INVESTIGATING THE MALE GERM CELL AND MOLECULAR REQUIREMENT OF RNF216 ON SPERMATOGENESIS AND FERTILITY IN MICE

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A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

Cell and Molecular Biology – Doctor of Philosophy

2025

ABSTRACT

Infertility effects 10% of the population with half of cases attributed to the male partner, resulting in financial, emotional, and social burdens when trying to conceive. Male infertility may be attributed to disruptions in spermatogenesis, which prevents spermatogonia from becoming mature spermatozoa necessary for fertilization. However, the mechanisms behind male infertility and contributing genetic factors are not fully elucidated. One such factor is Ring finger protein 216 (RNF216/TRIAD3), an E3 ubiquitin ligase in the RING-between-RING (RBR) subfamily with mutations identified in patients with Gordon Holmes Syndrome (GHS), a neurodegenerative disorder with male reproductive dysfunction. Additionally, global deletion of ubiquitous RNF216 in mice revealed essentiality of RNF216 in male fertility. However, the germ cell requirement and mechanism of RNF216 in male reproduction and GHS are unclear. To address this, I generated novel transgenic mouse lines to examine expression and localization of RNF216 *in vivo*, conditionally knockout RNF216 in male germ cells, and model the human GHS ubiquitin ligase inactivating RNF216 mutation. First, I characterized RNF216 expression in male germ cell populations that is seminiferous tubule stage-specific and localized within sub-nuclear domains. Furthermore, I discovered RNF216 is intrinsically required in male germ cells for spermatogenesis and fertility but is dispensable for spermatogonia function and survival. Finally, I determined the human GHS mutation led to progressive germ cell degeneration and infertility, demonstrating RNF216-directed ubiquitination is essential for spermatogenesis. These data definitively show RNF216 has a pivotal role in male germ cell biology, GHS reproductive etiology, and infertility.

ACKNOWLEDGEMENTS

My educational and scientific journey is not one of a single man, but rather the culmination of several individuals and groups that have supported, encouraged, and helped me in this endeavor. My decision to pursue a Ph.D. was significant and not without thought, since I knew the dedication and time required would be a sizable sacrifice from time with family and friends in the pursuit of addressing my research questions and passions. To begin, when I arrived at Michigan State University with all my options laid out before me, I felt welcomed and wanted by the BioMolecular Science (BMS) Ph.D. gateway program faculty, staff, and peers. I would like to extend a thank you to BMS faculty leadership, Dr. John Lapres and Dr. Claire Vieille, for offering me a position here at Michigan State University for my graduate education.

At Michigan State University, I got connected with my mentor, Dr. Chen Chen, who has been everything I could have ever asked for as my Ph.D. advisor. He welcomed me into his laboratory as his first Ph.D. student, which I cannot thank him enough for trusting me to contribute to his ongoing projects and establish my own dissertation research while financially supporting me all these years. Dr. Chen has been a valuable source of knowledge, guidance, and understanding, all of which were reflected in our interactions in lab meetings, one-on-one meetings, and casual passings. He always looked out for my best interests and was kind and gentle, yet effective, in his feedback of my work and always pushed me to achieve my full potential. A simple thank you in my acknowledgements is not enough for all he has done to sharpen me as a scientist, stimulate my passion for reproductive and developmental biology, and support my desire for mentoring and teaching opportunities. Furthermore, the Chen laboratory has been a

wonderful place to conduct my research and a major reason for this has been the consistency and hard work of our two post-doctoral researchers, Dr. Chao Wei and Dr. Xiaoyuan Yan, both of whom have been incredibly kind, patient, and helpful during training of new techniques, troubleshooting some experiments I could not get to work correctly, and coordinating coverage of my mice so I could have time to spend with family. All your contributions to my Ph.D. training could not be understated.

I had the utmost pleasure of joining the Cell and Molecular Biology (CMB) graduate program, which is full of inspiring graduate students, faculty mentors, and leadership, that has been a tremendous source of support and opportunities. Specifically, I would like to thank the CMB Director, Dr. Margaret Petroff, who was part of my interview process for the BMS program and for her recruitment of me into CMB. Your work to build the CMB program during my time at Michigan State University has been fruitful and appreciated. Additionally, I would like to thank the CMB Associate Director, Dr. Ron Chandler, the CMB Academic Program Coordinator, Alaina Burghardt, and the CMB Program Assistant, Geoffrey Grzesiak, for their help and participation in making CMB an ideal program to pursue my Ph.D. Lastly, I would like to thank all my peers in CMB, and those involved in CMB graduate student organization leadership, for making CMB what it is today, a program that values student input and celebrates their successes. I wish you all the best in your future endeavors.

Outside of the Chen laboratory and CMB program, I would like to extend a large thank you to the members of my guidance committee, Dr. Jose Cibelli, Dr. Bin Gu, Dr. Liangliang Sun, Dr. Jian Hu, and Dr. Sangbum Park. I am appreciative of your dedication and support throughout my projects and the turns and successes it has entailed. I

recognize that your involvement in my Ph.D. journey has taken time out of your already busy schedules, and I could not be more grateful of your sacrifice. Each of you has brought valuable experience and knowledge to the table, and I thank you for all your comments, suggestions, and kindness in your deliveries. I have felt very supported by you all as you have encouraged me to keep pushing in my projects, presentations, and writings. I have enjoyed all our interactions as collaborators, at conferences, or in committee meetings. I am inspired by all your research and wish you all well in your careers and beyond.

Pivoting away from my time at Michigan State University, I would like to thank and acknowledge the individuals in my educational and research experiences who have helped me position myself to pursue a Ph.D. before I stepped foot on campus. First, I would like to thank all my teachers during my time at Harper Creek Public Schools in Battle Creek, Michigan, for their encouragement and instruction. Additionally, during my split-time in high school, I would like to thank the Battle Creek Area Math and Science Center (BCAMSC) administration and faculty for providing me an opportunity to specialize in STEM prior to my undergraduate education. Your influence and support have not been forgotten. I will always hold a special place for my fellow Beavers and Phoenix.

As an undergraduate at the University of Michigan in Ann Arbor, I was blessed to find opportunities to enhance my knowledge in the classroom, participate in innovative research, and build my teaching and mentorship skills, all of which translated into my Ph.D. training. I would like to thank the University of Michigan Undergraduate Research Opportunity Program (UROP) for my acceptance into their program as a freshman and the chance to get plugged into my first serious research setting under Dr. Zora Djuric at

the University of Michigan in the Department of Family Medicine. Her support, guidance, and flexibility were paramount to my successes in her research group and I cannot thank her enough for believing in me. I also want to thank UROP for entrusting me to teach research courses and mentor fellow undergraduate researchers for two years as an upper-level undergraduate under the advisement of Dr. Luciana Nemtanu. This is where my passion for teaching and mentorship took shape and provided me an opportunity to encourage and enable the next generation of researchers. Last, I would like to extend a considerable thank you to my research mentor after graduation, Dr. Vesa Kaartinen, at the University of Michigan School of Dentistry. For two years, I was honored to be your research technician, only member of your research group, and right-hand man. I cannot thank you enough for giving me a chance to prove myself and grow in a wet laboratory setting and pursue mouse genetics and developmental biology research for the first time. I have used almost everything I learned under your guidance in my Ph.D. research. Most notably, I would like to thank you for your encouragement to pursue a Ph.D. and making me feel valued and appreciated. Overall, my time at the University of Michigan was full of moments of triumph, as well as momentary lows, that made me into the person I am today. I will always be a proud Michigan man.

Transitioning from individuals and institutions that have been impactful on me both academically and professionally, all of which deserve profound acknowledgement and gratitude, there remains another significant group that I would not have reached this point without: my friends and family. I cannot state how much all these individuals mean to me personally and how they all deserve their rightly due recognition.

I would like to thank my supportive friends in multiple locations and during

numerous times in my life that continue to encourage me to this day. My core friends from back home, Ben, Travis, and Zach, who have been like brothers to me and kept me smiling although miles apart. My college housemates and men of The Soil, Andrew, Jeff, Chandler, Chris, Zac, Marc, Peyton, Baker, and Devon, who helped shape me into the best man I can be. As iron sharpens iron, so one man sharpens another. Additionally, the breakaway post-graduation group of Andrew, Jeff, and Chandler during our Wednesday morning coffee sessions which have provoked thoughtful discussion, instilled wisdom, and offered unwavering support. Lastly, all the members of my church, Grace Bible Church, in Ann Arbor, Michigan, and my small group members, Erika, Andrew and Alexis, Jeff and Erin, Ryan and Ivy, John and Kristina, Bhumanyu and Emily, Jon and Katerina, Soren and McKenna, and Lauren. You all are continuing examples of faith and are never failing to show up in all arenas of life.

Turning to my family, I would like to extend the sincerest thanks to my loving parents, Douglas and Kathy. You are both tremendous examples and your generosity, character, kindness, reliability, and unconditional love is unmatched. I could not have been blessed with better parents who encourage me to fulfill my potential and find joy in my work and in life. To my siblings (and their spouses), Jeremy (and Ashley) and Rebecca (and Ryan), I have had the privilege to be your younger brother and watch you both grow and excel in your lives while having you reciprocate your love, trust, and confidence in me. I would also like to continue my thanks to my extended family of grandparents, aunts, uncles, and cousins for their support.

I would like to thank my beautiful wife, Erika, our goofy border collie, Patches, as well as Erika’s faithful and loving family (and their spouses), Carl and Jane, Nathan (and

Danielle), Jenna (and Taylor), and Tara. To Erika, you have been such a source of happiness and strength in my life, and you are the best of wives and best of women. I am inspired by your work ethic in the hospital as a physician and your gentleness and compassion towards others, including myself. Thank you for supporting me during my Ph.D. and the sacrifices that have come along with it. I could not have accomplished this achievement without you; I love you endlessly.

Lastly, I would like to share a more personal sentiment. When people ask about my research, I share my thought that while reproduction may not be essential for personal survival, it is paramount for future generations to ensue, thus justifying reproductive and developmental biology research. Over the final year and a half of my Ph.D. training, Erika and I sadly lost two early pregnancies, placing significant emotional strain on ourselves as we navigated this troublesome time. There were days when coming to the laboratory to investigate underlying genetic causes of reproductive failure weighed on me while we struggled to reason through our own losses and sought help. Through this experience, I have found a greater appreciation for the work that is done in the field of reproductive biology at all levels, as well as a greater sensitivity towards those who have been in our shoes and grieved, regardless of when their losses have happened or are happening – you are not alone.

Ending on a positive note, it is with tremendous joy and delight that I dedicate my Ph.D. dissertation to my firstborn daughter, Julia Grace Mann, and the gift that she is coming out of a difficult season of life. You are so loved and a true reflection of the faithfulness and goodness of our Lord and savior, Jesus Christ. It is only through Him that any of this would be possible.

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CHAPTER I.

INTRODUCTION

1.1 Overview of murine and human male reproductive biology

Sexual reproduction is at the core of multicellular eukaryotic life, as the need to pass down heritable genetic material to offspring is essential to maintain life beyond a single generation [1-3]. This process protects the longevity of the genetic code of life in these organisms that can be maintained for millennia, albeit with modifications to gene composition or expression, on a generation-to-generation basis. These changes can range from random genetic mutations to epigenetic modifications through environmental exposures that have accumulated in parental germline DNA [4-6]. All these factors will culminate when new allele combinations from the father and mother spawn a unique offspring that is genetically different from its parents, although it arose from the same genomes. From the cardinal to the wolverine, the buffalo to the alligator, and the mouse to the human, the process of sexual reproduction is evolutionary conserved in all mammals, reptiles, and birds [3, 7].

Both the female and male partner have their own respective reproductive organs, processes, and gametes that contribute to this equation, but for the sake of this dissertation, the male contribution to life will be the primary focus. Fortunately, the study of male reproduction is achievable due to advances in experimental cellular and molecular biology techniques and reagents, among these being the house mouse, *Mus musculus*, which has a high level of conservation in both male reproductive structure and function with modern humans, *Homo sapiens* [8-10]. Taking advantage of these similarities at the cellular, molecular, and structural levels of male reproduction, unique

research questions can be asked and addressed to uncover the remaining mysteries of male sexual reproduction to ensure the existence of future generations and treat fertility issues at the clinical level.

1.1.1 Male reproductive tract structure and function

Male reproduction starts within the primary sexual organ and gonad, the testis, which is housed in the scrotum. There are two primary functions occurring within these testes, which include a solely reproductive function to produce sperm and an endocrine function to produce testosterone [11, 12]. Testis contain a series of tube-like structures intertwined to form the bulk mass of the testis, called seminiferous tubules (**Figure 1.1**). Male germ cells reside within these tubules, and this is where the production of sperm occurs starting from stem cell populations to generate sperm cells through a process called spermatogenesis. Furthermore, there exists a series of blood vessels that lie between the seminiferous tubules in interstitial spaces providing nutrients and hormones to the testis that will aid spermatogenesis. These seminiferous tubules twist and turn to form what resembles a ball of yarn contained within a membranous sac called the tunica vaginitis. The seminiferous tubules all terminate at the rete testis before converging to efferent ductules connected to the secondary male sexual organ, the epididymis.

The epididymis is a long, crescent-shaped organ attached to the testis that is also composed of coiled tubules (although different from seminiferous tubules). The epididymis may be broken down into three distinct components, which are the head (caput), body (corpus), and tail (cauda), with the latter forming a small bulge at the end of the epididymis (**Figure 1.1**). The primary function of the epididymis is to store sperm produced in the testis and provide an ideal environment for sperm maturation prior to

ejaculation. Maturing sperm moves through the epididymis tubules and approximately 50 – 80% of all this motile sperm is within the cauda epididymis, which is connected to the vas deferens [13, 14]. Storage of sperm within the epididymis also provides a form of protection from foreign insults through the blood-epididymis-barrier and other protective factors secreted from epididymal epithelial cells that maintain optimal pH and produce antioxidant enzymes [13, 15-17]. The last, yet critical, function of sperm maturation provides these gametes with motility through additional structural changes so they can ultimately reach and fertilize an oocyte [18, 19]. Upon ejaculation during sexual intercourse, the mature sperm within the cauda will exit the epididymis first through the vas deferens.

As mature, motile sperm travels through the vas deferens, it passes through the seminal vesicles and prostate, both of which contribute essential nutrients and proteins to form semen [20, 21]. In addition to aiding sperm with valuable nutrients to meet high energy demands, these secreted fluids in semen also provide protection for sperm upon entry into the vaginal canal, which has a harsh, acidic environment [20-22]. From this point, a small proportion of surviving sperm will continue the journey through the fallopian tube to reach an ovulated oocyte, and merge with the egg in an acrosome reaction. Thus, the two haploid gametes can combine to form a diploid zygote, which upon successful implantation, will grow into a fetus. This fertilization process is nearly identical between humans and common model systems, such as mice, providing an ideal mammalian model to examine how the testis and epididymis function to ensure successful waves of spermatogenesis to form and mature the sperm required to continue life to the next generation.

1.1.2 Spermatogenesis

Male fertility is dependent on the constant supply of mature sperm to ensure that males are fertile throughout their entire adult lives. Populations of germ cells are responsible for this supply of sperm, and all arise from the same progenitors, primordial germ cells (PGCs), which begin from a small subset of epiblast cells [23, 24]. During embryonic development, PGCs migrate to the genital ridge, ultimately becoming the testes, thus making the testes the site of future sexual reproduction [24]. Within the testis, spermatogenesis occurs on a non-stop basis, cyclically replenishing sperm supply. The process of spermatogenesis has been well studied and can be broken down into three phases consisting of mitosis, meiosis, and spermiogenesis, all of which need to be completed to derive sperm from spermatogonia stem cells (SSCs) [11, 25-28]. One complete cycle of spermatogenesis, encapsulating multiple cell divisions, differentiation events, and cellular morphogenetic changes, occurs every 35 days in mice and 74 days in humans (**Figure 1.2**) [28, 29]. Furthermore, spermatogenesis (and oogenesis in females) is the only process that utilizes meiosis in multicellular eukaryotes and provides a means for genetic diversity in haploid gametes through meiotic recombination events [28, 30, 31]. Spermatogenesis consists of a balance of proliferation and differentiation, chromosomal separation, and morphogenetic changes and DNA repackaging to maintain proper male fertility.

1.1.2.1 Mitosis

Spermatogenesis begins during puberty in males, with hormones such as luteinizing hormone (LH), follicle stimulating hormone (FSH), and testosterone (see Chapter I, section 1.1.3) operating in somatic cell types within the testis to signal

undifferentiated (type A) spermatogonia, derived from gonocytes, for the next steps in spermatogenesis [12, 28, 32]. Undifferentiated spermatogonia are responsible for maintaining the germ cell linages since all sperm arise from these cells [28]. Since undifferentiated spermatogonia need to be always present, proliferative events occur to renew these cells [28, 33, 34]. Undifferentiated spermatogonia are maintained throughout the entire lifespan, which is how males can maintain fertility late into adulthood, unlike females who have a gradual decline of available gametes, significantly shortening their reproductive window of opportunity [34-36]. Undifferentiated spermatogonia reside within all seminiferous tubules at a relatively low concentration along the basement membrane, making these the rarest and most primitive postnatal germ cells [33, 34]. Undifferentiated spermatogonia need to survive and maintain their numbers, but also need to be able to progress into spermatogenesis through differentiation events [33, 34, 37].

This presents a delicate balance that needs to be maintained by undifferentiated spermatogonia to ensure both normal spermatogenesis and testis health. If proliferation occurs without the check of differentiation in these cells, then no sperm will be produced since meiosis and spermiogenesis will never occur. This can lead to the development of testicular cancer [38, 39]. On the other hand, a strict commitment to differentiation will inevitably exhaust the undifferentiated spermatogonia population leaving no stem cells to start fresh waves of spermatogenesis. This has been shown in mouse models knocking out genes essential to undifferentiated spermatogonia self-renewal resulting in male infertility [40-42]. If this balance is properly maintained, then an appropriately sized population of differentiated spermatogonia (type B) will arise from undifferentiated spermatogonia. The exact mechanism of the undifferentiated spermatogonia fate

decision is unclear since there are competing theories between symmetrical and asymmetrical division [34]. However, studies have been done to understand undifferentiated spermatogonia behavior using an undifferentiated spermatogonia marker, PLZF, and differentiated spermatogonia markers, C-KIT, DDX4, DAZL, which showed that asymmetric may be preferred *in vitro* [43]. Nevertheless, once proliferated spermatogonia are produced, there is a surge of retinoic acid (RA) produced by Sertoli cells that will prompt spermatogonia differentiation, which then leads these cells into the next step of spermatogenesis, meiosis [44-46].

1.1.2.2 Meiosis

Meiosis is exclusivity reserved in germ cells in both males and females and is the process in which diploid germ cells divide to form haploid gametes. Aside from the unique nature of equal chromosomal division into daughter cells, there are also meiotic recombination (crossing over) events that allow chromosomes to exchange genetic information while retaining complete sets of each gene [28, 30]. This process introduces genetic diversity into progeny since each haploid gamete should harbor unique patterning of alleles on each chromosome. However, this process in not perfect, as cases of aneuploidy and chromosome instability can lead to disorders in offspring and genes involved in meiotic spindle formation have been shown to contribute to this in mouse models [47, 48]. Furthermore, meiotic division encompasses several steps occurring in two rounds, including prophase, the pairing of chromosomes, metaphase, the midline arrangement of the chromosomes, anaphase, the separation of chromosomes, telophase, the movement of chromosomes to the poles, and finally, cytokinesis, the separation of the cytoplasm, producing haploid germ cells [28].

While the process of meiosis is conserved between males and females, there are strikingly different outcomes between the sexes. Females undergo two rounds of asymmetrical division, resulting in a single primary oocyte and three smaller polar bodies that serve no significant purpose in sexual reproduction since key nutrients and factors are invested into the one egg cell [35]. Males, however, undergo a symmetrical form of division, resulting in four equal haploid cells different in allele combinations only [28]. Meiosis in males occurs after the RA pulse drives differentiation of spermatogonia into differentiated spermatogonia, committing these cells to meiosis [44-46]. From this point, differentiated spermatogonia will go on to become preleptotene, leptotene, zygotene, pachytene, and diplotene spermatocytes prior to the final cytokinesis event. The final product of meiosis are round spermatids, which are the haploid precursors to sperm. This process has been well studied in mice, with several gene knockout models generated, including SYCP3 knockout mice, resulting in zygotene arrest due to improper chromosomal pairing, resulting in male infertility [49, 50]. Since meiosis has extensive chromosomal information exchanges, this process must be highly regulated and protected during spermatogenesis to property generate sperm that can fertilize an egg and provide genetic diversity to the next generation.

1.1.2.3 Spermiogenesis

After round spermatids are produced, these haploid cells contain the necessary genetic information to combine with an oocyte to produce a zygote, but these cells are not properly equipped to travel to the oocyte. As a result, the last phase of spermatogenesis, spermiogenesis, will complete this process by turning this round haploid cell into a sperm cell. Spermiogenesis features several structural changes

including cytoplasm shedding, nuclear condensation, and the growth of a flagellum [51, 52]. A key feature of this process occurs within the nucleus, in which histone proteins are exchanged for arginine-rich proteins called protamines (P1 and P2) [53-56]. These protamines help compact chromatin to a far denser and more protected state than histones, allowing for sperm head compaction [57]. While sperm appear functional at the end of this transformation within the testis seminiferous tubule lumen, they are not fully functional since proper motility has not been developed [18]. Once spermiogenesis is completed, sperm will passively move into the epididymis for the final maturation steps to become functional sperm, thus completing the transition from SSCs to an efficiently designed sperm cell ready for paternal DNA delivery to the oocyte.

1.1.3 Regulation of male reproduction

Sperm is produced in the testis of males and oocytes are produced in the ovaries of females, designating both gonads the center of sexual reproduction. However, reproductive development and the generation of gametes is controlled outside these organs through the Hypothalamic-Pituitary-Gonad (HPG) axis [58, 59]. The HPG axis regulates gamete production through the release of hormones that will lead to the production of sex-specific hormones, such as testosterone in males and estrogen in females [12, 58-60]. The three organs involved in this pathway, as its name suggests, have two within the brain (hypothalamus and pituitary gland) and the other being the testes (or ovaries) (gonad). The HPG axis works as a closed system of hormone production, secretion, and negative feedback to coordinate sperm and testosterone production in males. This cascade begins in the hypothalamus with neurosecretory cells producing gonadotropin-releasing hormone (GnRH) [58, 59, 61]. This in turn signals

endocrine cells in the anterior pituitary gland to produce and release luteinizing hormone (LH) and follicle-stimulating hormone (FSH) into the bloodstream, which will travel through the blood vessels of the testis [58, 59, 62]. However, the blood-testis-barrier prevents these hormones from directly interacting with post-mitotic male germ cells [16, 63]. Additionally, these hormones are not conducive to stimulate or restrict spermatogenesis in their present states. Therefore, for the brain to influence spermatogenesis, LH and FSH must work though somatic cell types in the testis to bring the final stage of the HPG axis to male germ cells.

Within the testis, germ cells are the primary cell type and are responsible for the transfer of genetic information to the next generation via sperm. However, other somatic cells exist in the testis, including peritubular myoid cells, Leydig cells, and Sertoli cells [11, 64, 65]. Of these cells, Leydig cells and Sertoli cells are the destinations for hormones produced by the pituitary gland. Leydig cells contain membrane LH receptors, allowing for LH transport into the cell and triggering the production of the male androgen, testosterone [64]. Testosterone is required for male fertility and low testosterone production has been heavily linked to spermatogenic failure [66, 67]. This male sex hormone cannot directly influence spermatogenesis from Leydig cells since it cannot pass the blood-testis-barrier, so it is transported into Sertoli cells along the basement membrane of the seminiferous tubules [16, 63-65, 68]. From here, testosterone can move into the seminiferous tubule lumen via Sertoli cell cytoplasm and promote spermatogenesis in post-mitotic germ cells. Additionally, testosterone can be transported back into the bloodstream and act as a negative regulator of LH release in the pituitary gland, resulting in the need for testosterone balance to maintain healthy sperm production

[69]. On the other hand, Sertoli cells are responsible for FSH and will use it for a myriad of functions including promoting Sertoli cell proliferation and health [65, 68]. Sertoli cells act as “nurse” cells that promote spermatogenesis through direct contact with germ cells and form the blood-testis-barrier, protecting valuable germ cell DNA from damage [16, 63, 65, 68]. One of the Sertoli cell functions is to generate another hormone, inhibin, which can act as a negative regulator of FSH release in the pituitary gland, thus completing the HPG axis loop [69, 70]. Therefore, the coordination of the HPG axis through the brain and somatic cells in the testis can influence sperm production and disrupting any of these portions of the HPG axis could be detrimental to male fertility. The relationships, secretions, and locations of the involved organs and cell types of the HPG axis on male reproduction are depicted in **Figure 1.3**.

1.2 Male infertility

In the general reproductive-aged population, approximately 10% of couples occur difficulties when trying to conceive [36, 71-73]. Of these couples, there is an even split of the primary source of dysfunction between male and females in cases with a recognized diagnosis [36, 71-73]. However, a large proportion of patients with difficulties conceiving and/or establishing pregnancy to the point of detectible levels of human chorionic gonadotrophin (hCG) in the female’s bloodstream (5 to 25 mIU/mL) are left without a clear diagnosis of the root source of the problem, leaving couples confused and dissatisfied [72, 74]. When heterosexual couples are unable to establish a viable pregnancy after a span of at least 12 months of frequent vaginal intercourse without the use of contraceptives, they may be clinically diagnosed as “infertile” [72, 75]. In response, a series of clinical tests can be administered for each sex to reach this diagnosis.

Briefly, these female-specific fertility tests may include a broader blood panel examining anti-Mullerian hormone (AMH), to estimate oocyte population count, progesterone, to evaluate ovulation, thyroid stimulating hormone (TSH), to examine thyroid function, and estradiol prolactin levels [76, 77]. Furthermore, other diagnostic approaches such as an abdominal ultrasound or transvaginal ultrasound can be used to examine cervix, ovary, and uterus morphology to determine if there are any structural defects presenting a barrier to sperm transport, oocyte release, or embryo implantation [78]. While these approaches are comprehensive to an extent, they do not always conclude with definitive results pointing to a cause for infertility but can rather be used as an eliminatory approach to improve understanding of the potential problem, which may reside in underlying genetic causes, the production of antibodies against sperm within the vaginal canal, or with the male component of the reproductive equation.

On the other side of the equation, the clinical evaluation of male reproduction at the sperm level is relatively more straightforward, with semen analysis being the current gold standard evaluation tool used by physicians. Semen analysis may be broken down into the examination of sperm count, the amount of sperm, sperm morphology, the shape of sperm, and sperm motility, the ability of sperm to move freely towards an oocyte for fertilization [73, 79]. This of achieved by collecting semen from a male individual and examining it in addition to measuring pH levels, the percentage of live sperm, and the amount of DNA fragmentation from sperm (damaged DNA) in the sample [73, 79, 80]. While each of these factors may point to the cause of infertility, it may not necessarily provide a clear answer to solve the mystery. Even so, patients may present with essentially normal semen samples, yet the persistence of the inability to impregnant their

partner ensues, prompting further investigation to understand how spermatogenesis and male fertility may be dysfunctional at the genetic level in these patients.

1.2.1 Classifications of male infertility and subfertility

Male infertility and subfertility, the absent or reduced ability to naturally conceive, comes in many different forms depending on the underlying cause of dysfunction. The final product of spermatogenesis, sperm, must be present and motile to fertilize an oocyte during sexual intercourse in order to be considered fertile. However, there are far more components to this equation that can cause fertility problems, ranging from the SSC pools to hormone production. The combination of multiple factors and cell types make it difficult to pinpoint the root cause for sperm production problems at the clinical level, leaving room for further basic science research to alleviate this burden though the identification of genes causing fertility problems using model systems in basic science research.

An overwhelming majority of male infertility cases stem from problems with sperm production [73]. This can be further divided into degrees of infertility, depending on the state or presence of sperm within the male reproductive tract. The most extreme form of male infertility is azoospermia, which describes virtually no motile sperm in semen produced by the male [73, 81]. This likely stems from a critical issue in spermatogenesis which prevents SSCs from becoming sperm. A milder form is oligospermia, where some sperm is produced, but at a concentration too low for successful conception to occur [73, 82]. An additional type of male infertility is asthenozoospermia, or reduced sperm motility. If the sperm cannot move through the female reproductive tract and reach the oocyte, then conception cannot occur. Finally, the last class of sperm production problems in males is teratozoospermia, which is the appearance of misshapen sperm in semen [73,

82]. These defects can range from kinked sperm, multiple heads or tails, or truncated flagella [18, 73, 82, 83]. While each of these cases can be observed through conventional semen analysis, they do not identify the cause of the problem, which could be working on a genetic or environmental level.

