., 2005).

1.3 HYPOTHESIS AND RESEARCH OBJECTIVE

RNF216 has been found to function as an E3 ubiquitin ligase. The overall objective of my thesis was to evaluate the expression and function of RNF216 in the mouse testes to better understand the role of RNF216 in spermatogenesis and male fertility. This objective includes evaluating the RNF216 knockout mouse phenotype, determining the protein pattern of RNF216 in the mouse testes, and testing potential interacting protein substrates with RNF216.

Chapter 2: EXPRESSION OF RNF216 IN THE MOUSE TESTES AND E3 LIGASE ACTIVITY

2.1 INTRODUCTION

RNF216 mRNA is highly expressed in the testes (GTEx, 2017), however the protein expression of RNF216 in the testes has not been characterized. It has also been found to act as an E3 ubiquitin ligase, yet the substrates it targets in the testes have not been established. Beclin-1 (BECN1) has been found to interact with RNF216 in cell lines, causing ubiquitination of BECN1 and degeneration via the proteasome (C. Xu et al., 2015). BECN1 is involved in autophagy, a cellular process that helps maintain homeostasis of the cell by degrading cytosolic components or protein aggregates (Liu et al., 2011). Autophagy ensures a cells survival and development, and its impairment leads to cell death and genetic disorders. Xu et al. suggests that RNF216 is interacting with BECN1 to promote its ubiquitination, thereby accelerating the degradation of BECN1 in the cell and inhibiting autophagy (C. Xu et al., 2015).

Mabb et al. demonstrated that RNF216 ubiquitinates Arc, leading to the degeneration of Arc via the proteasome. They also showed that RNF216 and Arc localize to clatharin-coated pits and associated with endocytic sites in spines and dendrites. When Arc is degraded by clatharin coated localization of RNF216, it regulates the availability of synaptic AMPA receptors. Additionally, it regulates Arc-mediated plasticity at glutamatergic synapses (Mabb et al., 2014).

Husain et al. demonstrated that neurological complications possessed by TRIAD3A mutations in human patients may have an effect on spatial learning, memory, and function. They found that missense mutations in TRIAD3A abolish the interaction of Arc and TRIAD3A, disrupting Arc ubiquitination and affecting the degradation of this protein (Husian et al., 2017).

Here, I will describe several assays intended to prove RNF216 is an E3 ubiquitin ligase and to evaluate its protein expression in the mouse testes.

2.2 MATERIALS AND METHODS

2.2.1 Tissue Collection and Preparation

Mouse testes were collected at zero (P0) days of age through adulthood and were fixed in 4% Paraformaldehyde (PFA) in Phosphate Buffered Saline (PBS) at 4°C overnight on a rocker. Testes were embedded in paraffin and cut with a microtome in 5µm sections.

2.2.2 Western Blotting

Mouse testes were collected and homogenized using RIPA buffer (50um Tris-HCL, pH 7.4, 1% Np-40, 0.5% Na deoxycholate, 0.01% sodium dodecyl sulfate (SDS), 1mM EDTA, and 150mM NaCl). A 4-20% SDS -polyacrylamide gel electrophoresis (PAGE) gel was used to separate protein lysates. Gels was transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad) and blocked for one hour in 5% Non-fat dry milk on a rocker at room temperature. Primary antibodies were then incubated with the membrane overnight at 4°C on a rocker. The membranes were washed the next day in Tris Buffered Saline with Tween (TBS-T) several times at room temperature and incubated with HRP-conjugated goat anti-rabbit IgG (1:5000; 1706515, Bio-Rad) or goat anti-mouse IgG (1:5000; 1706516, Bio-Rad) for 1 hour before detecting with chemiluminescence. The primary antibodies used included: anti-RNF216 (1:1000, A304-113A, Bethyl Laboratories) and HRP-conjugated mouse anti-β-actin (1:10,000; A3854, Sigma).

2.3 RESULTS

2.3.1 RNF216 Ubiquitinates Arc in HEK293T Cells

To determine whether RNF216 has E3 ubiquitin ligase activity, we overexpressed RNF216 and tested its ubiquitination activity against Arc, a reported RNF216 substrate in neurons. We transfected HEK293T cells with HA-ubiquitin and FLAG-Arc with GFP-RNF216 or without GFP-RNF216 (control). Immunofluorescence of GFP showed the successful expression of RNF216 in transfected cells (Figure 5, upper panel). After immunoprecipitation of FLAG-Arc,

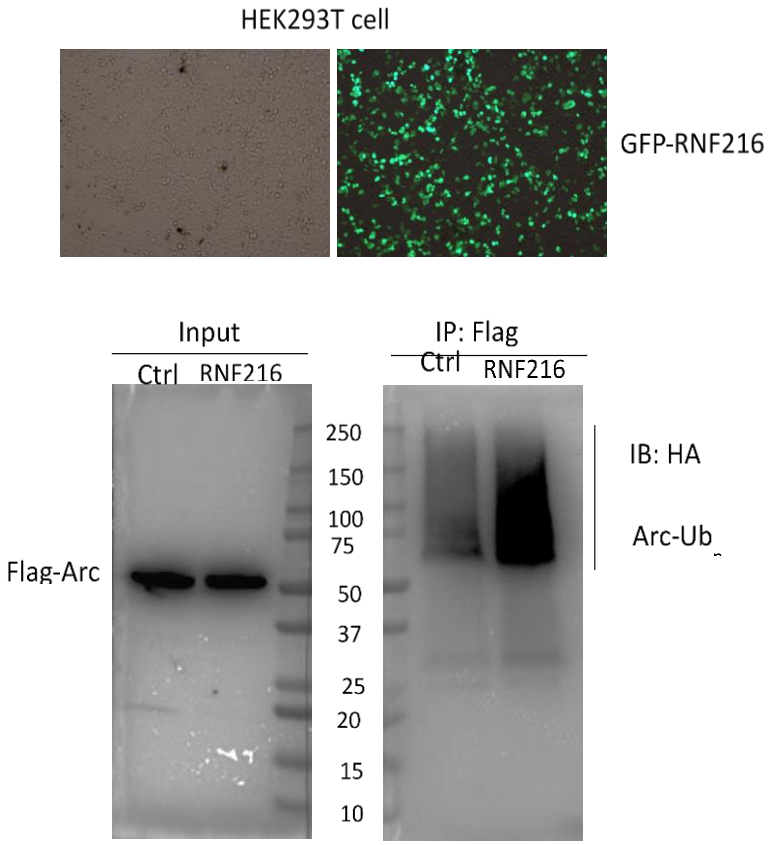

Figure 5: RNF216 Ubiquitinates Arc HEK293T cells were transfected with HA-ubiquitin, Flag-Arc, and GFP-RNF216 (control without GFP-RNF216). Co-IP was performed using flag-tagged beads. Immunofluorescence confirms successful transfection of GFP-RNF216. Western blot with anti-HA-ubiquitin shows high levels of ubiquitin when co-expressed with RNF216. Ladder was used to visualize protein sizes.

