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2012

SPAG16 is a Bifunctional Gene Regulating Male Fertility

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SPAG16 IS A BIFUNCTIONAL GENE REGULATING MALE FERTILITY

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

by

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Richmond, Virginia
July, 2012

Acknowledgment

I would like to take this opportunity to thank some of the many individuals who have supported and guided me during my graduate education. First of all, my graduate advisor, Dr. Jerome F. Strauss, III, who always managed to make my educational progress a priority. I have been extremely fortunate to train with a physician-scientist role model who understands the dual attractions of the clinic and the lab, and has provided a wonderful example that even an extremely successful career can still thrive on the routine (albeit sometimes sporadic) excitement of a good result. I thank him for always being willing to make time.

I would also like to thank Dr. Sarah Spiegel, who gave me my start in research at VCU and who has been a continuing source of inspiration. It takes both confidence and creativity to see the scientist in a recent Folklore & Mythology undergraduate, yet without her willingness to take a chance it would be hard to imagine another path by which I would have arrived where I am today.

I would like to extend these thanks to Dr. Gordon Archer, who also showed a willingness to work with a non-traditional MD-PhD applicant. Dr. Archer has been a tireless supporter of the MD-PhD program throughout my time here. His office door is always open to students, and he recognizes that both levity and gravity have their places in the world of academia.

To my co-workers in the Strauss lab I offer my sincerest thanks. To Dr. Zhibing Zhang in particular, for day-to-day advice and scientific discussion throughout my training. To Drs. Lori Hill, Maria Teves, Xuening Shen and to Jingmei Song, for fostering a collaborative atmosphere and a fun, friendly place to work.

My committee members have always made themselves available, and I thank them for both setting a fair, strong standard and for supporting me as I worked to attain it. Drs. Archer, Fawcett, Kordula, and Spiegel have each provided helpful insight and encouragement that have directly improved my ability to conduct research and to present my findings.

I would like to thank my parents, all of whom have been exceptionally strong role models in different ways. My father- and mother- in-law, Drs. Prakash and Mitzi Nagarkatti, helped me navigate entry into the world of biomedical research. They have provided constant encouragement, and the type of discerning advice that can only derive from years of experience combined with a continuing passion to explore and to

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List of Abbreviations

AD   autosomal dominant

AR   autosomal recessive

cAMP   cyclic adenosine monophosphate

CREM   cAMP-responsive element modulator

FS   fibrous sheath

HRP   horseradish peroxidase

ICSI   intracytoplasmic sperm injection

IMSI   modified ICSI

IVF   *in vitro* fertilization

LD   linkage disequilibrium

LH   luteinizing hormone

ODFs   outer dense fibers

PCD   primary ciliary dyskinesia

PCR   polymerase chain reaction

PKD   polycystic kidney disease

PVDF   polyvinylidene fluoride

qPCR   quantitative PCR

RACE   rapid amplification of cDNA ends

RT-PCR	reverse transcriptase PCR
RNP	ribonucleoprotein
SNP	single nucleotide polymorphism
TEM	transmission electron microscopy
UTR	untranslated region

Abstract

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SPAG16 is the murine orthologue of *Chlamydomonas reinhardtii* PF20, a protein known

to be essential to the structure and function of the "9 + 2" axoneme. The "9 + 2" axoneme

provides the cytoskeletal core of all eukaryotic motile cilia and flagella. In

Chlamydomonas, the *Pf20* gene encodes a single protein present in the central pair of

the axoneme. Loss of *Pf20* prevents central pair assembly and results in flagellar

paralysis. The murine *Spag16* gene encodes two proteins. While 71 kDa SPAG16L is

found in all murine cells with motile cilia or flagella, 35 kDa SPAG16S transcript and

protein are detected only in male germ cells, suggesting a unique role distinct from

general axoneme formation. Transgenic mouse studies published previously by our lab

have shown that abrogation of both SPAG16 isoforms causes arrest of spermatogenesis,

and the mutant allele is not transmitted to offspring by chimeric males. Mice homozygous

for a knock-out of SPAG16L alone are infertile, but show no abnormalities in

spermatogenesis. The defects seen in chimeric *Spag16* mutant mice, unaccounted for by loss of SPAG16L, indicate a possible role for SPAG16S in the specialized process of male germ cell development. Our results demonstrate that SPAG16S is predominantly found in specific regions within the nucleus of round spermatids. These nuclear sub-domains also contain SC35, a known marker of nuclear speckles enriched in pre-mRNA splicing factors. Putative interaction partners of SPAG16S are also shown to play critical roles in the peri-nuclear region during the round spermatid transition to the condensation and elongation stage of spermiogenesis, the final specialization point in sperm development. The distinct localization of SPAG16S at this critical juncture, its interaction with discretely localized proteins at a critical temporal junction in spermatogenesis, and its ability to modulate SPAG16L expression, suggest that SPAG16S plays an important role in the gene expression machinery of male germ cells, and represents an evolutionary distinction in axoneme gene function.

Chapter 1: General Introduction

Early Discoveries

In the mid-17th century, a one-time haberdasher and civic official in Delft began to perfect a proprietary approach to glass lens manufacture, enabling him to produce smaller, more perfect spheres than any previously available. At a time when most microscopic observations were still severely limited in magnification, Leeuwenhoek produced single lens, simple microscopes capable of up to 300X amplification (Huxley 2007). Leeuwenhoek's carefully honed spheres became one of the most important technological breakthroughs of modern science, granting powerful new opportunities to explore the sub-microscopic world. Over five decades of active investigation, Leeuwenhoek became the first scientist to describe muscle fibers, blood flow, and structures of bacteria, earning the title "Father of Microbiology" for his groundbreaking reports (Dobell and Leeuwenhoek 1932).

While he is most famous for his descriptions of bacterial structure, Leeuwenhoek produced a diverse body of work. After years of wrestling with one of the most shocking discoveries afforded by his inquiries, in 1677 he penned a letter to the Royal Society of London, excitedly describing a new finding:

"… and I have seen so great a number of living animalcules… in it, that sometimes more than a thousand were moving about in an amount of material the size of a grain of sand…" (letter to W. Brouncker, President of the Royal Society of London, Nov 1677, in (Leeuwenhoek and Rijnberk 1939))

Leeuwenhoek was describing, for the first time in history, the microscopic contents of human semen. His observations challenged Church doctrine that organisms were fundamentally divided into two groups: large, procreating, creatures, and small, spontaneously generating life forms (letter to Anthonio Magliabechi, Oct 1699, in (Leeuwenhoek and Rijnberk 1939)). Though Leeuwenhoek himself was a devout Calvinist, and indeed was profoundly troubled by the implications of his findings (Huxley 2007), his report posed a fundamental challenge to contemporary European beliefs on the nature of the human soul - procreation could no longer be assumed to result from a purely spiritual union. Leeuwenhoek's work suggested that human life might begin from just one of millions of tiny, single cells, each with the potential to give rise to a new being.

Strategies in Contraception

In bi-gendered species, males are defined as the gender producing numerous small, motile gametes, termed spermatozoa (Bishop and Walton "Spermatogenesis and the structure of mammalian spermatozoa" in (Marshall, Lamming et al. 1984))(Birkhead, Pellatt et al. 1988). Evolutionary biologists have interpreted this strategy as a consequence of the challenge of genetic competition; for the non-gestating partner, producing more gametes increases the likelihood of reproductive success (Kilgallon and

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Simmons 2005; Parker and Ball 2005). It has even been proposed that quality and quantity of male gamete output varies dynamically with perceived reproductive competition, a complex psychophysiological response to the challenge of ensuring genetic continuity (Friedmann 1981; Law 1986).

As a result of this prodigious and continuous production of viable sperm, male contraception requires fundamentally different approaches than those collectively referred to as "the Pill," the array of hormone regimens that suppress female ovulation. The human female menstrual cycle is a well characterized and highly regulated series of physiological changes focused on the production, typically, of a single ovum. Over the course of an average 28-29 days cycle (Clifton, Kaneda et al. 2008), the endocrine system primes the uterus to become a hospitable environment for implantation and gestation. If fertilization does not occur, the uterine lining is secreted and a new round of hormonal surges triggers the cycle to begin again.

Female oral contraceptives, which employ progesterone alone or in combination with estrogen as well, are employed by 18% of contraceptive users worldwide (National Prescribing Service 2007). When taken perfectly as prescribed (usually a daily administration in the case of oral dosing), pregnancy rates are around 0.3% (Fu, Darroch et al. 1999; Hatcher 2007; Halpern, Lopez et al. 2011). However, typical real world adherence results in a failure rate of approximately 8% during the first year of use (Hassan and Killick 2004).

Contraceptive failure is not the only drawback to these medications – reported side effects include impaired recovery of fertility (Shufelt and Bairey Merz 2009; Blanco-Molina and Monreal 2010), deep venous thrombosis (La Vecchia and Bosetti 2009; Cibula, Gompel et al. 2010), and certain types of neoplasm (Keith, Keith et al. 1975; Heinemann, Saad et al. 2005; Heinemann, Saad et al. 2005). While these medications are highly effective, and the vast majority of studies have demonstrated the health risks of hormonal contraceptives to be quite limited, both the reality of their side effects and patient concerns arising from perceived risk suggest that alternative approaches are highly desirable.

Male Hormone Contraception

Reports have indicated that the majority of men would be willing to take a perfected oral contraceptive pill (Oudshoorn 2003), and researchers have been optimistic about producing one for decades. Over 50 years have passed since leaders in China and India labeled male contraceptives as the critical missing element in population control programs (Zatuchni, Goldsmith et al. 1986). Early research sought a quick-acting agent to disrupt sperm production by direct inhibition of hormone function, and later by targeting various steps in the endocrine control pathway (reviewed in (Heckel, Rosso et al. 1951)), based on the early observation that testosterone itself was antispermatogenic in rats (Steinberger and Smith 1977; Swerdloff, Peterson et al. 1978). Many of the first studies focused on estrogens, which have long been used in treatment of prostate cancer and are known to disrupt spermatogenesis. However, the long-term feminizing effects of estrogen treatment are unacceptable, and require counter-administration of testosterone

(Oudshoorn 2003), which still does not fully overcome reduced libido, acne, testicular shrinkage, and breast growth (Farrell, Joshua et al. 1975). In addition to creating a complex clinical protocol for administration, androgens also carry their own risks, including hepatic damage (Muldoon, Mahesh et al. 1982) and neoplasm (Zatuchni, Goldsmith et al. 1986). Moreover, onset may take up to several years (NJ Alexander "Sites for Disruption of Male Fertility" in (Prasad and Diczfalusy 1983; Qian and Wang 1984)).

Gossypol

Clearly, an approach is necessary that targets male fertility more specifically, rather than mimicking the strategy of female oral contraceptives. In the 1980s, significant research efforts focused on gossypol, a naturally-occurring chemical derived from cotton seeds (Shepu, Shudong et al. 1980; Wen 1980). A polpyhenol, gossypol's direct cellular target remains controversial. Despite limited mechanistic data, gossypol was reported to be safe and effective following a trial in 4,000 Chinese men, who exhibited decrease in motile sperm and eventual oligozoospermia and azoospermia (Coutinho, Athayde et al. 2000). Subsequent studies have in fact demonstrated that gossypol is well tolerated during continuous dosing, and potential toxicities such as hypokalemia can be significantly diminished by routine clinical check-up (Liu and Lyle 1987; Liu, Lyle et al. 1987; Liu, Lyle et al. 1987). However, gossypol appears to be too potent; infertility is markedly prolonged in up to 50% of users, and, critically, is irreversible in 10-20% of users after administration is stopped (Ansbacher 1971; Shulman, Zappi et al. 1972).

Vasectomy

Vasectomy, a surgical severing and tying off of the vas deferens, is highly effective in preventing male fertility, and has been safely practiced for decades. The procedure is widely available, is performed on an out-patient basis, and requires less than half an hour. For men who desire a permanent approach to contraception, vasectomy offers a safe and convenient way to achieve this goal without major lifestyle changes.

However, numerous studies have raised concerns regarding unforeseen side effects of vasectomy. It is well established that circulating levels of anti-sperm antibodies are increased following vasectomy (Nagarkatti and Rao 1976; McDonald and Halliday 1992), and cell-mediated immune responses to spermatozoa have been observed as well (Witkin, Zelikovsky et al. 1982). Additionally, immune complexes in general are elevated in vasectomized men (Alexander and Clarkson 1978; Clarkson and Alexander 1980). Several reports have also proposed a link between vasectomy and cardiovascular disease (Ross, Paganini-Hill et al. 1983; Sidney 1987; Honda, Bernstein et al. 1988; Newell, Fueger et al. 1989). Early studies also suggested an increased risk of certain genitourinary concerns, including prostate (Goldacre, Clarke et al. 1978; Strader, Weiss et al. 1988; Thornhill, Conroy et al. 1988; Cale, Farouk et al. 1990) and testicular cancers (Schwingl and Guess 2000; Kohler, Fazili et al. 2009).

Follow-up studies and meta-analyses have repeatedly shown these links between vasectomy and pathologies to be unsubstantiated, and have suggested the surgery is safe for the vast majority of potential patients (reviewed in (Lee, Li et al. 2008)).