1.2.2 Causes of male infertility and subfertility

The first barrier in determining fertility is the examination of semen. However, there are cases where no sperm of present in semen, suggesting azoospermia. One reason for this observation may be due to a physical barrier preventing sperm transport. This can occur from blockages in the vas deferens, spontaneous growth of cysts, or the presence of varicoceles, causing obstructive azoospermia (AO) [73, 84]. In fact, procedures such as vasectomies take advantage of blocking sperm transport through the cutting of the vas deferens to prevent pregnancies yet leave sperm production unaltered like what is observed in clinical AO [85]. If no blockages are detected or if sperm are not present in the epididymis or testis via biopsy in humans, then AO can be ruled out at the clinical level and the search continues.

Other causes of infertility in males can stem from external sources. While the blood-testis-barrier serves to protect germ cells from unwelcome pathogens, a pathogen triggering an inflammatory response in the testis can disrupt this barrier created by Sertoli cell tight junctions, leaving germ cells susceptible [16, 63]. This is observed in sexually transmitted diseases such as chlamydia, gonorrhea, as well as Human Immunodeficiency Virus (HIV), which can negatively impact sperm production [85]. Furthermore, the accumulation of injuries to the testes, epididymis, or penis can impact spermatogenesis, sperm storage, transportation, and penetrative ability during sexual intercourse [73, 86].

Therefore, sexual problems like Erectile Dysfunction (ED) also pose a barrier to impregnation if the penis cannot be properly inserted into the vagina during ejaculation. Fortunately, medications have become available to combat ED, but other structural abnormalities associated with congenital defects may prevent the opportunity to conceive naturally as well. Lifestyle choices, such as using tobacco products or marijuana, drinking excessive alcohol, decreased physical fitness, and nutritionally poor diet have also all been linked to reproductive dysfunction in males [87, 88].

For a male to be deemed fertile, they need to be able to produce healthy sperm that can fertilize an oocyte [28, 36, 73]. The largest factor behind infertility lies within the genes responsible for male reproductive function. Several human diseases that have accompanying male infertility or subfertility have mutations or deletions identified in genes related to spermatogenesis. These can range from large scale deletions of the male sex chromosome (Y) and sex chromosome aneuploidies (Klinefelter's syndrome) to single amino acid alterations in proteins required for spermatogenesis in human males [89-93]. Since sperm production is controlled by numerous genes operating at each level of spermatogenesis, sperm maturation, and semen production, there are a multitude of factors that could be contributing to male fertility at the genetic level. Additionally, since spermatogenesis is regulated by hormone production via the HPG axis, genes involved in the production or transport of these hormones, such as testosterone, can cause male infertility [94]. With the genetic complexity of male reproduction, there are several areas in which genes can impact sperm production leading to infertility, resulting in the need for additional studies and models to understand the roles of genes at the male reproductive tract and male germ cell levels.

1.2.3 Current therapies and treatments for male infertility and subfertility

While sexual reproduction may not be essential for individual longevity and overall health, it is essential for future generation to ensue. Furthermore, the desire to pass down genetic information to progeny is programmed into almost all organisms and for humans, the longing for the creation of a family of their own may have deep rooted emotional ties [74, 95-97]. This places a high value on reproductive success that is further enforced through societal or relational expectations. As a result, medical advancements have progressed to where some cases of infertility can be rectified, and the afflicted individual can produce natural children. Although each case of male infertility or subfertility is clinically different to an extent, some treatments and therapeutics have been shown to be effective. Blockages in the male reproductive tract preventing sperm from entering the female reproductive tract may be corrected by surgery [84, 98]. Additionally, there are medications that can treat infections like chlamydia and gonorrhea, in addition to ED [99, 100]. Disrupted hormone production or imbalances may be addressed through supplementation in some cases [101, 102]. Lastly, physician-recommended lifestyle changes have been shown to improve male reproductive success though intervention in areas of substance use, diet, exercise, and sleep [103]. While some forms of male infertility and subfertility have been addressed with medical intervention, there remains a large proportion of genetic-based causes of male reproductive dysfunction that are not fully understood. This leaves these patients with minimal and/or expensive treatment options, should they exist. In summary, these approaches to treat male infertility have been made possible through decades of basic science research on male infertility, but as new genetic cases arise at the clinical level, further investigations into underlying genetic

causes of symptoms are essential for future male reproductive success in humans.

1.3 RNF216 is involved in male reproductive dysfunction and human disease

1.3.1 RNF216 is genetically linked to human Gordon Holmes Syndrome

Diseases can take many forms in humans with a wide range of symptoms, yet it may be hard to determine the exact causative factor(s). Manifestation of certain symptoms was the primary way to identify certain diseases before the era of modern medicine, as was the case in the early twentieth century. During this time, Dr. Gordon Holmes, an Irish neurologist, examined patients who suffered from a combination of neurological and reproductive dysfunction, classifying their condition as Gordon Holmes Syndrome (GHS) in 1908 [104]. However, since this was prior to the discovery of DNA as the genetic material, the underlying genetics of GHS were unknown. Fast-forwarding to 2013, a clinical report was published detailing multiple patients with GHS-like symptoms as well as their whole-exome sequencing, identifying two genes, OTU domain-containing protein 4 (*Otud4*), coding for a deubiquitinase, and Ring finger protein 216 (*Rnf216*), coding for an E3 ubiquitin ligase, each harboring mutations in afflicted patients [105]. Both genes produce proteins involved in ubiquitination with opposing functions. However, each patient that presented with a GHS phenotype of neurological dysfunction and hypogonadotropic hypogonadism had mutations in *Rnf216*, while only a few had mutations in *Otud4* [105]. This suggested that *Rnf216* was the primary underlying genetic driver for human GHS etiology.

In addition to this clinical report, several more GHS patients have been identified and sequenced for *Rnf216* mutations resulting in more mutations being identified. Interestingly, while both males and females with *Rnf216* mutations displayed neurological

symptoms, males seemed to display more severe reproductive symptoms, including missed puberty, azoospermia, and hypogonadism [105]. A complete list of reported GHS male patients with accompanying reproductive phenotypes may be found in a portion of **Table 1.4** [105-112]. While a majority of these patients had either homozygous or compound homozygous *Rnf216* mutations, one reported male patient had a heterozygous *Rnf216* mutation (*Rnf216 p.G138GfsX74/+*), but still had hypogonadotropic hypogonadism later in adulthood [105]. This male also had normal puberty, unlike other male GHS patients, suggesting that these different paced symptoms were the result of another unknown gene mutation, making GHS potentially oligogenic [105]. Even if this were the case, the evidence put forth across all these studies made a very strong case for RNF216 to be linked to human GHS, but how RNF216 mutations led to neurological and reproductive symptoms is still unknown.

1.3.2 RNF216 is a ring-between-ring E3 ubiquitin ligase with chain linkage specificity

An overwhelming majority of mutations recorded in GHS patients were within or near the predicted E3 ligase domain of RNF216 at its C-terminus [105-112]. Aside from the E3 ligase domain, RNF216 also contains other predicted domains near its N-terminus, including SUMO-interacting motifs, a TRAF-interacting motif, and ubiquitin binding motifs [113-115]. While these regions have been identified in RNF216 structure, there is limited knowledge of the roles these domains play in overall RNF216 function in humans. Upon further inspection of RNF216 structure, it was determined that RNF216 was classified as a Ring-between-Ring (RBR) E3 ubiquitin ligase based of the architecture of its E3 ligase domain (**Figure 1.5**) [113-116]. All E3 ubiquitin ligases occupy a portion of the ubiquitin system of reactions, in which coordinating proteins transfer ubiquitin, a protein modifying

molecule, onto a specific substrate protein. In this pathway, E1 protein, ubiquitin-activating enzyme, E2 protein, ubiquitin-conjugating enzyme, and finally E3 protein, ubiquitin ligase, work together to facilitate ubiquitin transfer onto substrate protein [117]. While there are eight E1 and approximately 40 E2 identified proteins in humans, there are thousands of E3 ligases [118, 119]. Since the E3 ligase recognizes specific substrates of ubiquitination based on its substrate recognition domain(s) located near the E3 ligase domain, it is understandable why there are so many E3 proteins in humans to account for thousands of substrates. Additionally, some E3 ligases are known to have multiple substrates [119-121].

The molecular mechanism of ubiquitin transfer differs based on the structural composition of the E3 ligase domain. There are three classes of E3 ligases, RING (Really Interesting New Gene), HECT (Homologous to the E6-AP Carboxyl Terminus), and RBR (RING-between-RING), with RBR being the smallest known class [118, 119]. The stepwise mechanism for each of these classes is depicted in **Figure 1.6**. Each E3 ligase requires the attachment of the E2-ubiquitin complex onto a domain (RING or HECT) within the ubiquitin ligase domain. Substrates are then recruited in proximity for ubiquitin attachment. It is in the mechanism responsible for the transfer of ubiquitin onto the substrate protein that is the main difference between E3 ligase classes. RING E3 ligases have direct ubiquitination from the E2 onto substrates [118, 119]. HECT E3 ligases use two lobes of the HECT domain to temporarily transfer ubiquitin from the E2 enzyme onto a catalytically active cysteine reside within one HECT lobe forming an intermediate thioester bond [118, 119, 122]. The ubiquitin is then attached to the substrate protein. Finally, RBR E3 ligases, like RNF216, are a hybrid of the two previous classes of E3

ligases [123]. RBR E3 ligases have two RING domains (RING1 and RING2) separated by an In-Between-Ring (IBR) domain. E2-ubquitin is recruited to RING1, and then ubiquitin is transferred to a catalytically active cysteine reside within the RING2 domain and connected via thioester bond [116, 118, 119]. This ubiquitin is then transferred to the target substrate, tagging it for a specific fate. Because of their unique mechanism, RBRs, like Parkin, which is genetically connected to another neurodegenerative disorder, Parkinson's Disease (PD), have been well studied [124-126]. Furthermore, disordered ubiquitination caused by mutations in E3 ligases have been shown to impact male reproduction and spermatogenesis, such as mutation of other Ring-finger proteins, RNF8 and RNF168 [127, 128]. Based on these models, there may be the potential of RNF216 playing an important role specifically as an E3 ligase in male reproductive development and function in human GHS male patients, but how it operates within cellular pathways is unknown.

RNF216 has been studied primarily in a neurological context and there have not been many studies examining how it may influence male reproduction. In cell lines as well as mouse models, brain specific RNF216 was suggested to directly ubiquitinate activity-regulated cytoskeleton-associated protein, Arc, to regulate synaptic strength through Arc degradation [129-131]. Additionally, other studies proposed RNF216 also ubiquitinates Beclin 1 (BECN1) to regulate autophagy via proteasomal degradation of BECN1 [132-134]. Another study found RNF216-directed ubiquitination impacted the stability of Staufen2 (STAU2) in the hypothalamus [135]. All these studies placed RNF216 into cellular pathways to help explain the neurological symptoms observed in human GHS but were reliant on RNF216 preferring lysine-48 (K48) ubiquitin chain linkages that would

target substrates for degradation through the ubiquitin-proteosome system (UPS) [116, 136, 137]. This detail is important since the ubiquitin chain-linkage type of a substrate protein dictates the fate of the tagged protein. For example, there are eight different ubiquitin chain linkage types based on exposed lysine residues (K6, K11, K27, K29, K33, K48, K63) as well as the first methionine (M1) of ubiquitin [138]. Recent structural and *in vitro* studies examining RNF216 have shown that RNF216 preferentially ubiquitinates artificial substrates with K63 ubiquitin chain-linkages, and to a lesser extent, K11 ubiquitin chain-linkages [113, 115]. K63 ubiquitination is not associated with target protein degradation, but rather influences protein interactions, DNA damage repair pathways, and transcriptional regulatory events [138, 139]. K11 ubiquitination on the other hand has been shown to operate in both proteasomal degradation as well as cell cycle regulation [138, 140]. While it is unclear as to what chain linkage type RNF216 may use on these proposed substrates in the brain, the preferred chain linkage type of RNF216 may be able to place it more accurately into cellular pathways based on *in vivo* substrate fate in both the brain for neurological GHS symptoms and well as in the testis to understand its molecular role in male reproduction.

1.3.3 RNF216 is required for male fertility and meiosis during spermatogenesis in mice

Since research surrounding RNF216 in human GHS had been focused on the neurological side of the disease, there was not much known on how RNF216 may influence reproduction. Melnick, et al. was the first to investigate the role of RNF216 on mammalian reproduction using a transgenic mouse model [141]. The generation of a global knockout RNF216 (*Rnf216 KO*) mice allowed for the examination of reproductive function *in vivo* without RNF216 expression. Like observations in human GHS patients,

there were severe reproductive defects in the testes, resulting in azoospermia [141]. However, there were no apparent reproductive problems in female *Rnf216 KO* mice, which is similar, yet not identical to the lesser reproductive phenotypes in human GHS females [105, 141]. This *Rnf216 KO* mouse model proved the essentiality of RNF216 for male reproductive development and function, since RNF216 loss led to spermatogenic failure, increased male germ cell apoptosis, and infertility. Shortly after this report, another group also generated a *Rnf216 KO* mouse with similar findings of male reproductive dysfunction [142]. Further analysis of *Rnf216 KO* testis histology suggested RNF216 global deletion caused meiotic defects at the zygotene spermatocyte stage, leading to incomplete spermatogenic arrest. Furthermore, proteomic analysis identified PKA catalytic subunit β (PRKACB) as a potential target for RNF216-mediated ubiquitination in mouse testis. A complete list of published RNF216 mouse models and their male reproductive phenotypes may be found in a portion of **Table 1.4** [141-143]. In summary, the data presented by both research groups confirmed that RNF216 ablation in mice leads to male reproductive defects, which prompts new questions surrounding RNF216 in spermatogenesis and human GHS.

Therefore, the purpose of this dissertation is to expand upon the work done by Melnick, et al. and Li, et al. to investigate RNF216 in male reproductive function at various levels [141, 142]. Utilizing what is known about male germ cell biology, male infertility, and GHS reproductive dysfunction in male patients, this research will address key questions of RNF216 in male reproductive biology. First, the cell specific expression of RNF216 in the testis is poorly defined and identifying what cell types RNF216 may have expression in could help answer questions as to how it impacts spermatogenesis. The

localization within these cells is of an utmost importance as well since this can refine potential pathways and substrates of RNF216 in male reproduction. Second, RNF216 has strong expression in both the brain and gonad, but it is unclear in which organs RNF216 is required for proper spermatogenesis [141]. Determining the requirement of RNF216 in the testis would provide new information that would be an improvement upon the global RNF216 knockout mouse models. Additionally, it is unclear if RNF216 plays a role on mitotic germ cell functions, which will also be investigated more thoroughly. Third, the development of male reproductive dysfunction in GHS patients has been genetically linked to RNF216 E3 ligase mutations, but no model exists to examine the need for the E3 ligase mechanism of RNF216 on male reproduction *in vivo*. Therefore, this dissertation seeks to address these questions regarding RNF216 to improve understanding of RNF216 function within the testis, male germ cell biology, male fertility, and GHS male reproductive etiology that can be used for future studies to improve reproductive outcomes.

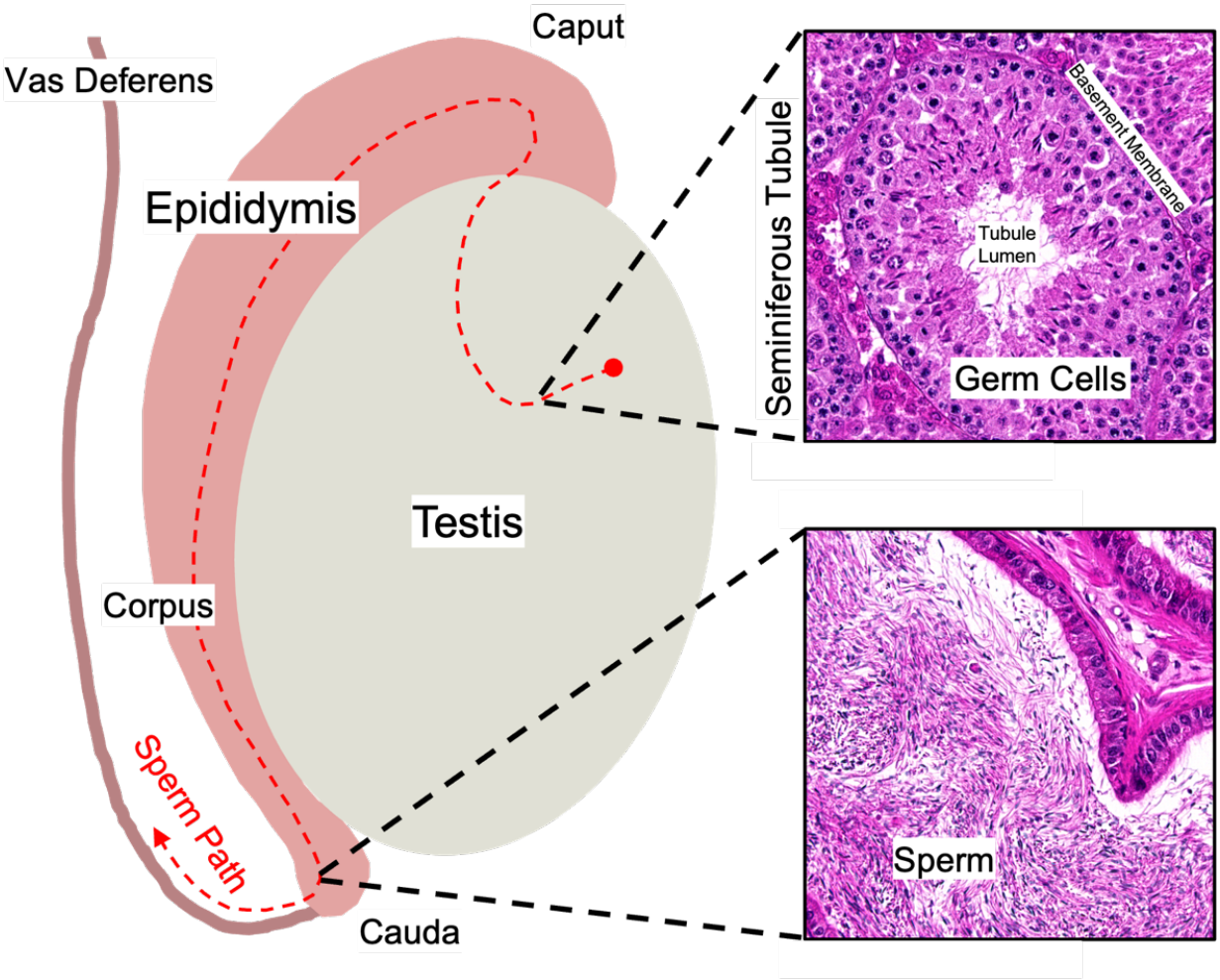

Figure 1.1. Testis and epididymis morphology.
Illustration of mammalian testis and epididymis with hematoxylin and eosin-stained (H&E) adult mouse testis and cauda epididymis (from Mann et al., data unpublished). Dotted red arrow represents the approximate path of sperm from the testis and epididymis to the vas deferens.

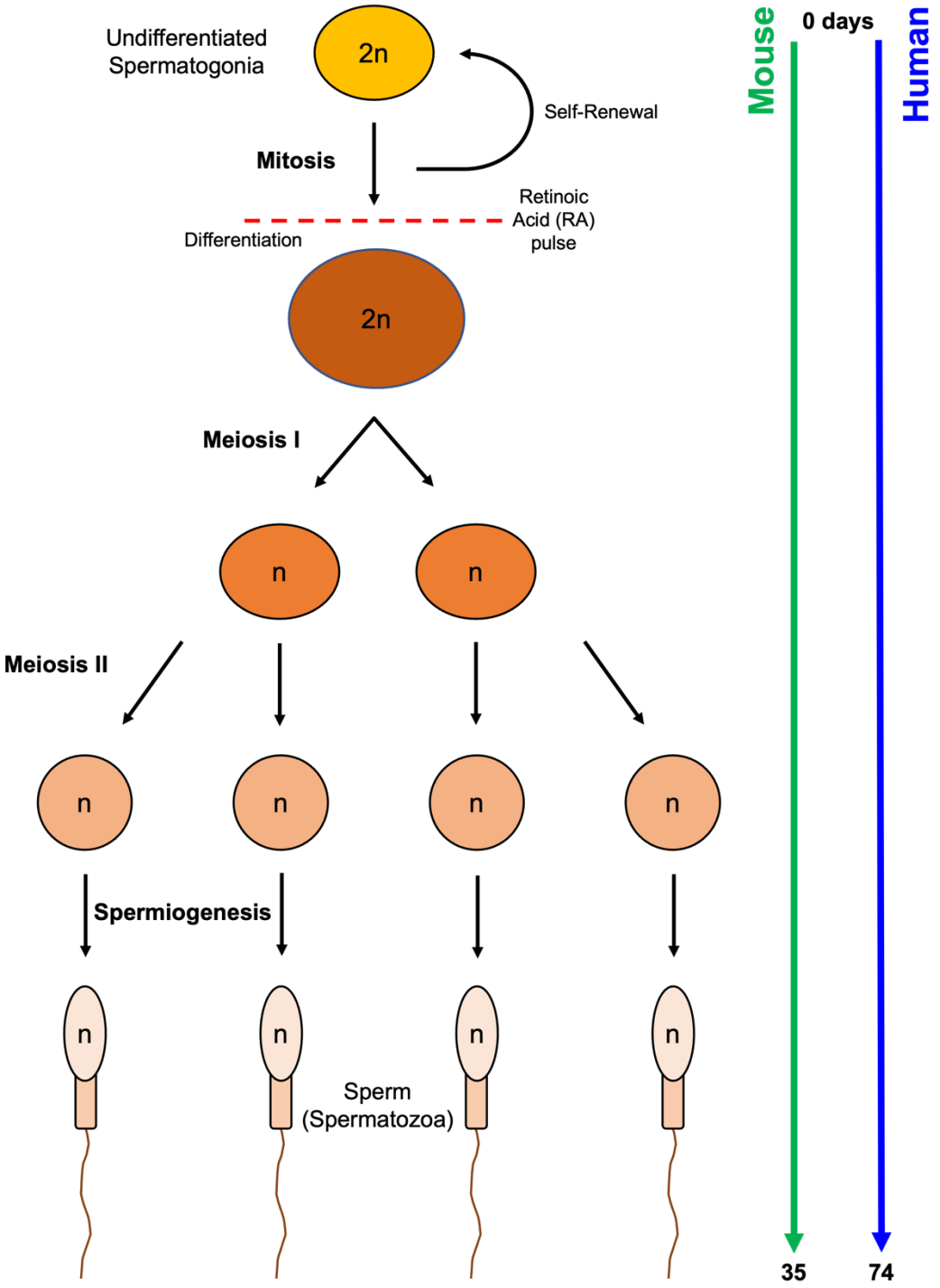

Figure 1.2. Timeline of murine and human spermatogenesis.
Schematic of one complete wave of mammalian spermatogenesis and its three distinct steps of mitosis, meiosis (I and II), and spermiogenesis starting with undifferentiated spermatogonia and ending with sperm. n, chromosome copy number. Timeline indicating duration required to complete one wave of spermatogenesis for mouse (green arrow) and human (blue arrow).

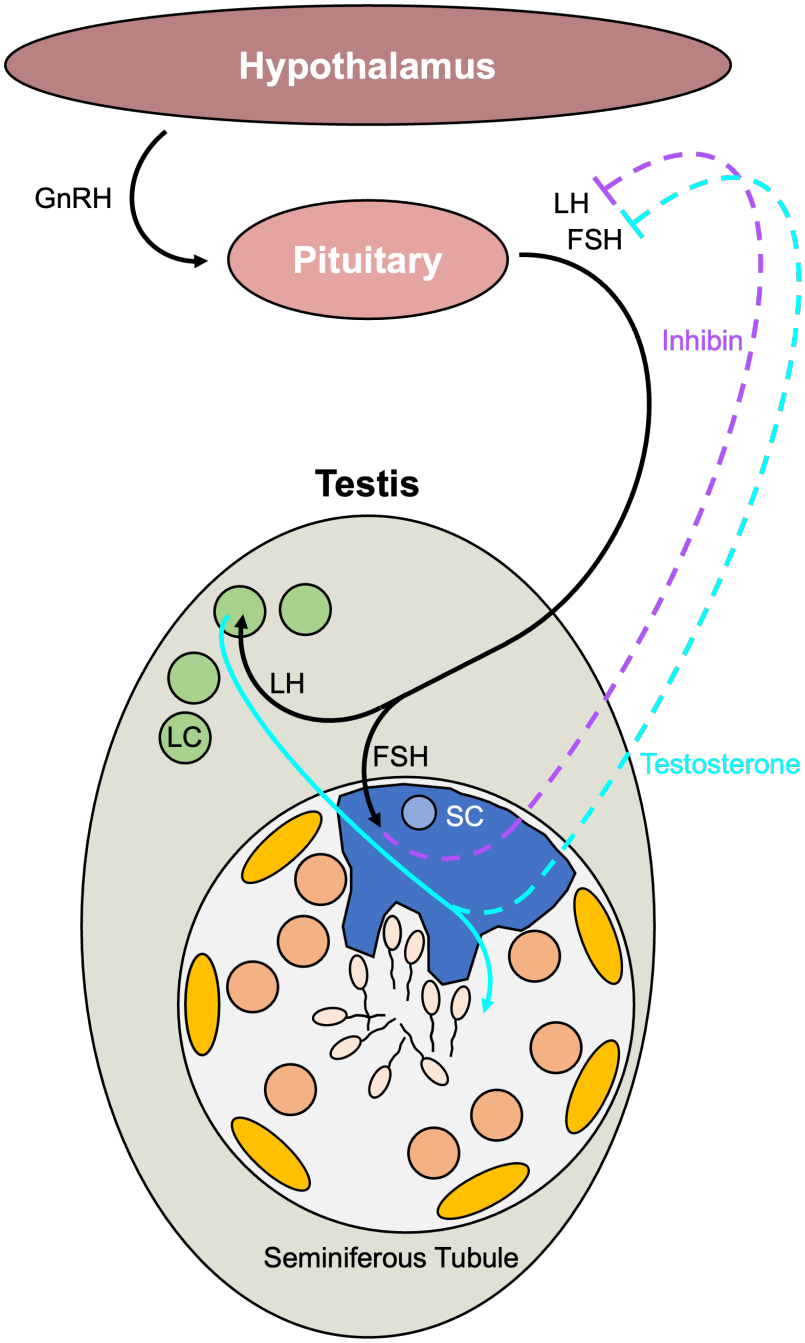

Figure 1.3. Hormonal control of mammalian spermatogenesis via the Hypothalamic-Pituitary-Gonadal (HGP) axis.
Schematic of the HGP axis showing hormones secreted from the brain (hypothalamus and pituitary gland) influencing mammalian spermatogenesis through the production of testosterone in the testis. Negative feedback from testosterone (cyan dotted line) and inhibin (purple dotted line) in testis balances the axis limiting pituitary hormone release. GnRH, gonadotropin-releasing hormone; LH, luteinizing hormone; FSH, follicle-stimulating hormone; LC, Leydig cell (green); SC, Sertoli cell (dark blue body with light blue nucleus). Image not to scale.

Table 1.4. Reported GHS mutations and mammalian models of RNF216 with associated male reproductive phenotypes.

Species	Mutation/Model	Male Reproductive Phenotype	Reference
Human	p.R751C/p.R751C	Hypogonadotropic hypogonadism	Margolin, et al., 2013 [105]
Human	p.E205DfsX15/p.C597X	No spontaneous puberty	Margolin, et al., 2013 [105]
Human	p.R717C/+	No spontaneous puberty	Margolin, et al., 2013 [105]
Human	p.G138GfsX74/+	Normal puberty, late developing hypogonadotropic hypogonadism	Margolin, et al., 2013 [105]
Human	p.G456E/p.G456E	Low gonadotropin serum levels	Santens, et al., 2015 [106]
Human	p.Q302*/p.Y539C	Low gonadotropin serum levels	Santens, et al., 2015 [106]
Human	p.A818G/p.A818G	Hypogonadism	Ganos, et al., 2015 [107]
Human	p.G687A/p.G687A	Hypogonadism	Alqwaifly, et al., 2016 [108]
Human	p.P606L/p.P606L	Hypogonadotropic hypogonadism	Calandra, et al., 2019 [109]
Mouse	*Rnf216 -/-*	Infertile	Melnick, et al., 2019 [141]
Mouse	*Rnf216 -/-*	Infertile, meiotic defect	Li, et al., 2021 [142]
Mouse	*NestinCre:Rnf216 cKO* (brain-conditional knockout)	None	Mabb, et al., 2022 [143]
Human	p.E650X/p.E650X	Azoospermia, low gonadotropin serum levels	Chen, et al., 2022 [110]
Human	p.C621SfsTer56/p.C621SfsTer56	Hypogonadotropic hypogonadism	Celik, et al., 2023 [111]
Human	p.L569Idel/p.L569Idel	Hypogonadotropic hypogonadism	Rochtus, et al., 2024 [112]

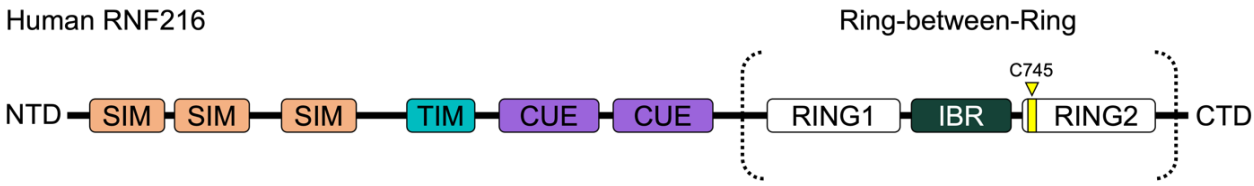

Figure 1.5. Predicted protein structure of human RNF216.
Protein architecture for human RNF216. Catalytically active cysteine residue (C745) (yellow) within in the RING2 domain of the greater Ring-between-Ring E3 ligase ubiquitination domain (dotted parenthesis) near the C-terminal domain (CTD). SIM, SUMO-interacting motif (orange); TIM, TRAF-interacting motif (turquoise); CUE, coupling of ubiquitin conjugation to ER degradation domain (purple); RING, really interesting new gene (white); IBR, in-between-RING (purple). Image not to scale.