we examined the Arc ubiquitination levels by Western blotting of anti-HA-ubiquitin. The results indicate that Arc ubiquitination was increased in RNF216 transfected, but not control, cells (Figure 5, lower panel). This suggests that RNF216 is able to ubiquitinate Arc, consistent with the results from Mabb et al.

2.3.2 RNF216 is Expressed in the Mouse Testes

To evaluate the expression level of RNF216 in the mouse testes, we performed Western blot analysis of protein lysates from one-week, two-week, three-week, four-week, five-week, and adult (>six weeks) wild type testes using an anti-RNF216 antibody. The results showed that RNF216 is expressed throughout mouse spermatogenesis in the testes. B-actin served as a control in this experiment (Figure 6).

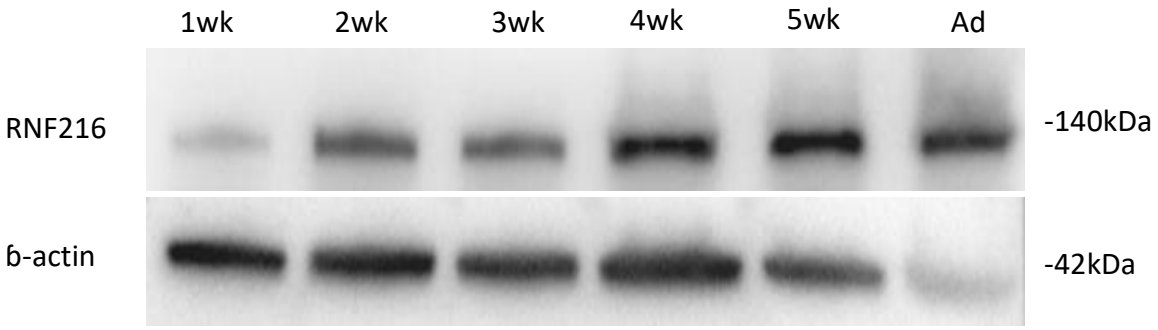

Figure 6: RNF216 is Expressed in the Mouse Testes Western blot analysis shows protein expression of RNF216 in the mouse testes from one week to adult (<6wk). Beta-actin served as a control in this assay.

2.4 DISCUSSION

RING domains, including RNF216, belong to a family of E3 ubiquitin ligases, which are responsible for controlled removal of protein in the cell. Our data demonstrate that RNF216 exhibits E3 ubiquitin ligase activity in vivo, in cultured cells. The characterization of RNF216 expression in the mouse testes indicates its constructive presence during spermatogenesis. If

RNF216 functions as an E3 ubiquitin ligase in the testes, it may regulate its substrates through protein degradation via the proteasome. The substrates of RNF216 in the testes is currently unknown. However, RNF216 has been shown to interact and ubiquitinate Arc in neuronal cells (Mabb et al., 2014). Although the expression and function of Arc in spermatogenesis is unknown, it would be interesting to directly test whether Arc is a potential substrate of RNF216 in the testes. In addition, future experiments are needed to identify other substrates of RNF216 in the testes. This can be achieved by isolating RNF216's protein complex by immunoprecipitation and identifying RNF216 binding proteins by mass spectrometry.

Chapter 3: RNF216 IS REQUIRED FOR SPERMATOGENESIS AND MALE FERTILITY

3.1 INTRODUCTION

RNF216 mutations have been identified in humans with Ataxia, Dementia, and Hypogonadotropism a disease classified as Gordon Holmes Syndrome (GHS) (Holmes, 1908). OTUD4 (OTU domain containing protein 4), a gene encoding a deubiquitinating enzyme, was also identified. Both genes play an important role in the ubiquitin pathway, however whether mutation of a single gene or both genes leads to neurodegenerative disease and simultaneous reproductive dysfunction is still unknown (Margolin et al., 2013).

Although mutations in RNF216 have been shown to be associated with ataxia, dementia, and hypogonadotrophism in human patients, there is still interfamilial variability within mutations of this gene alone. A case study shows results from two brothers in a Middle Eastern family of nine with RNF216 mutations. The first brother was 26 and started showing developmental problems at age 20. His secondary sexual characteristics were altered, had poor facial hair growth, mild cerebellar ataxia, gynecomastia (excessive breast development), hypogenitalism (subnormal development of genital organs), low testosterone and LH levels, unfused growth plates, and ossified bone. The second brother was 31 years old and started showing developmental issues at age 24. He also had altered secondary sexual characteristics, but had more severe cognitive dysfunction that his brother, where he couldn't navigate daily activities on his own. He had hypogenitalism, dysarthria (difficulty articulating words), no facial hair, broken saccadic eye movement, appendicular and truncal cerebellar ataxia, low LH and

testosterone levels, and unfused growth plates, among several other developmental abnormalities (Alqwaifly & Bohlega, 2016).

In this section, we address the physiological function of the RNF216 gene by generating RNF216 knockout mice. Specifically, we show that RNF216 is essential for spermatogenesis and male fertility.

3.2 MATERIALS AND METHODS

3.2.1 Mouse Care

All animals that were used in this project were approved by the Institutional Animal Care and Use Committee of Michigan State University (AUF 10/16-173-00). All experiments using these mice were conducted ethically and followed institutional guidelines according to the Guide for the Care and Use of Laboratory Animals.