Nevertheless, there are substantial costs to vasectomy. The procedure is irreversible in just under half of cases (Robles, Martinez et al. 1987; Santiso 1988; Sharma and Sharma 1996), and acceptance varies significantly amongst different cultures (Inhorn 2009). Even in groups where vasectomy is regularly employed as a contraception technique, it is not uncommon for men to feel somewhat diminished by knowledge of their own sterility, stating e.g. "It's a bit unmanly in a way" (Roberts 2000; Rowlands 2002; Pollack 2003). Both US and UK guidelines state that the surgery should be viewed as a permanent sterilization, and should be performed only on patients who definitely do not want to father children in the future (Fenster and McLoughlin 1981; Belker, Thomas et al. 1991; Potts, Pasqualotto et al. 1999; Sandlow, Westefeld et al. 2001). Despite careful pre-operational screening, 3-6% of vasectomy patients eventually seek reversal (Fenster and McLoughlin 1981; Belker, Thomas et al. 1991), and the more time that passes after vasectomy, the lower the chances are that fertility will be successfully restored (Birenbaum-Carmeli, Carmeli et al. 1995).

Male Infertility

Unlike the nascent state of male birth control, medical technology is somewhat more advanced in the treatment of impaired male fertility. Nevertheless, the basic framework of knowledge regarding andrology and male infertility remains limited (Cates, Farley et al. 1985; World Health Organization 1987; Outlook 2002; Potter and Hanin 2005). Estimates shown that approximately 1 in 7 couples will experience difficulty conceiving (Bhasin 2007), and that in instances of sub-fertility, male factor infertility is involved in roughly 50% of cases (Whorton, Krauss et al. 1977; Wyrobek, Gordon et al. 1983; Schrader,

Turner et al. 1991). Moreover, infertility rates have been widely reported to be increasing, with links drawn to environmental and occupational toxins (Auger, Kunstmann et al. 1995; Irvine, Cawood et al. 1996; Selevan, Borkovec et al. 2000; Jorgensen, Carlsen et al. 2002). Most studies have focused on industrialized nations, citing reduced sperm counts and increased rates of abnormal sperm morphology in young men (Olsen, Bodner et al. 1995; Fisch, Goluboff et al. 1996), though these findings have been somewhat controversial (Irvine, Cawood et al. 1996). Reports not only point out trends of reduced male fertility, but tend towards alarmist tones, calling "urgently" for more data (Katz 1994; Birenbaum-Carmeli, Carmeli et al. 1995; Inhorn 2004). Social scientists have reported widely that male infertility seems more strongly stigmatized (Carmeli et al. in (Inhorn 2009)) citing anecdotes of severe anxiety at the prospect of being completely infertile:

"The men feel so bad if they don't have sperm. They will be so happy to know that they have sperm, even if none of them are motile. When I tell them they have sperm, they are crying. Sometimes they hug me. They speak about [sperm] donation, and he feels miserable. I spoke to a man who was told he was azoospermic. I told him to bring me a slide of his testis. I put it on my electro-microscope and showed him where the sperm were. I told him, "You are not azoospermic." He was holding me and kissing me. (from H Goldberg in (Inhorn and Balen 2002; Teman 2003))

In fact, current fertility treatments have made possible the successful use of lower quality and even immotile sperm. While research has focused more strongly on female infertility (reviewed in (Rodriguez-Martinez 2006)) advances in fertility drugs and in the techniques

of in vitro fertilization (IVF) have reduced the need for quantity and quality of sperm that would traditionally be required for unperturbed fertility (Maggiulli, Neri et al.; Setti, Figueira et al. 2010). Micromanipulation and advanced screening of male gametes has allowed a targeted, "modified" approach to intracytoplasmic sperm injection (IMSI, a modified approach to ICSI) that has raised successful pregnancy rates in couples with clinically-diagnosed male infertility on par with those of couples requiring simple in vitro semination (Fortunato and Tosti 2011). While these advances have been welcomed by hopeful couples and by practitioners, concerns have been raised that the practice of in vitro fertilization produces more birth defects and subsequent pathologies than are observed with traditional procreation, perhaps owing to reduced quality of gametes used and imperfect conditions of the *in vitro* environment (Lokman and Moore; Nagano 2011). Researchers have continued to focus on methods to induce the production of improved sperm, but these techniques remain elusive (Austin and Short 1972).

Spermatogenesis

The most promising approaches to both targeted male contraception and enhanced male fertility lie in a deeper understanding of spermatogenesis, the highly specialized process that generates male gametes capable of: 1) directed motility; 2) recognition of the female ovum; 3) binding to the ovum; 4) penetration of and fusion with the ovum, i.e. capacitation (Clermont and Trott 1969). This process takes a total of 35 days in mice, and 74 days in humans (Brehm and Steger 2005). Male gametes are produced from a population of spermatogonial stem cells, and derive from a maturation process that can be divided into three distinct stages (Hess and Renato de Franca 2008).

In the first stage of spermatogenesis, spermatogonial stem cells divide by mitosis, with one daughter cell continuing the stem cell population while the other differentiates and matures. In the second stage of spermatogenesis, meiotic division of a primary spermatocyte results in the production of four round spermatids. In the final differentiation step, spermiogenesis, round spermatids produced by meiosis mature into functional spermatozoa. In this final step, differentiating sperm develop specialized structures, such as the flagellum, acrosome, and condensed nucleus, critical to their unique reproductive function.

Seminiferous Tubules

The majority of the process of spermatogenesis takes place within the seminiferous tubules of the testis (Skinner and Griswold 2005). Seminiferous tubules are arranged as rings, with undifferentiated cells at the periphery maturing as they move towards the lumen. This architecture is established by the seminiferous epithelium, consisting of germ cells interspersed with Sertoli cells, the only somatic cells present within the tubules and a critical participant in sperm maturation (R Hess and LR Franca in (Hess and Renato de Franca 2008))(Leblond and Clermont 1952).

While the seminiferous epithelium represents a mixture of Sertoli cells and various germ cell types, investigators have divided the epithelium into several stages based on the mixture of cells present in a given tubular cross section, defining twelve stages (I-XII) in rodents (Clermont 1963) and six stages (I-VI) in men (Russell 1990). This convention

provides a common framework and vocabulary for researchers to address pathologies that perturb the normal distribution within the cycle of cells undergoing spermatogenetic differentiation. Spermatogenesis has been studied most extensively in the mouse, where staging is commonly grouped into 'Early' stages I-V, 'Middle' stages VI-VIII, and 'Late' stages IX-XII (Grudzinskas and Yovich 1995).

Epididymis

As haploid round spermatids elongate and condense during spermiogenesis, the maturing spermatozoa are released into the seminiferous tubule lumen. Final sperm maturation takes place as spermatozoa move from the lumen into the epididymis, where they are stored until ejaculation (reviewed by HDM Moore in (Hess and Chen 1992)). Storage time varies among animals, and is known to be affected by scrotal temperature, which also modulates the duration of spermatogenesis (Bedford 1965; Bedford 1967; Cooper 1998; Toshimori 1998). As spermatozoa move into the epididymis, they are still not functionally mature – they lack forward motility and the ability to recognize the oocyte zona pellucida (Sullivan, Frenette et al. 2007). These functions are acquired within the epididymis under the direction of a group of molecules collectives designated "epididymosomes" (Toshimori, Kuwajima et al. 1999). Impairment of this process limits sperm motility and significantly reduces fertility (Connell 1976).

Leydig cells

Both Sertoli cells and spermatogonia are supported by paracrine signaling from Leydig cells residing in the interstitial space outside the seminiferous tubules. While other cells,

such as fibroblasts, macrophages, and mast cells, are also present in the interstitium (Schulze 1984), Leydig cells are the primary resident cell type with a direct and necessary effect on spermatogenesis. The primary responsibility of Leydig cells is to produce testosterone in response to luteinizing hormone (LH) (Andersson, Jorgensen et al. 2004). The testosterone released by Leydig cells diffuses to Sertoli cells and spermatogonia within the seminiferous tubule, effecting critical paracrine stimulation of spermatogenesis. As 12-15% of men with diminished spermatogenesis exhibit reduced testosterone levels, it can be inferred that proper Leydig cell function is highly important to male fertility (Dym 1994; Timpl 1996; Timpl and Brown 1996).

Sertoli cells

Interspersed between spermatogonial stem cells, Sertoli cells form physical contact with the seminiferous epithelium's basement membrane of type IV collagen and laminins (Bawa 1963; Flickinger and Fawcett 1967). Sertoli cells play several important roles during spermatogenesis, including: nutrition and support of germ cells; maintenance of tight junctions for seminiferous tubule compartmentalization as a protected microenvironment (Russell and Griswold 1993; Hess and Renato de Franca 2008); release of spermatids into the lumen; secretion of growth factors; phagocytosis of degenerating germ cells and of residual body cytoplasm shed by mature sperm (Van Haaster, De Jong et al. 1992; Hess, Cooke et al. 1993; Sharpe, McKinnell et al. 2003). It has been established in numerous studies that the number of Sertoli cells shows a strong correlation with the maximal resulting sperm production (Kormano 1967; Setchell 2008).

The tight junctions of Sertoli cells are critical to the integrity of the "blood-testis barrier" that generates a specific and controlled environmental within seminiferous tubules of post-pubertal mammalian testes (Witkin, Jeremias et al. 1996). This barrier creates an immunologically-privileged compartment from which circulating antibodies and immune cells are excluded (Teuscher, Wild et al. 1982; Yule, Montoya et al. 1988; Tung and Teuscher 1995). Some reports have suggested that this barrier is incomplete, with the result that germ cells occasionally pass the barrier and become subject to immune-mediated attack (Russell 1978). Critically, this barrier must occasionally open to allow developing spermatids into the seminiferous tubule lumen (Mruk and Cheng 2004), with competing theories (reviewed in (Russell 1990; Chiarini-Garcia, Hornick et al. 2001)) debating the mechanism of this controlled traffic flow. As a critical step in sperm maturation, this juncture represents a potential bottleneck target for contraception.

Mitosis in Spermatogonia

Spermatogonia are diploid (2n) cells comprising the earliest stages of spermatogenesis as well as the fully undifferentiated spermatogonial stem cell population. In rodents, electron microscopy evaluation of heterochromatin distribution in developing spermatogonia has resulted in sub-classification into four groups: undifferentiated (stem) cells; differentiated type A (further sub-divided into A1-A4); intermediate (In); and type B (B) (Brinster 2002; Ogawa, Ohmura et al. 2005; Oatley and Brinster 2006). Three such classifications are used in humans: undifferentiated, type A differentiated, and type B (de Rooij and Russell 2000; Aponte, van Bragt et al. 2005; Hofmann, Braydich-Stolle et al. 2005; Hess, Cooke et al. 2006; Oatley and Brinster 2006; Brinster 2007).

The spermatogonial stem cell population is maintained by mitotic division, under which one daughter cell differentiates while the other maintains the stem cell line. During mitosis and maturation of differentiated spermatogonia, a relatively limited number of factors (e.g. c-kit, Vitamin A) and proteins have been identified as initiators of key signaling events (Clermont 1972). The number of regulated mitotic divisions varies characteristically by species (Russell, Chiarini-Garcia et al. 2002), and it is during this step in maturation that apoptotic loss of germ cells occurs, as a normal facet of spermatogenesis in all mammals thus far investigated (Franca, Avelar et al. 2005; Luiz Renato, Russell et al. 2006). For each differentiated type A spermatogonium, only 2-3 spermatocytes result from mitotic divisions capable of producing 8-12 (Hess and Renato de Franca 2008), demonstrating that apoptotic culling of the developing spermatogonial population is a critical regulator of gamete production. It has been proposed (Sharpe, McKinnell et al. 2003) that programmed cell death may result from a density-dependent limit on spermatogonial cell expansion established by the number of available Sertoli cells, which would explain the observation that Sertoli cell population is a critical determinant of maximal sperm production (Toshimori 2009).

Meiosis

The end products of spermatogonial maturation, spermatocytes, represent the beginning of meiotic prophase I. Unlike female oocytes, which do not complete meiosis until ovulation, spermatocytes progress through all steps of meiosis prior to spermiation (Russell 1977; Weber, Russell et al. 1983). While in early stages they still rest on the

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basement membrane, as they develop spermatocytes move through the blood-testis barrier of Sertoli cell junctions (as discussed earlier), entering the seminiferous tubule lumen for further development (Franca and Cardoso 1998; Franca, Avelar et al. 2005). During this process, spermatocytes also increase significantly in size as they begin preparing the appropriate cellular contents necessary at the haploid spermatid stage (Hess and Renato de Franca 2008).

During meiosis I, 4n primary spermatocytes divide to produce secondary 2n spermatocytes, a relatively transient step in spermatozoan differentiation (Sassone-Corsi 1997; Eddy 2002). Meiosis II sees the division of secondary spermatocytes into haploid (1n) round spermatids, which begin the profound biochemical and cytological restructuring of spermiogenesis.

Spermiogenesis

Following meiosis, haploid round spermatids initiate a programmed system of robust transcriptional activity, which has been extensively reviewed in the literature (Schmidt, Ohbayashi et al. 1997). Dramatic increases in expression of transcription factors and polymerases correspond with a need to rapidly produce necessary mRNAs and proteins before chromatin is condensed in the tightly packaged nucleus of elongating spermatids (Blendy, Kaestner et al. 1996; Nantel, Monaco et al. 1996).

While several testis-specific genes and isoforms play important roles, perhaps none are so important as cAMP-responsive element modulator (CREM), a master transcription

factor that regulates promoter function of many haploid spermatid genes (Steger, Klonisch et al. 1999). In studies of infertile men with spermatogenesis arrest at the round spermatid stage, CREM mRNA (Weinbauer, Behr et al. 1998) and protein (Braun, Peschon et al. 1989) have both been reported to be severely reduced.