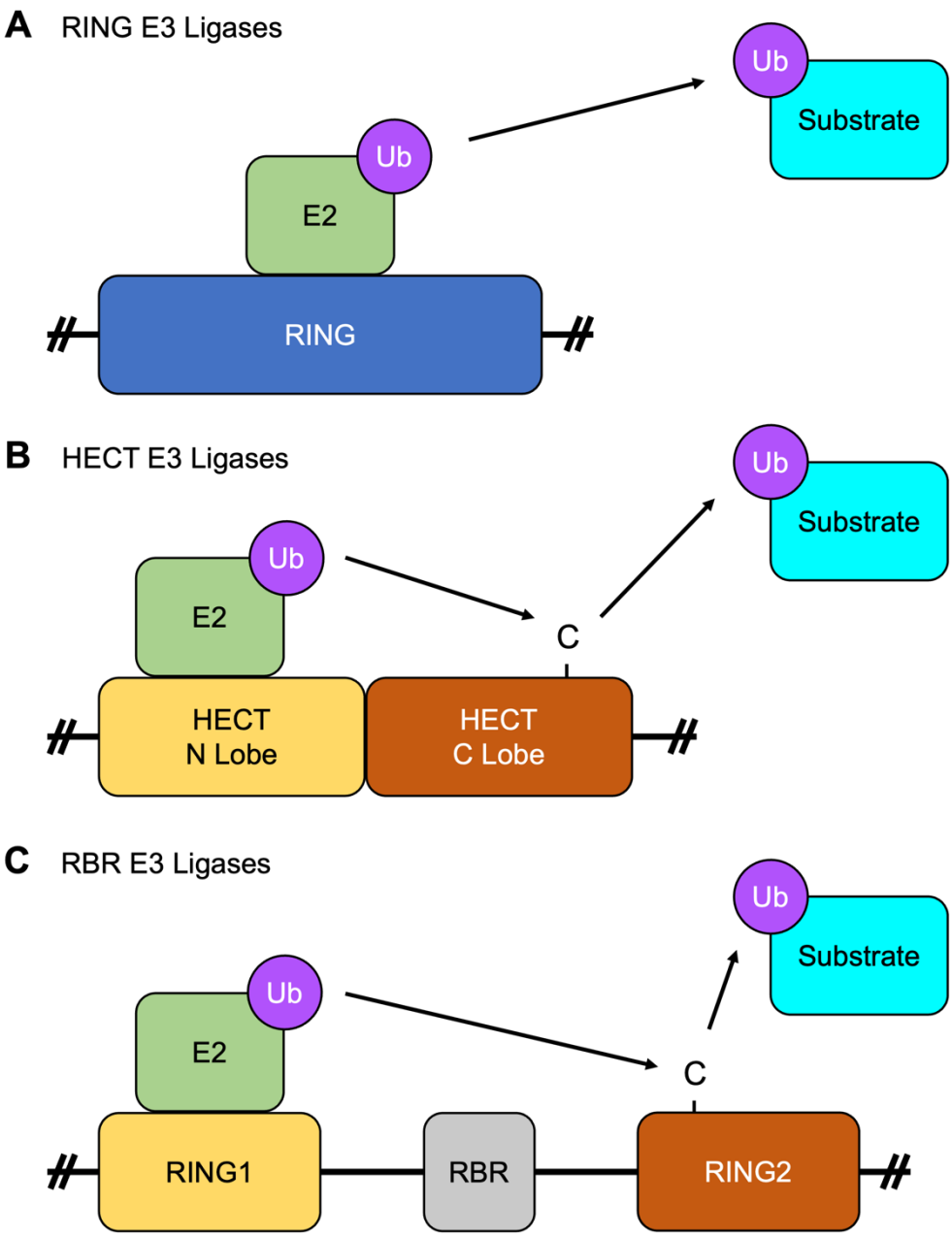

Figure 1.6. Structure and molecular mechanism of E3 ubiquitin ligase classes. **A.** Molecular mechanism for RING E3 ligase ubiquitination of substrate. RING, Really Interesting New Gene. **B.** Molecular mechanism for HECT E3 ligase ubiquitination of substrate using an intermediary thioester bond with catalytic cysteine residue (C) in C lobe of HECT domain. HECT, Homologous to the E6-AP Carboxyl Terminus. **C.** Molecular mechanism for Ring-between-Ring (RBR) E3 ligase ubiquitination of substrate using an intermediary thioester bond with catalytic cysteine residue (C) in RING2 domain. RBR, RING-between-RING. **A. – C.** Ub, ubiquitin; //, truncated protein outside region of interest. Arrows indicate ubiquitin transfer in molecular mechanism.

CHAPTER II.

RNF216 HAS UNIQUE SUB-NUCLEAR EXPRESSION IN MALE GERM CELL POPULATIONS INDICATIVE OF POTENTIAL PATHWAYS AND FUNCTION IN MOUSE SPERMATOGENESIS

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Chapter II will be combined with Chapter III and an adapted version will be submitted as a single manuscript for publication.

This study was supported by the National Institutes of Health R01GM132490 and R01HD084494, and the National Institute of Food and Agriculture MICL02690.

2.1 Introduction

Male germ cells are the carriers of genetic material that will be passed down to future generations through sexual reproduction. These germ cells progress from spermatogonia stem cells (SSCs) to sperm that will ultimately go on to fertilize an ovulated oocyte. In mammals such as mice and humans, this process is ongoing once sexual maturity is reached in males (~35 days in mice, ~9-14 years in humans) [11, 28, 144]. Male germ cell populations undergo mitosis, meiosis, and morphogenetic changes during spermiogenesis, that will produce haploid sperm. This production of sperm needs to happen around the clock to meet the high demand of male reproduction, since the chance for fertilization could happen at any time. Indeed, adult males can produce up to 100 million sperm per day to meet these demands, which can pose strain on male germ cell populations if unchecked [28, 92, 145].

The mechanism in place to maintain ongoing spermatogenesis and populations of mature sperm ready for fertilization is made possible due to spermatogenesis occurring at different stages within the testis. Within the seminiferous tubules, spermatogenesis is occurring at a different stage depending on what germ cell populations are present. For example, a cross-section of a seminiferous tubule may show sperm within the tubule lumen, yet a seminiferous tubule cross-section next to it may only have elongating spermatids closest to the lumen. This phenomenon has been well characterized and can be broken down into twelve seminiferous tubule stages (I-XII) [146, 147]. As a result, fresh sperm are produced at any given moment, keeping spermatogenesis occurring on a non-stop basis in adulthood. The coordination of male germ cells undergoing mitosis, meiosis, and spermiogenesis during spermatogenesis ensures that the process is not disturbed,

which prevents gaps in the fertile window for males. As these events occur, each germ cell population needs to maintain cellular functions and molecular mechanisms for proper spermatogenesis, with dysfunction in any germ cell population extremely consequential for male fertility.

Recently, deletion of an E3 ubiquitin ligase, RNF216, has shown RNF216 to be required for male fertility in mouse models with confirmation by multiple research groups [141, 142]. Additionally, RNF216 has been connected to neurodegeneration and male reproductive dysfunction in humans, further suggesting that RNF216 plays an important role in reproductive physiology in human disease [105-112]. However, the expression of RNF216 at the organismal level showed RNF216 has high expression within the brain and gonads, accurately accounting for the phenotypes observed in both humans and mice [105-112, 141-143]. However, the cell specific expression of RNF216 and its function leading to male reproductive dysfunction are not known. Understanding the expression of RNF216 at the cellular and sub-cellular levels would provide better knowledge of the pathways of action that RNF216 may be involved in males to maintain proper reproductive physiology.

To address this gap in RNF216 cellular expression within highly expressed tissue types, a transgenic mouse model to accurately target and identify intrinsic RNF216 was created. These mice were genetically altered to add a recognizable peptide sequence to RNF216 to examine its cell specific expression within the male reproductive tract to improve knowledge on where RNF216 is potentially required for spermatogenesis and fertility. Furthermore, this mouse model will provide a means to map RNF216 expression with high specificity within its expressed cell types using a combination of

immunofluorescence tagging and high-power microscopy, further refining its potential areas of function and cellular pathways of involvement. Using these techniques, RNF216 was shown to be expressed only within male germ cells in the testis. Surprisingly, this expression was not in all germ cell types throughout spermatogenesis, but turned on and off during spermatogenetic progression, suggesting unique windows of function. Notably, RNF216 was primarily confined to the nuclei of male germ cells and compartmentalized within the nucleus, suggesting that RNF216 may play a critical role as an E3 ubiquitin ligase in this setting for spermatogenesis and male fertility.

2.2 Materials and Methods

2.2.1 Ethics Statement

All animal procedures were approved by the Institutional Animal Care and Use Committee of Michigan State University (AUF 202200230). All experiments with mice were conducted ethically following institutional guidelines according to the Guide for the Care and Use of Laboratory Animals.

2.2.2 Mouse Models

KI-Rnf216 mutant mice were generated by CRISPR-Cas9 targeting of the mouse *Rnf216* locus (ENSMUSG00000045078). Wild-type NLS-Cas9 protein, synthetic single guide RNA (gRNA) and a single strand DNA oligo (ssODN) donor template were used (Synthego, CA) to insert 3xFLAG/HA (hereby “KI”) peptide sequence within *Rnf216* exon 2. Protospacer (N)20 and PAM sequences corresponding to the gRNA used were 5’-TCTGAATTTGAAAATGGCAG -AGG -3’ and the sequence of the ssODN is 5’-CAAGTGAATGACCTCCTCTTTGTTGTTTCCCTCTGCcataccaccagcgtaatctggaacatcgt atgggtagccgcccttgtcatcgtcatccttgtaatcgatatcatgatctttataatcaccgtcatggtctttgtagtcCATTTT

CAAATTCAGATACAAACATGTGTGATTG -3'. The *Rnf216* sequence, gRNA, donor template sequences, and Sanger sequencing results alignment were annotated and visualized on Benchling online platform (benchling.com). The gRNA was incubated with Cas9 protein for 5 minutes at 37°C to pre-form ribonucleoprotein (RNP) complexes. RNPs and the donor ssODN were introduced into C57BL/6J mouse zygotes using a Gene Editor electroporator (BEX CO., LTD, Tokyo, Japan). Embryos were implanted into pseudo-pregnant recipients according to standard procedures. Editing of founder offspring was assessed using polymerase chain reaction (PCR) and Sanger sequencing of the target region. The sequences of primers used for PCR genotyping are listed in **Table A1** on tail biopsies lysed with proteinase K to release genomic DNA. Reaction products were run on 2% agarose gel.

2.2.3 Western Blot

Mouse testis were homogenized and lysed in RIPA buffer (50 mM Tris-HCl, pH 7.4, 0.01% SDS, 1mM EDTA, 1% Nonidet P-40, 0.5% Na deoxycholate, and 150 mM NaCl). Protein lysates were separated by 4-20% SDS-PAGE gel, transferred onto polyvinylidene difluoride (PVDF) membranes (Bio-Rad), and blocked in 5% non-fat milk solution in 1x PBS. PVDF membranes were incubated with α-HA (1:2000; C29F4, Cell Signaling) and mouse HRP-conjugated α-β-actin (1:5000; A3854, Sigma) in blocking solution at 4°C overnight and washed with 1 x Tris Buffered Saline with Tween (TBS-T) three times. The PVDF membranes were then incubated with HRP-conjugated goat anti-rabbit IgG (1:5000; 1706515, Bio-Rad) for 1 hour, then rinsed three times in TBS-T. Chemiluminescent detection of protein was performed using the XRS+ imaging system (Bio-Rad).

2.2.4 Fertility Tests

Rnf216 KI homozygous (*Rnf216 KI/KI*, "*KI-Rnf216*") males were bred with *Rnf216* +/+ (control) females for four continuous months (*n*=3). The number of litters and number of pups for each cage were recorded during this time. All mice were given identical diets, access to water, and bedding materials. Expanded data regarding these matings can be found in **Table A3**.

2.2.5 Histology

Mouse testes and epididymides were harvested, washed briefly in 1 x Phosphate-Buffered Saline (PBS), fixed overnight in either 4% paraformaldehyde (PFA) or Bouin's fixative at 4°C on a rocker, then embedded in paraffin. For morphology analysis, Bouin's-fixed and embedded tissues were sectioned at 5 μm and stained with hematoxylin and eosin after dewaxing and rehydration. For immunofluorescence staining assays, 4% PFA-fixed and embedded tissues were sectioned at 5 μm.

2.2.6 Immunofluorescence

Testis were fixed in 4% PFA overnight on a rocker at 4°C and then embedded in paraffin. Testis were sectioned at 5 μm and incubated overnight at 37°C, then dewaxed and rehydrated. Antigen retrieval was performed in Citrate buffer (pH 6.0). Testis sections were blocked in 5% Normal Goat Serum (NGS) for 1 hour at room temperature. Testis sections were incubated with α-RNF216 (1:100; A304-111A, Bethyl Laboratories) or α-HA (1:100; C29F4, Cell Signaling) in 5% NGS at 4°C overnight. After washing with 1 x PBS, sections were incubated with goat α-rabbit IgG Alexa Fluor 555 (1:500; A21429, Invitrogen) and α-FITC-conjugated γH2AX (1:500; 16-202A, Millipore) for 1 hour and mounted with Vectashield mounting media with DAPI. A complete list of antibodies may

be found in **Table A2**. Fluorescence microscopy was performed using Fluoview FV1000 confocal microscope (Olympus) at the Michigan State University Center for Advanced Microscopy, East Lansing, MI, USA.

2.2.7 Statistical Analysis

All data are presented as mean ± SEM. The two-sample *t* test was used for all statistical analyses. Statistical significance was determined by $p < 0.05$.

2.3 Results

2.3.1 Recognizable epitope tag was successfully introduced into mouse Rnf216

To investigate the *in vivo* expression and localization of RNF216, an epitope tag knock-in (KI) mouse model was developed. Using CRISP-Cas9 technology, a unique peptide tag consisting of a triple FLAG peptide tag coupled with a single HA peptide tag in series (3xFLAG/HA, hereby "KI") was inserted into exon 2 of mouse *Rnf216* (**Figure 2.1 A**, **Figure B1 A**) and confirmed Sanger sequencing (**Figure B1 B**). This insertion was downstream of the *Rnf216* start codon (ATG) of all known isoforms and did not interrupt any known protein domains. Additionally, its insertion into exon 2 near the N-terminal domain (NTD) resided further away from most known RNF216 regions of interest located near the C-terminal domain (CTD) for a higher probability that it will not disturb protein localization or function. Founder mice (F0) were established and crossed to produce heterozygous pups (F1) (*Rnf216 KI/+*), which were crossed again to establish homozygous pups (F2) (*Rnf216 KI/KI*, hereby "*KI-Rnf216*") (**Figure B1 C**). These F2 genotypes were confirmed via PCR (**Figure B1 D**). The validity of the epitope tag was tested using commercially available α-HA primary antibody on an adult whole testis lysate immunoblot using *KI-Rnf216* and *Rnf216 +/+* mice (**Figure 2.1 B**). KI-RNF216 was in fact

recognized in *KI-Rnf216* testis and was absent in *Rnf216 +/+* testis, as expected. (KI-RNF216 was also recognized using α-FLAG primary antibody to a similar result (data not shown)). Additionally, immunofluorescence staining of *Rnf216 KI/+* heterozygous and *KI-Rnf216* homozygous adult testis with α-HA primary antibody proved effective with no difference between KI-RNF216 staining intensity, expression, or localization (**Figure B1 E**). Therefore, *KI-Rnf216* male homozygous mice were to be used for the remainder of experiments as the preferred KI genotype.

2.3.2 KI-Rnf216 male mice are fertile

To determine if the inserted KI peptide tag impacted spermatogenesis and male fertility, *KI-Rnf216* males were raised to adulthood and evaluated. One concern was that the introduction of the KI peptide would render a nonfunction protein that would result in a similar phenotype to *Rnf216 KO* males [141, 142]. Upon initial observation, the gross anatomical appearance of *Rnf216 +/+* and *KI-Rnf216* adult males was identical (**Figure 2.1 C**) as were the dissected testis of both mice (**Figure 2.1 D**). Additionally, dissected adult testis weights of *Rnf216 +/+* (0.103 g ± 0.002 g, n=6) and *KI-Rnf216* (0.105 g ± 0.001 g, n=7) were not statistically significant ($p > 0.05$, p=0.2984), suggesting no developmental or spermatogenic defects (**Figure 2.1 E**). Additionally, male *Rnf216 +/+* and *KI-Rnf216* adult mice were paired with *Rnf216 +/+* females for 4-month mating tests. *Rnf216 +/+* males were able to father pups, as expected (7.4 pups/litter ± 1.2 pups/litter, n=3), and *KI-Rnf216* males were also fertile (7.5 pups/litter ± 0.7 pups/litter, n=3) ($p > 0.05$, p=0.1875) (**Figure 2.1F**), confirming proper spermatogenesis and reproductive function with the inserted KI peptide in RNF216. This finding was further supported with H&E staining of *Rnf216 +/+* and *KI-Rnf216* adult testis and cauda epididymis showing

normal spermatogenesis and sperm present in normal numbers (**Figure 2.1 G**).

2.3.3. KI-RNF216 is nuclear and only expressed in germ cells within the testis and epididymis.

Previous reports of RNF216 in murine male reproduction have isolated RNF216 expression to the whole testis but lacked further cell-specific refinement [141]. To address this, *KI-Rnf216* adult testis were harvested and immunofluorescently stained with α-HA primary antibody to examine its expression and localization within the male reproductive tract. Immunofluorescence staining displayed a germ cell-specific staining pattern of KI-RNF216 within the testis (**Figure 2.2 A**). Furthermore, the KI-RNF216 signal was predominantly nuclear in nature, which suggests RNF216 may have nuclear functions in male germ cells. While KI-RNF216 was expressed in male germ cells, there was no expression in the two primary somatic cell populations involved in spermatogenesis, Leydig cells (**Figure 2.2 B**) and Sertoli cells (**Figure 2.2 C**), further confining RNF216 involvement within the testis to the germ cell populations. Additionally, KI-RNF216 was also not expressed in caput epididymis (**Figure 2.2 D**) or cauda epididymis (**Figure 2.2 E**) sperm populations stemming from KI-RNF216 positive germ cell linages, suggesting that RNF216 may not play a role in sperm maturation outside the testis. Another concern was if the KI peptide tag altered RNF216 expression or localization in any way compared to native RNF216 in *Rnf216 +/+* mice. To ensure this KI-RNF216 staining pattern was identical to native RNF216, adult *Rnf216 +/+* and *KI-Rnf216* testis sections were stained with commercial α-RNF216 primary antibody, albeit with increased non-specific staining, to ensure the KI peptide tag did not interfere with expression (**Figure B2**). The expression pattern and localization were identical between RNF216 and KI-RNF216.

2.3.4 KI-RNF216 is temporally expressed in specific male germ cell populations.

To better understand how RNF216 may be influencing spermatogenesis, high resolution confocal images were captured using *KI-Rnf216* adult testis for each germ cell type (**Figure 2.3 A**). KI-RNF216 was expressed within undifferentiated spermatogonia (type A) and expression continued throughout mitosis and differentiation into differentiated spermatogonia (type B) at a high relative intensity. Moving forward, KI-RNF216 was continually expressed in each spermatocyte population during meiosis including early stage pachytene spermatocytes (Early Pach). After early pachytene spermatocytes, KI-RNF216 expression was absent for the remainder of meiosis and division events resulting in haploid round spermatids. Interestingly, KI-RNF216 was expressed for a brief window during spermatogenesis in late round spermatids just prior to elongation, but not during elongation (stage VIII). From this point forward, KI-RNF216 was not expressed in sperm at any seminiferous tubule stage. Additionally, KI-RNF216 is predominately nuclear across all RNF216-positive cell germ cell types. To better visualize RNF216 expression within male germ cells throughout spermatogenesis, a summary of RNF216-positive cells was compiled for each seminiferous tubule stage (**Figure 2.3 B**) with conserved nomenclature to confocal images in **Figure 2.3.A**. This summary illustrates the primary window of RNF216 expression in spermatogenesis starting with stage I type A spermatogonia through stage IV early pachytene spermatocytes, and a smaller secondary window of expression in stages VI – VII late round spermatids, suggesting RNF216 may have a role in these populations for proper spermatogenesis.

2.3.5 KI-RNF216 is has unique sub-nuclear localization in male germ cells

Since KI-RNF216 is expressed in select male germ cells and during specific

windows of spermatogenesis, further inspection of these RNF216-postive germ cell populations was required. The nucleus is not a homogenous organelle, but rather composed of several subdomains that serve specific biological processes and house different genetic materials [148, 149]. Therefore, the staining pattern of RNF216 within germ cell nuclei may provide more information on potential pathways of function for RNF216 in male germ cells. Upon further high-resolution microscopy of immunofluorescently stained *KI-Rnf216* adult testis sections, KI-RNF216 had a unique localization pattern within the nuclei of spermatogonia and spermatocytes (**Figure 2.4**), as well as less defined, but similar, localization in late round spermatids. A more detailed examination shows KI-RNF216 was absent in areas of high DAPI expression, most indicative of higher AT-rich regions and a higher DNA concentration [150, 151]. This unique nuclear localization pattern may suggest nucleus region-specific pathways and potential interacting molecules, including possible ubiquitination substrates, for RNF216 in these male germ cell populations that may influence spermatogenesis.

2.4 Discussion

Male germ cells need to undergo constant rounds of spermatogenesis that will replenish SSC populations and produce sperm able to fertilize an oocyte. This process has been shown to be disrupted by RNF216 loss, resulting in male infertility in mice [141, 142]. Although RNF216 was known to be expressed within the testis, little was known as to where RNF216 was acting at the cellular level. Through the generation of the *KI-Rnf216* mouse model, KI-RNF216 has been shown to have expression in different male germ cells throughout spermatogenesis. This restricts RNF216 function to male germ cells with no expression observed in testis somatic cells, such as Leydig or Sertoli cells in the testis.

KI-RNF216 was expressed within the nuclei of male germ cell populations, suggesting it may be involved with nuclear-specific molecular pathways. Furthermore, its nuclear sub-localization displays distinct areas void of KI-RNF216 expression in RNF216-positive germ cell populations, further refining its potential functions in these cells to maintain spermatogenesis in mice.

While RNF216 is expressed within male germ cells, it remains unclear as to its exact role in maintaining spermatogenesis. RNF216 was shown to have expression from undifferentiated spermatogonia through early pachytene spermatocytes undergoing meiosis. This window of expression requires further refinement since the only RNF216-related defect observed was incomplete zygotene arrest in male *Rnf216 KO* mice [142]. However, the nature of an incomplete arrest in spermatogenesis is complicated, since some zygotene populations can progress despite RNF216 loss, requiring further investigation. Since there is strong expression of RNF216 in undifferentiated spermatogonia, it may be possible for RNF216 to play a crucial role in these cell types, or other differentiated pre-zygotene germ cells, that may attribute to the large-scale germ cell degeneration observed in *Rnf216 KO* male mice [141, 142]. The second brief expression of KI-RNF216 in late round spermatid is intriguing, since this expression suggests a role of RNF216 prior to round spermatid elongation. This marks the beginning of spermiogenesis, which includes the growth of flagella, cytoplasm shedding, and protamine replacement of histones [51-54, 56, 57]. Likewise, RNF216 may have a role in the preparation of these processes during the extensive remodeling process to produce sperm. The combination of this RNF216 expression-window, along with the meiotic defects observed in *Rnf216 KO* mice, may prevent post-zygotene germ cells from

progressing any further, resulting in the infertile phenotype observed [142].

With KI-RNF216 expression present in two distinct windows of spermatogenesis in adult mice and the deletion of RNF216 leading to male infertility, RNF216 has been shown to play an essential role in male reproduction. However, the exact molecular role is unclear. Li, et al., suggested that RNF216 may act as an E3 ubiquitin ligase to modulate PKA catalytic subunit β (PRKACB) levels in testis, leading to the meiotic defects in these mice [142]. While this interaction may occur, it is unclear if this is the only substrate of RNF216 in male germ cells. With additional data showing KI-RNF216 is nuclear, potential interactors or substrates can be further refined to primarily nuclear molecules and improved reagents could pull-down RNF216 interactors with increased efficiency and specificity. This could be done using the recognizable peptide tag of the *KI-Rnf216* mouse model as a target for proteomic analysis of RNF216 interactors in male germ cells. Unfortunately, this could come with pitfalls, since RNF216 is a large protein (~100 kDa) with additional protein binding domains located near the N-terminus that could impact protein interactions, including transient interactions such as the RING2-Ub transfer to a substrate. An alternate approach to identify potential RNF216 substrates *in vivo* could be whole testis proteome analysis of *Rnf216 +/+* and *Rnf216 KO* testis of the same age to see if any differences in protein expression exist, which may suggest RNF216-mediated degradation of elevated proteins in *Rnf216 KO* could have RNF216 interactions. An additional approach to investigate germ cell-specific RNF216 substrates, including those in KI-RNF216-positive late round spermatids, could be achieved through combined cell sorting and proteomic analysis. Nevertheless, the segmented expression of RNF216 in spermatogenesis leaves additional questions to be explored to appreciate how RNF216

may be working in these germ cell populations.

KI-RNF216 was observed to have nuclear expression in male germ cells, including unique localization within the nuclei of spermatogonia and spermatocytes. KI-RNF216 was absent in areas with high DAPI-concentration, such as heterochromatin regions, which are transcriptionally inactive [150-152]. Based on the diffuse KI-RNF216 expression within the remainder of the nucleus, it may be possible for RNF216 to play a role in transcriptional regulation, alternative splicing pathways, or DNA-damage response outside of these inactive regions. This sub-nuclear expression can be teased out further to place RNF216 in specific cellular pathways. For example, co-localization with known nuclear markers in these germ cell populations could shed more information to RNF216-positive nuclear domains. Additionally, E3 ligase expression in the nucleus places RNF216 in a unique position for molecular pathways not found in the cytoplasm or on the cellular membrane, proposing RNF216 may impact DNA repair-related pathways during mitosis and/or meiosis, which have not been investigated.

Altogether, this data is the first to show RNF216 expression in male germ cells and provides a detailed germ cell expression profile throughout spermatogenesis and seminiferous tubule stages of the testis. The *KI-Rnf216* mouse model was able to produce functional KI-RNF216 protein that matched expression and localization of native RNF216 and allowed for proper spermatogenesis. High-power microscopy of RNF216 revealed sub-nuclear expression of KI-RNF216 in male germ cells, providing the setting of RNF216 function in male germ cells that is required for spermatogenesis and male fertility. The *KI-Rnf216* mouse model can serve as a useful tool for future investigation of RNF216 at male germ cell and molecular levels, identification of new candidates for protein

interactions and E3 ubiquitination substrates, and help elucidate how RNF216 operates in male germ cells to maintain constant waves of spermatogenesis and preserve male fertility.