3.2.2 RNF216 Knockout Mice

C57BL/6J RNF216 mutant mice were generated by the International Mouse Phenotyping Consortium.

3.2.3 Histology

Mouse testes and epididymis were fixed in 4% PFA or Bouins fixative in PBS, rocking at 4°C overnight. The tissue was embedded in paraffin and 5µm sections were sliced using a microtome. Sections were stained with hematoxylin and eosin after dewaxing and rehydration for histological and morphological analysis.

3.2.4 Immunofluorescence

Testes were fixed in 4% PFA in PBS at 4°C overnight and embedded in paraffin. 5µm sections were cut with a microtome, dewaxed, and rehydrated using Xylene solution and several ethanol dilutions. We preformed antigen retrieval by microwaving the prepared slides in 0.01M sodium citrate buffer (pH 6.0). The sections were rinsed with PBS several times and blocked with 5% normal goat serum (NGS) for 30 minutes at room temperature. The testes sections were incubated with anti-PLZF (1:30; sc-22839, Santa Cruz Biotechnology) or anti-ACRV1 (1:50; 14040-1-AP, Proteintech) diluted in 5% NGS for two hours. Sections were washed with PBS and incubated with Alexa Fluor 555 goat anti-rabbit IgG (1:500; A21429, Life Technologies) for one hour, rinsed in PBS, and mounted using Vectorshield mounting media with DAPI (H1200, Vector Laboratories). Fluorescence microscopy was performed using a Fluoview FV1000 confocal microscope (Olympus, Japan).

3.2.5 Western Blotting

Mouse testes were collected and homogenized using RIPA buffer (50um Tris-HCL, pH 7.4, 1% Np-40, 0.5% Na deoxycholate, 0.01% sodium dodecyl sulfate (SDS), 1mM EDTA, and 150mM NaCl). A 4-20% SDS -polyacrylamide gel electrophoresis (PAGE) gel was used to separate protein lysates. Gels were transferred to a polyvinylidene difluoride (PVDF) membranes (Bio-Rad) and blocked for one hour at room temperature in 5% Non-fat dry milk. Primary antibodies were incubated with the membrane overnight at 4°C. The membranes were washed the next day in TBST and incubated with HRP-conjugated goat anti-rabbit IgG (1:5000; 1706515, Bio-Rad) or goat anti-mouse IgG (1:5000; 1706516, Bio-Rad) for one hour before

detecting with chemiluminescence. The primary antibodies used included: anti-RNF216 (1:1000, A304-113A, Bethyl Laboratories) and HRP-conjugated mouse anti-B-actin (1:10000; A3854, Sigma).

3.2.6 Polymerase Chain Reaction (PCR)

Total RNA was extracted from mouse tissue using Trizol reagent (Thermo Scientific). Complementary DNA was synthesized using 1µg of RNA treated with DNase I (M0303S, NEB) and transcribed with iScript cDNA Synthesis Kit (Bio-Rad). PCR was performed using the primers listed below (Table 1).

Primer Name	**Primer Sequence**
RNF216-RT-PCR-Forward	AGAAAGTGAGCCTTTGGAAGTT
RNF216-RT-PCR-Reverse	AGTATGGATGGTCAAGCCAGTA

Table 1: RNF216-PCR Primers

3.3 RESULTS

3.3.1 Targeted Deletion of RNF216 in Mice

To investigate whether RNF216 is essential for spermatogenesis, we characterized mice with a targeted deletion of RNF216 obtained from International Mouse Phenotype Consortium

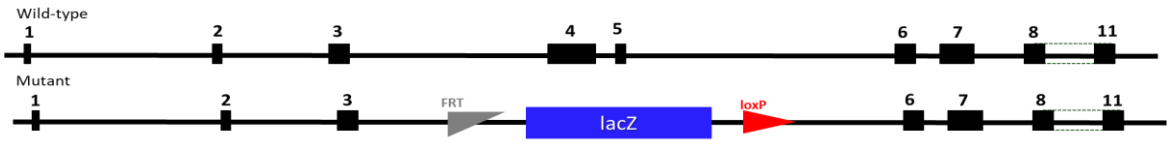

Figure 7: RNF216 Knockout Strategy RNF216 wild type allele has 11 exons. RNF216 mutant mice have exons four and five removed and replaced with a LacZ cassette.

(IMPC). RNF216 knockout mice were created using a “knockout-first” strategy. This strategy used a LacZ cassette to replace exons four and five of the RNF216 gene (Testa et al., 2004). The resulting mutant allele is expected to generate a short-truncated N-terminal protein segment if translated but lacking two critical Ring domains that are essential for E3 ligase activity. Thus, this mutant allele is predicted as a null mutation that abolishes RNF216 protein function in cells.

3.3.2 RNF216 Knockout Mice Do Not Contain the RNF216 Transcript

To verify that RNF216 was successfully mutated in knockout mice, genotyping was performed. Briefly, genomic DNA was isolated from RNF216 wildtype, heterozygous and mutant mice tail snips. DNA was amplified using genotyping PCR with a series of multiplex primers including a forward, reverse, and a TCP LacF primer (Table 2). An RNF216 wildtype band (0.3kb) was detected in both wildtype and heterozygous mic. RNF216 mutant band (0.6kb) was detected in both the knockout and heterozygous mice (Figure 8).

Primer Name	**Primer Sequence**
RNF216 Forward	TTGGTAGGAAGAGGATCCTAGAAAG
RNF216 Reverse	AGGCTCTTCATGCAGTAACCAAGCC
TCP LacF	CCATTACCAGTTGGTCTGGTGTC

Table 2: RNF216 Genotyping Primers

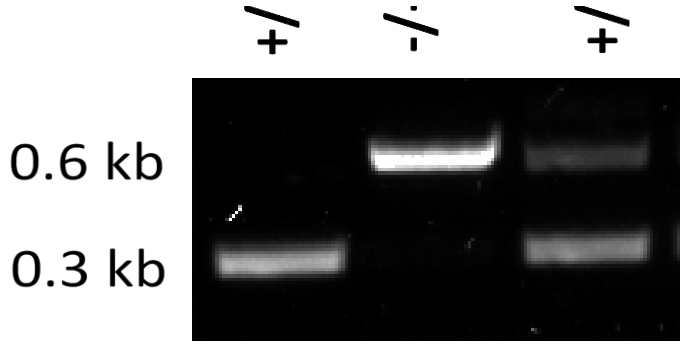

Figure 8: RNF216 Mouse Genotyping Genotyping results show wild-type (+/+), Heterozygous (+/-), and mutant (-/-) mice, confirming successful mutation of RNF216 and creation of RNF216 knockout mice.