Concurrent with spermiogenesis-specific transcription, translation of resulting mRNAs is regulated by the binding of cytoplasmic repressor proteins to the 3'-UTRs (Kwon and Hecht 1993). A 17-nucleotide motif within the 3'-UTR of protamine mRNAs has been pinpointed as a critical signaling element dubbed the "Z-box," which coordinates expression of genes involved in nuclear re-packaging (Steger, Failing et al. 2001) and mediates temporary storage of transcripts in ribonucleoprotein (RNP) particles (Singh and Rao 1987; Kundu and Rao 1995; Caron, Veilleux et al. 2001).

Nuclear Packaging

As haploid spermatids elongate and condense within the seminiferous tubules, re-modeling of nuclear DNA allows for tighter compaction and protects against mutation. Histones are the predominant protein binding genomic DNA in somatic cells, and they play this role in male germ cells as well up to the end of meiosis. However, spermatid elongation calls for the replacement of histones, first by transition proteins TP-1 and TP-2 (Bedford and Calvin 1974; Balhorn, Weston et al. 1984), and ultimately by highly basic protamine proteins (Aitken and Krausz 2001; Aitken and Baker 2004). By binding to protamines, genomic DNA is remodeled to a toroid "doughnut-like" structure that is

resistant to the numerous stresses encountered during prolonged storage and journey to the oocyte (Toshimori 2009).

Tail Formation

Chromatin remodeling to create the unique structure of the sperm head allows for efficient and stable transport of haploid genomic material, accomplishing one of the primary structural goals of sperm development. Head shapes vary somewhat across species, being generally classified as: 1) sickle-shaped (falciform), common in rodents, and 2) paddle-shaped (spatulate), seen in humans and many other species (Inaba 2011).

While there is some variation in the structure of the sperm head, evolution has more strongly shaped the spermatozoan tail. Transit of genomic material (i.e. the sperm head) within the female reproductive tract is the end goal of spermatogenesis, and the structure of eukaryotic sperm flagellum is remarkably conserved across species (Baccetti and Afzelius 1976), being a nearly universal approach to the challenge of male gamete motility (Oko 1988).

Flagellum formation is based on the assembly of a cytoskeletal core, known as the axoneme, which supplies structure and coordinates movement. Surrounding the axoneme, robust mitochondrial development fuels waveform movement, while outer dense fibers (ODFs) and a fibrous sheath (FS) form accessory cytoskeletal components (Tesarik, Sousa et al. 1998). While ICSI has opened the door to fertilization using non-

motile sperm (Pazour, Agrin et al. 2005), under normal conditions the building and maintenance of these flagellar structures is essential.

Basic Research on Cilia & Flagella

From a structural perspective, the terms 'cilia' and 'flagella' are used interchangeably. Function and formation have been so strongly conserved by evolution that these structures are eukaryotic life's sole option for a directed, rapidly moveable cellular appendage.

Strong conservation of cilia structure and components has allowed for significant molecular biology insight based on proteomic and gene mutation analyses in model organisms, most notably the unicellular algae *Chlamydomonas reinhardtii* (overview in (Lefebvre and Rosenbaum 1986)). The first critical ciliary genes were identified by investigation of proteins markedly up-regulated following biochemical removal of cilia – protein products produced after this disruption were hypothesized to be needed for replacement of the missing organelle (Luck, Huang et al. 1982; Blacque, Cevik et al. 2008). From this initial set of candidate flagellar components, single-gene mutation studies have highlighted several genes that are critical to ciliary motility; gene mutations resulting in flagellar paralysis, misassembly within macromolecular complexes, or perturbed interactions between large cytoskeletal components have been reported. This extensive body of work (reviewed in (Wargo and Smith 2003)) has highlighted the importance of the "9 + 2" axoneme, the core cytoskeletal structure that gives shape to eukaryotic and allows for coordinated movement.

The "9 + 2" axoneme consists of a circular arrangement of 9 outer microtubule doublets surrounding two central, singlet microtubules dubbed the "central pair." In *Chlamydomonas*, these singlet microtubules are designated "C1" and "C2," and each possesses asymmetric projections to the surrounding doublets, allowing regulation of the outer microtubules' sliding movements (Dutcher and Lux 1989). In addition, the central pair have direct connections to each other, allowing them to coordinate in their manipulation of the outer doublets. Critically, this central pair is not present in the "9 + 0" axoneme found in eukaryotic sensory, or "primary" cilia, and thus is deemed defining and essential to ciliary motility.

Gene mutation and ultrastructural analyses in *Chlamydomonas* have allowed characterization of specific domains of the axoneme, such that proteins can be assigned in one of five groupings: 1) β-tubule projections, running between microtubules of a given doublet; 2) Outer dynein arms, which extend from one doublet towards another along the periphery of the axoneme; 3) Inner dynein arms, which extend from one doublet towards another along the medial portion of the axoneme; 4) Radial spokes, which extend from the outer doublets towards the central pair; 5) Central pair components, which facilitate connections within one microtubule or between the pair (Duldulao, Li et al. 2010).

Ciliary dysfunction in disease

While their functions are not yet fully understood, cilia are found on almost all mammalian cells (Nonaka, Tanaka et al. 1998; Nonaka, Shiratori et al. 2002). Though research

efforts have previously examined ciliary functions in organisms such as *Chlamydomonas* due to the relative simplicity of biochemical manipulations in such models, recent work has focused more strongly on mammalian pathologies and development. It has been shown that cilia in mice are essential for proper left-right asymmetry during embryonic development (Chizhikov, Davenport et al. 2007; Davenport, Watts et al. 2007; Haycraft, Zhang et al. 2007; Lehman, Laag et al. 2009; McDermott, Liu et al. 2010). The advent of Cre-Lox conditional gene knockout technology has enabled targeted probing of ciliary function in particular cell types, leading to the discovery of ciliary involvement in functions such as hair follicle development, bone formation, mammary gland branching, central nervous system morphogenesis, and satiety responses (Huangfu, Liu et al. 2003). These somewhat unexpected observations have generated significant interest in the roles cilia play not only as structural organelles but also as components of signal transduction. The vertebrate Hedgehog pathway, which governs gene expression during development, is dependent on cilia (Otto, Schermer et al. 2003; Simons, Gloy et al. 2005). Wnt signaling is also affected by cilia function, thought in part due to the fact that the Wnt master switch Inversin localizes to cilia (Hildebrandt, Benzing et al. 2011).

In humans, cilia have been associated with a numerous diseases, collectively termed ciliopathies. The ubiquity of cilia expression suggests that cilia function is important in a wide variety of cell types and tissues, though we will focus here on several important examples (for a full review see (Wodehouse, Kharitonov et al. 2003; Olin, Burns et al. 2011)).

Primary Ciliary Dyskinesia/Kartagener's Syndrome

Primary Ciliary Dyskinesia (PCD) often manifests immediately in affected neonates, reflecting that it is a disorder with origins during embryonic development. However, diagnosis requires identification of a complex clinical presentation and subsequent TEM (transmission electron microscopy) analysis of the patient's cilia, making it likely that at least some patients are missed and do not receive treatment (Zhu, Belmont et al. 2006). Diagnosed by electron microscopy evidence of specific ciliary ultrastructural defects or genetic testing revealing mutation in one of eight known genes, PCD has a somewhat variable presentation centered around a trio of classic symptoms. Ciliary dysmotility during embryogenesis commonly leads to situs inversus – complete left-right asymmetry of the visceral organs – without apparent consequence. 50% of PCD patients have situs inversus without observed clinical sequelae, while 6% have heterotaxy, abnormal distribution of the viscera with paired organs (e.g. lungs, kidneys) mirrored rather than uniquely "left" or "right" (Noone, Leigh et al. 2004; Kendig and Wilmott 2006). The most common clinical symptom is severe respiratory distress, requiring neonatal oxygen supplementation and leading to chronic respiratory infection. Ciliary defects lead to reduced mucus clearance, creating a fertile ground for these repeated bacterial infections. In adulthood, these early problems generally lead to bronchiectasis, a dilation of the bronchial tree where constant inflammation limits airway adaptability, as in COPD commonly suffered by long-term cigarette smokers (Afzelius 2004). Male PCD patients additionally suffer from infertility in 50% of cases, owing to similar ultrastructural defects in the sperm flagellum (Saeki, Kondo et al. 1984).

Polycystic Kidney Disease

A strong association between cilia and Polycystic Kidney Disease (PKD) has been known for decades (Yoder, Hou et al. 2002; Lin, Hiesberger et al. 2003; Otto, Schermer et al. 2003), with many PKD-related genes isolated to cilia (Torres, Harris et al. 2007). PKD may result from autosomal dominant (AD) or recessive (AR) mutations, with the autosomal dominant forms representing the most common lethal autosomal dominant mutations in the US, at 1 in 1000 people affected (Pazour, Dickert et al. 2000; Yoder, Hou et al. 2002; Sun, Amsterdam et al. 2004). The genes affected in AD PKD, *PKD1* and *PKD2*, are both important in differentiation and maintenance of renal tubules. Disruption of these genes, and of cilia-related genes within the kidneys in general, leads inevitably to kidney cyst formation (Kim and Walz 2007; Torres, Harris et al. 2007). These pathological changes are not always detected in youth, as their clinical manifestations – hypertension, abdominal pain, urinary tract infections – are non-specific (Alam and Perrone 2010; Hogan and Norby 2010). Without treatment, however, PKD leads to end-stage renal disease requiring dialysis and eventual kidney transplant, an outcome that can be at least postponed with early detection and treatment (Ibanez-Tallon, Gorokhova et al. 2002; Sapiro, Kostetskii et al. 2002; Banizs, Pike et al. 2005).

Hydrocephalus

Hydrocephalus is a pathological accumulation of cerebrospinal fluid within brain's ventricular spaces. Under normal conditions, cerebrospinal fluid is constantly moved, resorbed and processed by ependymal cells, which form a ciliated epithelium surrounding the ventricles. When ependymal cilia are unable to perform this task, fluid

builds up within the ventricular spaces and forces expansion, compressing cerebral tissue and raising intra-cranial pressure to unsafe levels (Inaba 2011).

The diversity of clinical pathologies deriving from cilia dysfunction demonstrates the complexity of roles played by cilia in the human body. Addressing these clinical challenges requires a better understanding of the basic molecular biology of the highly conserved ciliary structure.

Spag16 and the Axoneme

The studies discussed here will focus primarily on a gene of the axoneme's central apparatus. The central apparatus contains at least 17 proteins (Randall, Warr et al. 1964; McVittie 1972; Adams, Huang et al. 1981; Dutcher, Huang et al. 1984; Brokaw and Luck 1985), including products from six of the 'pf' genes, so designated because their loss in *Chlamydomonas reinhardtii* results in paralyzed flagella: *pf6*, *pf15*, *pf16*, *pf18*, *pf19*, and *pf20* (Xu and Min 2011).

SPAG16, homologous to *pf20* found in *Chlamydomonas*, produces a 631 amino acid protein. The only identified conserved domains in the SPAG16 protein are seven WD-repeat domains located on the C-terminal half. These WD domains of approximately 40 amino acids in length give rise to a tertiary beta propeller structure, and are thought to mediate macromolecular assembly through protein-protein interactions (reviewed in (Zhang, Sapiro et al. 2002)). In all studied eukaryotic organisms, the pattern of protein structure and function is highly conserved: the protein is located exclusively in the

cytoplasm of ciliated cells, and specifically is found in the axoneme central apparatus; the amino acid sequence is comprised of an N-terminal half with no known conserved domains, and a C-terminal half with seven WD repeats.

In mice, the classic *Spag16* protein product is known as SPAG16L, or "long" SPAG16, as a second, shorter protein product has been identified and labeled SPAG16S (Strausberg, Feingold et al. 2002). Whereas SPAG16L is found in all ciliated cells, SPAG16S was isolated from testis, and has not been detected in any tissue outside the male reproductive system to date. The amino acid sequences in both proteins are exactly the same, except that SPAG16S lacks the N-terminal half of SPAG16L, having instead only the portion with WD repeats. Unlike SPAG16L, SPAG16S is found in the nucleus (as well as the cytoplasm), and is enriched in specific sub-nuclear clusters.

While SPAG16S has been most extensively characterized in mice, including the work presented here, we hypothesize that expression of SPAG16S represents an evolution of the *Spag16* gene in mammals. We have previously identified a putative *SPAG16S* promoter and 5'-UTR in humans [GenBank: EF591776.1], and the identified rat version of SPAG16 – isolated from testis – resembles murine SPAG16S (Clark 2007).

Phenotypes of Spag16 disruption

Though it has been one of the major goals of the field of male reproduction research for decades, a reliable male germ cell model line has yet to be established (reviewed in

(Zhang, Kostetskii et al. 2006)). As a result, studies of gene function are largely carried out using transgenic mouse models.

SPAG16L

We have previously reported that male mice with a gene mutation disrupting production of SPAG16L (but not SPAG16S) are infertile, with reduced sperm counts and profound disruption of sperm motility, demonstrating the importance of SPAG16L to sperm flagellar function (Lesich, Zhang et al. 2010). Interestingly, SPAG16L-deficient mice do not display signs of hydrocephalus, bronchial abnormality, or kidney function failure. Additionally, the axoneme ultrastructure of SPAG16L-deficient mice appears normal when examined by transmission electron microscopy. However, recent studies have demonstrated that sperm flagella from SPAG16L-deficient mice show greatly reduced bending during attempted waveform movement, and that molecular responses to calcium are significantly diminished (Zhang, Kostetskii et al. 2004). In light of these recent data, we hypothesize that the role of SPAG16L in the axoneme is essential for coordinated movement within the structure, rather than for structural assembly. The critical point, however, is that loss of SPAG16L specifically affects flagellar motility, whereas spermatogenesis is largely undisturbed.