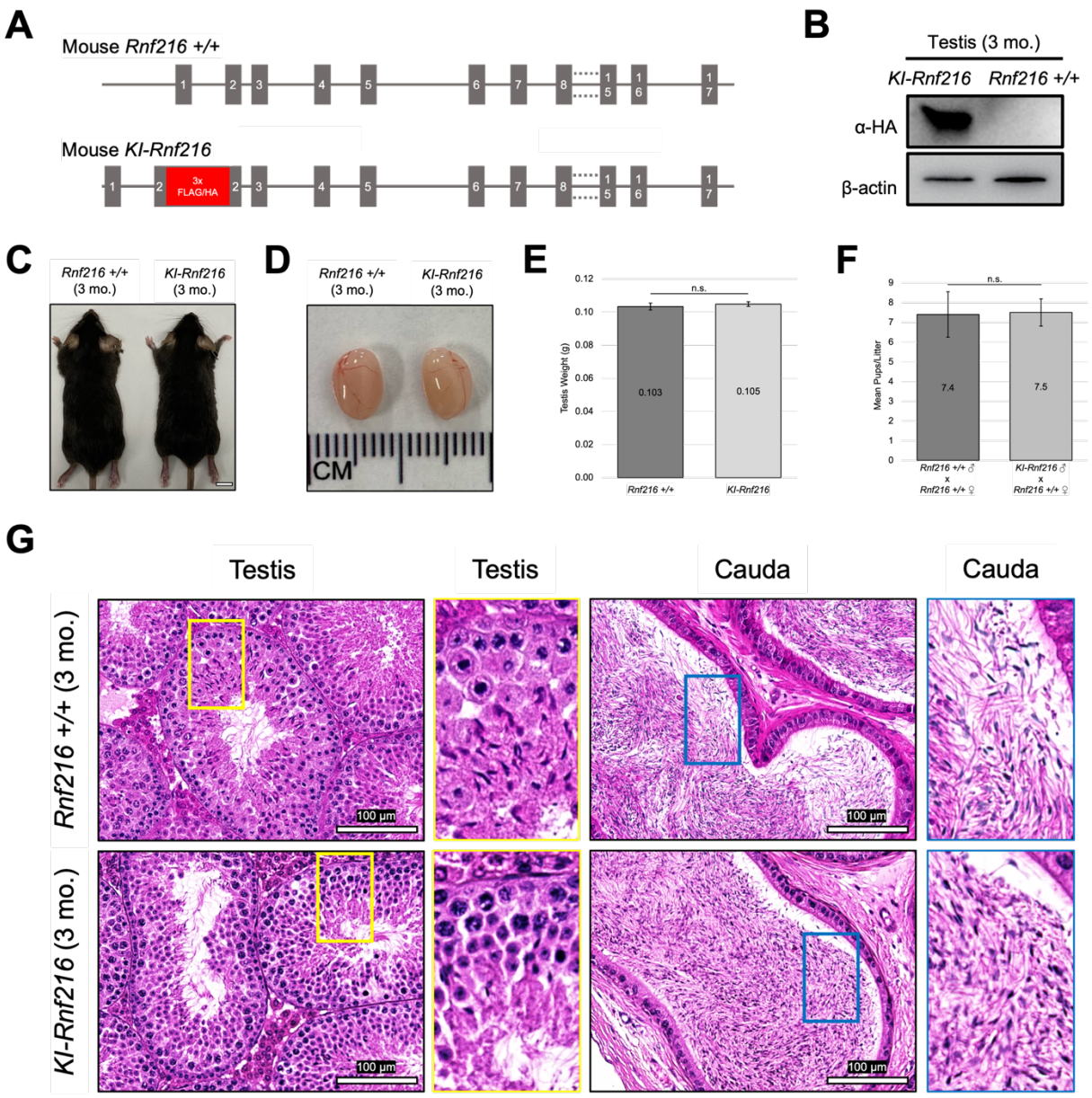


Figure 2.1. *Rnf216* epitope tag knock-in (*KI-Rnf216*) male mice are fertile.
A. Gene locus of *Rnf216* +/+ (wild-type) compared to epitope tagged *KI-Rnf216* (knock-in) with inserted N-terminal domain (NTD) 3xFLAG/HA peptide tag within *Rnf216* exon 2. **B.** Western blot comparing *KI-Rnf216* and control (*Rnf216* +/+) littermate adult testis with commercial α-HA primary antibody recognizing KI-RNF216. **C.** Gross comparison of adult *Rnf216* +/+ and *KI-Rnf216* mice. Scale bar 1 cm. **D.** Comparison of adult *Rnf216* +/+ and *KI-Rnf216* testis. **E.** Statistical comparison of *Rnf216* +/+ and *KI-Rnf216* testis wet weights after dissection. *$p < 0.05$; n.s., not statistically significant. **F.** Statistical comparison of adult *Rnf216* +/+ and *KI-Rnf216* mice during 4-month breeding trials. *$p < 0.05$; n.s., not statistically significant. **G.** Hematoxylin and eosin (H&E)-stained adult *Rnf216* +/+ and *KI-Rnf216* testis, testis insert (yellow), cauda epididymis, and cauda insert (blue) sections. Scale bars 100 µm. Images are representative of three biological repeats.

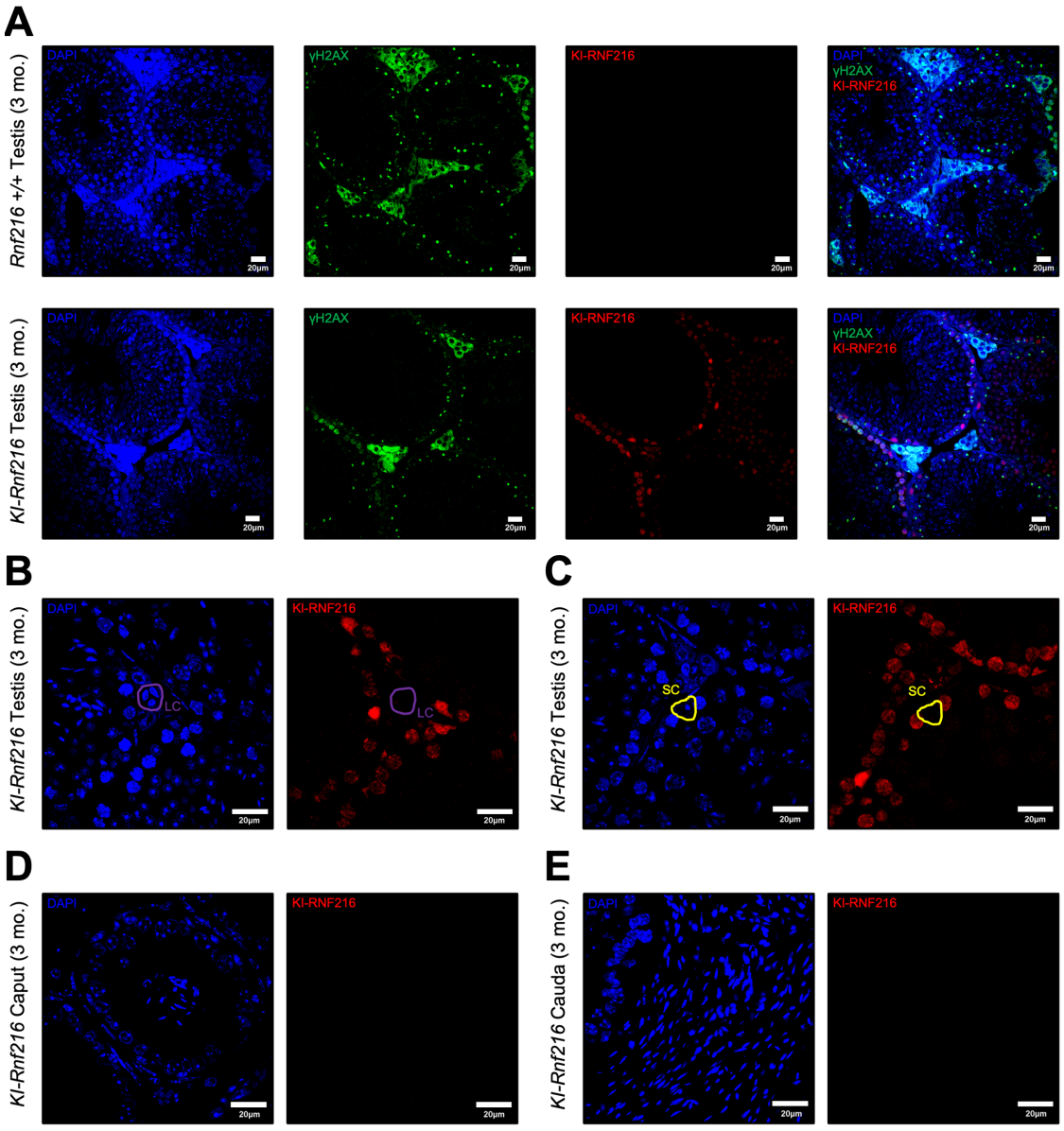
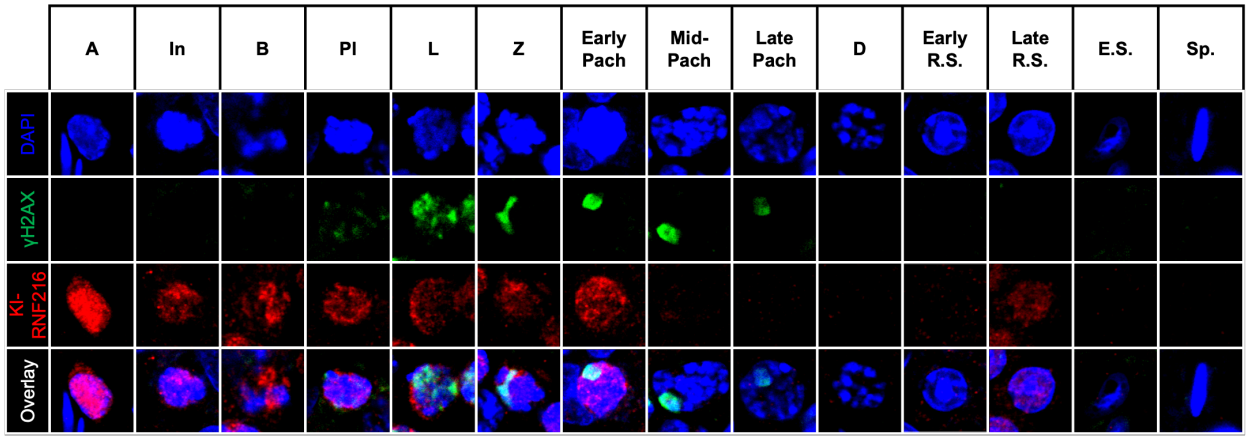


Figure 2.2. Immunofluorescence of KI-RNF216 displays nuclear germ cell-only expression within male reproductive tract in mice.
A. Confocal images of *Rnf216* +/+ (control) and *KI-Rnf216* adult testis sections immunofluorescently stained with α-HA antibody recognizing KI-RNF216. Native RNF216 is not detected by α-HA antibody. KI-RNF216 (red) is expressed within select male germ cell populations. Scale bars 20 μm. **B.** Confocal images *KI-Rnf216* adult testis sections showing Leydig cells (LC, purple circle) are negative of KI-RNF216 (red) expression. Scale bars 20 μm. **C.** Confocal images *KI-Rnf216* adult testis sections showing Sertoli cells (SC, yellow circle) are negative of KI-RNF216 (red) expression. Scale bars 20 μm. **D.** Confocal images *KI-Rnf216* adult caput epididymis sections showing no KI-RNF216 expression. Scale bars 20 μm. **E.** Confocal images *KI-Rnf216* adult cauda epididymis sections showing no KI-RNF216 expression. Scale bars 20 μm. All images in **A. – E.** are representative of three biological repeats.

A

KI-Rnf216 Testis (3 mo.)



B

Sp.	Sp.	Sp.	Sp.	Sp.	Sp.	Sp.				
Early R.S.	Early R.S.	Early R.S.	Early R.S.	Late R.S.	Late R.S.	E.S.	Sp.	Sp.	Sp.	Sp.
Early Pach	Early Pach	Early Pach	Mid-Pach	Mid-Pach	Mid-Pach	Mid-Pach	Late Pach	Late Pach	D	4n → 2n → n
A	A/In	In/B	B	B/Pl	Pl	Pl/L	L	L/Z	Z	Z
A	A	A	A	A	A	A	A	A	A	A
A	A	A	A	A	A	A	A	A	A	A
I	II-III	IV	V	VI	VII	VIII	IX	X	XI	XII

Seminiferous Tubule Stage

Figure 2.3. KI-RNF216 is temporally expressed in select male germ cell nuclei during spermatogenesis in mice.
A. Confocal images of *KI-Rnf216* adult testis male germ cell lineage progression with individual cells magnified displaying protein markers. KI-RNF216, red; DAPI, blue; γH2AX, green. Images are representative of three biological repeats. **B.** RNF216 expression summary during spermatogenesis across each seminiferous tubule stage for adult male mice. RNF216 expression, red; no RNF216 expression, colorless. For **A. – B.**: A, type A spermatogonia; In, intermediate spermatogonia; B, type B spermatogonia; Pl, pre-leptotene; L, leptotene; Z, zygotene; Early Pach, early pachytene; Mid-Pach, mid-pachytene; Late Pach, late pachytene; D, diplotene; Early R.S., early round spermatid; Late R.S., late round spermatid; E.S., elongating spermatid; Sp., sperm.

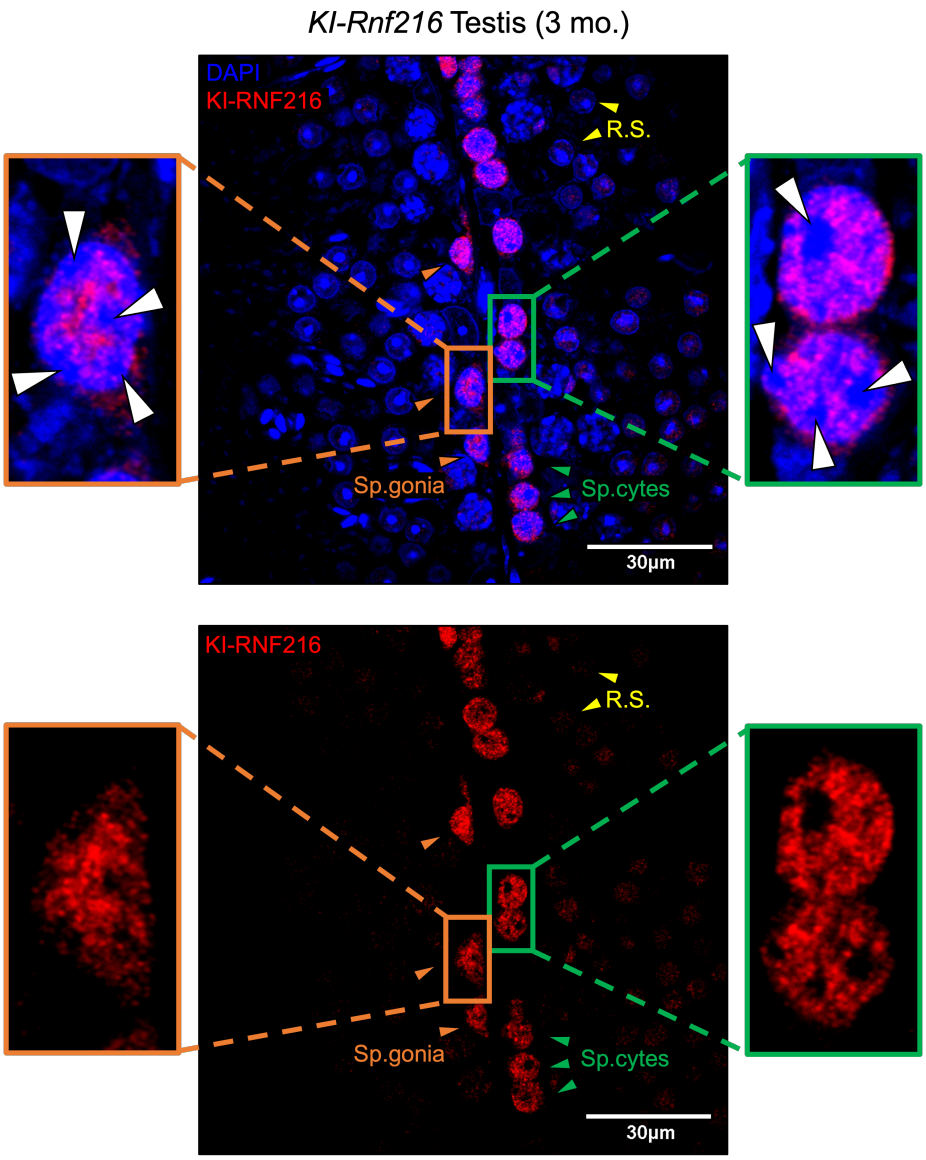


Figure 2.4. Mouse KI-RNF216 is localized within sub-nuclear domains in select male germ cell populations.
Confocal images of *KI-Rnf216* adult tests sections displaying predominantly nuclear expression pattern of KI-RNF216 within select male germ cell populations. Magnified inserts show germ cells (orange and green boxes) with defined areas within nucleus that are DAPI-positive yet void of KI-RNF216 expression (white arrowheads). Sp.gonia, spermatogonia (orange arrowheads); Sp.cytes, spermatocytes (green arrowheads); R.S., round spermatid (yellow arrowheads). Scale bars 30 μm. Images are representative of three biological repeats.

CHAPTER III.

RNF216 IS INTRINSICALLY REQUIRED IN MALE GERM CELLS FOR SPERMATOGENESIS AND FERTILITY IN MICE

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Chapter III will be combined with Chapter II and an adapted version will be submitted as a single manuscript for publication.

This study was supported by the National Institutes of Health R01GM132490 and R01HD084494, and the National Institute of Food and Agriculture MICL02690.

3.1 Introduction

Infertility is a worldwide problem that effects approximately 10% of individuals independent of sex [36, 71-73]. Males contribute to half of these cases, which usually stems from problems with sperm production [72, 73]. Defects in spermatogenesis can lead to hypogonadism in males, resulting in medical, social, and relational hardships with limited treatments options available, especially when underlying genetic causes are the source of presented infertility. Numerous genes have been identified to play an important role in the maintenance of mammalian spermatogenesis, with one of those being RNF216, an E3 ubiquitin ligase [105-112, 141-143]. Mutations in RNF216 have been observed in human Gordon Holmes Syndrome (GHS), which individuals, more strikingly in males, show reproductive dysfunction along with neurological defects [105-112]. With a causative connection to human disease established at the clinical level, RNF216 has been investigated heavily on the neurological side and *in vitro,* but has been limited in terms of understanding its role in male reproduction [113-115, 129, 132-135, 143]. To date, two research groups have explored the requirement of RNF216 on spermatogenesis using mouse models, leaving room for further research questions to be addressed [141, 142].

The generation of *Rnf216 -/-* (*Rnf216 KO*, knockout) mice with RNF216 deleted in the entire organism demonstrated an essential role of RNF216 in male mice, since *Rnf216 KO* males displayed azoospermia along with decreased testis size (hypogonadism) [141, 142]. Furthermore, these mice showed severe germ cell loss through increased apoptosis, leading to extensive seminiferous tubule degeneration in adulthood [141, 142]. Since these male *Rnf216 KO* mice were unable to father pups, they

were deemed infertile [141, 142]. Interestingly, an expanded early panel of *Rnf216 -/-* testis histology suggested meiotic defects leading to incomplete zygotene spermatocyte arrest because of RNF216 loss [142]. While these studies enhanced knowledge of RNF216 function in testis biology, there was no explanation for the seemingly agametic seminiferous tubules in observed in adulthood, leaving room for further understanding of RNF216 functions in spermatogonia or post-zygotene germ cells that prevent cells with incomplete meiotic arrest from developing into sperm. While there is more information to be gained of RNF216 function in male reproduction, the *Rnf216 KO* mouse model highlighted the importance of RNF216 in spermatogenesis and set the stage for future interrogation of RNF216 in a more refined setting within the testis.

These studies of RNF216 global deletion in mice were groundbreaking for RNF216-related biological phenomenon in reproduction but were also limited in where RNF216 was essential for spermatogenesis in mice. Immunoblot analysis of RNF216 across various tissues showed strong expression in the brain and testes of mice, which accurately corresponded to the two main organs of disorder seen in human GHS patients [141]. However, spermatogenesis is also controlled by the brain and testes somatic cells through hormonal signaling in the Hypothalamic-Pituitary-Gonadal (HPG) axis. This axis relies on hormones produced in the hypothalamus and pituitary gland of the brain to influence testosterone production in Leydig cells, which is transported to Sertoli cells in the seminiferous tubule to promote spermatogenesis [64, 65, 67, 68]. Since hypogonadism is observed in human GHS male patients, as well as *Rnf216 KO* male mice, one needs to consider the root cause for this reproductive dysfunction. Given its testes expression, it is possible that RNF216 may play a role in male germ cell functions,

the production or shuttling of testosterone in somatic cells of the testis, the production of brain hormones in this axis, or a combination of these events. A straightforward way to answer this conundrum would be to conditionally ablate RNF216 expression in a portion of the HPG axis for further study. In fact, a neurological research group undertook this endeavor through the conditional knockout (cKO) of RNF216 in the brain, resulting in hypothalamic and pituitary gland *Rnf216 cKO* of a mouse model using *NestinCre* as a Cre driver [143]. While these *NestinCre:Rnf216 cKO* mice had neurological symptoms, no reproductive defects resulting in infertility were observed for either sex [143]. Thus, this data set the stage for testis specific RNF216 to play an essential role for male gonadal development, spermatogenesis, and fertility.

To determine the requirement of RNF216 within the testis for male reproduction, further refinement would need to be done to determine if RNF216 plays an important role in testis somatic or germ cell populations. Since *NestinCre:Rnf216 cKO* mice showed no consequential male reproductive defects, suggesting RNF216 may not be acting in the upstream part of the HPG axis, it could be reasoned that brain RNF216 loss does not likely impact the latter part of testosterone production in testis somatic cell types [64, 67, 143]. Therefore, a mouse model to conditionally knockout RNF216 in male germ cells was created using *Stra8Cre* as the Cre driver for *Rnf216* germ cell-specific deletion. This approach would postnatally delete *Rnf216* at 3 days of age (P3) in mice, prior to spermatogonia proliferation or differentiation [11, 28, 153, 154]. Using this mouse model, RNF216 was shown to be intrinsically required in male germ cells for spermatogenesis and fertility. Furthermore, there was germ cell loss and seminiferous tubule degeneration similar to what was observed in *Rnf216 KO* mouse testis, including increased germ cell

apoptosis. Additionally, germ cell loss was drastic in late adulthood, but germ cell RNF216 deletion seemed to not impact spermatogonia survival or functions, which agreed with observations in early *Rnf216 KO* spermatogenesis [142]. Altogether, the conditional knockout of RNF216 in male germ cells resulted in male reproductive dysfunction in mice, which further isolated critical function of RNF216 on spermatogenesis to male germ cell populations that is separate from hypothalamic and pituitary gland influence on male reproduction.

3.2 Materials and Methods

3.2.1 Ethics Statement

All animal procedures were approved by the Institutional Animal Care and Use Committee of Michigan State University (AUF 202200230). All experiments with mice were conducted ethically following institutional guidelines according to the Guide for the Care and Use of Laboratory Animals.

3.2.2 Mouse Models

Rnf216 male germ cell conditional knockout (*cKO*) mutant mice were generated by crossing *Stra8Cre* [154] or *DDX4Cre* [155] (*VasaCre*) male mice with *Rnf216 Flox* (*Rnf216 F/F*) female mice. The resulting cross yielded either *Stra8Cre*(*or DDX4Cre*)*:Rnf216 F/+* or *Stra8Cre*(*or DDX4Cre*)*:Rnf216 F/-* (*cKO*) mice. *Rnf216 Flox* mice were generated using CRISPR-Cas9 targeting of the mouse *Rnf216* locus (ENSMUSG00000045078) to insert two *loxP* sites (5'- ATAACTTCGTATA-GCATACAT-TATACGAAGTTAT -3') flanking *Rnf216* exons 4 and 5, and within *Rnf216* intron sequences, respectively, as to not disrupt predicted protein functions. The *Rnf216 Flox* sequence, donor template sequences, and Sanger sequencing results alignment were

annotated and visualized on Benchling online platform (benchling.com). The sequences of primers used for PCR genotyping *Rnf216 Flox* and *Stra8Cre*(*or DDX4Cre*)*:Rnf216 cKO* mice are listed in **Table A1** on tail biopsies lysed with proteinase K to release genomic DNA. Reaction products were run on 2% agarose gel. For *KI-RNF216* mice generation, see Chapter II, section 2.2.2.

3.2.3 Fertility Tests

Stra8Cre(*or DDX4Cre*)*:Rnf216 F/-* ("*Stra8Cre*(or *DDX4Cre*)*:Rnf216 cKO*") and control (*Rnf216* +/+) males were bred with *Rnf216* +/+ (control) females for four continuous months (*n*=3). The number of litters and number of pups for each cage were recorded during this time. All mice were given identical diets, access to water, and bedding materials. Expanded data regarding these matings can be found in **Table A4** and **Table A5**.

3.2.4 Histology

Mouse testes and epididymides were harvested, washed briefly in 1 x Phosphate-Buffered Saline (PBS), fixed overnight in either 4% paraformaldehyde (PFA) or Bouin's fixative at 4°C on a rocker, then embedded in paraffin. For morphology analysis, Bouin's-fixed and embedded tissues were sectioned at 5 μm and stained with hematoxylin and eosin after dewaxing and rehydration. For immunofluorescence staining and TUNEL assays, 4% PFA-fixed and embedded tissues were sectioned at 5 μm.

3.2.5 Cauda Sperm Extraction

Epididymides were isolated from testis and the cauda (tail) portion was removed using micro scissors. Cauda were placed into 2 ml 1 x PBS and micro scissors were used to mince the cauda. The solution was mixed and then incubated at 37°C for 5 minutes.

After the solution had settled, 5 µl of supernatant was placed onto a microscope slide and coverslipped. Light microscopy was performed using EVOS FLc digital microscope (Invitrogen).

3.2.6 Immunofluorescence

Testis were fixed in 4% PFA overnight on a rocker at 4°C and then embedded in paraffin. Testis were sectioned at 5 µm and incubated overnight at 37°C, then dewaxed and rehydrated. Antigen retrieval was performed in Citrate buffer (pH 6.0) or Tris-EDTA buffer (pH 9.0). Testis sections were blocked in 5% Normal Goat Serum (NGS) for 1 hour at room temperature. Testis sections were incubated with α-HA (1:100; C29F4, Cell Signaling), α-PLZF (1:100; SC-28319, Santa Cruz Biotechnology), α-PLZF (1:100; SC-22839, Santa Cruz Biotechnology), or α-C-KIT (1:100; D13A2, Cell Signaling) in 5% NGS at 4°C overnight. After washing with 1 x PBS, sections were incubated with goat α-rabbit IgG Alexa Fluor 555 (1:500; A21429, Invitrogen) or goat α-mouse IgG Alexa Fluor 488 (1:250; A11029, Life Technologies) for 1 hour and mounted with Vectashield mounting media with DAPI. A complete list of antibodies may be found in **Table A2**. Fluorescence microscopy was performed using either Lionheart FX fluorescence microscope (BioTek) or Fluoview FV1000 confocal microscope (Olympus) at the Michigan State University Center for Advanced Microscopy, East Lansing, MI, USA.

3.2.7 TUNEL Assay

In Situ Cell Death Detection Kit - Fluorescein (11684795910, Roche) was used to evaluate cell death in 4% PFA-fixed paraffin-embedded 6-week testes sections according to manufacturer's instructions. Fluorescence images were captured by Fluoview FV1000 confocal microscope (Olympus) at the Michigan State University Center for Advanced

Microscopy, East Lansing, MI, USA.

3.2.8 Statistical Analysis

All data are presented as mean ± SEM. The two-sample *t* test was used for all statistical analyses. Statistical significance was determined by $p < 0.05$.

3.3 Results

3.3.1 RNF216 was conditionally knocked out in mouse male germ cells

To investigate the role of germ cell specific RNF216 expression on male reproduction in mice, a *Cre-loxP* conditional knockout approach was designed. A male germ cell specific Cre driver, *Stra8Cre*, was used to express Cre recombinase beginning at postnatal day 3 with the onset of *Stra8* transcription activation [154, 156]. These *Stra8Cre* male mice were crossed with *Rnf216 Flox/Flox* (*Rnf216 Flox*, or *Rnf216 F/F*) females, which had two *loxP* sites flanking *Rnf216* exons 4 and 5, respectively, that would be recognized by Cre and excised (**Figure 3.1**). These *Rnf216 Flox* mice were generated using CRISPR-Cas9 technology and were validated using Sanger sequencing and PCR to detect the *loxP* insertions as previously shown [141]. The *loxP* sites were designed to reside next to *Rnf216* exons 4 and 5 since these exons were targeted for deletion in the global *Rnf216 KO* mouse model, which showed male infertility [141]. From this initial cross, the resulting F1 *Stra8Cre:Rnf216 F/+* males were crossed again with female *Rnf216 Flox* mice to generate F2 *Stra8Cre:Rnf216 F/-* males (*StraCre:Rnf216 cKO*) harboring one *Rnf216 Null* allele and one *Rnf216 Flox* allele. Upon *Stra8Cre* activation in male germ cells, only the *Rnf216 Flox* allele in the male germ cells would be excised, leaving behind a second *Rnf216 Null* allele, therefore completing the *Rnf216* conditional knockout only in male germ cells.