To examine the RNF216 mRNA expression level in RNF216 knockout mice, we preformed RT-PCR using cDNA generated from both heterozygous and knockout testes. GADPH served as a control in our PCR assay (Figure 9). Results showed that RNF216 mRNA was not detectable in RNF216 knockout mice.

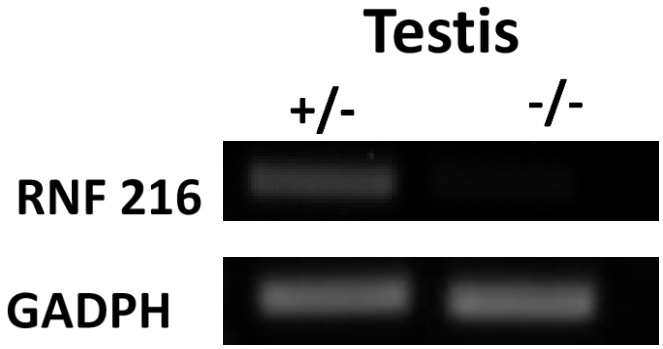

Figure 9: RNF216 Knockout Mice Do Not Contain RNF216 Transcript PCR results showing RNF216 knockout mice (-/-) do not contain the RNF216 RNA transcript. Heterozygous (+/-) RNF216 mice do show RNA transcription. GADPH served as a control in this assay.

3.3.3 RNF216 Protein Expression is Absent in the Knockout Mouse Testes

To validate that RNF216 protein expression is abolished in knockout mice, we performed Western Blotting using a rabbit anti-RNF216 antibody on wildtype, heterozygous

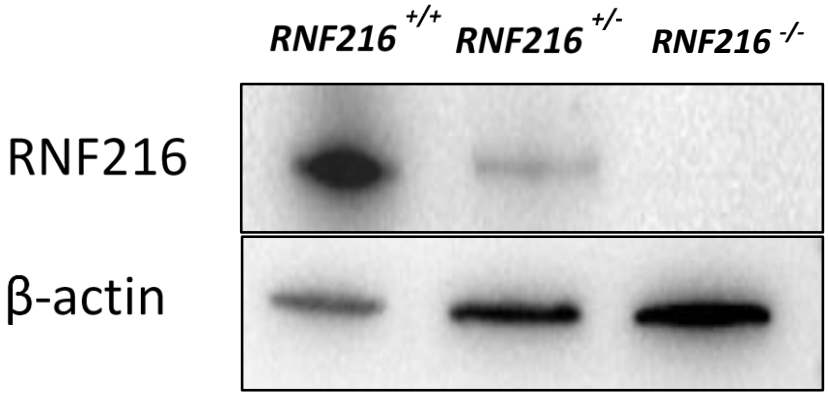

Figure 10: RNF216 Protein Expression is Absent in the Mouse Testes Western blot analysis shows RNF216 wildtype (+/+) and heterozygous (+/-) mice express the RNF216 protein and RNF216 knockout (-/-) mice do not. β-actin served as a control in this assay.

and knockout testes lysates (Figure 10). The expected band size (~130kd), for the RNF216 protein was seen in both RNF216 wildtype and heterozygous mice, but not the RNF216 knockout mice. Beta-actin served as a loading control in this experiment (~42kd) (Figure 10). This result indicates that the targeted RNF216 mutant allele is a protein null allele.

3.3.4 RNF216 is Not Required for Animal Viability but Knockout Males Display Reduced Testicular Size

RNF216 knockout mice are viable and can grow to adulthood without apparent health and behavioral problems (Figure 11). Examination of various tissues and organs did not reveal obvious abnormality. RNF216 knockout and heterozygous mice had similar body weights. Strikingly, RNF216 knockout males displayed a significant decrease in testicular size and weight when compared to their heterozygous control littermates (Figure 11 and 12). This data indicates that RNF216 is dispensable for animal survival but is essential for male gonad

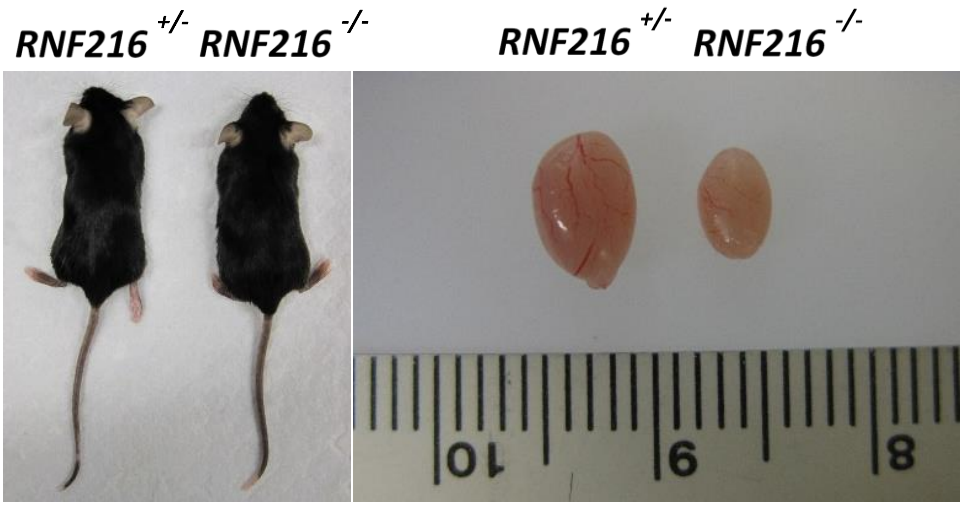

Figure 11: RNF216 is Essential for Spermatogenesis RNF216 heterozygous (+/-) and knockout adult (-/-) mice show no difference in body appearance (left), but RNF216 knockout mice show a reduced testicular size compared to its heterozygous littermate (right).

development. This reveals for the first time the physiological function of the RNF216 gene in mammals.