SPAG16L and SPAG16S

Disruption of both isoforms of SPAG16 results in a severe phenotype that manifests even in the chimeric state of transgenic animal breeding: spermatogenesis is significantly disrupted, and the flagellar axoneme is significantly disrupted (Zhang, Kostetskii et al.

2004). Notably, the *Spag16* mutant transgene was never transmitted by fertile male carriers, suggesting that sperm carrying the mutant copy of the gene were unable to complete spermatogenesis and/or fertilize a female ovum. Haploinsufficiency is rarely encountered in male reproduction, as such profound defects are unable to be passed to offspring and thus do not remain in the population. The difference between the flagellar-specific disruption seen with loss of SPAG16L alone and the spermatogenetic arrest observed with disruption of both isoforms suggests a fundamental role for *Spag16* in sperm development and function. We hypothesize that the localization of SPAG16S in the nucleus is necessary for the coordination of molecular signals that direct the progress of spermatogenesis.

SPAG16 protein interactors

As SPAG16 proteins lack identifiable effector domains, they are believed to function through interaction with other proteins, likely through the assembly of macromolecular complexes. We have previously reported that SPAG16S interacts with MEIG1, a 10-kDa protein initially believed to play a role in regulation of meiosis (Zhang, Shen et al. 2009). We have recently found that male mice deficient in MEIG1 are infertile due to arrest of spermatogenesis at the stages of elongation and condensation (Salzberg, Eldar et al. 2010), a conclusion which has been reached by an independent group as well (Stirnimann, Petsalaki et al. 2010). While the mechanism for spermatogenesis arrest in the absence of MEIG1 cannot be conclusively linked to SPAG16S, this finding provides additional support for the hypothesis that a SPAG16S-related pathway is necessary to spermatogenesis and independent of the structural role of SPAG16L.

Both SPAG16S and SPAG16L most likely exert their influence through interaction with several different effector partners. WD domains have been referred to as among the most promiscuous interactors (Lau, Bachorik et al. 2009), as they are commonly retrieved candidates in protein interaction studies. Further, it has recently been reported that at least some WD domains confer RNA binding specificity as well (Luck 1984; Porter and Sale 2000). The diversity of potential interaction partners means that relatively little is known about the evolution and function of the *SPAG16* gene.

The present work will discuss the roles of *SPAG16* in mammals by examining: the sub-localization of murine SPAG16S; the functional roles of the SPAG16S protein; the status of the *SPAG16* gene in azoospermic human subjects.

Chapter 2: *Spag16*, an Axonemal Central Apparatus Gene, Encodes a Male Germ Cell Nuclear Speckle Protein that Regulates SPAG16 mRNA Expression

Abstract

Spag16 is the murine orthologue of *Chlamydomonas reinhardtii* PF20, a protein known to be essential to the structure and function of the "9 + 2" axoneme. In *Chlamydomonas*, the *PF20* gene encodes a single protein present in the central pair of the axoneme. Loss of PF20 prevents central pair assembly/integrity and results in flagellar paralysis. Here we demonstrate that the murine *Spag16* gene encodes two proteins: 71 kDa SPAG16L, which is found in all murine cells with motile cilia or flagella, and 35 kDa SPAG16S, representing the C terminus of SPAG16L, which is expressed only in male germ cells, and is predominantly found in specific regions within the nucleus that also contain SC35, a known marker of nuclear speckles enriched in pre-mRNA splicing factors. SPAG16S expression precedes expression of SPAG16L. Mice homozygous for a knockout of SPAG16L alone are infertile, but show no abnormalities in spermatogenesis. Mice chimeric for a mutation deleting the transcripts for both SPAG16L and SPAG16S have a profound defect in spermatogenesis. We show here that transduction of SPAG16S into cultured dispersed mouse male germ cells and BEAS-2B human bronchial epithelial cells increases SPAG16L expression, but has no effect on the expression of several other axoneme components. We also demonstrate that the *Spag16L* promoter shows increased activity in the presence of SPAG16S. The distinct nuclear localization of

SPAG16S and its ability to modulate *Spag16L* mRNA expression suggest that SPAG16S plays an important role in the gene expression machinery of male germ cells. This is a unique example of a highly conserved axonemal protein gene that encodes two protein products with different functions.

Introduction

The "9+2" axoneme, a cytoskeletal structure found in motile cilia and flagella, is composed of nine outer doublet microtubules linked to a central microtubule pair via dynein arms to form a motor complex allowing coordinated force generation (Adams, Huang et al. 1981; Dutcher, Huang et al. 1984). The central pair of microtubules is critical to the integrity and the motility of this structure. One essential element of the central apparatus, PF20, was first identified in *Chlamydomonas reinhardtii* (Smith and Lefebvre 1997; Smith and Lefebvre 1997), and has since been shown to exhibit strong conservation amongst a wide variety of organisms and ciliated cell types (Zhang, Sapiro et al. 2002).

We have reported that the murine orthologue of *PF20*, *Spag16*, encodes two distinct proteins: SPAG16L, which is a component of the axoneme central apparatus (Zhang, Kostetskii et al. 2004), and SPAG16S, a smaller protein representing the WD repeat region of SPAG16L, identified only in male germ cells (Zhang, Kostetskii et al. 2004). Chimeric mice carrying a mutation that disrupted the *Spag16* gene at a locus shared by transcripts encoding both SPAG16L and SPAG16S displayed a phenotype of haploinsufficiency; the mutant allele was never transmitted to offspring by chimeric males

(Zhang, Kostetskii et al. 2006). Furthermore, these mice exhibited significant germ cell loss at the round spermatid stage. In contrast, transgenic mice homozygous for a deleterious mutation in the SPAG16L-specific region of the gene were infertile, with normal spermatogenesis but resulting in sperm showing marked motility defects despite an axonemal structure devoid of significant ultrastructural defects (Lamond and Spector 2003). The deficits observed with ablation of both SPAG16 isoforms, not accounted for by loss of SPAG16L alone, suggest that SPAG16S may play a critical and previously un-described role in spermatogenesis.

We show here that SPAG16S is localized to nuclear subdomains called nuclear speckles. Nuclear speckles are non-nucleolar domains within the nucleus that contain splicing factors as well as transcription factors, RNA processing units, and structural scaffold proteins (reviewed by Lamond and Spector (Ricciardi, Kilstrup-Nielsen et al. 2009; Hu, Plutz et al. 2010)). Though not generally believed to be centers of active transcription, speckles have been implicated as compartments that can provide splicing factor contents to active transcription sites (Shopland, Johnson et al. 2003). Speckles are enriched in SC35, which is used as a marker for these distinct domains. SC35 domains have been linked to the development of a cell-type specific genomic organization and to the mapping of distinct "euchromatic neighborhoods" (Zhang, Kostetskii et al. 2006; Zhang, Zariwala et al. 2007). Though nuclear speckles have been shown to play central roles in management of gene expression, their role in male germ cell differentiation has not been previously reported.

Results

Identification of the 5' UTR of mouse SPAG16S

To identify the 5' UTR of *Spag16S* mRNA, 5' RACE was performed with a primer located close to the 3' end of mouse *Spag16L* mRNA (Fig. 2-1A). Two PCR products were amplified (Fig. 2-1B), and each one was cloned into the pCR2.1 Topo TA vector (Invitrogen). 10 clones of each PCR product were sequenced after vector insertion, demonstrating that *Spag16S* sequence is identical to that of *Spag16L* exons 11-17, with the addition of a 5' untranslated exon, not found in *Spag16L*, named exon 10a (Fig. 2-1C). This exon 10a is located in the middle of intron 10 of the *Spag16* gene, approximately 50 kb from *Spag16L* exon 10 and 50 kb from *Spag16L* exon 11. Sequencing results demonstrated multiple potential transcription start sites for *Spag16S* transcription; the exon is situated in a TC-rich locus that lacks a standard TATA box (Fig. 2-10).

Spag16S message is expressed only in the testis and male germ cells

SPAG16L is present not only in testis, but also in other murine tissues containing cells with a "9 + 2" axoneme structure (Zhang, Kostetskii et al. 2004). Primer sets were designed to specifically amplify *Spag16L* (exons 2-4) or *Spag16S* (exons 10a-12) (Fig. 2-1C). In adult mice, *Spag16L* mRNA was detected in testis, brain, lung, and oviduct; however, *Spag16L* mRNA was not detected in heart tissue (Fig. 2-1D), which does not contain cells with motile cilia. *Spag16S* mRNA was detected only in testis (Fig. 2-1D), and in further testing was not detected in kidney, liver, or spleen. *Spag16S* expression also appears to be exclusive to males, rather than being a general germ cell factor, as

Spag16S mRNA was never detected by PCR using up to 10 oocyte equivalents in qPCR amplification of up to 40 cycles.

Expression pattern of SPAG16S during the first wave of spermatogenesis

RNA and protein were isolated from mouse testis at 6, 8, 12, 16, 20, 30, and 42 days after birth. cDNA was generated by RT-PCR, and testis extracts were probed by PCR for *Spag16* isoform expression using specific primers. *Spag16S* mRNA was detected at day 16, whereas *Spag16L* mRNA was detected later, at day 20 (Fig. 2-2B). Western blotting was performed as well, using a polyclonal antibody that recognizes both isoforms of SPAG16 (Zhang, Shen et al. 2008). Consistent with PCR results, SPAG16S protein was detected at day 16, while SPAG16L was detected at day 20 (Fig. 2-2C). Additionally, both isoforms appeared to be up-regulated at days 30 and 42, consistent with the end of the first wave of spermatogenesis. The immunoreactive bands other than 71 kDa SPAG16L and 35 kDA SPAG16S seen on days 20, 30 and 42 may represent post-translational processing of SPAG16, including proteolytic cleavage of SPAG16L and phosphorylation or other modifications (Smith and Lefebvre 1997; Zhang, Tang et al. 2007; Lesich, Zhang et al. 2010).

Sub-cellular localization of SPAG16 isoforms

Cytoplasmic and nuclear fractions of adult mouse testis were isolated, and equivalent amounts of protein from each were probed by Western blot using the C-terminal SPAG16 antibody that recognizes both isoforms. SPAG16L was detected abundantly in the cytoplasm, while SPAG16S was detected in both cytoplasm and nucleus (Fig. 2-3A). The

cytoplasmic localization of SPAG16L is consistent with its identified role as a structural component of the "9 + 2" axoneme (Zhang, Kostetskii et al. 2006). In order to further characterize the sub-cellular localization of SPAG16S, immunohistochemistry was performed on tissue slices from adult mouse testis. Using the C-terminal SPAG16 antibody, the strongest signal was detected from discrete structures within the nucleus (Fig. 2-3B, see arrows). Protein expression was most clearly visualized approximately halfway through spermatogenesis, at the round spermatid stage.

A mixed population of mouse male germ cells was prepared from adult mouse testis, and immunocytochemistry performed to allow for single-cell imaging to compare with immunohistochemistry results. Consistent with previous data, the C-terminal SPAG16 antibody produced the strongest fluorescence in discrete sub-nuclear, non-nucleolar structures, approximately 2-6 per cell (Fig. 2-4). The N-terminal SPAG16 antibody, recognizing SPAG16L only, produced an exclusively cytoplasmic signal. Germ cells from SPAG16L-KO mice (Bastin, Pullen et al. 2000; Kohl and Bastin 2005) were isolated and immunolabeled as well, demonstrating the specificity of the antibodies (Fig. 2-4). As in wild-type germ cells, the C-terminal SPAG16 antibody produced sub-nuclear immunolabelling, which we interpret to represent SPAG16S, while the N-terminal antibody produced no signal, as expected following the deletion of SPAG16L.

SPAG16S co-localizes with nuclear speckles

In order to characterize the discrete nuclear structures identified by immunohistochemical analysis of SPAG16S localization, wild-type germ cells were co-immunolabeled with both

the C-terminal SPAG16 antibody and a monoclonal antibody directed against SC35, a marker for nuclear speckles. SC35 and SPAG16S signals strongly overlapped (Fig. 2-5A). They were determined to have a significant co-localization (Fig. 2-5B – Pearson's coefficient = 0.40; p = 0.0079; N = 43). Analysis demonstrated that 79% of SC35 signal co-localized with SPAG16S, while 44% of SPAG16S signal co-localized with SC35; in other words, most SC35-containing domains also contained SPAG16S, but SPAG16S had a wider distribution as well.

Role of SPAG16S in SPAG16L expression

Given the timing of SPAG16S expression (Fig. 2-2B-C) and its specific nuclear localization (Fig. 2-3 – 2-5), it was hypothesized that SPAG16S might play a role in regulation of gene expression. Mixed mouse male germ cells were isolated from testis and cultured, with exposure to a SPAG16S-transducing adenovirus or a control adenovirus. Following 48 hour culture, RNA was isolated for analysis of gene expression, and several highly conserved axoneme genes were assessed for mRNA levels. While *Spag6*, *Spag17*, and *Akap4* were unaffected, *Spag16L* message level was significantly increased (Fig. 2-6).

SPAG16S was also shown to induce SPAG16L expression in BEAS-2B human bronchial epithelial cells. This cell line does not express SPAG16L at levels detectable by qPCR or Western blotting (data not shown). Expression of SPAG16S was induced in cultured BEAS-2B cells by plasmid (Fig. 2-7A) or adenovirus (Fig. 2-7B), and primers specific for human SPAG16L were used to measure transcript levels, which were normalized to 18S

rRNA. Immunocytochemistry performed with the C-terminal SPAG16 antibody confirms that SPAG16 proteins are expressed in the transduced cells, but not in cells exposed to a control adenovirus (Fig. 2-7C). Following adenoviral transduction, protein was also isolated from cultured BEAS-2B cells, and SPAG16L was demonstrated by Western blot to be present in cells transduced with SPAG16S adenovirus, but not cells transduced with a control adenovirus (Fig. 2-7D).