3.3.2 Male germ cell RNF216 is intrinsically required for fertility

After *StraCre:Rnf216 cKO* mice were successfully generated and sequentially validated, they were allowed to mature along with *Rnf216 +/+* control males to adulthood. The overall appearance between *Rnf216 +/+* and *StraCre:Rnf216 cKO* adult males was similar (**Figure 3.2 A**), but the testis of *StraCre:Rnf216 cKO* males were much smaller than *Rnf216 +/+* testis (**Figure 3.2 B**). Indeed, the wet weight of *Rnf216 +/+* testis (0.104 g ± 0.002 g, n=6) compared to *Stra8Cre:Rnf216 cKO* testis (0.031 g ± 0.001 g, n=7) was statistically significant ($p < 0.0001$) (**Figure 3.2 C**). *Rnf216 +/+* and *StraCre:Rnf216 cKO* adult males were tested for fertility through 4-month paired mating tests with *Rnf216 +/+* females of a similar age. Through these mating tests, *Stra8Cre:Rnf216 cKO* males were unable to father pups (0.0 pups/litter ± 0.0 pups/litter, n=3) compared to *Rnf216 +/+* males (7.5 pups/litter ± 1.0 pups/litter, n=3) ($p < 0.0001$) (**Figure 3.2 D**). Furthermore, extracted cauda epididymis sperm of *Rnf216 +/+* males, which showed motile sperm (**Figure 3.2 E**), were compared to *Stra8Cre:Rnf216 cKO* males, which showed no motile sperm (**Figure 3.2 F**). This supported the finding of infertility for *Stra8Cre:Rnf216 cKO* males.

3.3.3 Male germ cell Rnf216 deletion leads to progressive germ cell loss and increased apoptosis in seminiferous tubules

To obtain a larger perspective of the impact of germ cell RNF216 loss on spermatogenesis, testes of *Rnf216 +/+*, *Stra8Cre:Rnf216 cKO*, and *Rnf216 KO* male mice were collected from early life to late adulthood (**Figure 3.3**). These testes were sectioned, H&E-stained, and visualized under light microscope to evaluate germ cell populations and spermatogenic progression. There were no notable defects at 3 days of age between *Rnf216 +/+* and *Stra8Cre:Rnf216 cKO* mice, which was expected since *Stra8Cre* was not

active prior to this timepoint. However, this phenotype also matched *Rnf216 KO* 3-day testis, which had global RNF216 deletion from conception, suggesting RNF216 may not play a significant role in late embryonic development of gonocytes or early postnatal gonocyte transition to spermatogonia. Prominent germ cell degeneration began at 2-weeks for *Stra8Cre:Rnf216 cKO* and *Rnf216 KO* males and progressed at a similar rate throughout life (**Figure 3.3**). The cauda epididymis sections of adult *Stra8Cre:Rnf216 cKO* and *Rnf216 KO* male mice were also similar with increased cellular debris and lack of sperm, which corresponds with observed infertility. Additionally, *Stra8Cre:Rnf216 cKO* adult males showed increased apoptosis within seminiferous tubules, which was on par with elevated apoptosis seen in *Rnf216 KO* adult males (**Figure 3.4**). This observation indicated RNF216 loss within male germ cells, causing germ cell degeneration, was due to germ cell death during spermatogenesis.

3.3.4 Fetal male germ cell RNF216 conditional knockout mice are infertile and match Stra8Cre:Rnf216 cKO testis phenotype in adulthood

While no notable differences were observed between *Rnf216* +/+, *Stra8Cre:Rnf216 cKO*, and *Rnf216 KO* mouse testis at 3 days of age, the role of RNF216 in male germ cell precursors during late embryogenesis and early postnatal life were unclear. To address this, an earlier germ cell *Rnf216* conditional knockout mouse model was generated concurrently to *Stra8Cre:Rnf216 cKO* generation. However, this additional conditional knockout used *DDX4Cre* (*VasaCre*) as a Cre driver, with Cre recombinase expression beginning at embryonic day 15.5 (E15.5) in gonocytes [155]. These *DDX4Cre* male mice were also bred with *Rnf216 Flox* female mice like *Stra8Cre:Rnf216 cKO* generation, resulting in F2 *DDX4Cre:Rnf216 cKO* male mice (**Figure B3 A**). Furthermore,

DDX4Cre:Rnf216 cKO adult males looked identical to *Rnf216 +/+* controls (**Figure B3 B**), and *DDX4Cre:Rnf216 cKO* adult testis were significantly smaller in size (0.031 g ± 0.001 g, *n*=6) compared to adult *Rnf216 +/+* testis (0.103 g ± 0.001 g, *n*=6) ($p < 0.0001$) (**Figure B3 C-D**). *DDX4Cre:Rnf216 cKO* males were also infertile (0.0 pups/litter ± 0.0 pups/litter, *n*=3) compared to *Rnf216 +/+* males (7.1 pups/litter ± 0.9 pups/litter, *n*=3) ($p < 0.0001$) (**Figure B3 E**). Histological examination of *DDX4Cre:Rnf216 cKO* adult testis and cauda epididymis showed germ cell loss and a lack of sperm in the epididymis (**Figure B3 F**). These phenotypes were hallmarks of *Stra8Cre:Rnf216 cKO* and *Rnf216 KO* males of the same age. The direct comparison of *DDX4Cre:Rnf216 cKO and Stra8Cre:Rnf216 cKO* adult testis and cauda epididymis were identical with extensive germ cell loss, leading to both male mice being infertile (**Figure B4**). Therefore, the *DDX4Cre:Rnf216 cKO* mouse model served as an effective model to understand the requirement of testis RNF216 in late fetal development and early life for male fertility.

3.3.5 Undifferentiated spermatogonia express RNF216 at an early age, but germ cell RNF216 is not required for spermatogonia functions

While it appeared that RNF216 was not required in late embryonic germ cells or early postnatal gonocytes for spermatogenesis, it was unclear if RNF216 was required for spermatogonia functions and survival. Immunofluorescence staining of *KI-Rnf216* postnatal day 3 testis showed strong nuclear expression in undifferentiated (type A) spermatogonia prior to proliferation and differentiation events (**Figure 3.5 A**). Since KI-RNF216 was strongly expressed in these early germ cell populations at the onset of spermatogenesis, it was hypothesized that RNF216 may be essential for spermatogonia functions and loss of germ cell RNF216 in spermatogonia would contribute to the germ

cell loss observed in adult *Stra8Cre:Rnf216 cKO* testis with some tubules being nearly agametic in late adulthood (**Figure 3.3**). Additionally, the germ cell conditional knockout of RNF216 at day 3 in *Stra8Cre:Rnf216 cKO* males would be an effective model to understand RNF216 function in spermatogonia proliferation and differentiation given RNF216 expression in these cells (**Figure 3.5 B**). To address this, adult *Rnf216 +/+*, *Stra8Cre:Rnf216 cKO*, and *Rnf216 KO* testis were examined for spermatogonia presence and functions. These testes were immunofluorescently stained with an undifferentiated spermatogonia marker, PLZF, to assess if these spermatogonia populations were able to remain established through proliferation events and survive in the absence of germ cell RNF216 expression (**Figure 3.6 A**). Undifferentiated spermatogonia were still present in all mice regardless of germ cell RNF216 expression and even survived through seminiferous tubule degeneration and germ cell loss. To balance the evaluation of spermatogonia function in spermatogenesis, the same mouse testes were immunofluorescently stained with a differentiated spermatogonia marker, C-KIT, to determine if these undifferentiated spermatogonia could still progress into spermatogenesis (**Figure 3.6 B**). Surprisingly, there was an abundance of differentiated spermatogonia present in all three mouse models during adulthood. These observations suggested that germ cell RNF216 was not required in the undifferentiated spermatogonia populations for survival, proliferation, or differentiation despite the observed strong intrinsic expression of undifferentiated spermatogonia RNF216.

3.4 Discussion

RNF216 has been shown to be important for male reproductive development and spermatogenesis in both humans and mice [105-112, 141-143]. However, the germ cell

specific requirement of RNF216 separate from HPG axis hormonal control of reproduction has been unknown. In this study, the development of a germ cell specific RNF216 conditional knockout mouse model (*Stra8Cre:Rnf216 cKO*) has shown that RNF216 is intrinsically required in male germ cells for spermatogenesis and fertility. Moreover, the deletion of RNF216 in male germ cells causes a testis phenotype that is like *Rnf216 KO* male mice, suggesting that potential influence of RNF216 on the HPG axis upstream from the testis may not be essential for fertility. In this vein, RNF216 could have different roles dependent on the tissue and cells in which it is expressed, causing neurological symptoms through action within the brain and reproductive dysfunction through pathways in male germ cells, respectively. Therefore, the development and study of this *Stra8Cre:Rnf216 cKO* mouse model has contributed new information on RNF216 involvement in male reproduction.

The use of *Stra8Cre* as a driver for Cre expression in postnatal germ cells effectively ablated RNF216 after 3 days (P3) of life in mice in all germ cell populations. At 3 days of age, male germ cells are near the end of the transition from gonocytes to undifferentiated spermatogonia and have yet to start proliferation or differentiation events, with the latter occurring after the pulsating of retinoic acid (RA) after the first week of life in mice [28, 44, 45]. While this timepoint of deletion was sufficient for this study, a second *Rnf216 cKO* mouse model was concurrently made with an earlier, prenatal Cre driver (embryonic day 15.5, E15.5), *DDX4Cre* (*VasaCre*) to generate *DDX4Cre:Rnf216 cKO* mice. This mouse could be used to examine if RNF216 loss impacted the transition from gonocytes to undifferentiated spermatogonia, a postnatal event, that could have drastic impacts on spermatogenesis starting with the establishment of undifferentiated

spermatogonia. The *DDX4Cre:Rnf216 cKO* phenocopied *Stra8Cre:Rnf216 cKO* testis in terms of germ cell loss and infertility, indicating that either RNF216 was not required during the E15.5 – P3 timeframe, or that RNF216 is not expressed in germ cell precursors during this time. However, P3 spermatogonia showed strong RNF216 expression, indicating that this germ cell RNF216 had a potential crucial role during spermatogenesis.

One report of *Rnf216 KO* mice suggested the presence of meiotic defects upon RNF216 deletion in male mice and did not observe any apparent mitotic or spermatogonia differentiation defects in early spermatogenesis [142]. However, both *Stra8Cre:Rnf216 cKO* and *Rnf216 KO* late adults (6 months) had excessive germ cell loss and seminiferous tubule degeneration, leaving some seminiferous tubules without any germ cell populations present, but leaving Sertoli cells intact (**Figure 3.3**). Additionally, P3 undifferentiated spermatogonia showed strong nuclear expression of RNF216, suggesting a RNF216 function in these germ cells. However, although RNF216 was expressed in early spermatogonia, these cells were able to proliferate and survive during adulthood, as well as maintain the ability to differentiate into differentiated spermatogonia and progress forward in spermatogenesis. This finding supports the previous report by Li, et al. of no apparent mitotic defects in *Rnf216 KO* mice [142]. Therefore, RNF216 may have a dispensable function, or may be compensated by other factors, in spermatogonia, yet an essential role in meiosis or potentially late meiosis/early spermiogenesis, which would require further study.

An interesting observation in *Stra8Cre:Rnf216 cKO and DDX4Cre:Rnf216 cKO* included further examination of cauda epididymis, where a majority of mature sperm would reside prior to ejaculation. Previous analysis of *Rnf216 KO* mice, including those

mice used in this study, showed azoospermia, confirming RNF216 played an essential role in maintaining proper spermatogenesis [141, 142]. With the separation of germ cell RNF216 function from the HPG axis control of reproduction, *Rnf216* was deleted in all germ cells and the resulting male mice were infertile. However, an occasional sperm cell would be observed in the cauda epididymis at an extremely low concentration in both *Stra8Cre:Rnf216 cKO and DDX4Cre:Rnf216 cKO* adult males. It was unclear if these sperm were motile, as none could be detected in extracted cauda sperm. This miniscule amount of sperm is not nearly the amount required for fertilization, as shown through repeated mating trials for these mouse lines. Given this data, it is possible that while germ cell RNF216 is essential for fertility and normal spermatogenesis, RNF216 influence in the brain portion of the HPG axis may rescue the occasional germ cell linage to progress to sperm, albeit in extremely low quantities. It is also possible that the same phenomenon is present in *Rnf216 KO* males, but to a lesser extent if spermatogenesis cannot be partially rescued in the absence of global RNF216, which could be examined further. Another theory is incomplete Cre deletion may have occurred, allowing a germ cell lineage to progress to sperm that lacked RNF216 deletion [157, 158]. Though these could all be possibilities, the overwhelmingly majority testis phenotype indicated that *Stra8Cre:Rnf216 cKO and DDX4Cre:Rnf216* led to severe germ cell loss and infertility with high penetrance, showing germ cell RNF216 is critically impactful on spermatogenesis.

In summary, the conditional knockout of RNF216 in male germ cells led to germ cell loss, seminiferous tubule degeneration, and infertility in adult male mice. RNF216 loss in male germ cells increased germ cell apoptosis, leading to the accumulation of

cellular debris in cauda epididymis. These germ cell *Rnf216 cKO* male mice had smaller testis and had a severe reproductive phenotype, opposite of conditional knockout of brain RNF216 in mice, which showed no significant reproductive dysfunction in males [129]. Although expression of RNF216 was present in early undifferentiated spermatogonia, there were no defects in spermatogonia proliferation or differentiation events, suggesting RNF216 may not have an essential role in these germ cell populations. An essential role of RNF216 in male germ cells is apparent, but the extent to which germ cell populations specifically require RNF216 for fertility requires further examination. One potential realm of interest would be the germ cell-specific substrate(s) of RNF216 as an E3 ubiquitin ligase, which could place it into a cellular pathway that may be germ cell type-specific, further explaining the unique role of RNF216 in spermatogenesis. The conditional knockout of RNF216 in male germ cells revealed that RNF216 function in the testis is essential for spermatogenesis and fertility, providing a better understanding to how reproductive dysfunction may be caused in human disease and infertility.

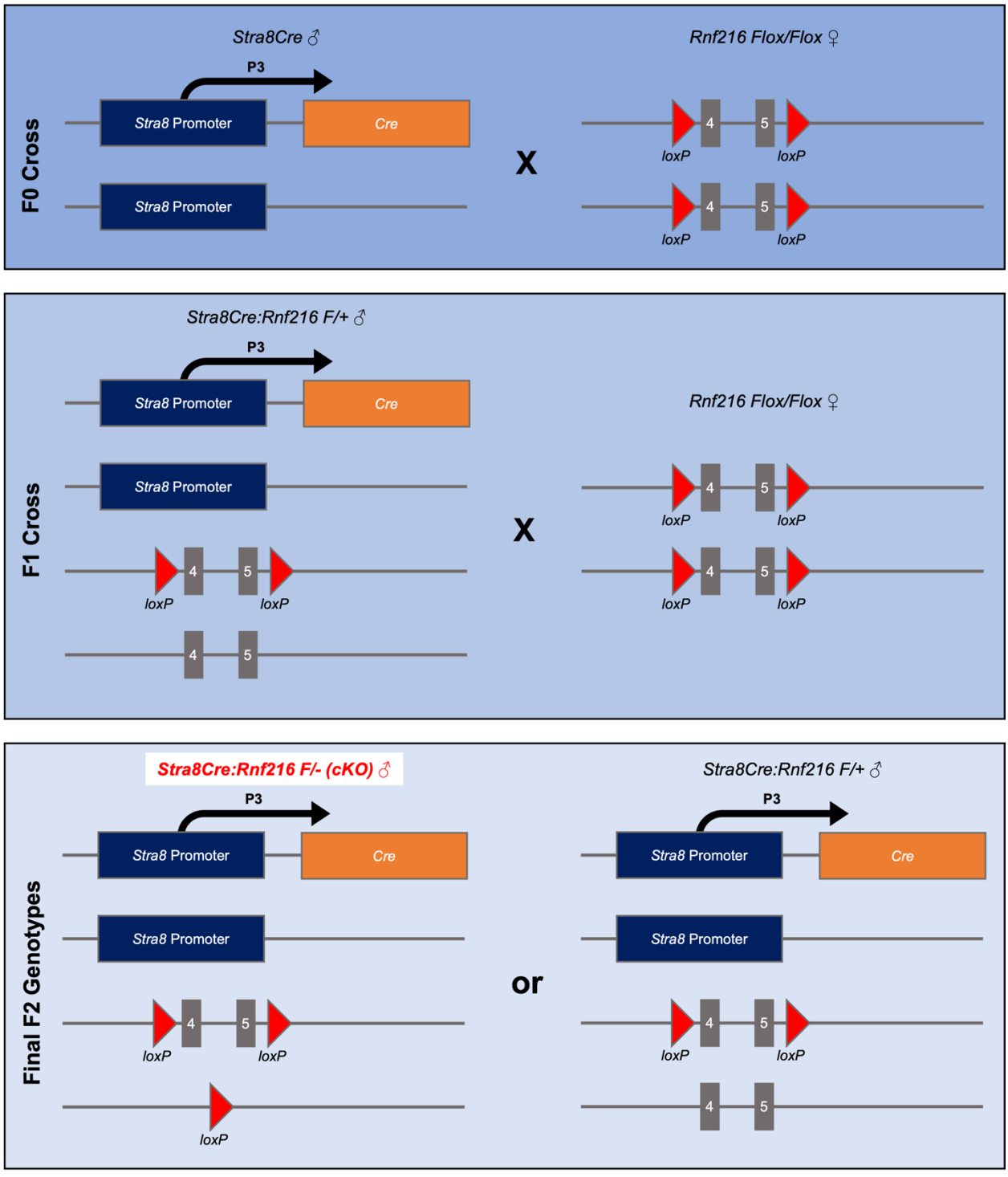

Figure 3.1. Gene and breeding schematic of germ cell specific RNF216 conditional knockout mouse model.
Stra8Cre males were bred with homozygous *Rnf216 Flox* (*Rnf216 Flox/Flox*) females to generate F1 *Stra8Cre:Rnf216 F/+* males. These F1 males were crossed with *Rnf216 Flox/Flox* females to generate F2 *Stra8Cre:Rnf216 F/-* (red text) (*Rnf216 cKO* in male germ cells) or *Stra8Cre:Rnf216 F/+* (control) males. *Rnf216* exons 1-3, 6-17 not shown.

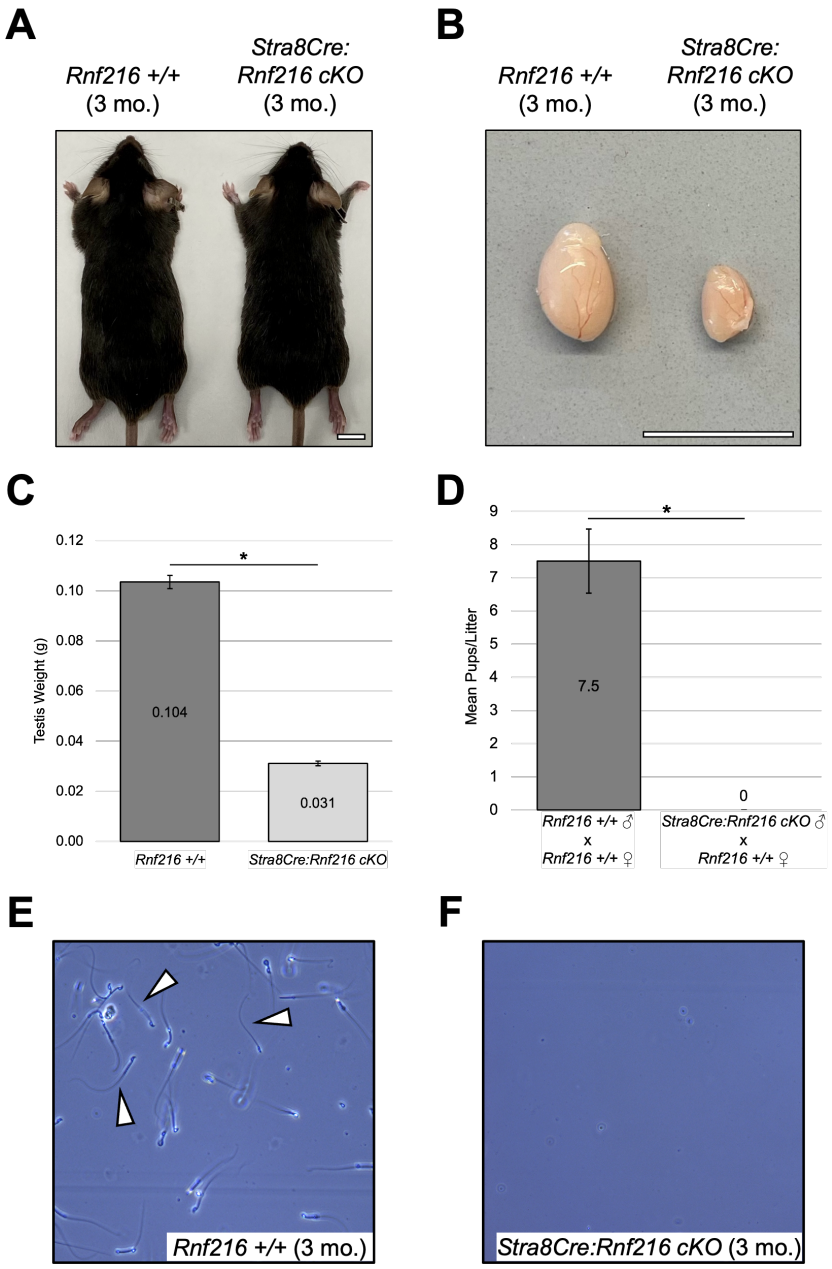

Figure 3.2. RNF216 is intrinsically required in male germ cells for fertility in mice. **A.** Gross comparison of adult *Rnf216 +/+* and *Stra8Cre:Rnf216 cKO* mice. Scale bar 1 cm. **B.** Comparison of adult *Rnf216 +/+* and *Stra8Cre:Rnf216 cKO* testis. Scale bar 1 cm. **C.** Statistical comparison of *Rnf216 +/+* and *Stra8Cre:Rnf216 cKO* testis wet weights after dissection. *$p < 0.05$. **D.** Statistical comparison of adult *Rnf216 +/+* and *Stra8Cre:Rnf216 cKO* mice during 4-month breeding trials. *$p < 0.05$. **E. – F.** Extracted sperm from adult *Rnf216 +/+* and *Stra8Cre:Rnf216 cKO* cauda epididymis, respectively. Motile sperm, white arrowheads.

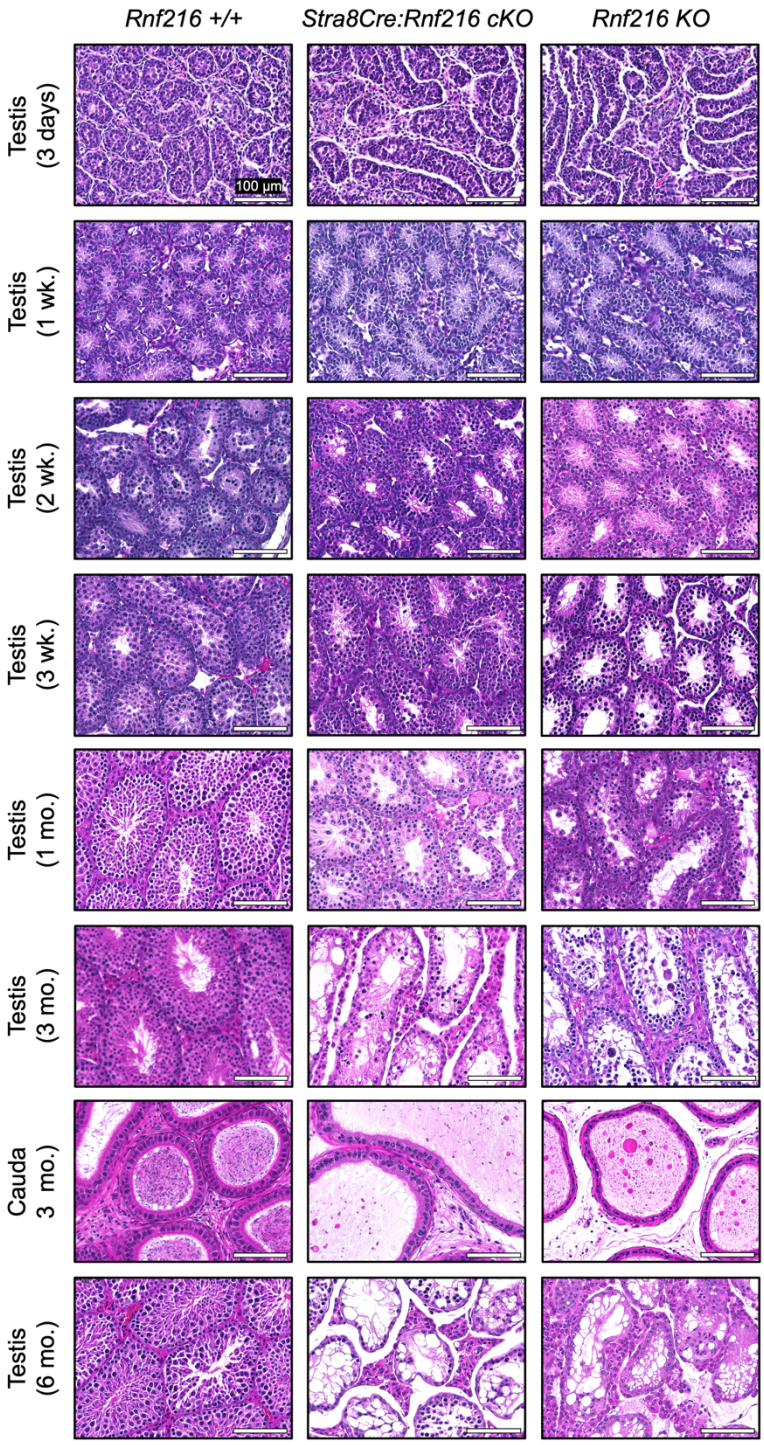

Figure 3.3. *Rnf216* deletion in male germ cells leads to progressive germ cell loss and seminiferous tubule degeneration.
Hematoxylin and eosin (H&E)-stained testes and cauda epididymides of *Rnf216 +/+* (control), *Stra8Cre:Rnf216 cKO*, and *Rnf216 KO* mice across various timepoints starting with *Stra8Cre* expression at postnatal day 3 (P3). Scale bars 100 µm. Images are representative of three biological repeats.

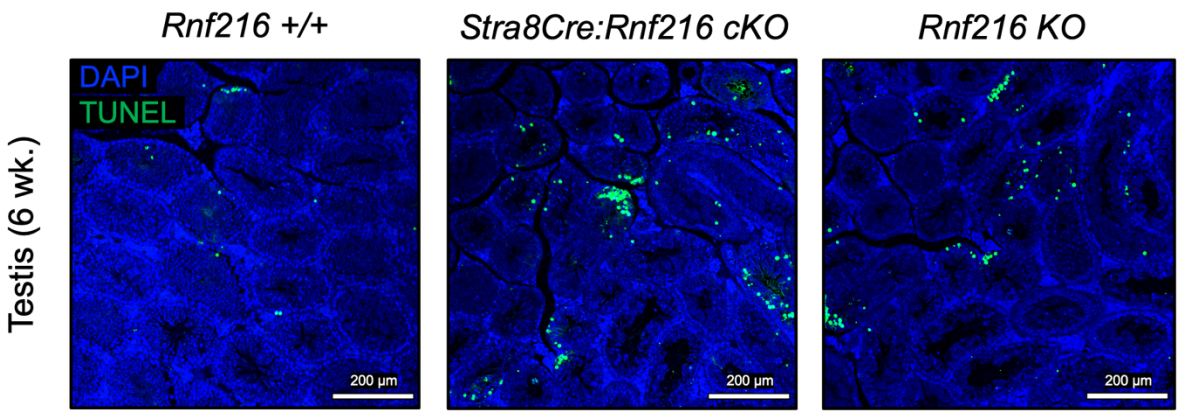

Figure 3.4. RNF216 loss in male germ cells is the result of elevated apoptosis. Confocal images of mouse *Rnf216 +/+, Stra8Cre:Rnf216 cKO*, and *Rnf216 KO* adult (6 week) testis immunofluorescently stained for apoptosis (green). Scale bars 200 µm. Images are representative of three biological repeats.