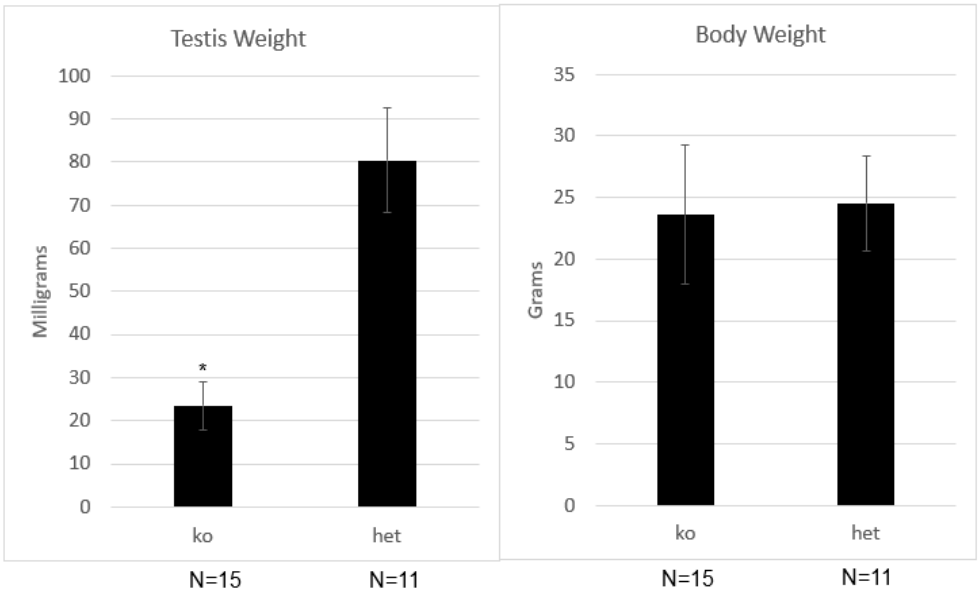

Figure 12: RNF216 Knockout Mice Have Significantly Reduced Testicular Weight Both RNF216 heterozygous (het) and knockout (ko) mice have similar body weights (right), however RNF216 knockout mice show a significant decrease in testicular weight, when compared to their heterozygous littermate (left).

3.3.5 RNF216 Knockout Mice Show Severe Germ Cell Degeneration

Targeted deletion of the RNF216 gene results in defective spermatogenesis in homozygous male mice. We observed that RNF216 knockout mice display germ cell arrest at meiotic or post-meiotic stages of spermatogenesis, leading to germ cell loss in seminiferous epithelium. In adult RNF216 males, the epididymis contained only some round spermatid-like cells and a few mature spermatozoa. Even spermatozoa appeared abnormal, exhibiting bent heads, bent necks, or detached heads. This phenotype is similar to non-obstructive azoospermia in human males.

The seminiferous tubules of RNF216 knockout mice display a heterogeneous spread of germ cell phenotype. Some tubules are Sertoli cells only, while others contain an array of germ cells. However, these tubules containing germ cells were not morphologically normal. For example, some tubules contained spermatocytes as the most advanced cell type and some tubules were arrested at round spermatid stage. This indicates that germ cell development is severely compromised due to RNF216 deficiency.

We further examined RNF216 knockout testes at different developmental stages from postnatal week one to postnatal week 12. Spermatogenesis in RNF216 knockout mice appeared to progress normally during the first week of development. The first signs of spermatogenic arrest are visible around two weeks of age. Mice continue to show germ cell arrest and apoptosis as they age. By 11-12 weeks almost no tubules contained elongated spermatids and there was a drastic increase in Sertoli-cell only tubules. Sertoli cell populations appeared normal throughout development in knockout mice.

3.3.6 RNF216 Knockout Mice Show Varying Degrees of Germ Cell Defect

RNF216 knockout mice exhibit a heterogeneous array of degenerative tubules in the testes. To better determine the degree of germ cell loss within the seminiferous tubules in mice, I categorized the different tubules into six different classes (Class one through six) (Figure 13.) Class six is the most severe case of degeneration and Class one is the least extensive case of degeneration. Classes two through five range from mild to medium degrees of degeneration

(Figure 13). I will refer to these classes, from here on forward, when describing the phenotype of the mice.