Following the observation that SPAG16L protein and mRNA levels were increased in the presence of SPAG16S, *Spag16L* promoter activity was tested as well. While other axoneme gene promoters (*Spag17*, *Spag6*) did not show significantly altered activity, *SPAG16L* promoter activity was significantly higher in the presence of plasmid-induced SPAG16S when compared to co-transfection with a control empty vector plasmid (Fig. 2-8).

In order to identify a specific region within the *SPAG16L* promoter showing increased activity with SPAG16S expression, luciferase plasmid constructs were made with progressively shorter sequences of the *SPAG16L* proximal promoter, ranging from 2 kb to 100 bp upstream of the transcription start site. Promoters ranging from 2 kb to 200 bp upstream of the transcription start site demonstrated significantly higher activity when co-transfected with a SPAG16S plasmid, but a 100 bp upstream *SPAG16L* promoter did not show increased activity in the presence of SPAG16S plasmid (Fig. 2-9).

Discussion

Flagella and cilia are strongly conserved structures, having maintained a highly specific structure and function throughout eukaryotic life, from unicellular protists to mammals (Inaba 2003; Inaba 2007). It has been suggested that molecular structures of each component in the axoneme are not always conserved, but protein *domains* within axoneme structures are preferably conserved, allowing for overall morphological and functional conservation (Kleene 1996; Fajardo, Haugen et al. 1997). Although the *Chlamydomonas pf20* gene encodes only one transcript, its mouse orthologue encodes two mRNAs for two proteins, SPAG16L and SPAG16S, each of which have conserved WD-repeat domains. To study the expression pattern of the two mRNAs and proteins, testicular total RNAs and proteins were isolated from mice at different ages. Given that *Spag16S* harbors an untranslated exon 10a – which is not present in Spag16L – a *Spag16S*-specific forward primer was designed to selectively amplify the *Spag16S* transcript. Both SPAG16S mRNA and protein are expressed earlier than *SPAG16L* message and SPAG16L protein. For both SPAG16L and SPAG16S, mRNAs and proteins appear simultaneously (SPAG16S at day 16, followed by SPAG16L at day 20), suggesting that there is no translation delay (Zhang, Tang et al. 2007). Furthermore, *Spag16L* is expressed in other tissues, such as brain and lung, all of which contain motile cilia (Reddi, Flickinger et al. 1999; Eddy 2002; Reddi, Shore et al. 2002), but *Spag16S* is expressed only in the mouse testis, indicating that *Spag16S* is a testis-specific gene product. Moreover, *Spag16S* sequences identified in GenBank in a variety of mammalian species (Mouse: NM_025728.3; Rat: BC158602.1; Human: EF591776.1) have each been detected from testis samples.

Testis-specific genes can be grouped into three clusters. Homologous genes are those which are expressed only in spermatogenic cells, but which are closely related to genes expressed in somatic cells, and are often members of gene families. Unique genes are those without significant similarity to any other genes in the genome. Variant transcripts are transcribed from genes also expressed in somatic cells but are often smaller or larger than their somatic cell counterparts. They are the result of the utilization of one or more alternate transcriptional start sites, splice sites, or polyadenylation signals. Since spermatogenesis is a highly coordinated process, different genes are expressed during the three different stages of spermatogenesis, and gene expression can be used as a tool to assess progression through the stages of spermatogenesis (Wang, McCarrey et al. 2001). There are genes that have been shown to be expressed solely in the mitotic phase (spermatogonia) (Eddy and O'Brien 1998). Some are expressed during the meiotic phase (spermatocyte) (Beissbarth, Borisevich et al. 2003) and others during the post-meiotic phase in spermatids (Zhang, Kostetskii et al. 2006). Some of these genes are expressed solely in germ cells, while others are also expressed in somatic cells as well.

It appears that the *Spag16S* transcript is unique, as no related transcripts were identified in somatic cells. It is unlikely to be a spliced isoform of the *Spag16L* transcript, since it is detected in testis of mice homozygous for a mutation that ablates the Spag16L mRNA (Strausberg, Feingold et al. 2002). Similarly, evidence suggests that the SPAG16S protein is not a processed form of SPAG16L, since the SPAG16S protein is present in

SPAG16L-deficient mice, and the expression of SPAG16S occurs earlier than SPAG16L (Figure 2-2).

The mouse *Spag16S* 5'-UTR and upstream putative promoter region are highly conserved in rat (see Fig. 2-10). It is worth noting that only a single *Spag16* transcript has been identified in rat (BC158602.1), and its coding sequence corresponds to the region shared by both mouse *Spag16* isoforms. However, the rat *Spag16* mRNA includes a 410 bp 5'-UTR that is nearly identical to the mouse exon 10a plus its immediate upstream genomic sequence (Zhang, Kostetskii et al. 2006). Moreover the rat *Spag16* transcript was identified specifically in testis, leaving open the possibility that a *Spag16L*-like transcript may be likely to exist in testis and other tissues.

Different subcellular localization of SPAG16L and SPAG16S suggests that the two proteins play different roles. This was subsequently confirmed by two knockout models generated previously in our laboratory. SPAG16L regulates ciliary motility (Zhang, Kostetskii et al. 2004), while SPAG16S controls spermatogenesis (Escalier 2006). Loss of both SPAG16 isoforms produced a non-transmitted allele and led to significant arrest and cell death at the round spermatid stage in chimeric male mice carrying the mutant gene. While homozygosity for a SPAG16L-deletion mutation produced male infertility, the mutant allele was transmitted in the chimeric and heterozygous states, and the perturbation of spermatogenesis observed with total SPAG16 knockout was not seen. It has been observed that genetic mutations contributing to male infertility are often discovered to take effect through unexpected or unexplored cellular pathways. Thus,

while *Spag16* is known to be important to the axoneme through SPAG16L, the existence of an additional protein of unknown – and potentially more essential – function is consistent with published findings (Neer, Schmidt et al. 1994; Smith, Gaitatzes et al. 1999).

Both SPAG16L and SPAG16S contain 7 WD-repeat domains, semi-conserved 40 amino acid-regions ending with tryptophan-aspartate (W-D). These regions are known to mediate protein-protein interactions by giving rise to a ß–propeller tertiary structure. By interacting with effector partners, WD repeat proteins have been shown to play important roles in an extensive variety of cellular activities, including cell division, gene transcription, mRNA modification, and transmembrane signaling (reviewed in (Gallenberger, Meinel et al. 2011)). More recently, it has been reported that WD domains may also mediate specific processing of small RNAs (Lau, Bachorik et al. 2009) and direct binding of RNAs (Caceres, Misteli et al. 1997; Lallena and Correas 1997), further diversifying the range of potential roles these structures can play in eukaryotic cells. Because SPAG16S contains no obvious nuclear localization signals, its importation into the nucleus may be effected by its association with other proteins including transcription factors or nuclear speckle components.

We have demonstrated that while SPAG16L is exclusively in the cytoplasm of male germ cells – consistent with its structural role in the axoneme – SPAG16S is present in both nucleus and cytoplasm, and exhibits enhanced sub-localization within the nucleus. A monoclonal antibody directed against SC35, a canonical marker for nuclear speckles

(Lamond and Spector 2003), maps to the same nuclear regions that show enhanced SPAG16S signal, suggesting that SPAG16S is located in nuclear speckles of male germ cells, specifically at the round spermatid stage. Nuclear speckles are enriched in splicing-related factors, and though they are not active centers of transcription (Brown, Green et al. 2008; Hu, Kwon et al. 2008; Takizawa, Gudla et al. 2008; Zhao, Bodnar et al. 2009), they have been shown to associate with active alleles (Spector, O'Keefe et al. 1993).

The role of nuclear speckles has yet to be described in mammalian spermatogenesis. Though somatic cells nuclei contain 30-50 nuclear speckles, these structures are known to condense following inhibition of transcription (Marchler-Bauer and Bryant 2004; Marchler-Bauer, Lu et al. 2009); thus, the presence of only a few, larger speckles at the round spermatid stage is consistent with the down-regulation of transcription during sperm development. The unique nature of transcript and protein packaging during nuclear condensation in spermatogenesis suggests the intriguing possibility that nuclear speckles may play a distinct role in this process, and that SPAG16S may be a germ cell-specific factor necessary to guide this process.

SPAG16L mRNA and SPAG16L protein are up-regulated *in vitro* in the presence of SPAG16S, suggesting that one role of SPAG16S involves regulation of the SPAG16L isoform. These data are consistent with the observation that SPAG16L appears after SPAG16S. We have also shown that the *Spag16L* promoter displays significantly enhanced activity in the presence of SPAG16S.

While we cannot rule out the possibility that SPAG16S is involved in SPAG16L regulation through transcript processing events in nuclear speckles, these data strongly suggest that a primary responsibility of SPAG16S is activation of *SPAG16L* transcription. Since SPAG16S does not have an identified DNA-binding domain, it is unlikely to directly bind the *Spag16L* promoter, but rather to effect this interaction by binding with one or several protein partners. WD-repeat proteins are known to interact dynamically and reversibly with multi-protein complexes, thus identification of SPAG16S-associating structures presents a challenging but enticing area for future study.

While the *in vitro* promoter studies presented here strongly suggest a role for SPAG16S in direct transcription of *SPAG16L*, future studies should seek to clarify the exact role of SPAG16S in male germ cell transcript regulation, as the speckle localization of the protein is more suggestive of a role in transcript processing. Such studies would also clarify the role of nuclear speckles in round spermatids, as these structures are of particular interest given the unique transcriptional requirements of round spermatids as spermiogenesis takes place.

The 2kb *SPAG16L* proximal promoter was also assessed for putative binding sites of other transcription factors using ConSite open source software, but current transcription factor lists are limited in scope, particularly regarding germ cell-specific relevant transcription factors, some of which are known to activate the *SPAG16L* promoter. Given currently limited *in silico* capabilities, we believe that further molecular data are required

before generalized transcription factor prediction is a sufficiently reliable in a germ cell system.

In summary, SPAG16S is a testis-specific protein found in nuclear speckles that appears to regulate spermatogenesis by controlling testis-specific target gene expression, one of the target genes being *Spag16L*. Thus, the murine *Spag16* gene has dual functions. It encodes a structural protein at the axoneme, which is essential for sperm motility, and a nuclear speckle-associated factor that regulates *Spag16L* gene expression. To the best of our knowledge, this is the first example of a gene's evolution conferring the ability to regulate its own, conserved products.

Materials and Methods

Ethics Statement

No human or primate subjects were used in this work. All rodent work was approved by Virginia Commonwealth University's Institutional Animal Care & Use Committee (protocol permit #AM10297) in concordance with all federal and local regulations regarding the use of non-primate vertebrates in scientific research. Research animals were humanely housed and care was taken to prevent undue distress.

5' Rapid amplification of cDNA ends (5' RACE)

5' RACE was carried out to define the 5' non-translated region sequence and transcriptional start site of the mouse Spag16S mRNA using mouse Marathon cDNA amplification kit (Clontech) according to the manufacturer' instructions. Briefly, a reverse

primer was designed within the coding region of Spag16 (5'-AGAAGCCACGAAGTCACCACAGGAGT-3') and used together with the Marathon cDNA adaptor primer to generate 5'-RACE products. The smaller product was cloned into the pCR2.1-TOPO TA vector and subjected to DNA sequence analysis.

Conserved domain analysis

Mouse SPAG16S (NP_080004.1) and SPAG16L (NP_083436.2) protein sequences were analyzed using NCBI Conserved Domains tool (Zhang, Sapiro et al. 2002; Zhang, Kostetskii et al. 2004).

Antibodies

SPAG16-specific antibodies directed against the common C-terminus (SPAG16L amino acids 330-639) and the SPAG16L-specific N-terminus (amino acids 1-212) have been previously described (Abramoff, Magelhaes et al. 2004). α-tubulin (cytoplasmic marker) and Lamin B (nuclear marker) were from Santa Cruz Biotechnology. SC35 was purchased from Sigma-Aldrich.

Microscopy and colocalization analysis

Confocal laser scanning microscopy was performed using a Leica TCS-SP2 AOBS (Leica Microsystems). Colocalization calculations were performed using Volocity Quantitation software (Perkin-Elmer).

Mixed germ cell preparation

A male adult mouse was anesthetized and euthanized, and the testis decapsulated and placed in 5 mL PBS. Collagenase IV (Sigma-Aldrich) was added to a final concentration of 0.5 mg/mL and DNase I (Sigma-Aldrich) to a final concentration of 1.0 mg/mL. Testis solution was incubated 30 min at 32° C to dissociate cells, and then centrifuged 5 min at 1000 rpm. Cells were fixed by 15 min incubation in 1% paraformaldehyde/PBS at room temperature, and then washed three times with PBS. Prior to plating, cells were re-suspended in 12.5 mL PBS and 50 µL of cell suspension was spread on SuperFrost/Plus microscope slides (Fisher Scientific) and allowed to air-dry. Slides were used immediately for immunocytochemistry or stored at -80° C until use.

Cell fractionation into nuclei and cytoplasm

Freshly isolated mouse male germ cells (see germ cell slide preparation protocol) were separated into nuclear and cytoplasmic fractions using a Nuclear/Cytosol Fractionation Kit (BioLine, Inc.) per manufacturer's instructions.