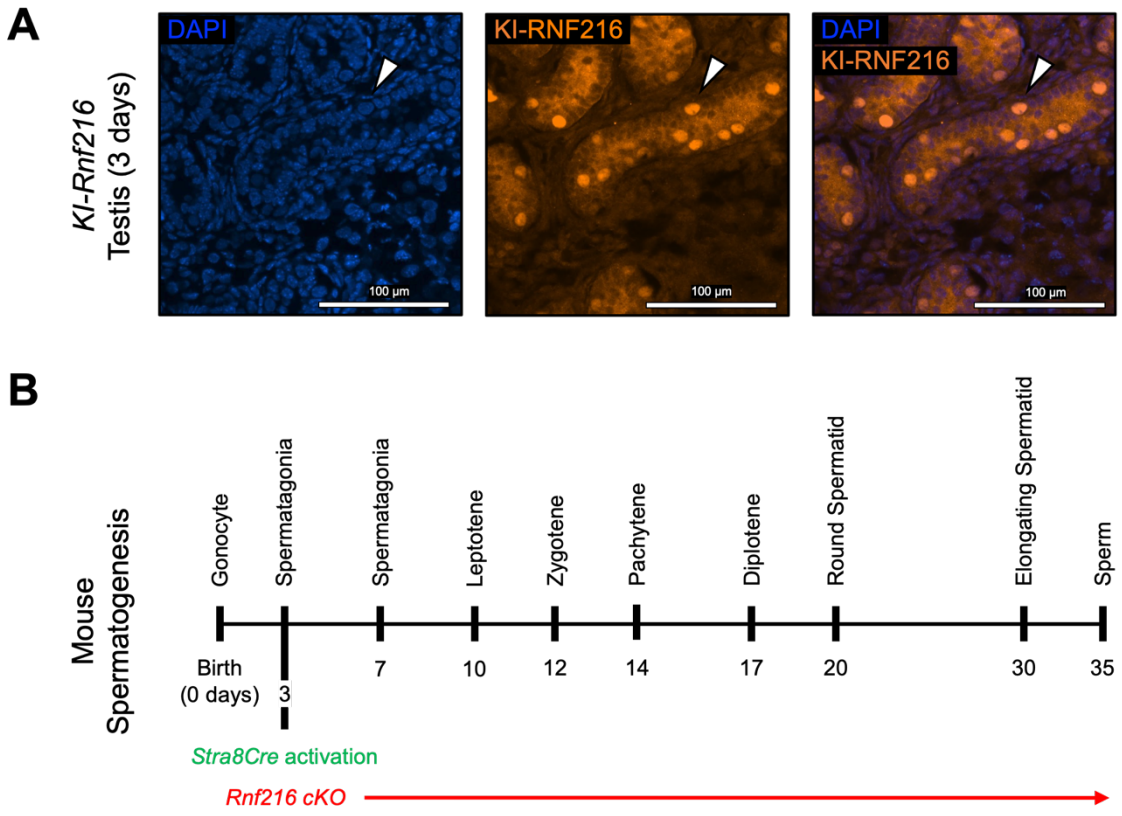

Figure 3.5. Mouse KI-RNF216 is expressed in early postnatal undifferentiated spermatogonia prior to differentiation or proliferation.
A. Microscope images of mouse *KI-Rnf216* (3 days) testis immunofluorescently stained with α-HA (orange) showing KI-RNF216 expression is present in early spermatogonia before proliferation or differentiation events. Undifferentiated spermatogonia, white arrowheads. Scale bars 100 µm. Images are representative of three biological repeats. **B.** Timeline of mouse spermatogenesis from birth to the completion of the first wave of spermatogenesis (35 days). *Stra8Cre* activation (green), and subsequent RNF216 conditional knockout in male germ cells (red text and arrow), shown at postnatal day 3 prior to undifferentiated spermatogonia self-renewal or differentiation.

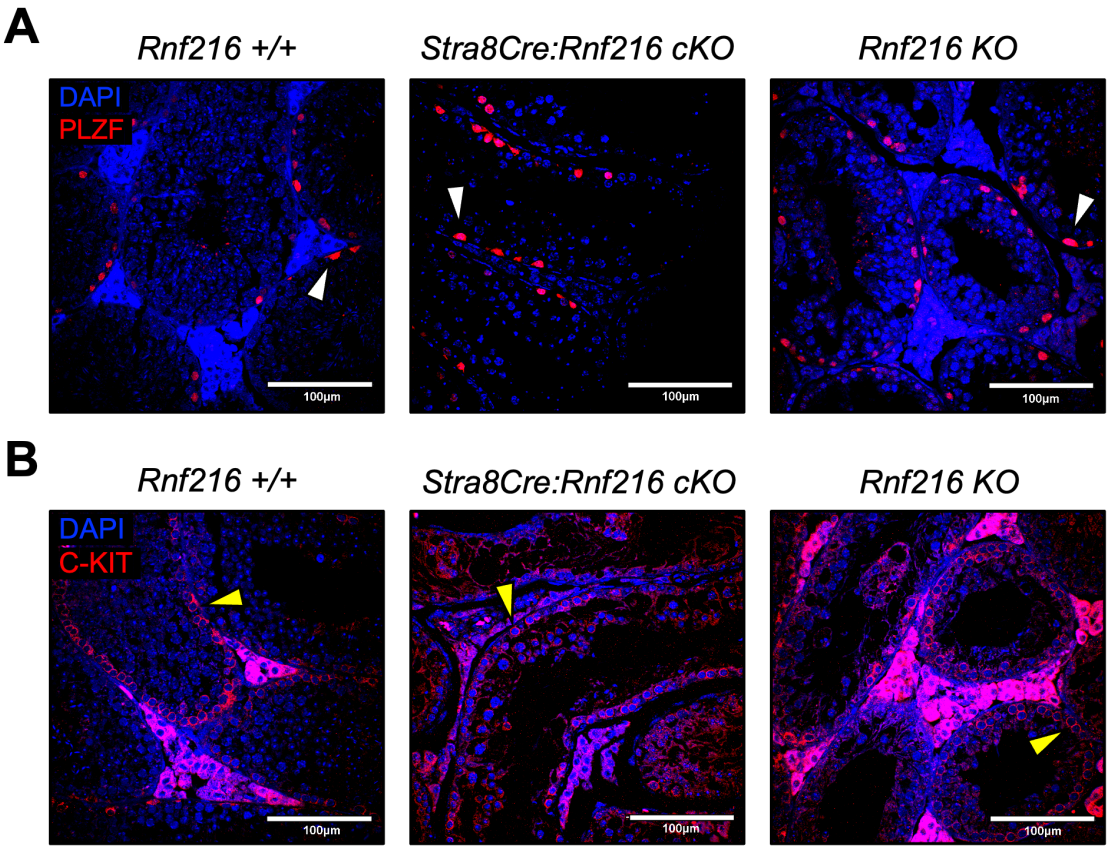

Figure 3.6. Germ cell RNF216 is not required in undifferentiated spermatogonia for proliferation, self-renewal, or survival.
A. Confocal images of mouse *Rnf216 +/+, Stra8Cre:Rnf216 cKO*, and *Rnf216 KO* adult (3 mo.) testes immunofluorescently stained with undifferentiated spermatogonia, white arrowheads, marker, PLZF (red). Scale bars 100 µm. Images are representative of three biological repeats. **B.** Confocal images of mouse *Rnf216 +/+, Stra8Cre:Rnf216 cKO*, and *Rnf216 KO* adult (3 mo.) testes immunofluorescently stained for differentiating spermatogonia, yellow arrowheads, marker, C-KIT (red membrane staining). Scale bars 100 µm. Images are representative of three biological repeats.

CHAPTER IV.

HUMAN GORDON HOLMES SYNDROME MODELING IN MICE REVEALS ESSENTIAL FUNCTION OF RNF216 DIRECTED UBIQUITINATION IN SPERMATOGENESIS AND MALE FERTILITY

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Chapter IV has been modified from Mann et al., 2025, submitted to *Genes & Diseases*.

This study was supported by the National Institutes of Health R01GM132490 and R01HD084494, and the National Institute of Food and Agriculture MICL02690.

4.1 Introduction

Gordon Holmes Syndrome (GHS) is a devastating disease characterized by neurological and reproductive dysfunction, including ataxia, dementia, and hypogonadotropic hypogonadism [104-112]. While neurological symptoms occur in both males and females, more severe reproductive phenotypes have been observed in male patients at the clinical level [105]. Currently, these patients have few positive outcomes, and little is known on disease etiology and progression at the cellular and molecular levels. However, a breakthrough occurred when whole-genome sequencing of GHS patients identified mutations in Ring finger protein 216 (*Rnf216*), coding for an E3 ubiquitin ligase, and OTU domain-containing protein 4 (*Otud4*), coding for a deubiquitinase [105]. Of these male patients, those harboring homozygous or compound homozygous mutations of RNF216 alone presented both neurological and reproductive disorders, suggesting RNF216 is the primary underlying genetic factor in GHS onset and progression [105]. Interestingly, a male patient with a heterozygous RNF216 mutation also showed slower developing symptoms, suggesting GHS may be oligogenic in nature [105]. Nevertheless, additional case reports describing GHS in patients with RNF216 mutations have been published, solidifying the genetic link between RNF216 and human GHS development [105-112]. These RNF216 GHS mutations primarily surrounding the catalytically active ubiquitination region, the Ring-between-Ring (RBR) domain, located near the C-terminus of RNF216, making the function of RNF216 as an E3 ubiquitin ligase an interesting area of study.

The presence of the RBR domain places RNF216 into a small subset of E3 ubiquitin ligases, RBRs, due to its molecular mechanism of action for targeted protein

ubiquitination, using both RING domains to effectively coordinate protein ubiquitin-tagging in a stepwise fashion [113, 116, 123, 159]. Briefly, the E2-ubiquitin complex is recruited by the RING1 domain, and the ubiquitin would be transferred to a catalytically active cysteine residue within the RING2 domain to form a temporary thioester bond intermediate. From here, a substrate-recognizing domain on the RBR E3 ligase (like RNF216) would recruit substrate and then the ubiquitin would be attached to either the substrate or an existing ubiquitin on the substrate, forming a ubiquitin chain. The ubiquitin chain linkage type, based on lysine (K) or methionine (M1) attachment position on ubiquitin, would direct the ubiquitin-tagged substrate to a specific fate [138]. This mechanism of action is also observed and well documented in a RBR family member, Parkin, which has been identified in Parkinson's Disease (PD), another well characterized neurodegenerative disease [124-126]. At the molecular level, RNF216 has been described, with various research groups characterizing its *in vitro* behavior, which included a preference for non-canonical protein ubiquitin-chain linkages, including lysine-63 (K63) and K11, which are associated with signaling and DNA-damage pathways rather than just proteasomal degradation (K48-ubiquitination) via the ubiquitin-proteosome system (UPS) pathway [113-115]. In addition, *in vitro* analysis of human GHS RNF216 mutations, notably near the catalytically active cysteine residue within the RING2 domain (human C745), showed these human GHS mutations completely disrupt ubiquitin ligase activity, rendering RNF216 unable to ubiquitinate substrates [113, 114]. These data suggest that the human GHS mutation within this region is detrimental to RNF216-mediated ubiquitination function and could effectively create a catalytically dead mutant protein.

This discovery provided a unique opportunity to examine the role of RNF216 ubiquitination as an RBR E3 ligase in male reproduction, which would improve understanding of the role RNF216 plays on spermatogenesis. RNF216 has been shown to be essential for spermatogenesis and male infertility in mice, but this was with the generation of a protein null mouse model through global *Rnf216* knockout [141, 142]. Since RNF216 contains several protein-binding domains near its N-terminus, including ubiquitin-binding and SUMOylation domains, it is unclear if the function of RNF216 as a ubiquitin ligase alone is the driver for maintaining spermatogenesis in mice [113-115]. To address this question, a transgenic mouse model harboring the human GHS ubiquitin ligase inactivating mutation to investigate the role of RNF216-directed ubiquitination on reproductive development and function was generated. These *Rnf216 GHS* male mice had the human GHS arginine to cysteine mutation (mouse R739C) six residues downstream for the RING2 catalytically active cysteine residue (mouse C733) required for ubiquitin attachment to RNF216. The RNF216 GHS mutant protein was still expressed and localized to male germ cell nuclei, which was the same as native RNF216 expressed in control littermates, hereby only having the altered residue being the sole difference. Using this human GHS mouse model, disrupted spermatogenesis and male infertility were observed and were similar in germ cell degenerative pacing to *Rnf216 KO* mice of identical timepoints. These data are the first to examine the impact of the human RNF216 GHS mutation on male reproduction *in vivo* and indicate that the mechanism of protein ubiquitination by RNF216 is essential for spermatogenesis and male fertility in mice and may lead to the hypogonadotropic hypogonadism phenotypes observed in male GHS patients.

4.2 Materials and Methods

4.2.1 Ethics Statement

All animal procedures were approved by the Institutional Animal Care and Use Committee of Michigan State University (AUF 202200230). All experiments with mice were conducted ethically following institutional guidelines according to the Guide for the Care and Use of Laboratory Animals.

4.2.2 Mouse Models

Rnf216 GHS mutant mice were generated by CRISPR-Cas9 targeting of the mouse *Rnf216* locus (ENSMUSG00000045078). Wild-type NLS-Cas9 protein, synthetic single guide RNA (gRNA) and a single strand DNA oligo (ssODN) donor template were used (Synthego, CA). Protospacer (N)20 and PAM sequences corresponding to the gRNA used were 5'- AACCGCATGTCTTGCCGCTC -TGG -3' and the sequence of the ssODN is 5'- CATAGCCATTGATAGAAACTCGACAGAGGTAGCACATCTGGGCACCA CAGCaGCAAGACATGCGGTTGCAGCCTTCAGACTTGAT -3'. The *Rnf216* sequence, gRNA, donor template sequences, and Sanger sequencing results alignment were annotated and visualized on Benchling online platform (benchling.com). The gRNA was incubated with Cas9 protein for 5 minutes at 37°C to pre-form ribonucleoprotein (RNP) complexes. RNPs and the donor ssODN were introduced into C57BL/6J mouse zygotes using a Gene Editor electroporator (BEX CO., LTD, Tokyo, Japan). Embryos were implanted into pseudo-pregnant recipients according to standard procedures. Editing of founder offspring was assessed using PCR and Sanger sequencing of the target region. The sequences of primers used for PCR genotyping are listed in **Table A1** on tail biopsies lysed with proteinase K to release genomic DNA. PCR products underwent further

restriction digestion using a mixture of restriction enzyme MspA1I (R0577S, New England Biolabs) (CA/CG:CG/TG, blunt ends), CutSmart Buffer, and ddH_2O and incubation at 37°C for 1 hour, followed by 65°C for 20 minutes. Reaction products were run on 2% agarose gel. Wild-type allele containing the restriction site yielded two PCR products at 267 base pairs (bp) and 213 bp. *Rnf216 GHS* point mutation allele without the restriction site generated a single PCR product at 480 bp. Male founder 480 bp product bands were excised from agarose gels using E.N.Z.A Gel Extraction Kit (D2500-01, Omega) following manufacturer instructions. Extracted PCR products underwent Sanger sequencing and were used to confirm the correct mutation and location using UGENE v50.0 software (Unipro).

4.2.3 Fertility Tests

Rnf216 GHS homozygous (*Rnf216 R739C/R739C*, "*Rnf216 GHS*") and control (*Rnf216* +/+) male mice were bred with *Rnf216 +/+* (control) female mice of a similar age for four continuous months (*n*=3). The number of litters and number of pups for each mating cage were recorded during this time. All mice were given identical diets, access to water, and bedding materials. Expanded data regarding these matings can be found in **Table A6**.

4.2.4 Histology

Mouse testes and epididymides were harvested, washed briefly in 1 x Phosphate-Buffered Saline (PBS), fixed overnight in either 4% paraformaldehyde (PFA) or Bouin's fixative at 4°C on a rocker, then embedded in paraffin. For morphology analysis, Bouin's-fixed and embedded tissues were sectioned at 5 μm and stained with hematoxylin and eosin after dewaxing and rehydration. For immunofluorescence staining and TUNEL

assays, 4% PFA-fixed and embedded tissues were sectioned at 5 μm.

4.2.5 Immunofluorescence

Testis were fixed in 4% PFA overnight on a rocker at 4°C and then embedded in paraffin. Testis were sectioned at 5 μm and incubated overnight at 37°C, then dewaxed and rehydrated. Antigen retrieval was performed in Tris-EDTA buffer (pH 9.0). Testis sections were blocked in 5% Normal Goat Serum (NGS) for 1 hour at room temperature. Testis sections were incubated with α-RNF216 (1:100; A304-111A, Bethyl Laboratories) or α-TRA98 (1:100; ab82527, Abcam) in 5% NGS at 4°C overnight. After washing with 1 x PBS, sections were incubated with goat α-rabbit IgG Alexa Fluor 555 (1:500; A21429, Invitrogen) or goat α-rat IgG Alexa Fluor 555 (1:500; A21434, Life Technologies) for 1 hour and mounted with Vectashield mounting media with DAPI. A complete list of antibodies may be found in **Table A2**. Fluorescence microscopy was performed using Fluoview FV1000 confocal microscope (Olympus) at the Michigan State University Center for Advanced Microscopy, East Lansing, MI, USA.

4.2.6 TUNEL Assay

In Situ Cell Death Detection Kit - Fluorescein (11684795910, Roche) was used to evaluate cell death in 4% PFA-fixed paraffin-embedded 6-week testes sections according to manufacturer's instructions. Fluorescence images were captured by Fluoview FV1000 confocal microscope (Olympus) at the Michigan State University Center for Advanced Microscopy, East Lansing, MI, USA.

4.2.7 Statistical Analysis

All data are presented as mean ± SEM. The two-sample *t* test was used for all statistical analyses. Statistical significance was determined by $p < 0.05$.

4.3 Results

4.3.1 Human RNF216 GHS mutation was successfully introduced into mouse Rnf216

To investigate the impact of RNF216-directed ubiquitination *in vivo*, a human GHS mouse model was developed. Using CRISPR-Cas9 technology, the human GHS E3 ubiquitin ligase inactivating point mutation of *RNF216* resulting in the substitution of arginine 751 (R751) to cysteine (R751C) in RNF216, was successfully introduced into mouse *Rnf216* gene loci (C57BL/6 background) (**Figure 4.1 A**). The mouse *Rnf216* locus was targeted with single guide RNA (gRNA) in part to introduce the identically located point mutation (GGC → TGC) at amino acid residue 739 (R739C) (mouse R739 = human R751) (**Figure B5 A**). This residue lies six residues downstream from the catalytically active cysteine residue (mouse C733, human 745) required for ubiquitin attachment to the RNF216 RING2 domain via thioester intermediate during the ubiquitination cascade [113, 114, 116, 123]. Using nucleotide sequence alignment, this catalytically active cysteine residue and mutated arginine reside in human GHS are conserved between human and mice and reside within the E3 ligase Ring-between-Ring (RBR) domain (**Figure 4.1 B**). Furthermore, to validate the transferability of the human GHS mutation in the mouse germline, F0 founders were bred with *Rnf216* +/+ to produce heterozygous F1 *Rnf216 R739C/*+ pups. Heterozygous *Rnf216 R739C/*+ males and females showed no reproductive defects and we able to produce offspring (data not shown). These F1 pups were crossed once more to generate F2 homozygous *Rnf216 R739C/R739C* males (hereby "*Rnf216 GHS*") pups (**Figure B5 B**). *Rnf216 GHS* mice DNA were genotyped, restriction digested, and underwent Sanger sequencing, confirming the correct human GHS mutation location (**Figure B5 C-D**).

4.3.2 Rnf216 GHS male mice have disrupted spermatogenesis and are infertile

Upon initial examination, the overall appearance of *Rnf216 GHS adult male* mice was identical to *Rnf216* +/+ littermates (**Figure 4.1 C**). However, *Rnf216 GHS* testis size was significantly decreased in adulthood (0.029 g ± 0.001 g, *n*=10) compared to *Rnf216* +/+ testis of the same age (0.100 g ± 0.002 g, *n*=8) ($p < 0.0001$) (**Figure 4.1 D-E**). To examine the impact of human GHS mutation on male fertility in mice, *Rnf216 GHS* males were bred with *Rnf216* +/+ (control) females for 4 continuous months. Likewise, *Rnf216* +/+ males were also bred for the same 4-month span with *Rnf216* +/+ females. The *Rnf216* +/+ males showed no fertility problems (6.8 pups/litter ± 0.9 pups/litter, *n*=3), while *Rnf216 GHS* males were unable to father pups and no live litters were recorded (0.0 pups/litter ± 0.0 pups/litter, *n*=3) ($p < 0.0001$) (**Figure 4.1 F**), thus suggesting defects in spermatogenesis and/or male reproductive development and function.

4.3.3 RNF216 GHS protein is still expressed in male germ cells

One concern with *Rnf216 GHS* mice was the production of RNF216 GHS protein *in vivo*. To accurately evaluate the impact of the human GHS mutation on RNF216 ubiquitination function in male reproduction, *Rnf216 GHS* male testis sections, along with *Rnf216* +/+ and *Rnf216 KO*, were immunofluorescently stained with α-RNF216 to see if RNF216 GHS protein was still expressed, rather than generating a null protein (like in *Rnf216 KO* mice). RNF216 GHS was expressed within select male germ cell populations and localized to the nucleus in *Rnf216 GHS* adult mice, which was identical to native RNF216 expression in *Rnf216* +/+ male mice (**Figure 4.2**). This suggests that the human GHS mutation in *Rnf216 GHS* mice did not ablate RNF216 GHS expression nor alter its overall localization, but only prevented its ability to ubiquitinate substrates, as shown *in*

vitro in reported studies [113, 114].

4.3.4 Rnf216 GHS male mice display progressive germ cell degeneration throughout life

With RNF216 GHS expression confirmed, hematoxylin and eosin (H&E)-stained *Rnf216 GHS* testis and epididymis sections were examined for spermatogenesis progression at several timepoints. Histological analysis of *Rnf216 GHS* testis showed extensive germ cell loss along with azoospermia in cauda epididymis sections (**Figure 4.3**). This resounded with the germ cell loss and cellular debris-filled cauda epididymis observed in *Rnf216 KO* mice, suggesting the human GHS *RNF216* mutation was detrimental to mouse male reproduction. *Rnf216 GHS* germ cell degeneration began around 2-weeks of life and continued at a heterogenous pace into late adulthood (6 mo.) (**Figure 4.3**). Not all *Rnf216 GHS* seminiferous tubules had similar degeneration patterns, with some retaining some meiotic spermatocyte populations into early adulthood (3 mo.). On the other hand, some *Rnf216 GHS* seminiferous tubules degenerated at a faster rate, leaving little to no male germ cells present. Ultimately, this phenotype was similar to *Rnf216 KO* male mice, suggesting RNF216-directed ubiquitination was essential for spermatogenesis and male fertility *in vivo*.

4.3.5 RNF216 GHS mutation causes decreased germ cell populations via increased apoptosis

To characterize germ cell loss in *Rnf216 GHS* adult testis, testis sections were immunofluorescently stained with male germ cell marker, TRA98 (**Figure 4.4 A**) [160]. Male germ cell populations were decreased in *Rnf216 GHS* adult male mice compared to *Rnf216 +/+* male littermates, confirming extensive germ cell loss due to the human GHS *RNF216* mutation. To determine the mechanism of this germ cell depletion, TUNEL

assays were performed on 6-week *Rnf216 GHS* testis, prior to considerable germ cell loss seen in later adulthood (3 mo.) (**Figure 4.4 B**). Indeed, the abundance of apoptosis was increased in *Rnf216 GHS* mice germ cells compared to *Rnf216 +/+* male testis. This increased rate of germ cell apoptosis in *Rnf216 GHS* testis may be connected to the appearance of cellular debris and lack of sperm within the cauda epididymis (**Figure 4.3**).

4.4 Discussion

The introduction of reported human GHS RNF216 mutation (R751C) into the E3 ubiquitin ligase domain of mouse RNF216 (*Rnf216 R739C/R739C*, "*Rnf216 GHS*"), shown to be ubiquitin ligase inactivating *in vitro*, led to male infertility [113, 114]. Spermatogenesis was incompletely arrested and led to the absence of mature sperm in the cauda epididymis. The human GHS mutation in mice led to substantial germ cell loss and apoptosis, suggesting RNF216 ubiquitination function alone is essential for male fertility. This further narrows the key molecular function of RNF216 in spermatogenesis and the reproductive dysfunction observed in these male GHS patients. This was supported by RNF216 GHS still being expressed and localizing in male germ cells like *Rnf216 +/+* male RNF216 protein yet still having a consequential impact of spermatogenesis like global RNF216 loss (*Rnf216 KO*) in mice [141, 142]. The increased apoptosis and decreased germ cell counts occurring in *Rnf216 GHS* seminiferous tubules suggest that RNF216-mediated ubiquitination of substrates in male germ cells is essential to maintain germ cell viability during spermatogenesis. This mouse model was the first *in vivo* model of disordered RNF216 ubiquitination seen in human GHS and its impact on male reproduction, making it a useful tool for understanding how RNF216 mutations may contribute to human GHS development and disease progression.

While this *Rnf216 GHS* mouse model demonstrated the essentiality of RNF216-mediated ubiquitination on spermatogenesis, further questions remain as of the role of RNF216 in human disease. While most human mutations observed in GHS males were located near or within the RBR region, there were some that existed closer to RNF216 N-terminal protein domains. RNF216 protein structure predicts additional functions outside of protein ubiquitination, but it is unclear if these are also essential for male reproductive function, leaving room for these mutations to be better studied *in vitro* and *in vivo* [113-115]. Additionally, the generation of a RNF216 GHS mutation model could be useful in a neurological context since the *Rnf216 GHS* mutation is global and RNF216 has shown to be expressed and function within the brain [141, 143]. Whether RNF216 E3 ligase ubiquitination is essential in the brain for proper neurological function is still unknown, although brain specific conditional knockout of RNF216 in mice showed neurodegenerative symptoms [143]. Lastly, the function of a E3 ubiquitin ligase is dependent on having substrate(s) to ubiquitinate, yet only one potential substrate has been suggested in testis to date [142]. However, this substrate was shown to have higher expression in the absence of RNF216, with the authors suggesting RNF216 ubiquitinated it for proteasomal degradation [142]. The emergence of RNF216 ubiquitin chain-link specificity of K63 and K11 *in vitro*, which are less associated with the ubiquitin-proteosome system (UPS) protein degradation, but rather cellular pathways including DNA damage repair, protein complex assembly, and other signaling pathways, suggest other potential substrates may still exist in male germ cells [113-115]. Therefore, it is important to further investigate the germ cell specific, as well as brain specific, pathways of RNF216 since substrates reliant on K48 ubiquitin chain-linkages have been proposed

so far. While these previous proposed substrates may still be accurate to an extent, it is possible that an indirect interaction of RNF216 through ubiquitination of another protein ultimately leads to UPS degradation rather than direct RNF216 K48 ubiquitin chain-linkages.

In summary, this work is first to reveal consequences of human GHS RNF216 ubiquitin ligase domain inactivation mutation *in vivo*. RNF216 E3 ubiquitin ligase function is essential for spermatogenesis and fertility, and loss of RNF216-mediated ubiquitination led to both germ cell loss and increased apoptosis in testis. The introduction of the human GHS mutation did not alter RNF216 expression and localization in male germ cells, with only the E3 ubiquitin ligase function disrupted rather than interference with localization signals, predicted N-terminal domains, or the generation of a null protein. *Rnf216 GHS* mice provide another useful model to investigate targets of RNF216 ubiquitination and validate ubiquitination of proteins in germ cell development at the molecular level and can be used for future experiments to identify new potential RNF216 substrates of K63 or K11 ubiquitin chain-linkages. Therefore, RNF216 ubiquitin ligase inactivating human GHS mutation in *Rnf216 GHS* male mice contributes to reproductive dysfunction and may be used to advance understanding for both GHS etiology and male reproduction.

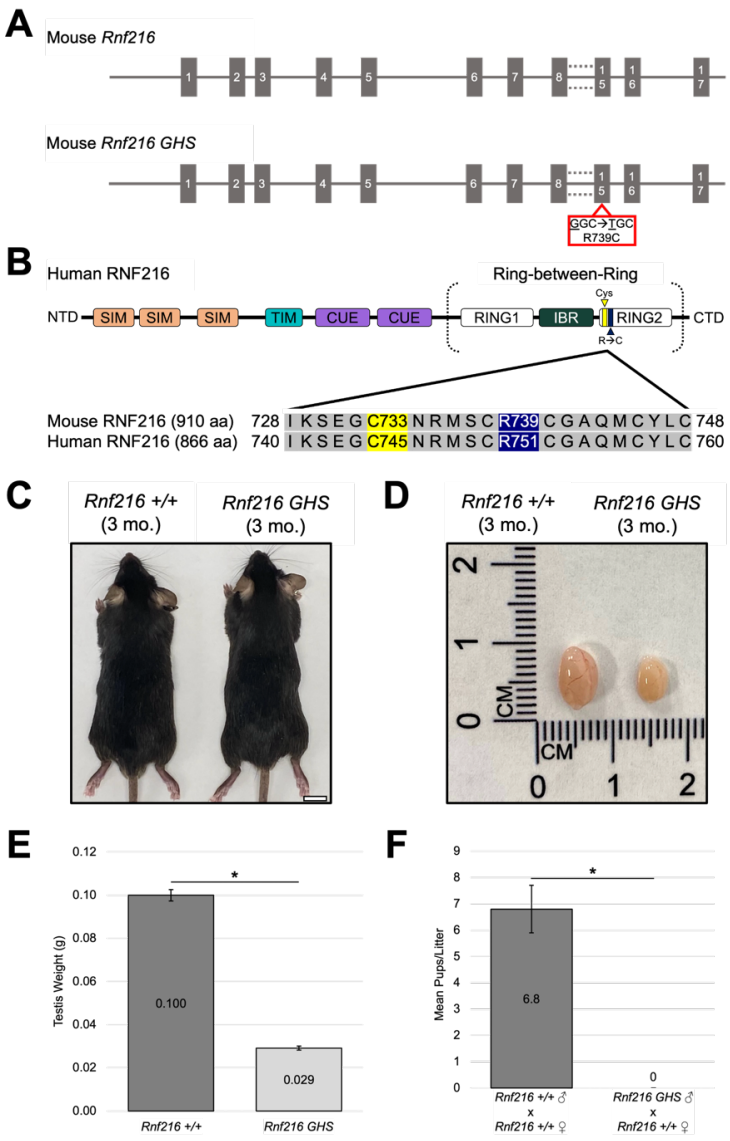

Figure 4.1. Human GHS E3 ubiquitin ligase inactivating RNF216 mutation causes male infertility in mice.