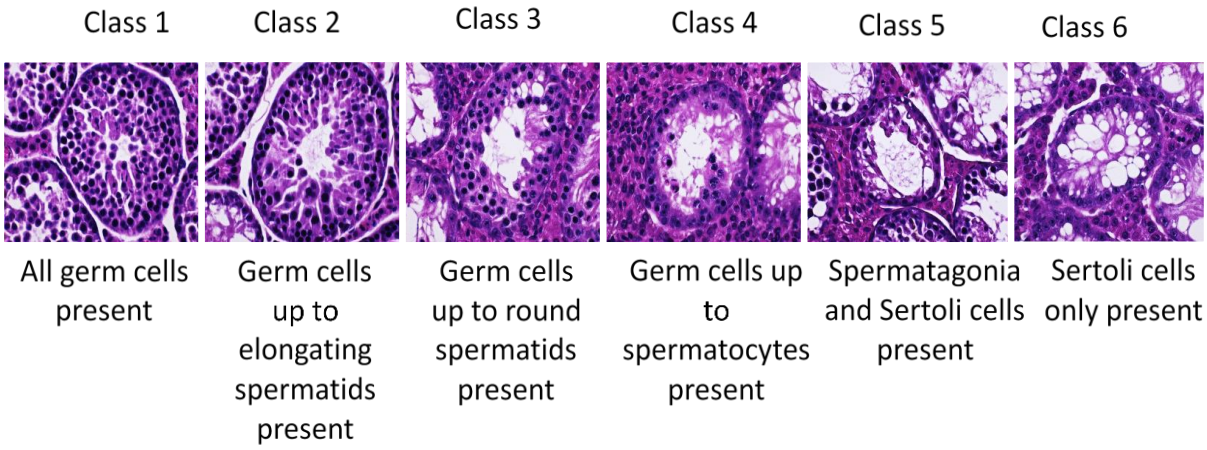

Figure 13: Classes of RNF216 Knockout Phenotype Classes of RNF216 knockout mice seminiferous tubules, determined based off cells present in the tubules. Class one represents the least severe case of germ cell loss with all germ cells present including elongated spermatids, round spermatids, spermatocytes, spermatagonia, and Sertoli cells, with little to no signs of germ cell degradation. Class two is like class one with the same cells present, however exhibits signs of degradation and more white space in the lumen of the tubules. Class three represents tubules possessing round spermatids, spermatocytes, spermatagonia, and Sertoli cells. Class four represents tubules containing spermatocytes, spermatagonia, and Sertoli cells. Class five represents tubules containing spermatagonia and Sertoli cells. Class six represents tubules containing Sertoli cells only, the most severe degree.

3.3.7 RNF216 Knockout Mice Show Germ Cell Loss Starting at Two Weeks

Mice under six weeks of age have not reached sexual maturity. Spermatogenesis does not make a full wave until six weeks, therefore mice under five-six weeks do not contain mature spermatozoa. Mice were collected at one, two, three, four, and five weeks of age. They were fixed and stained with Hematoxylin and Eosin. Knockout mice displayed germ cell degeneration

starting at two weeks of age. As the mice aged, germ cell degradation becomes more severe (Figure 14).

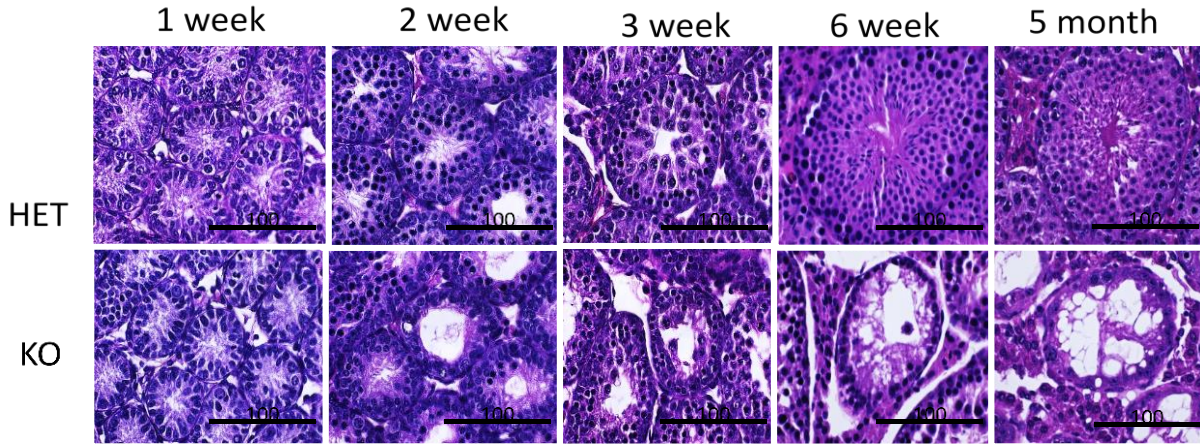

Figure 14: RNF216 Knockout Mice Display Morphological Abnormalities RNF216 knockout mice histology sections compared to RNF216 heterozygous histology sections before and after sexual maturity at one week, two weeks, three weeks, six weeks, and five months.

3.3.8 Young Adult RNF216 Knockout Mice Display Severe Germ Cell Atrophy (6-12 weeks)

We evaluated RNF216 heterozygous and knockout testes histology at six weeks old, when mice reach sexual maturity. Testes were collected, fixed, and stained with Hematoxylin and Eosin. RNF216 Knockout mice display moderate to severe germ cell degeneration. Tubules ranged from severe germ cell loss to moderate germ cell arrest/loss, classified as class four to class six tubules. Elongated spermatids were seen, but not in the right placement within the tubules. Some tubules were Sertoli-cell only, while others showed varying layers of germ cells (Figure 14). No sperm was found in the epididymal tubules and only round cells were present (Fig 15). At 10 weeks, only a few tubules displayed abnormal elongated spermatids and other tubules showed class three round spermatid tubules. Most other tubules displayed class six Sertoli-cell-only, or class five abnormal layering of spermatocytes. By 11 and 12 weeks, very few tubules displayed elongated spermatids and rare sightings of round spermatids. Most tubules

were completely empty, showing Sertoli-cell-only, stage six tubules. At 12 weeks old, sperm was typically not visible in the epididymis (Figure 15).

3.3.9 Adult RNF216 Knockout Mice Show the Most Severe Germ Cell Loss

Older adult mice display the most germ cell loss. Almost no spermatids were seen in any of the seminiferous tubules. Tubules varied in degree of degeneration. There were almost no