Western blotting analysis

Equal amounts of protein (50 µg/lane) were heated to 95° C for 10 minutes in 4X sample buffer, loaded onto 10% sodium dodecyl sulfate-polyacrylamide gels, electrophoretically separated, and transferred to PVDF membranes. The membranes were blocked and then incubated with primary antibody at 4° C overnight. After being washed, the blots were incubated appropriate HRP-conjugated secondary antibody (GE Healthcare UK) for 1 hour at room temperature. After washing, protein was detected with Super Signal

Chemiluminescent Substrate (Pierce). Densitometry performed using ImageJ (Horowitz, Zhang et al. 2005)

Immunocytochemistry and immunohistochemistry

Slides were first blocked 1 hour at room temperature with 10% goat serum/PBS. Following overnight incubation with primary antibody (diluted 1:100-300 in blocking medium) at 4º C, slides were washed with PBS and incubated 1 hour at room temperature with Alex 488-conjugated anti-mouse IgG secondary antibody (1:500; Jackson ImmunoResearch Laboratories) or Cy3-conjugated anti-rabbit IgG secondary antibody (1:1000; Jackson ImmunoResearch Laboratories). Following secondary antibody incubation, slides were washed in PBS and sealed using VectaMount with DAPI (Vector Laboratories).

Generation of adenovirus to express SPAG16S

Adenovirus expressing SPAG16S (AdSpag16s) was generated with AdEasyTM Adenoviral Vector System (Stratagene) following the instruction manual. Briefly, mouse Spag16S cDNA was subcloned into adenovirus shuttle vector pShuttle-CMV and the cDNA was transferred into pAdEasy-1 virus genome by means of homologous recombination in an adenovirus packaging cell line HEK-293 cells (ATCC; Manassas, VA). The expression of SPAG16S was tested with western blotting using proteins from COS-1 cells (ATCC) and BEAS-2B cells (ATCC) infected with AdSpag16S or control Ad virus. The AdSpag16S and control Ad virus were amplified and titered in the Macromolecule Core Facility of Virginia Commonwealth University.

Generation of plasmid to express SPAG16S

Mouse testis cDNA was used as a template to amplify *Spag16S* sequence by PCR. Amplified PCR product was cloned in a pCR2.1 Topo TA vector for sequencing, then released by restriction enzyme digestion and ligated in a pTarget vector. Primers used: Forward (*BamHI)*: 5'-<u>GGATCC</u>CCTGTAGATATGCAACCAGATCC-3'; Reverse (*SalI*): 5'-<u>GTCGAC</u> GCTGATCAGATCCACAACCGAATG-3'

RT-PCR and real-time PCR

Total RNA was isolated from cultured cells and indicated tissues with Trizol (Life Technologies, Inc., Grand Island, N.Y.), the RNA was reversed transcribed, and the cDNAs were used for RT-PCR or real-time PCR.

For real-time PCR, cDNAs from BEAS-2B cells transfected with pcDNA3 or Spag16S/pcDNA3 plasmids or infected with AdSpag16S or control Ad virus were utilized for PCR. Primers were designed for detection of human *SPAG16L* using the software from GeneScript Corporation (http://www.genscript.com/). Real-time PCR reactions were carried out using the 2X SYBR green master mix (BioRad). Akap4, Spag17, and Spag6 primers have been previously reported (Horowitz, Zhang et al. 2005).

(human) *SPAG16L* – Forward: 5'-TTCAGACTGCTGCTTCCATC-3'; Reverse: 5'-TCGCCTGTACATAGATCCCA-3'

(mouse) *Spag16L* – Forward: 5'-AGCAAGCCAGAGACATCCAT-3'; Reverse: 5'-CCAGAAATCTTCCCAACAGC-3'

(mouse) *Spag16S* – Forward: 5'-CTCTGACACAATGAGTATGG-3' (exon 10a); Reverse: 5'-CTACAGGAAATTCTGAATCC-3' (exon 11)

18S – Forward: 5'-GGCCCTGTAATTGGAATGAGTC-3'; Reverse: 5'-CCAAGATCCAACTACGAGCTT-3'

Luciferase promoter activity assay

Promoter plasmids were designed using a PGL3 basic vector (Promega). For each experiment, BEAS-2B cells were co-transfected with empty vector or promoter plasmid and a control (pTarget - Promega) or test (SPAG16S) plasmid using Fugene 6 transfection reagent (Roche) then cultured 48 hours. Cells were lysed using 1X passive lysis buffer according to manufacturer's instructions (Promega) and luciferase activity measured using freshly-prepared reagents. Data are represented as relative fold difference from PGL3 control promoter co-transfected with control pTarget vector plasmid.

Generation of promoter constructs for promoter function assays

Promoter sequences were amplified by PCR from mouse DNA and sub-cloned in a PGL3 basic vector after sequences were confirmed by first cloning in a Topo Vector (Invitrogen) for sequence analysis. Primers were designed to specifically amplify regions upstream of the transcription start site (Fig. 2-9). 2 kb, 1.5 kb, and 0.5 kb promoter constructs were previously generated in our lab (Boivin, Bunting et al. 2007). 0.3 kb, 0.2 kb and 0.1 kb promoter constructs were generated as described using a common reverse primer (5'-

CTCGAGGCTTGCAACTGCGGCCCCTCGGTGCC-3') with the following forward primers:

0.3 kb *Spag16L* –5'-<u>GGTACC</u>CGCAAGCAAGCAAGCAAGCAAGCAAGC-3'

0.2 kb *Spag16L* – 5'-<u>GGTACC</u>GGTTCTGGGCTTCAGGTCTGCAGTCC-3'

0.1 kb *Spag16L* – 5'-<u>GGTACC</u>CGCTTGACCGGGGCCTTTTGGTGC-3'

Statistical Analysis

Statistical analyses were performed using Student-Newman-Keuls post-hoc analyses with a significance level of $\alpha = 0.05$.

Acknowledgments
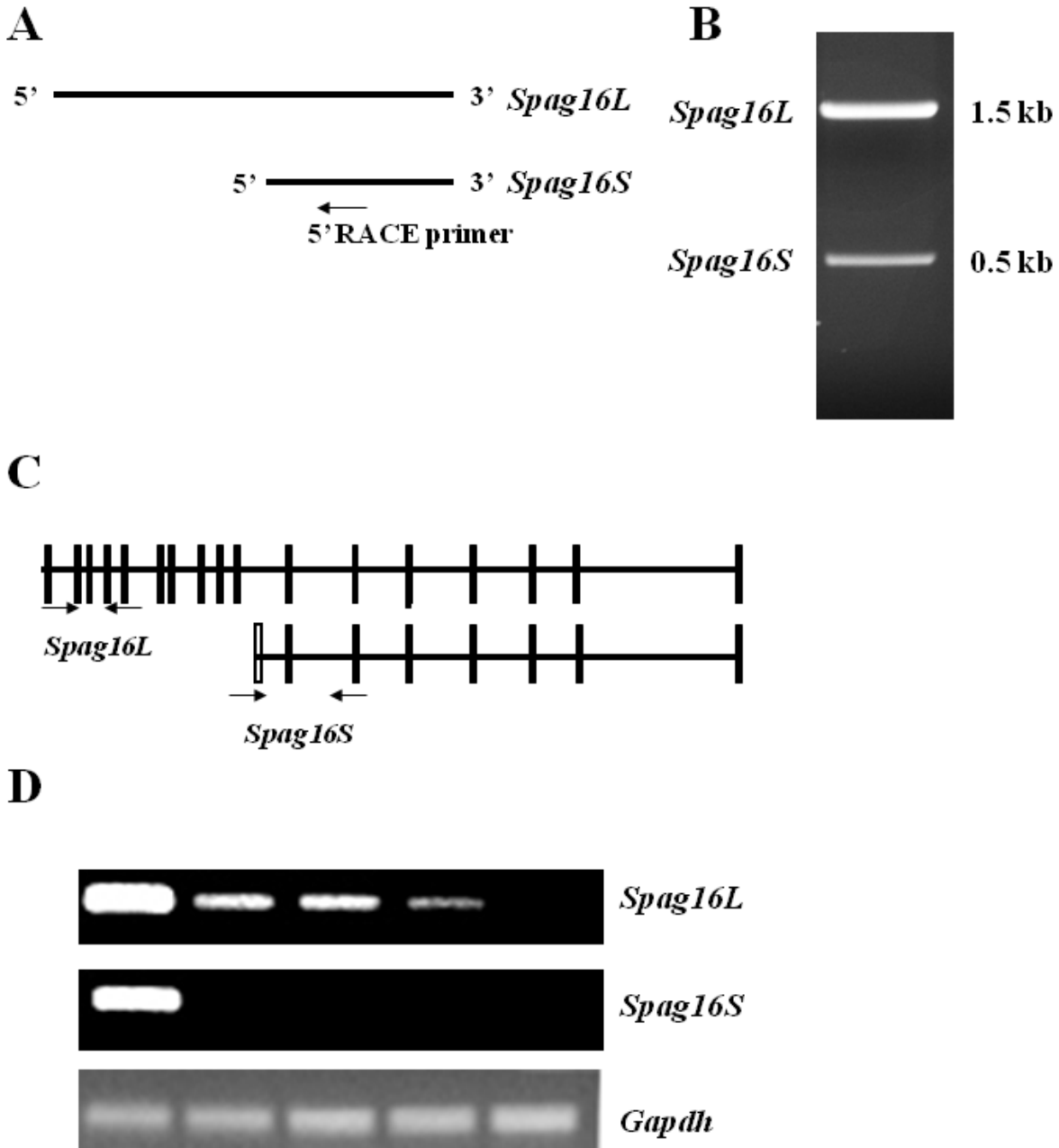
Figure 2-1. The murine *Spag16* gene encodes two transcripts. *Spag16L* is expressed in all tissues with ciliated cells, while *Spag16S* is expressed only in testis