A. Gene loci for mouse *Rnf216 +/+* and *Rnf216 GHS* with human GHS point mutation inserted into *Rnf216* exon 15 via CRISPR-Cas9 technology. **B.** Upper: protein architecture for human RNF216. Catalytically active cysteine (yellow) is six residues upstream from human GHS mutation residue (blue). SIM, SUMO-interacting motif (orange); TIM, TRAF-interacting motif (turquoise); CUE, coupling of ubiquitin conjugation to ER degradation domain (purple); RING, really interesting new gene (white); IBR, in-between-RING (green). Lower: protein alignment for human and mouse RNF216 showing catalytically active cysteine residue (yellow) and human GHS mutation residue (blue) are conserved along with other conserved resides (grey). **C.** Gross comparison of adult *Rnf216 +/+* and *Rnf216 GHS* mice. Scale bar 1 cm. **D.** Comparison of adult *Rnf216 +/+* and *Rnf216 GHS* testis. **E.** Statistical comparison of *Rnf216 +/+* and *Rnf216 GHS* testis wet weights after dissection. **p* < 0.05. **F.** Statistical comparison of adult *Rnf216 +/+* and *Rnf216 GHS* mice during 4-month breeding trials. **p* < 0.05.

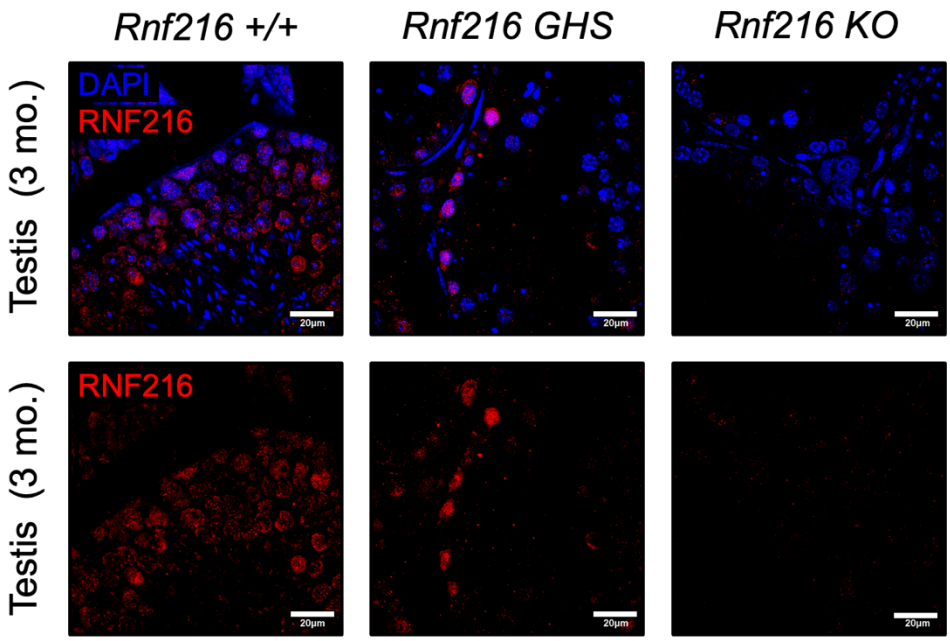

Figure 4.2. RNF216 GHS expression and localization matches native RNF216 in mouse male germ cells.
Confocal images of mouse *Rnf216 +/+*, *Rnf216 GHS*, and *Rnf216 KO* adult testis immunofluorescently stained with α-RNF216 primary antibody (red). Scale bars 20 μm. Images are representative of three biological repeats.

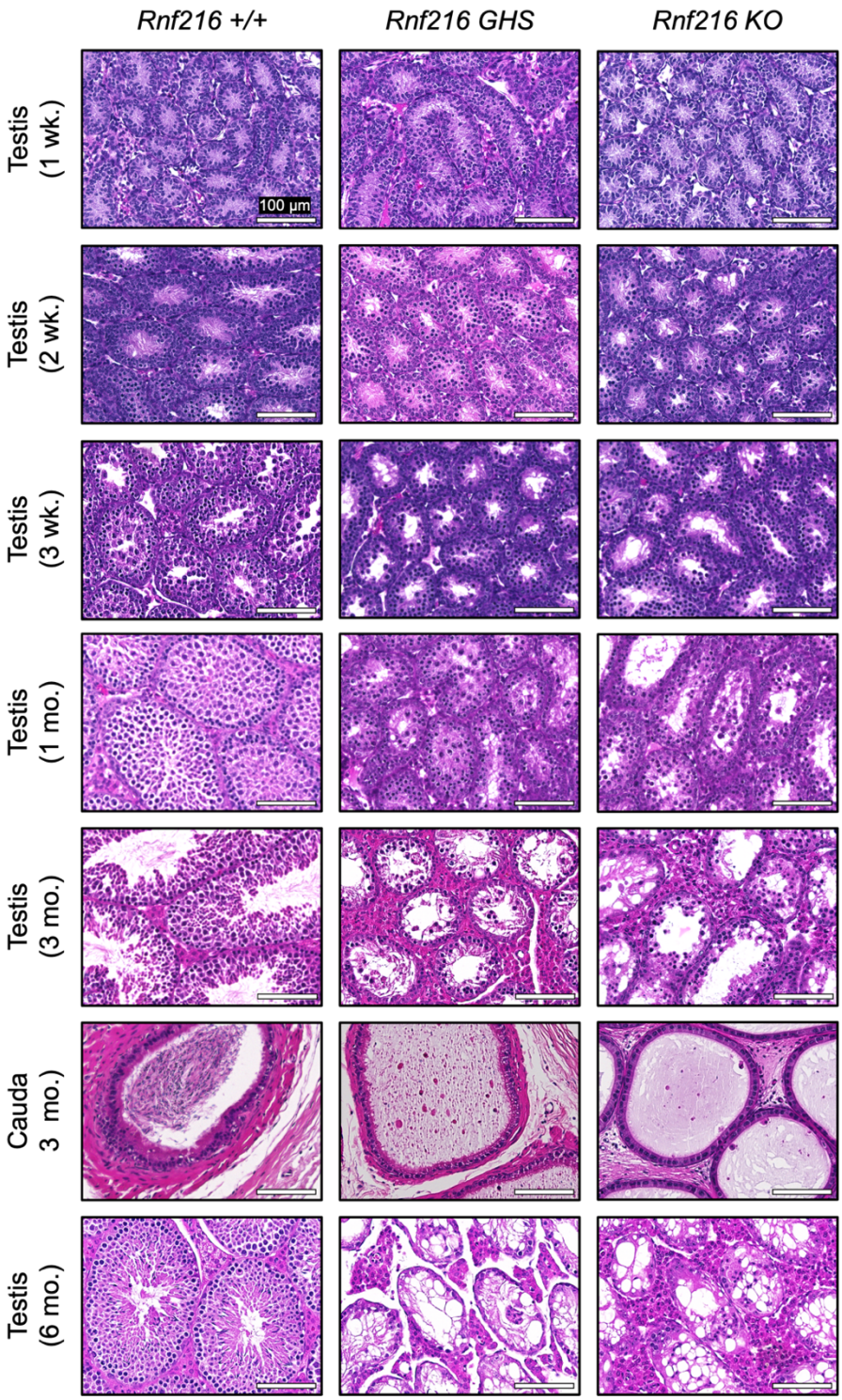

Figure 4.3. *Rnf216 GHS* male mice display progressive germ cell degeneration throughout life.

Hematoxylin and eosin (H&E)-stained testis and epididymis of *Rnf216* +/+ (control), *Rnf216 GHS*, and *Rnf216 KO* mice across various timepoints. Scale bars 100 µm. Images are representative of three biological repeats.

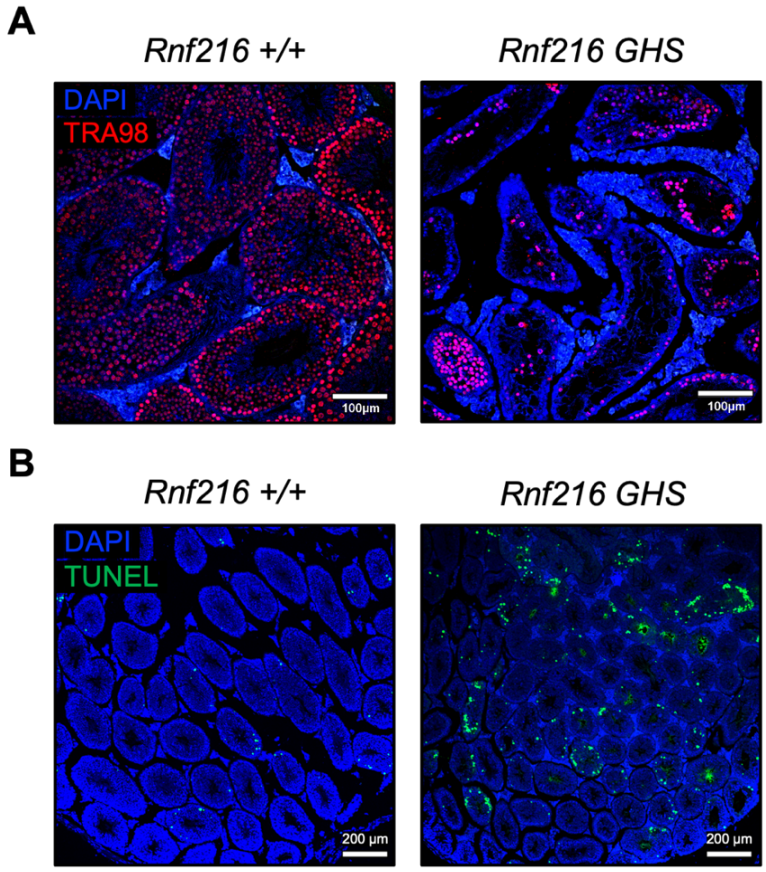

Figure 4.4. RNF216 GHS mutation causes decreased germ cell populations and increased apoptosis in mice.
A. Confocal images of mouse *Rnf216 +/+* and *Rnf216 GHS* adult (3 mo.) testes immunofluorescently stained with germ cell marker, TRA98 (red). Scale bars 100 μm. Images are representative of three biological repeats. **B.** Confocal images of mouse *Rnf216 +/+* and *Rnf216 GHS* young adult (6-week) testes immunofluorescently stained for apoptosis (TUNEL) (green). Scale bars 200 μm. Images are representative of three biological repeats.

CHAPTER V.

DISCUSSION AND FUTURE DIRECTIONS

5.1 Germ cell Rnf216 conditional knockout and human GHS Rnf216 mutation mirror Rnf216 global knockout reproductive phenotype in male mice

Through this dissertation research, the use of mouse models of RNF216 presented an opportunity for comparison. For example, KI-RNF216 expression was shown to be identical to native RNF216 in male germ cells (**Figure B2**), serving as an alternative model to examine RNF216 expression and localization in male germ cells. For the other mouse models involving *Rnf216* mutation or deletion, each mouse was viable can could be raised to identical timepoints. Global deletion of *Rnf216* was previously established (*Rnf216 KO*) and the same *Rnf216 KO* mouse line used by Melnick, et al. was used in these studies [141]. Additionally, the germ cell specific deletion of *Rnf216* in *Stra8Cre:Rnf216 cKO* males (identical in phenotype to *DDX4Cre:Rnf216 cKO* males (**Figure B4**)) and human GHS E3 ligase inactivating mutation mouse model, *Rnf216 GHS*, were designed to examine the impact of altered RNF216 function or expression on spermatogenesis *in vivo*. Though the studies in Chapters III and IV, each of these novel germ cell conditional knockout or mutant *Rnf216* mouse lines had a direct comparison and predominantly matching phenotype to previously published *Rnf216 KO* male mice [141, 142]. Likewise, female *Stra8Cre:Rnf216 F/-, DDX4Cre:Rnf216 F/-, and Rnf216 GHS* female mice were all fertile, consistent with *Rnf216 KO* females (**Figure B6**) [141].

KI-Rnf216 adult male mice were completely fertile and showed no signs of disrupted spermatogenesis, which was identical to *Rnf216 +/+* adult males. The only difference was the insertion of a N-terminal peptide tag that did not alter RNF216 protein

expression or function. On the other hand, *Stra8Cre:Rnf216 cKO* and *Rnf216 GHS* adult male mice had strikingly similar reproductive phenotypes as *Rnf216 KO* males (**Figure 5.1**). All three of these mouse *Rnf216* germ cell conditional knockout or mutation models (four including *DDX4Cre:Rnf216 cKO*) exhibited germ cell loss, seminiferous tubule degeneration, and infertility. Likewise, the germ cell loss was progressive throughout the lifespan of each of these mice, leading to extensive germ cell population exhaustion in late adulthood. While the cauda epididymis histology was very similar between these lines, the occasional sperm (motility unconfirmed) was present in *Stra8Cre:Rnf216 cKO* (and *DDX4Cre:Rnf216 cKO)* males at an extremely low frequency and concentration, still rendering these males infertile. Each *Rnf216* germ cell conditional knockout or mutation male mouse was ultimately infertile, which allowed for the role of RNF216 to be better defined with the data from each of these lines. This data can be used to better understand the requirement of RNF216 at both the cellular and molecular levels and provide new information that can improve knowledge of germ cell biology, fertility, and human disease surrounding RNF216 and other RBR E3 ligases involved in male reproduction.

5.2 Summary

In summary, the work performed in this dissertation investigated RNF216 expression, its germ cell specific requirement, and molecular mechanism surrounding spermatogenesis and fertility in mice. Each of these mouse models, and the experiments done in their corresponding Chapters of this dissertation, contributed new information on the overall function of RNF216 in male germ cell biology and reproduction. The key conclusions derived from data collected in Chapters II – IV are visually summarized in **Figure 5.2**.

Chapter II (**Figure 5.2 A**) established a novel epitope tag knock-in (KI) *Rnf216* mouse model (*KI-Rnf216*) that was fertile and showed no reproductive dysfunction. KI-RNF216 had identical expression and localization in male germ cells as native RNF216, with enhanced specificity using primary antibodies targeting the KI peptide tag. Immunofluorescence staining of *KI-Rnf216* adult mouse testis revealed male germ cell specific RNF216 expression and absence of expression within the somatic cells of the testis or epididymis. Furthermore, RNF216 was expressed in specific male germ cell types across seminiferous tubule stages, with a large window of expression from undifferentiated (type A) spermatogonia through early pachytene spermatocytes, then brief expression in late round spermatids prior to elongation in spermiogenesis. The localization of KI-RNF216 within the nuclei of spermatogonia and spermatocytes showed a compartmentalized pattern. This staining pattern suggests that RNF216 may be acting in cellular pathways within the nuclei of male germ cells and can be further refined to isolate RNF216 pathways in this setting.

Chapter III (**Figure 5.2 B**) generated two different germ cell conditional knockout (cKO) *Rnf216* mouse models, one in late embryonic development (*DDX4Cre:Rnf216 cKO*) and another in early postnatal life prior to undifferentiated spermatogonia proliferation or differentiation (*Stra8Cre:Rnf216 cKO*). Although earlier deletion of *Rnf216* occurred in *DDX4Cre:Rnf216 cKO* male mice, it phenocopied *Stra8Cre:Rnf216 cKO* testis in adulthood with severe germ cell loss, decreased size, and infertility, suggesting that RNF216 was not required, or potentially not expressed, in gonocytes for spermatogenesis. The seminiferous tubule degeneration in these mice was progressive throughout life and led to some seminiferous tubules apparently void of germ cells,

suggesting that germ cells may be impacted by RNF216 loss all the way back to undifferentiated spermatogonia, which were shown to have high RNF216 expression. However, deletion of *Rnf216* in undifferentiated spermatogonia did not impact the ability for these germ cells to proliferate and survive into adulthood and differentiate to continue spermatogenic progression. This suggests that RNF216 may have a more essential role in later germ cell types, especially during and/or after meiosis. Importantly, these mouse models highlighted the necessity of RNF216 in male germ cells, separate from HPG axis control of reproduction, to maintain proper spermatogenesis and fertility.

Chapter IV (**Figure 5.2 C**) examined the impact of a human RNF216 mutation within the catalytically active Ring-between-Ring (RBR) domain required for E3 ligase ubiquitination of substrates. This mutation was observed in human patients suffering from Gordon Holmes Syndrome (GHS), which presents neurological defects and reproductive defects in males. This mutation was confirmed to disrupt RNF216-directed ubiquitination *in vitro*, thus prompting the generation of an *in vivo* model of this mutation in mouse *Rnf216* (*Rnf216 GHS*). *Rnf216 GHS* male mice were infertile and had testis defects including progressive germ cell loss and increased apoptosis. This model proved that RNF216-driven ubiquitination of substrates was essential for spermatogenesis and fertility in mice and provided an explanation for the reproductive etiology of human GHS in males.

5.3 Future directions for RNF216-focused research

While these studies of RNF216 in male reproduction did answer key biological questions about its expression, localization, germ cell requirement, and mechanism of action, there are additional paths to pursue for future research and applications. The most

prominent gap in knowledge remaining is the placement of RNF216 into specific cellular pathways within male germ cells and identifying substrates in these cells. While one germ cell substrate has been suggested, *in vitro* data regarding RNF216 ubiquitin chain-linkage preferences that are less connected to canonical ubiquitin-proteosome system (UPS) degradation suggest there may be additional substrates to be discovered [113-115]. Furthermore, the discovery of RNF216 being a nuclear protein further restricts potential interactors, and therefore substrates, of ubiquitination in male germ cells. The placement of RNF216 into a cellular pathway would provide valuable information as to how RNF216 modulates spermatogenesis as an E3 ligase. Within these dissertation studies, attempts of RNF216 immunoprecipitation coupled with mass spectrometry have been made to identify new nuclear substrate candidates of RNF216 in mouse testis but have not produced a promising candidate to date (data not shown). Therefore, a different proteomic approach may be required to identify RNF216 substrate(s) in germ cells to address increase this area of knowledge.

Outside of identifying molecular pathways of RNF216 in spermatogenesis, further investigations of RNF216 in male reproduction could be undertaken. While the *in vitro* data of RNF216 ubiquitin chain-linkage type preferences are groundbreaking, they have yet to be confirmed *in vivo*. Additionally, new cases of GHS are documented around the world with novel mutations of RNF216 each year, with some of them residing outside the RBR region. Further research into the requirement of more N-terminal RNF216 protein domains in male reproduction could improve understanding of RNF216 as a whole molecule rather than an E3 ligase alone. With a need for better treatments and therapeutic options for reproductive dysfunction in individuals suffering from GHS, more research on

RNF216 could lead to advances that could be clinically applicable. Lastly, there remains a substantial need for an effective, safe, non-hormonal, and reversible form of male contraceptive for public consumption. While RNF216 may not be an ideal candidate based on the extreme reproductive phenotypes observed in humans with GHS and mutant or deleterious *Rnf216* mouse models, understanding RNF216 as an RBR E3 ubiquitin ligase in a reproductive context could spark new ideas to address this need. Although additional research regarding RNF216 in male reproduction is required to further improve comprehension on its contribution in spermatogenesis, male fertility, germ cell biology, and GHS, the work successfully completed in this dissertation provides convincing data and a strong foundation for future research endeavors regarding RNF216.

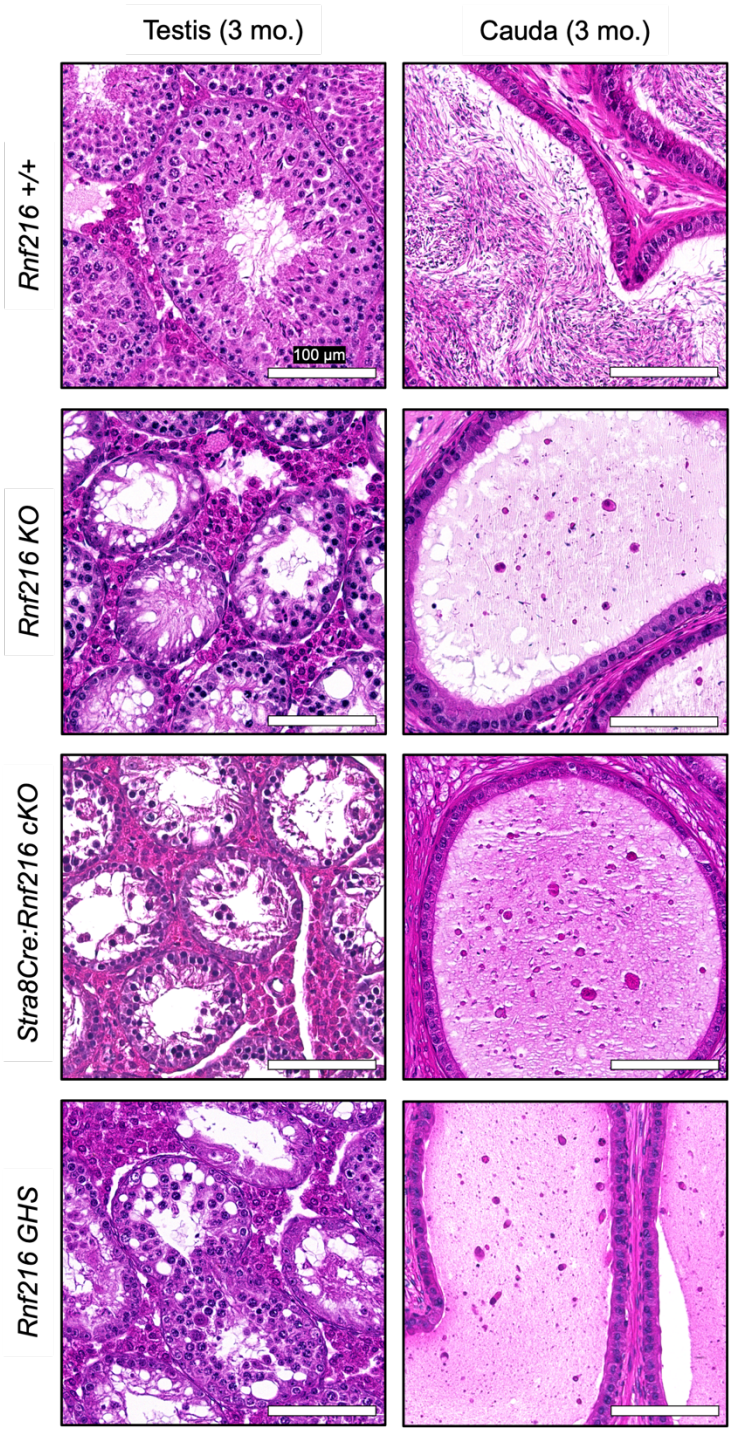

Figure 5.1. Mouse models of *Rnf216 KO*, germ cell *Stra8Cre:Rnf216 cKO*, and *Rnf216 GHS* display similar phenotypes.
Hematoxylin and eosin (H&E)-stained testes and cauda epididymis sections of *Rnf216 +/+* (control), *Rnf216 KO*, *Stra8Cre:Rnf216* cKO, and *Rnf216 GHS* adult mice. Each *Rnf216* mouse line (expect *KI-Rnf216*) exhibits progressive germ cell and seminiferous tubule degeneration leading to male infertility caused by RNF216 ablation or GHS E3 ligase mutation. Scale bars 100 µm. Images are representative of three biological repeats.

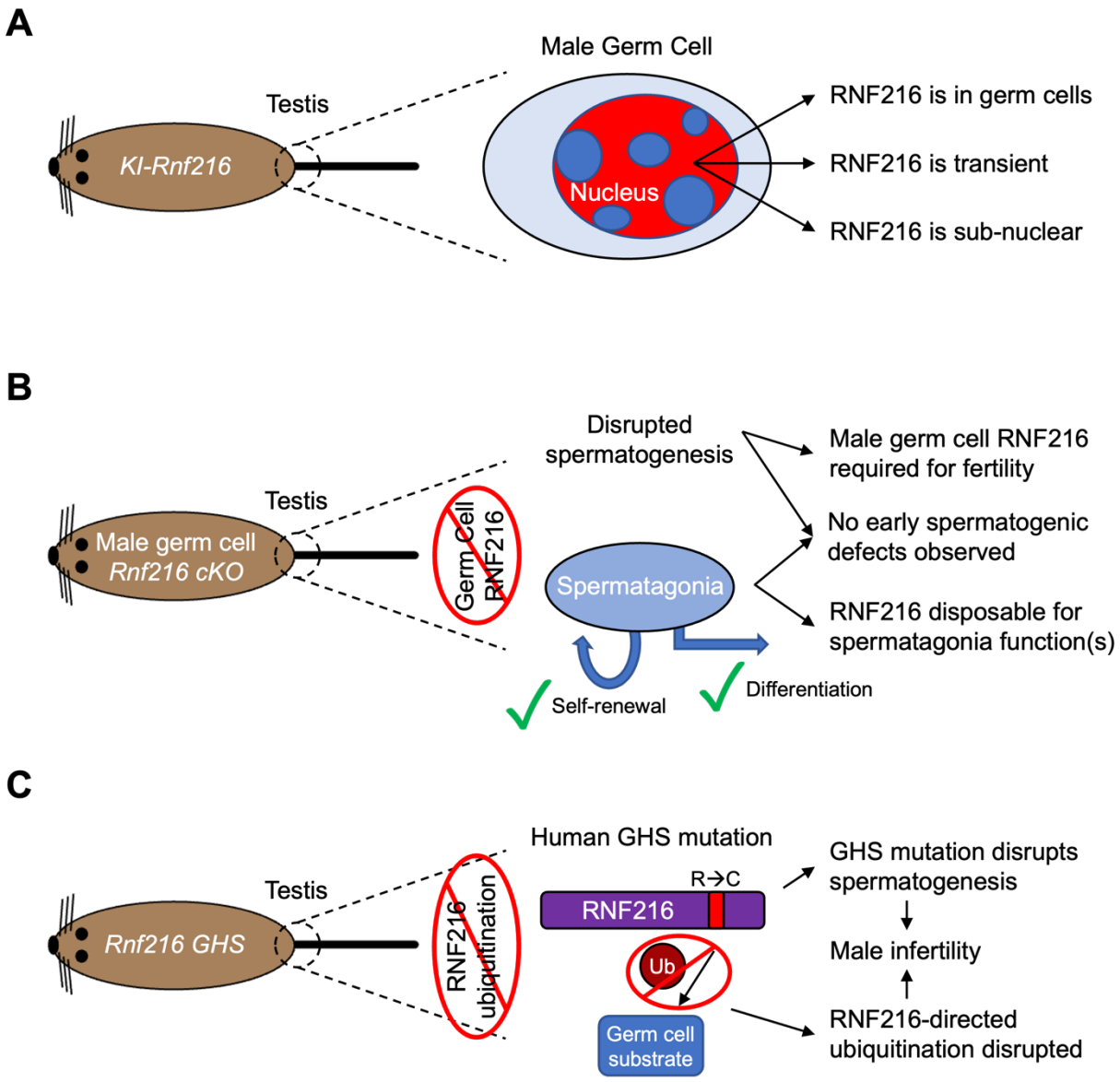

Figure 5.2. Summary of mouse RNF216 involvement in male reproduction.
A. Chapter II schematic of *KI-Rnf216* male mice with expression in select male germ cells. Red color in male germ cell nucleus represents KI-RNF216 expression. **B.** Chapter III schematic of *Stra8Cre:Rnf216 cKO* with reproductive phenotype in testis and undifferentiated spermatogonia. **C.** Chapter IV schematic of *Rnf216 GHS* with human GHS E3 ubiquitin ligase inactivating mutation (R→C, red bar) in RNF216 (purple) and consequential reproductive phenotype. Ub, ubiquitin.

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APPENDIX A.

ADDITIONAL TABLES

Table A1. Primers and primer sequences used in all studies.

Gene	Application	Forward Sequence (5' → 3')	Reverse Sequence (5' → 3')
DDX4-Cre	PCR	GCG GTC TGG CAG TAA AAA CTA TC	GTG AAA CAG CAT TGC TGT CAC TT
Stra8-Cre	PCR	GTG CAA GCT GAA CAA CAG GA	AGG GAC ACA GCA TTG GAG TC
KI-Rnf216	PCR	CCT GCC TCA ACC ACC GAA TT	ACA GTA GAA TCG CTC TGG CT
Rnf216 Flox	PCR	ACC TCG TGG TGC TTG CAT TTG TGT TC	TGG ACC CTC ACA TTG GTT CAC ACA C
Rnf216 GHS	PCR	ATG TCT CGG AAA GGC TCA GG	CTC CGT TTT CCC TGT GGC TA
Rnf216 Null	PCR	GGC GCA TAA CGA TAC CAC GA	AAG CCA AAT CAG AGG ACG GG

Table A2. Antibodies used in all studies.