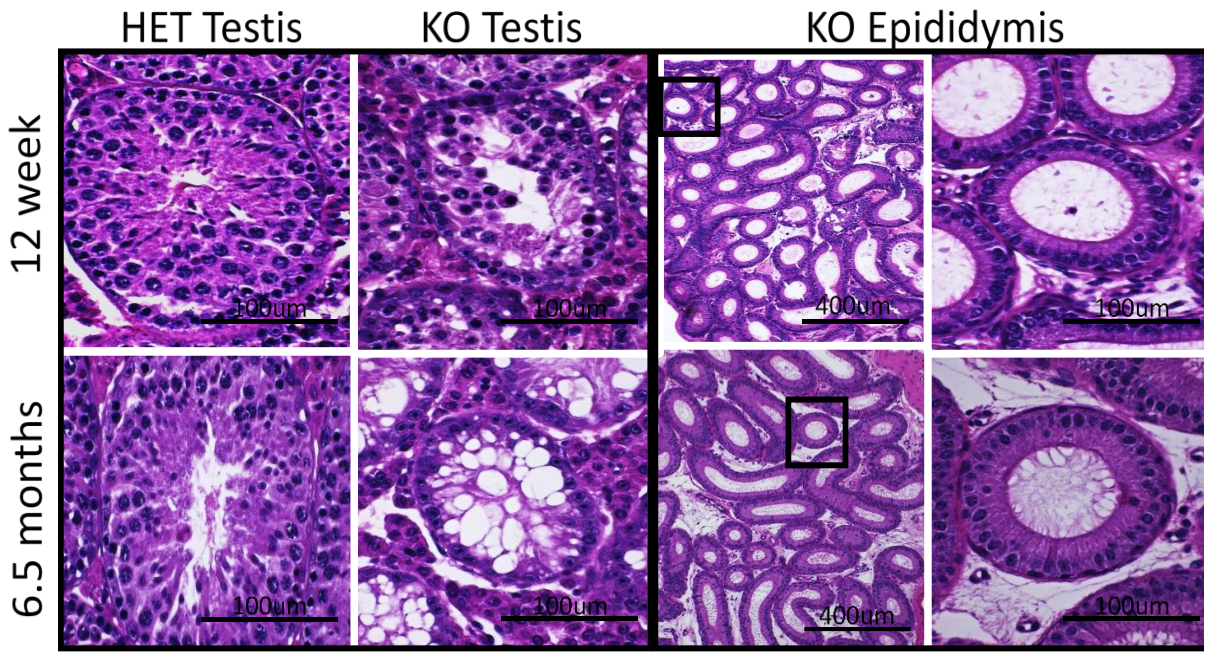

Figure 15: Adult RNF216 Knockout Mice Display Germ Cell Atrophy Histology sections showing RNF216 heterozygous and knockout testes (left) and epididymis (right) at 12 weeks old and 6.5 months old.

class one tubules with elongated spermatids and a very rare sighting of class two round spermatids. Most tubules were class six, completely empty with Sertoli-cell-only tubules (Figure 15). Thus, these histological findings demonstrate that RNF216 knockout mice display progressive germ cell loss over time that leads to testicular atrophy and male infertility.

3.3.10 Spermatagonial Stem Cells are Present in RNF216 Knockout Mice

We preformed immunofluorescence staining of germ cell markers in six-week-old heterozygous and knockout mouse testes sections and various germ cell markers to see if there was a difference in expression or presence of germ cells in the heterozygous and knockout seminiferous tubules. We used PLZF, a marker for spermatogonia and saw the spermatagonial stem cell population in both the heterozygous and the knockout mice seemed equivalent. We stained the testes sections with ACRV1, a marker of the acrosomal vesicle/acrosome. Staining with ACRV1 showed some tubules exhibited round spermatid populations, however we failed to see progression into elongated spermatid stages (Figure 16). This data suggests RNF216 is

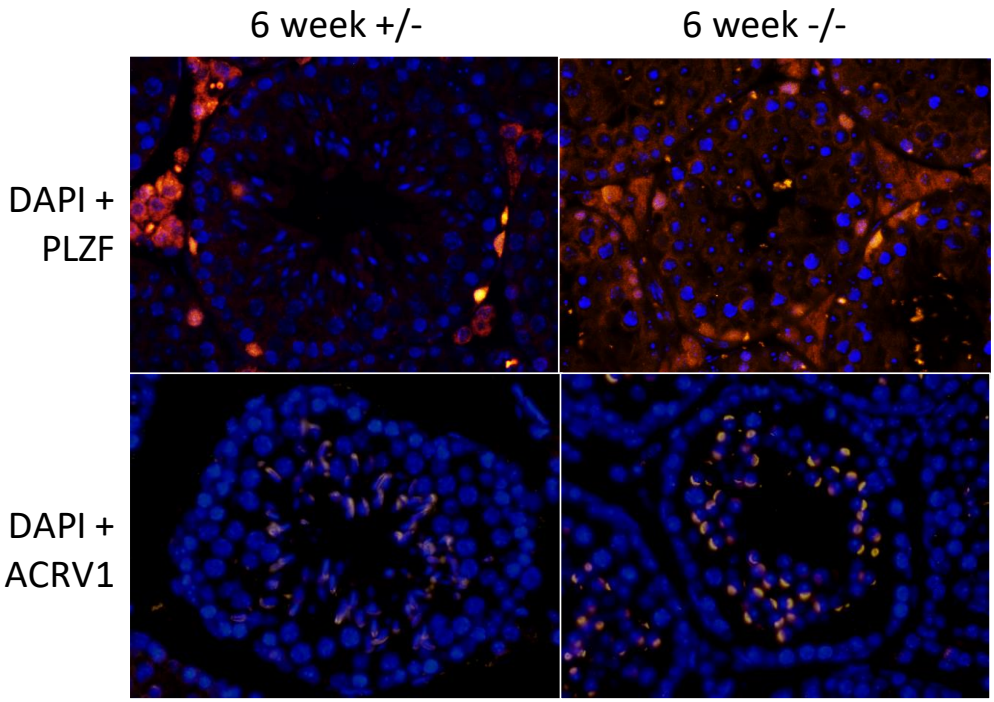

Figure 16: Spermatagonial Stem Cells are Present in RNF216 Knockout Mice
Immunofluorescence results showing GATA4 signal in the heterozygous and knockout testis (top), PLZF signal in the heterozygous and knockout testis (middle), and ACRV1 signal in the heterozygous and knockout testis (bottom).

not essential for spermatagonial stem cell survival, and that many of the tubules exhibiting germ cell defect is due to meiotic or post meiotic arrest.

3.3.11 RNF216 Female Knockout Mice are Fertile

RNF216 knockout male mice show a severe infertility phenotype and we wanted to check whether RNF216 knockout females are fertile or infertile. We set up a fertility test using wildtype males breeding with RNF216 knockout females. After observing these mice for three months, knockout females were able to achieve a normal pregnancy every month. Additionally, the average number of pups they produced was equivalent to the average number of pups RNF216 heterozygous female mice produced (Figure 17). RNF216 heterozygous and knockout females were similar in body appearance and weight, and the pups produced appeared healthy.