(A) 5' RACE was performed with a primer as indicated. (B) Products of 5' RACE separated on 1% agarose gel. (C) Exon map of Spag16 transcripts, unfilled box indicating untranslated exon 10a present only in Spag16S. Arrows indicate primers used to amplify specifically Spag16L or Spag16S message. (D) Specific primer sets were used as indicated for PCR amplification of cDNA from adult mouse tissues: Testis (T), Brain (B), Lungs (Lu), Oviduct (Ov), Heart (H).
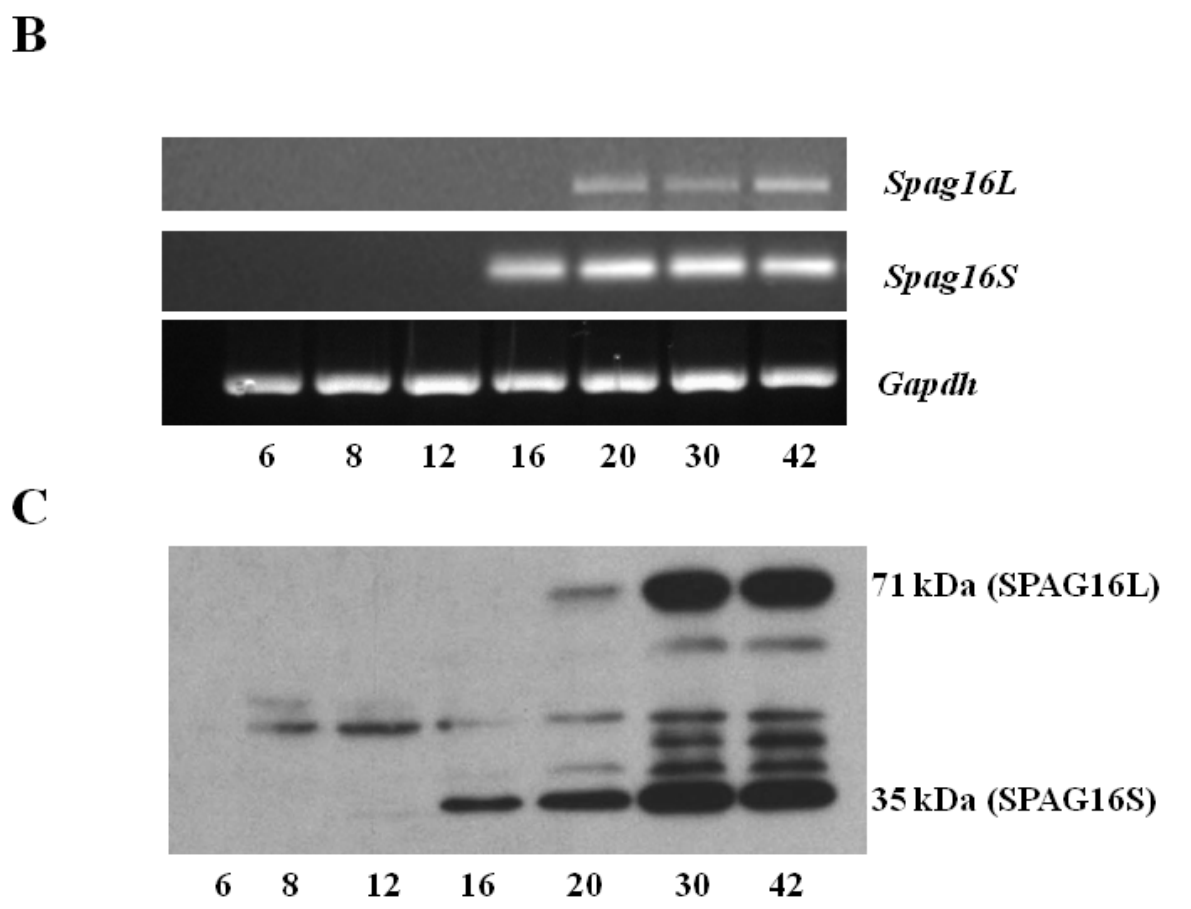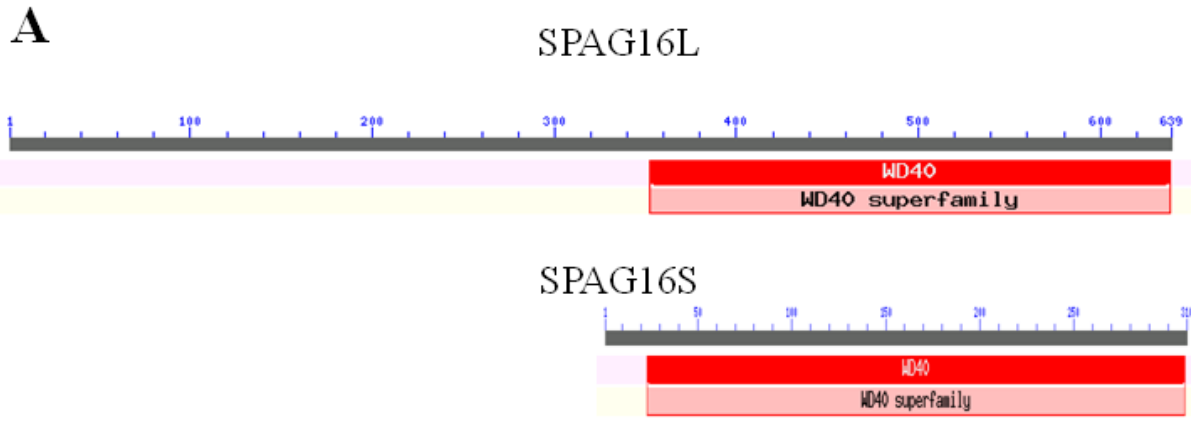
Figure 2-2. SPAG16isoforms have identical conserved domains. SPAG16S appears before SPAG16L during the first wave of mouse spermatogenesis
(A) Alignment and conserved domain analysis of SPAG16L and SPAG16S proteins. RNA (B) and protein (C) were isolated from mouse testis at the indicated day (bottom row) after birth. (B) Specific primer sets were used to probe gene expression by PCR of cDNA. (C) Protein samples were separated by SDS-PAGE and probed by Western blotting with a C-terminal SPAG16 antibody that recognizes both SPAG16L (71 kDa band) and SPAG16S (35 kDa band). Additional bands are not specific.
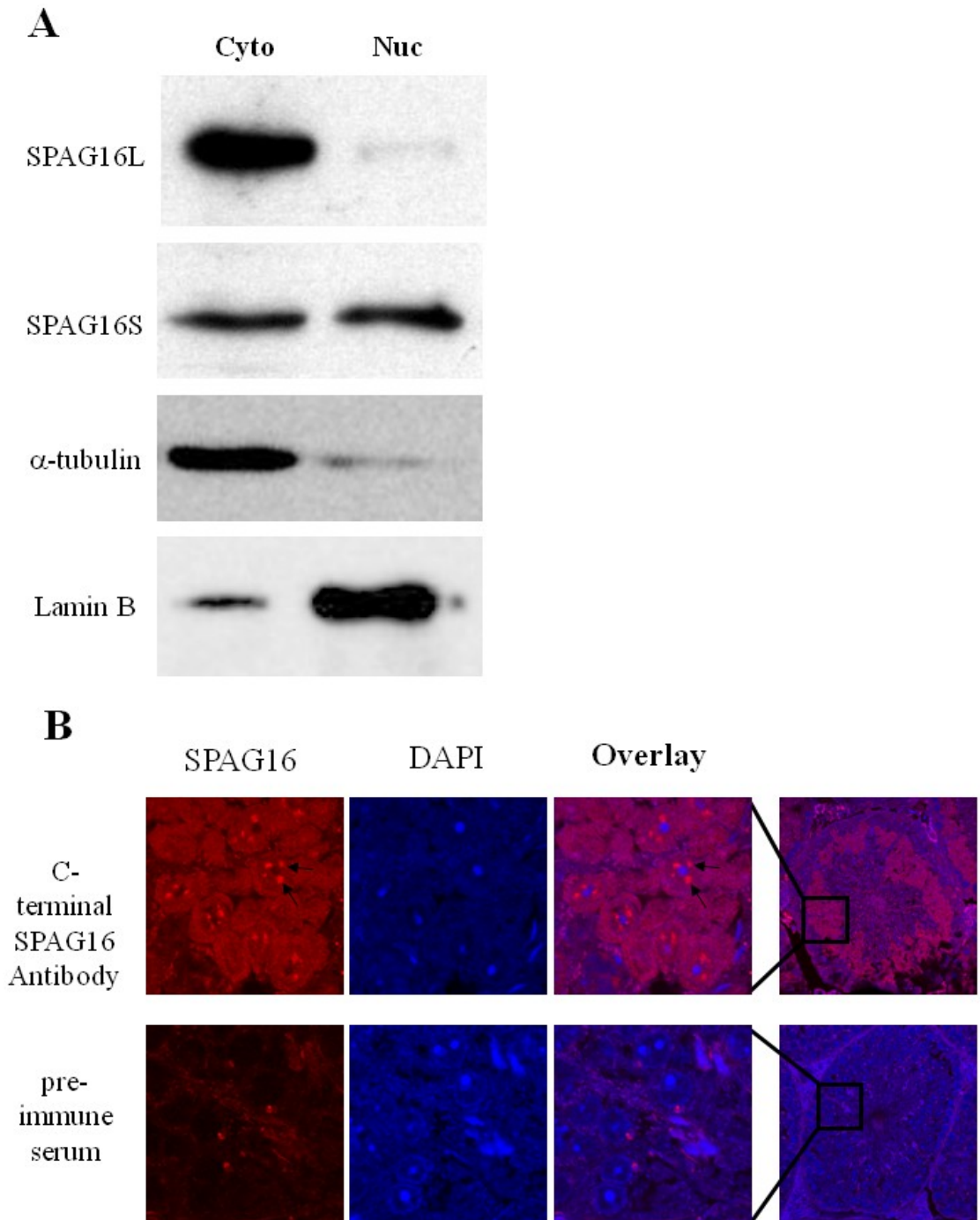
Figure 2-3. SPAG16L is in the cytoplasm of male germ cells, SPAG16S is in both the nucleus and the cytoplasm

(A) Cytoplasmic and nuclear fractions of adult mouse testis probed by Western blot for SPAG16 (C-terminal antibody recognizing both isoforms) or markers of cytoplasm (α-tubulin) or nucleus (Lamin B). (B) Sections of adult mouse testis immunolabeled with SPAG16 C-terminal antibody or pre-immune serum (negative control). Arrows indicate sample nuclear regions of heightened SPAG16 antibody immunoreactivity.
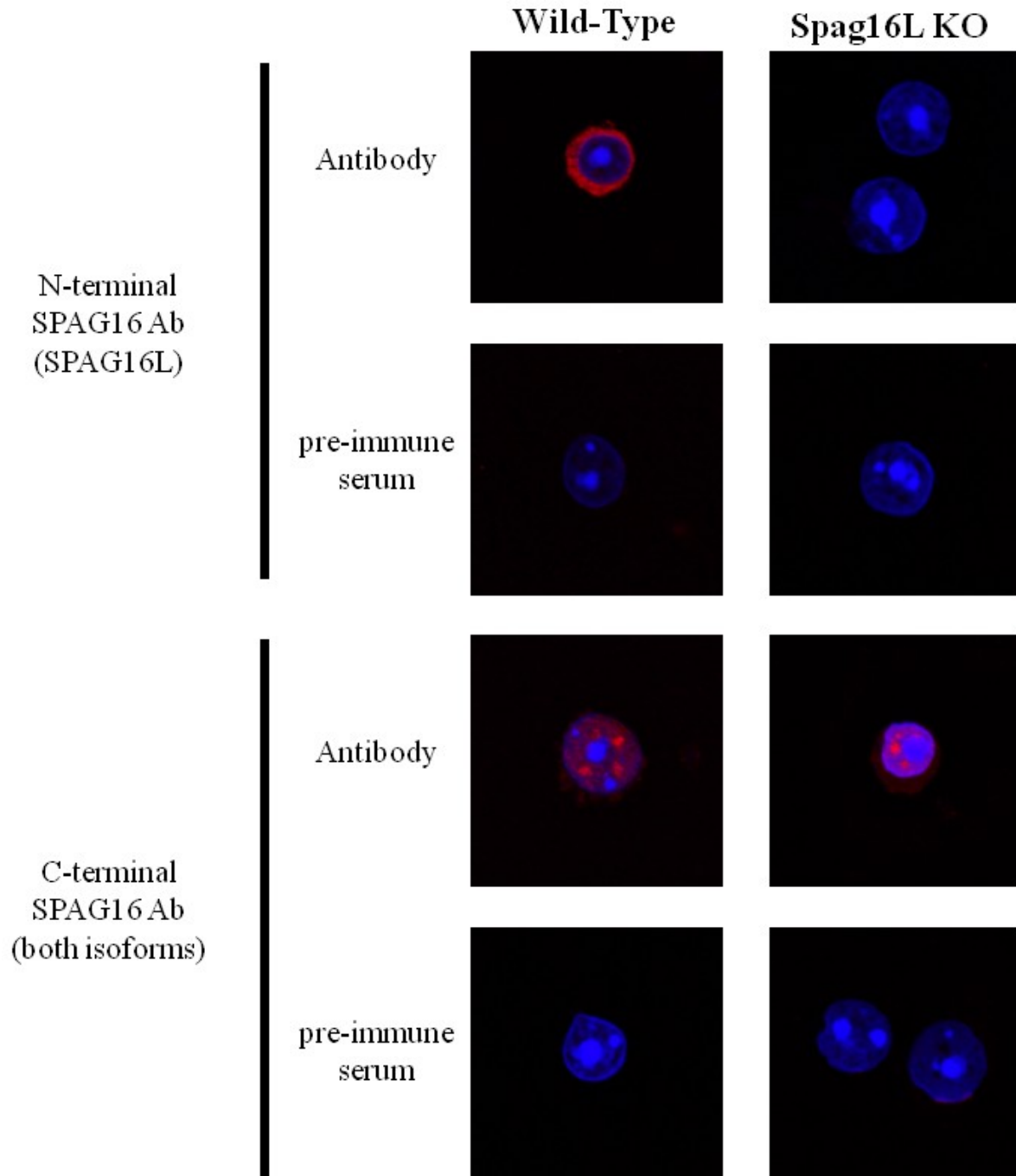
Figure 2-4. SPAG16S shows enriched nuclear sub-localization
Mixed male germ cells from wild-type or transgenic SPAG16L-KO mice immunolabeled with SPAG16 antibodies (red; N-terminal = SPAG16L only, C-terminal = both isoforms) and nuclear-stained with DAPI (blue). Pre-immune serum for each antibody is included as a negative control.

A

	SC35	SPAG16	DAPI	Overlay
SC35 + SPAG16 pre-immune				
anti-mouse IgG + SPAG16				
SC35 + SPAG16				

B
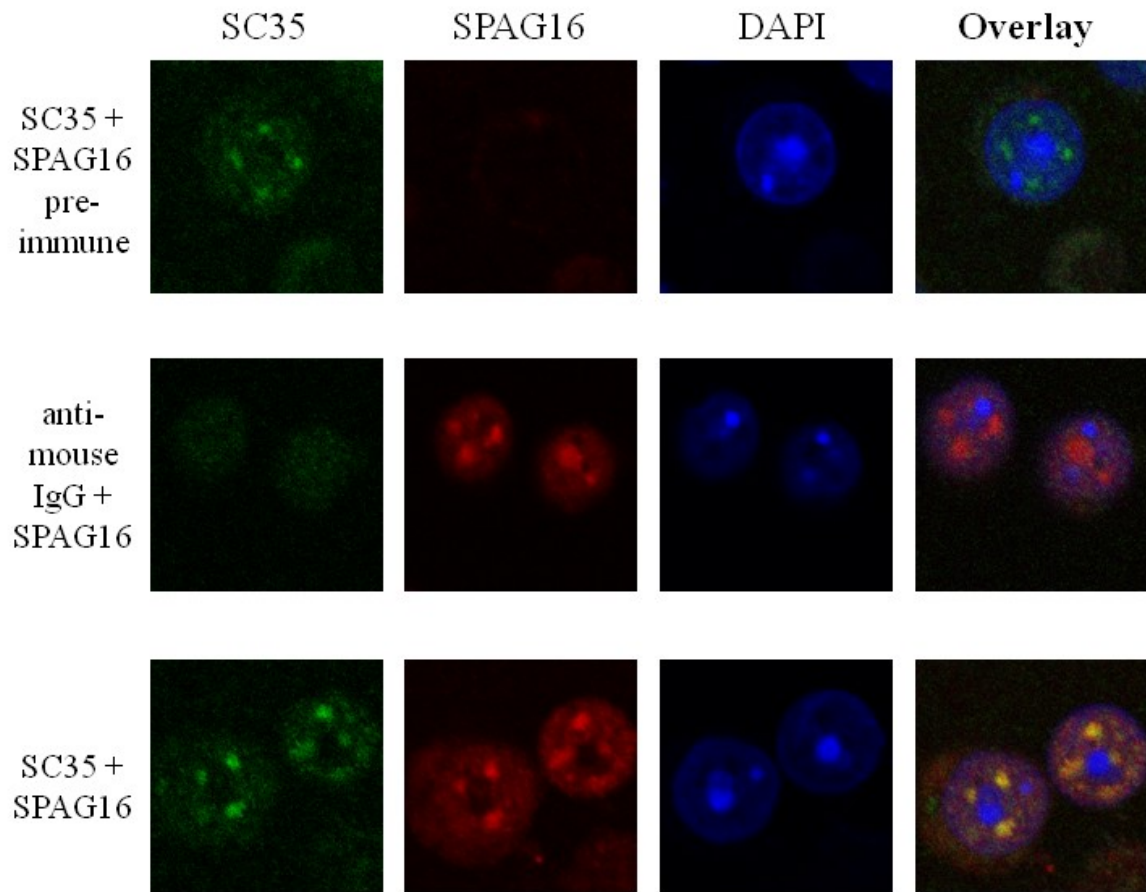
	Pearson's coefficient	SC35 colocalization coefficient	SPAG16S colocalization coefficient
SC35 + SPAG16S	0.40	0.79	0.44
SE	0.017	0.025	0.026

Figure 2-5. SPAG16S co-localizes with SC35 in nuclear speckles of mouse male germ cells

(A) Mixed male germ cells immunolabeled for SC35 (green) or SPAG16 (red), with DAPI as a nuclear marker. (B) Co-localization analysis of SC35 + SPAG16, *n* = 43.