Antibody	Manufacturer	Catalog Number	Host Species
C-KIT	Cell Signaling	D13A2	Rabbit
FITC-conjugated γH2AX	Millipore	16-202A	Mouse
HA	Cell Signaling	C29F4	Rabbit
PLZF	Santa Cruz Biotechnology	SC-28319	Mouse
PLZF	Santa Cruz Biotechnology	SC-22839	Rabbit
RNF216	Bethyl Laboratories	A304-111A	Rabbit
TRA98 (GCNA1)	Abcam	ab82527	Rat
Anti-mouse IgG Alexa Flour 488	Life Technologies	A11029	Goat
Anti-rat IgG Alexa Flour 555	Life Technologies	A21434	Goat
Anti-rabbit IgG Alexa Flour 555	Invitrogen	A21429	Goat
HRP-conjugated β-actin	Sigma	A3854	Mouse
HRP-conjugated anti-rabbit IgG	Bio-Rad	1706515	Goat

Table A3. *KI-Rnf216* mice four-month fertility tests.

Male Genotype	Female Genotype	n	Total Number of Litters	Total Number of Pups	Mean Pups Per Litter ± SEM	Mean Litter Per Mating Pair ± SEM
Rnf216 +/+	*Rnf216 +/+*	3	7	52	7.4 ± 1.2	2.3 ± 0.7
Rnf216 +/+	*KI-Rnf216*	3	6	48	8.0 ± 1.3	2.0 ± 0.6
KI-Rnf216	*Rnf216 +/+*	3	9	63	7.0 ± 0.8	3.0 ± 0.0
KI-Rnf216	*KI-Rnf216*	3	9	68	7.6 ± 0.7	3.0 ± 0.0

Table A4. *Stra8Cre:Rnf216 cKO* mice four-month fertility tests.

Male Genotype	Female Genotype	n	Total Number of Litters	Total Number of Pups	Mean Pups Per Litter ± SEM	Mean Litter Per Mating Pair ± SEM
Rnf216 +/+	*Rnf216 +/+*	3	10	75	7.5 ± 1.0	3.3 ± 0.3
Rnf216 +/+	*Stra8Cre: Rnf216 F/-*	3	8	56	7.0 ± 0.8	2.7 ± 0.3
Stra8Cre: Rnf216 cKO	*Rnf216 +/+*	3	0	0	0.0 ± 0.0	0.0 ± 0.0
Stra8Cre: Rnf216 cKO	*Stra8Cre: Rnf216 F/-*	3	0	0	0.0 ± 0.0	0.0 ± 0.0

Table A5. *DDX4Cre:Rnf216 cKO* mice four-month fertility tests.

Male Genotype	Female Genotype	n	Total Number of Litters	Total Number of Pups	Mean Pups Per Litter ± SEM	Mean Litter Per Mating Pair ± SEM
Rnf216 +/+	*Rnf216 +/+*	3	10	71	7.1 ± 0.9	3.3 ± 0.3
Rnf216 +/+	*DDX4Cre: Rnf216 F/-*	3	9	70	7.8 ± 0.7	3.0 ± 0.6
DDX4Cre: Rnf216 cKO	*Rnf216 +/+*	3	0	0	0.0 ± 0.0	0.0 ± 0.0
DDX4Cre: Rnf216 cKO	*DDX4Cre: Rnf216 F/-*	3	0	0	0.0 ± 0.0	0.0 ± 0.0

Table A6. *Rnf216 GHS* mice four-month fertility tests.

Male Genotype	Female Genotype	n	Total Number of Litters	Total Number of Pups	Mean Pups Per Litter ± SEM	Mean Litter Per Mating Pair ± SEM
Rnf216 +/+	*Rnf216 +/+*	3	8	54	6.8 ± 0.9	2.7 ± 0.3
Rnf216 +/+	*Rnf216 GHS*	3	9	62	6.9 ± 0.6	3 ± 0.6
Rnf216 GHS	*Rnf216 +/+*	3	0	0	0.0 ± 0.0	0.0 ± 0.0
Rnf216 GHS	*Rnf216 GHS*	3	0	0	0.0 ± 0.0	0.0 ± 0.0

APPENDIX B.

ADDITIONAL FIGURES

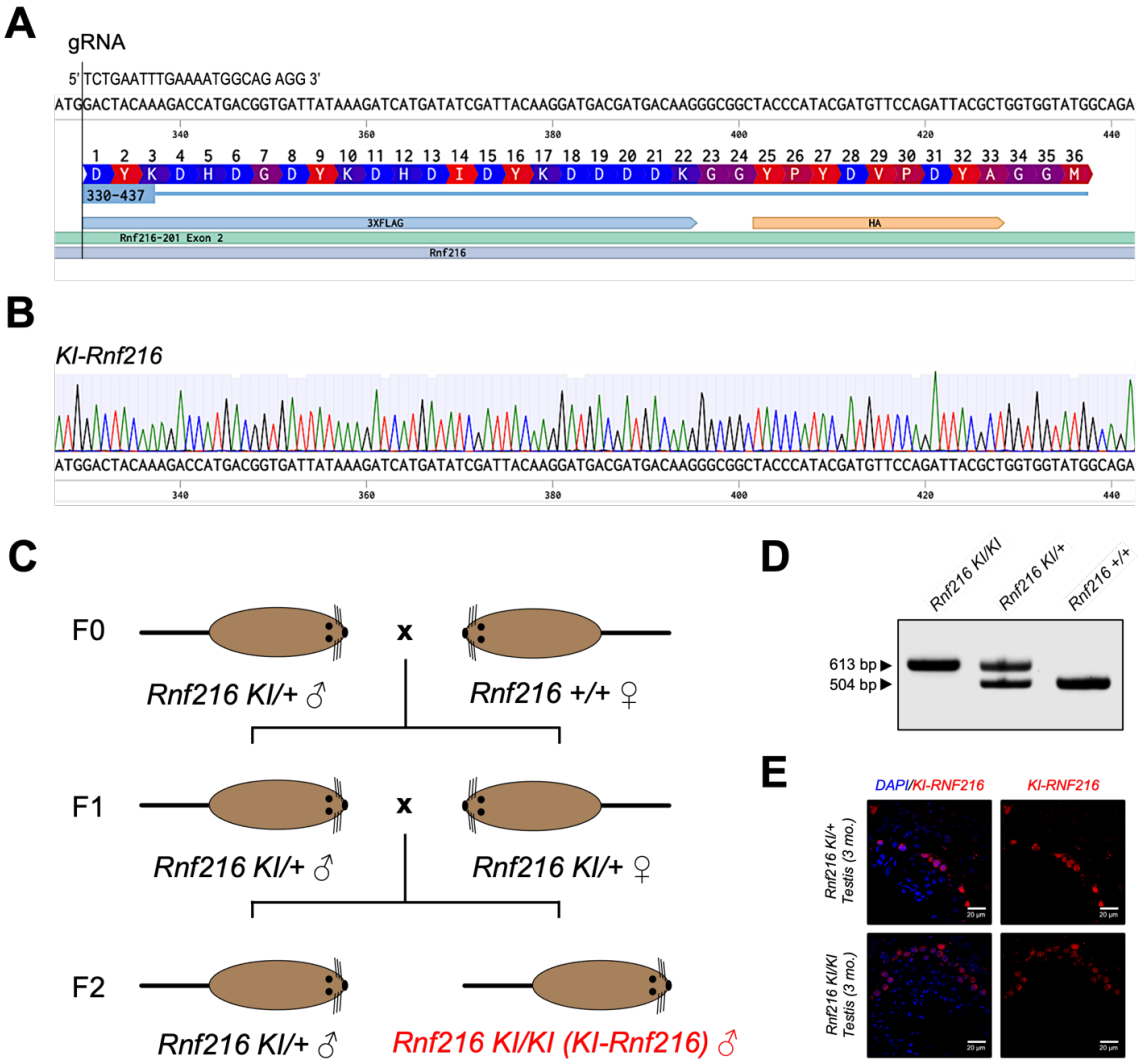

Figure B1. Generation and validation of *KI-Rnf216* mice.
A. Strategy for generation of *KI-Rnf216* (*Rnf216 KI/KI*) mice using single guide (gRNA), ssODN donor template, and CRISPR-Cas9 to introduce 3xFLAG/HA peptide tag sequence (DYKDHDGDYKDHDIDYKDDDDKGGYPYDVPDYA GGM) in mouse *Rnf216* exon 2. **B.** *KI-Rnf216* allele Sanger sequencing results confirming correct 3xFLAG/HA peptide tag insertion and location in F0 mice *Rnf216*. **C.** Mating schematic to generate homozygous F2 *Rnf216 KI/KI* (red) (*KI-Rnf216*) mice using F1 heterozygotes derived from F0 founders. **D.** Representation of PCR to genotype *KI-Rnf216* mice using agarose gel electrophoresis. **E.** Confocal images of heterozygous *Rnf216 KI/+* and homozygous *KI-Rnf216* adult testis showing no difference in KI-RNF216 expression (red) between these mice. Scale bars 20 μm. Images are representative of three biological repeats.

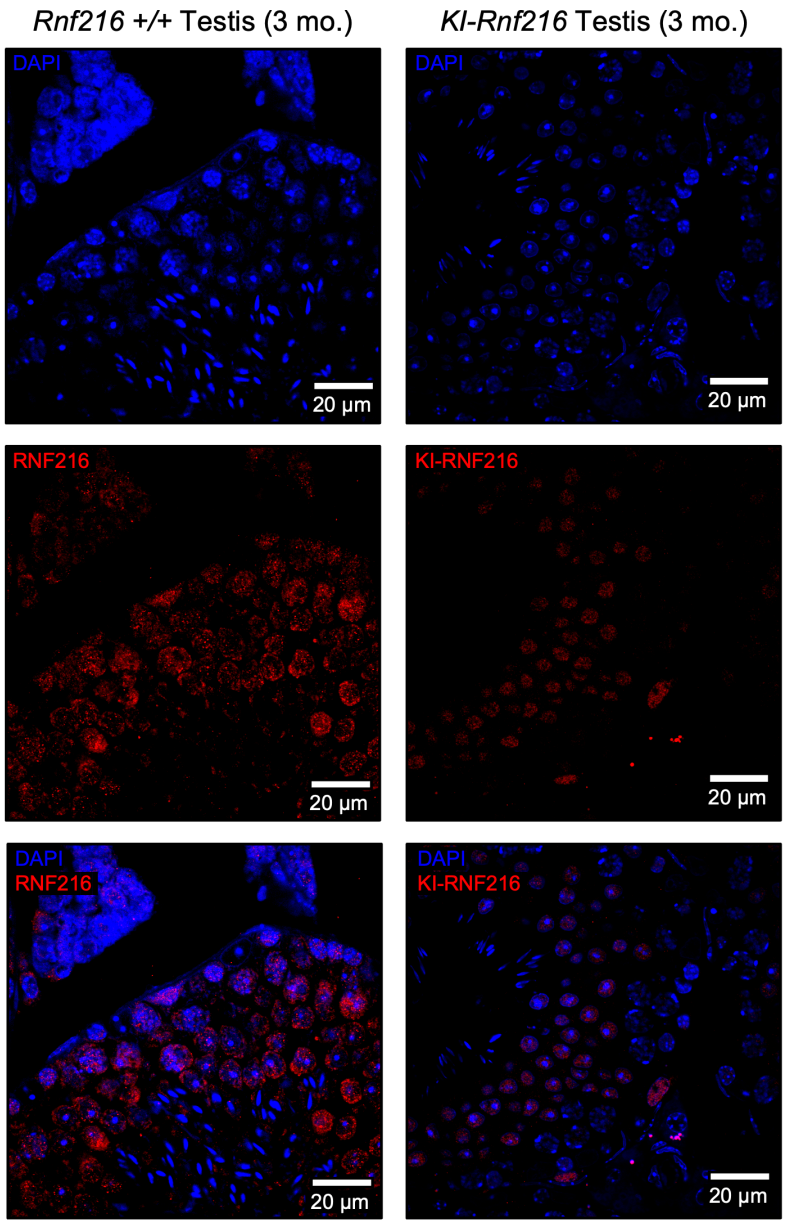

Figure B2. KI-RNF216 expression and localization matches native RNF216 *in vivo* with increased specificity.
Confocal images of *Rnf216* +/+ (control) and *KI-Rnf216* adult testis sections immunofluorescently stained with commercial α-RNF216 and α-HA primary antibodies, respectively. Both native RNF216 (red) and KI-RNF216 (red) are expressed and localized in select male germ cell nuclei with decreased non-specific background signal with α-HA primary antibody immunofluorescence staining. Scale bars 20 µm. Images are representative of three biological repeats.

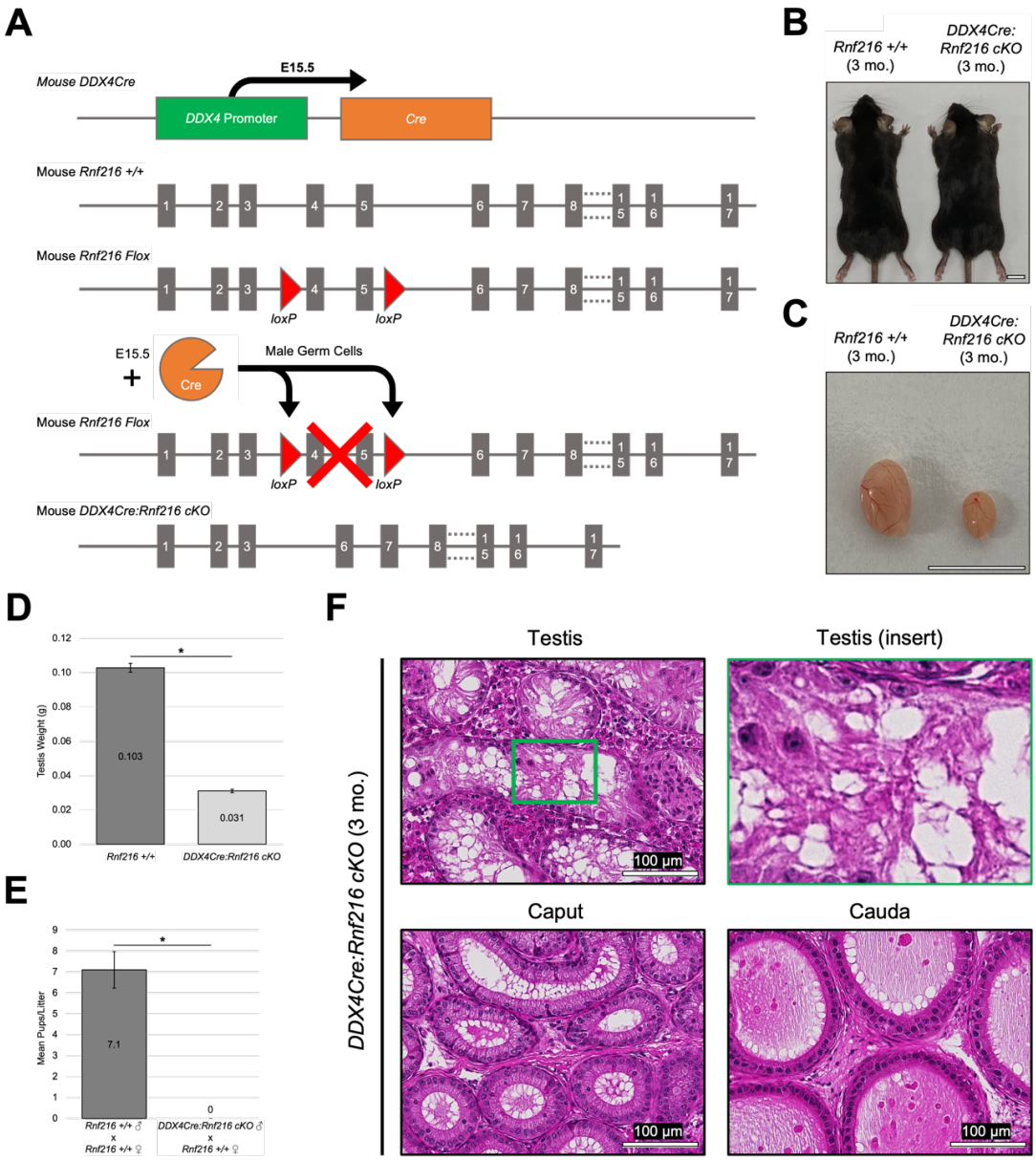

Figure B3. *DDX4Cre:Rnf216 cKO* male mice are infertile.
A. Gene loci for generation of *DDX4Cre:Rnf216 cKO* mice using the *DDX4* promoter as a Cre driver in male germ cells at E15.5 (embryonic day 15.5). Cre recombinase targets inserted *loxP* sites (red triangles) flanking *Rnf216* exons 4 and 5, excising them, to generate a *Rnf216 Null (-)* allele lacking exons 4 and 5 in *DDX4Cre:Rnf216 cKO* male germ cells. **B.** Gross comparison of adult *Rnf216 +/+* and *DDX4Cre:Rnf216 cKO* mice. Scale bar 1 cm. **C.** Comparison of adult *Rnf216 +/+* and *DDX4Cre:Rnf216 cKO* testis. **D.** Statistical comparison of *Rnf216 +/+* and *DDX4Cre:Rnf216 cKO* testis wet weights after dissection. **p* < 0.05. **E.** Statistical comparison of adult *Rnf216 +/+* and *DDX4Cre:Rnf216 cKO* mice during 4-month breeding trials. **p* < 0.05. **F.** Hematoxylin and eosin (H&E)-stained adult *DDX4Cre:Rnf216 cKO* testis, testis insert (green), and caput and cauda epididymis sections. Scale bars 100 µm. Images are representative of three biological repeats.

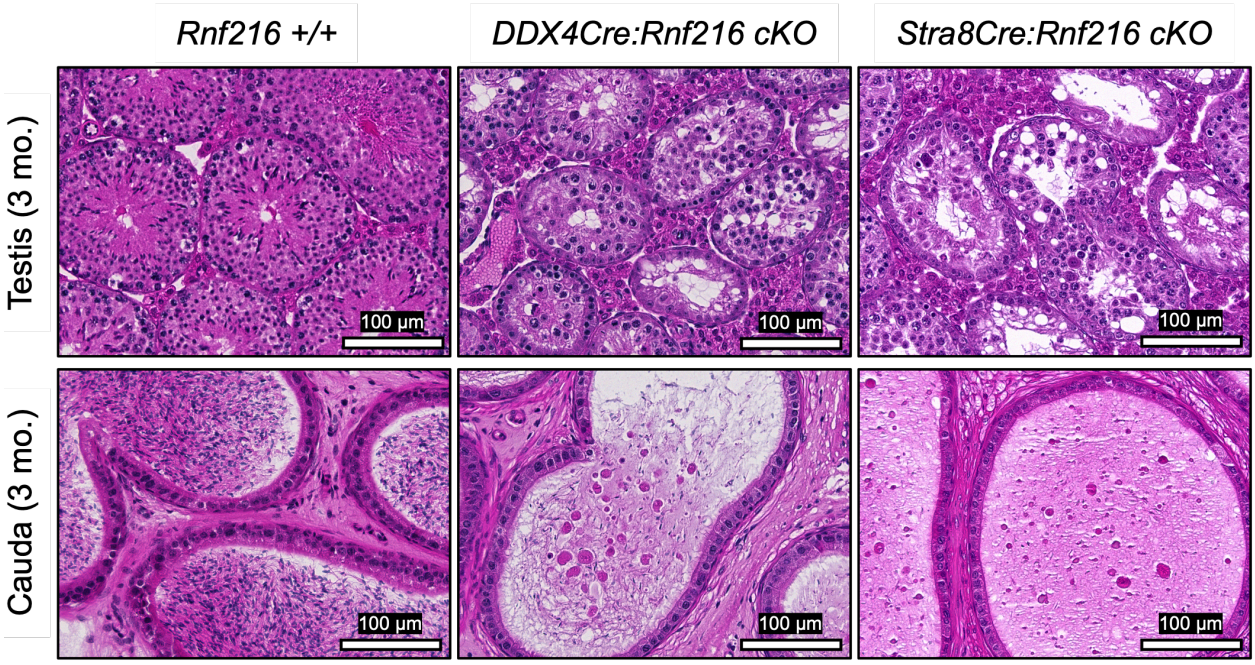

Figure B4. *Stra8Cre:Rnf216 cKO* and *DDX4Cre:Rnf216 cKO* male mice have identical phenotypes of seminiferous tubule degeneration, spermatogenic failure, and infertility.
Hematoxylin and eosin (H&E)-stained adult testis and epididymis of *Rnf216 +/+* (control), *DDX4Cre:Rnf216 cKO*, and *Stra8Cre:Rnf216 cKO* mice showing germ cell loss and incomplete arrest during spermatogenesis leading to male infertility. Scale bars 100 µm. Images are representative of three biological repeats.

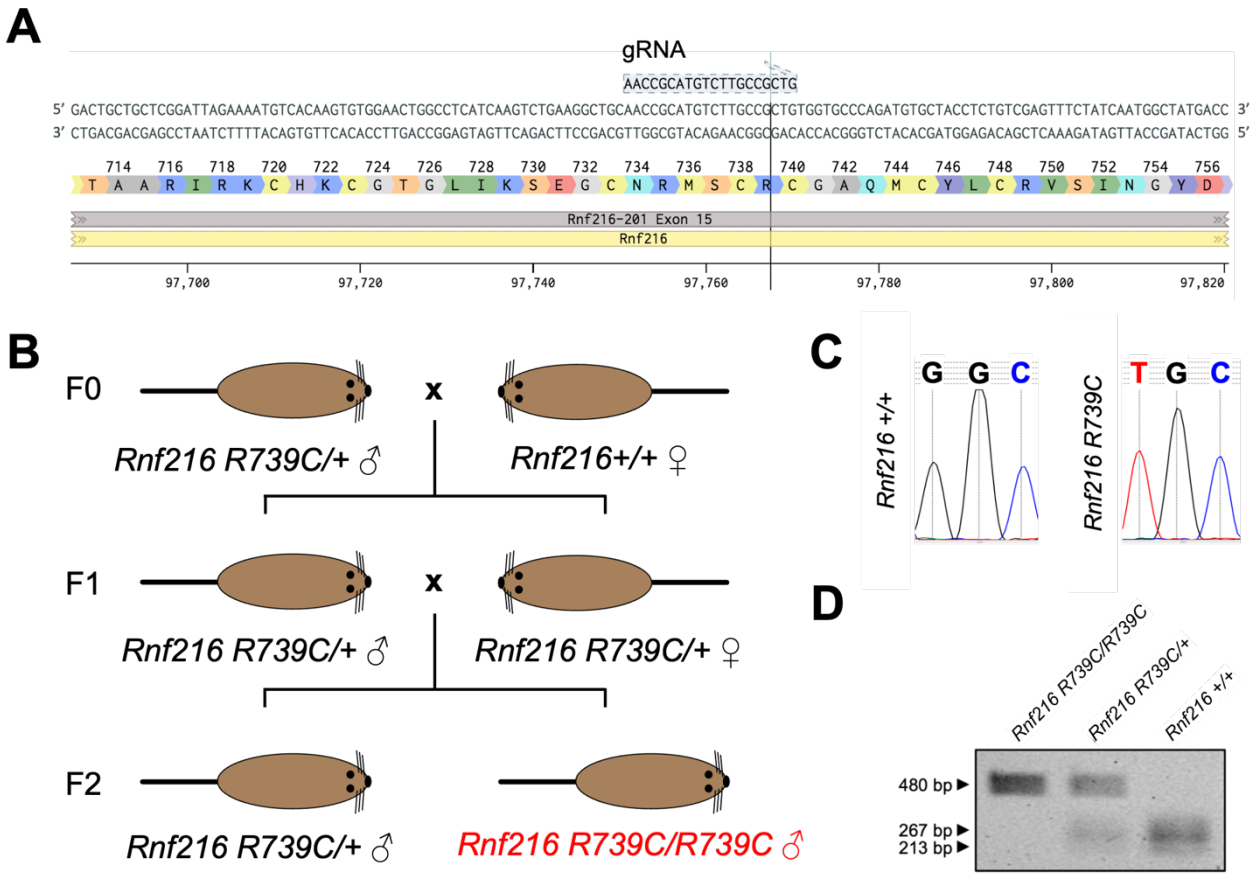

Figure B5. Generation and validation of *Rnf216 GHS* mice.
A. Strategy for generation of *Rnf216 GHS* (*Rnf216 R739C/R739C*) mice using single guide RNA (gRNA), ssODN donor template, and CRISPR-Cas9 to introduce human GHS *RNF216* point mutation (p.R739→C) into mouse *Rnf216* exon 15. **B.** Mating schematic to generate homozygous F2 *Rnf216 R739C/R739C* (red) (*Rnf216 GHS*) mice using F1 heterozygous mice derived from F0 founders. **C.** *Rnf216* +/+ and *Rnf216 GHS* allele Sanger sequencing results, respectively, confirming correct human GHS point mutation location. **D.** Representation of PCR and restriction digestion used to genotype *Rnf216 GHS* mice using agarose gel electrophoresis.

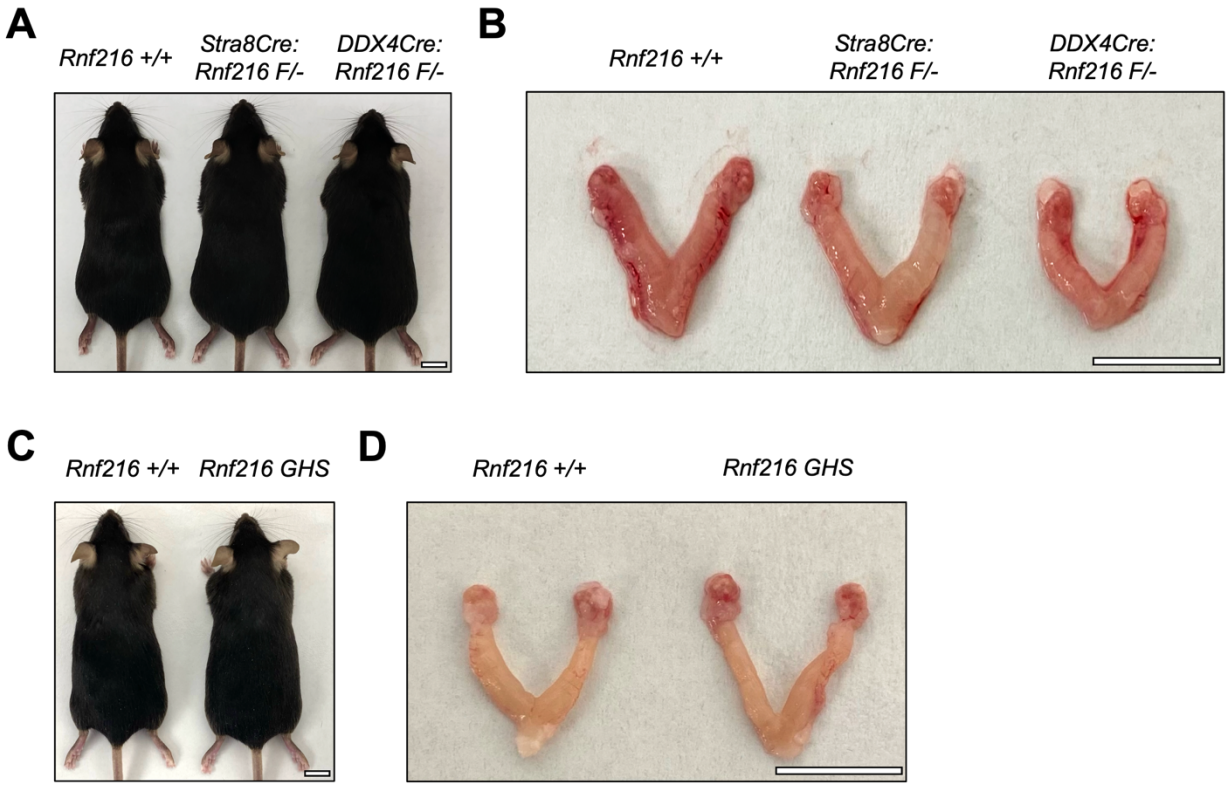

Figure B6. Female *Stra8Cre:Rnf216 cKO*, *DDX4Cre:Rnf216 cKO*, and *Rnf216 GHS* females show no reproductive defects, unlike male counterparts.
A. Gross comparison of adult *Rnf216* +/+ and germ cell conditional knockout lines *Stra8Cre:Rnf216 cKO* and *DDX4Cre:Rnf216 cKO* female mice. Scale bar 1 cm. **B.** Comparison of adult *Rnf216* +/+, *Stra8Cre:Rnf216 cKO*, and *DDX4Cre:Rnf216 cKO* ovaries and uteri. Scale bar 1 cm. **C.** Gross comparison of adult *Rnf216* +/+ and *Rnf216 GHS* female mice. Scale bar 1 cm. **D.** Comparison of adult *Rnf216* +/+ and *Rnf216 GHS* ovaries and uteri. Scale bar 1 cm. **A. – D.** All female mice, regardless of *Rnf216* genotype, were able to produce offspring when paired with *Rnf216* +/+ male mice (see **Tables A4**, **A5**, and **A6**).