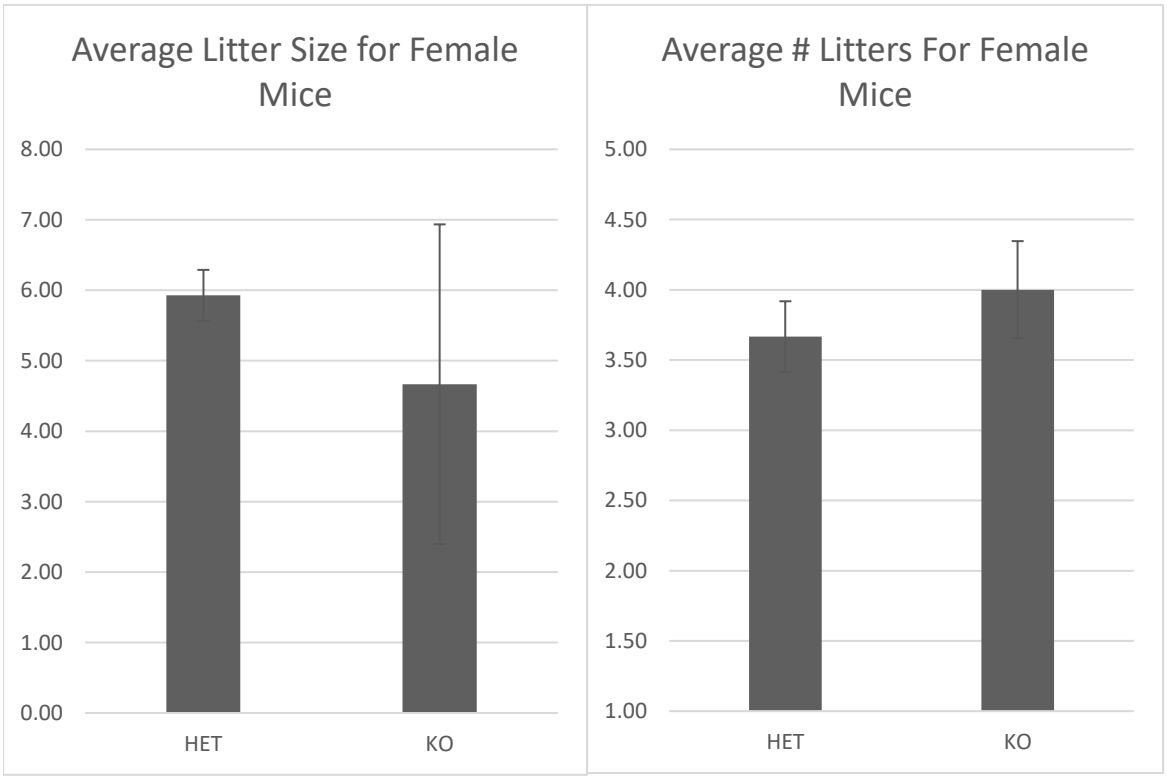

Figure 17: RNF216 Female Knockout Mice are Fertile Graph showing the average litter size and average number of litters for both heterozygous and knockout mice. N=5.

3.4 DISCUSSION

RNF216 mutations have been associated with human disease and reproductive dysfunction. Mutations in RNF216 and another gene named OTUD4, are linked to hypogonadotrophism, ataxia and dementia in human patients (Mabb et al., 2014). Other than this paper, RNF216 has not been studied in a mammalian model. In this study, we show for the first time, the physiological function of RNF216 in a mammalian animal model. We found RNF216 is essential for spermatogenesis because when this gene is knocked out from the mouse genome, the mice display decreased testicular size and weight, as well as a severe loss in germ cell population as they age. The knockout testis weight displays about a three-fold decrease in size, causing a highly significant difference compared to its control littermates, however the body weight of these mice remains the same. RNF216 female knockout mice, on the other hand, are fertile. Therefore, RNF216 is specifically required for male reproduction.

Severe germ cell degeneration in RNF216 male knockout mice is an indicator that spermatogenesis is disrupted at various stages. Germ cell defect was noticed as early as two weeks of age. At this age, intermediate and B-type spermatogonia are starting to form with the Sertoli cells already present. Sertoli cells are still observed in the seminiferous tubules well into adult-hood and some tubules are Sertoli cell only. However, as these male mice age, degeneration occurs mostly in spermatocytes. Only a few round spermatids, and especially rare elongated spermatids, are seen. This leads us to believe the germ cell arrest is occurring at a post meiotic stage.

To better understand how RNF216 knockout mice differ from RNF216 wildtype or heterozygous mice, we preformed staining using germ cell specific markers, including PLZF and ACRV1. No difference in PLZF signal in RNF216 knockout and heterozygous mice suggests RNF216 has a specific function in the spermatocyte or spermatid. ACRV1 staining shows knockout mice do not contain many acrosomes, but do contain some acrosomal vesicles, found during the round spermatid stage. This further helps us conclude that spermatogenesis is altered and that most germ cells cannot progress past the round spermatid stage.

Chapter 4: CONCLUDING REMARKS

In conclusion, we have determined that RNF216 is essential for male germ cell development and spermatogenesis. When RNF216 is knocked out in the mouse genome, male knockout mice display reduced testicular size, germ cell developmental defect, and degeneration, resulting in an azoospermia-like infertility phenotype. Although the expression pattern and mechanistic action of RNF216 in the testes is unclear, we have shown for the first time that RNF216 is an essential gene for spermatogenesis and male fertility.

4.1 FUTURE DIRECTIONS

The mechanism by which RNF216 functions in the testes is unclear. Future research for this project will include characterizing where the RNF216 protein is expressed in the testes. Additional research is needed to dissect the molecular mechanism by which RNF216 works as an E3 ubiquitin ligase. To identify the substrates of RNF216 will be a priority. Determining the stages of germ cell arrest and if RNF216 does possess multiple functions at different stages of development is another future direction.

Infertility in men is largely idiopathic. Our work shows that RNF216 is essential for male fertility in mice, which supports the notion that RNF216 is an essential gene for human male fertility, as shown by Margolin et al. Studying genes essential for spermatogenesis will lead to advances in understanding fertility and infertility and treatments for male reproductive disorders. Additionally, this research could provide directions for novel strategies for the development of male birth control.

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