55
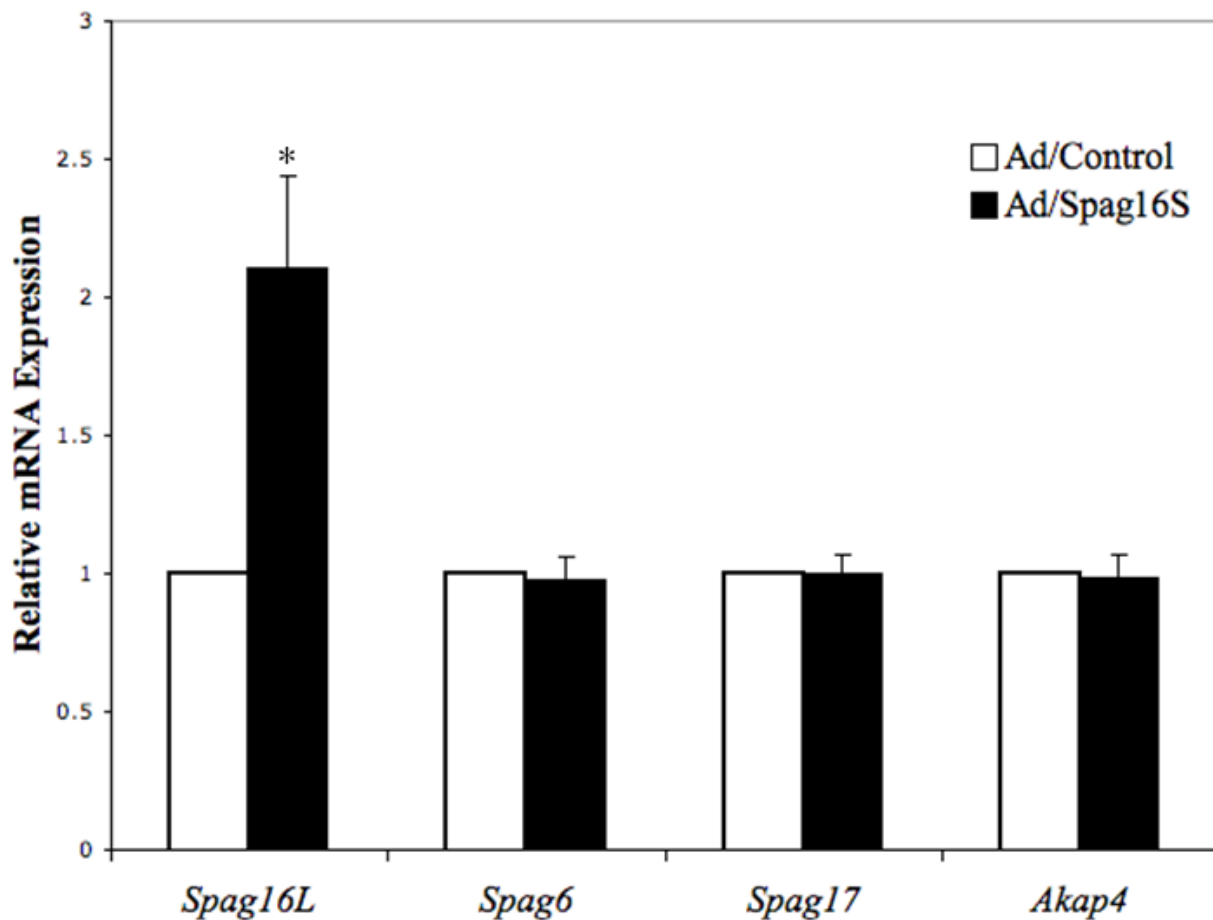
Figure 2-6. Transduction of SPAG16S induces SPAG16L expression in cultured mouse male germ cells

Isolated adult mouse male germ cells were infected with an adenovirus causing SPAG16S transduction or a control adenovirus. Following 48 hours in culture, RNA was isolated and relative expression levels of indicated transcripts were measured. (* $p < 0.05$ compared with Ad/Control)
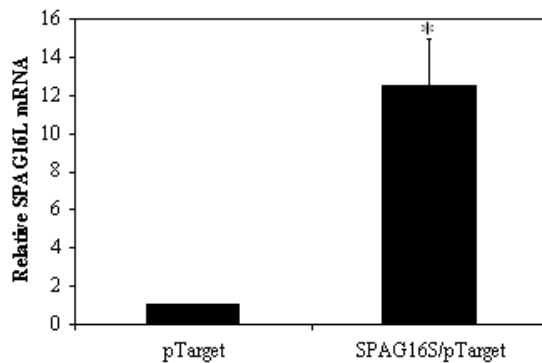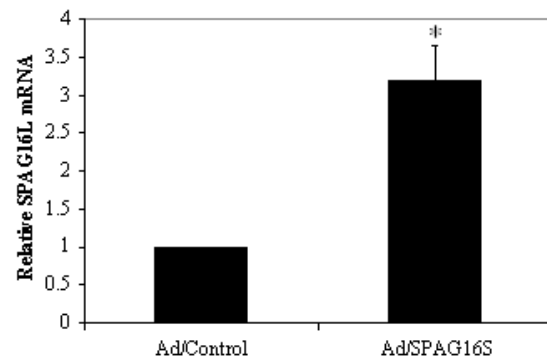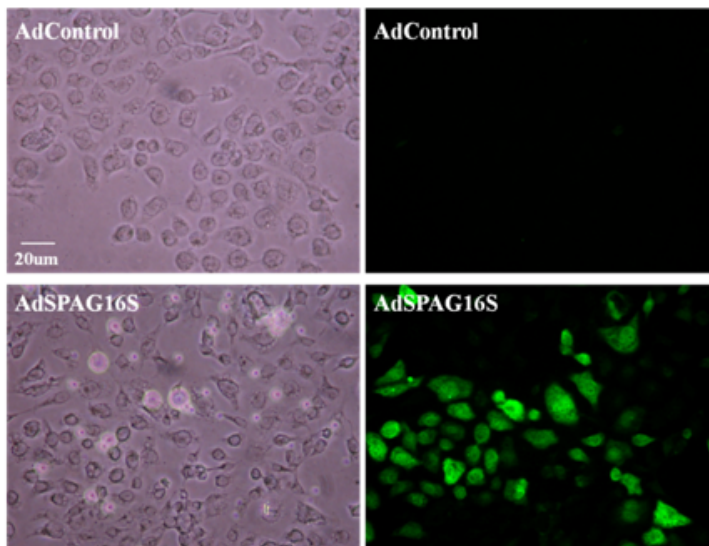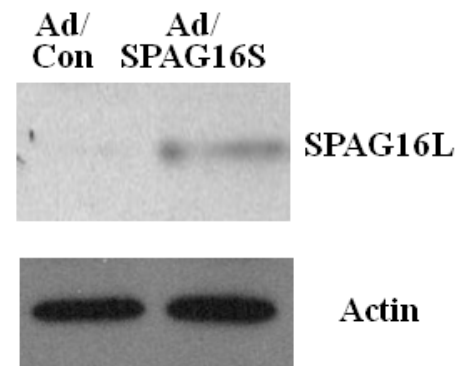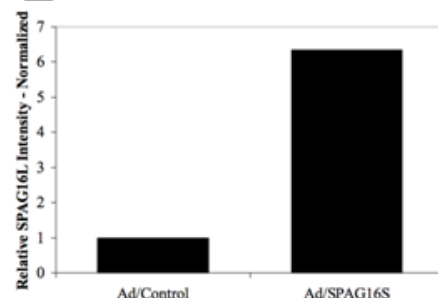
Figure 2-7. SPAG16S stimulates Spag16L mRNA and SPAG16L protein expression in BEAS-2B cells

Analysis of *Spag16L* mRNA expression by real-time PCR in BEAS-2B cells stably expressing SPAG16S (A) or transduced with Ad/SPAG16S (B). (C) Adenovirus-transduced BEAS-2B cells immunolabeled with a C-terminal SPAG16 antibody. (D) Analysis of SPAG16L protein expression by Western blotting in BEAS-2B cells infected by Ad/SPAG16S. This panel demonstrates two independent experiments. (E) Relative intensity of SPAG16L signal in panel D, normalized to Actin loading control for each sample (relative Actin signal for Ad/Control = 47.5%, for Ad/SPAG16S = 52.5%) (* p<0.05 compared with pTarget or Ad/Control)

57
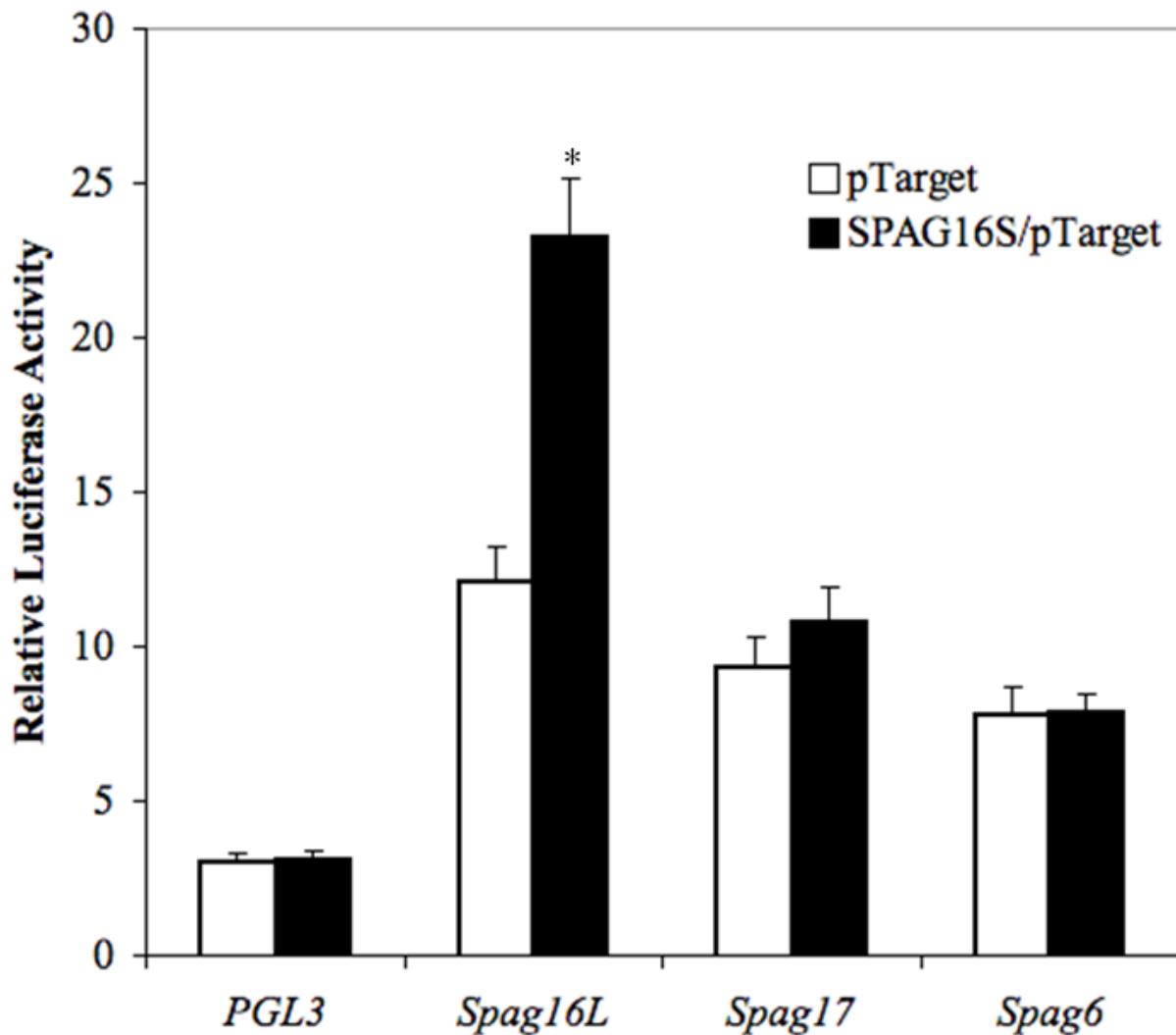
Figure 2-8. SPAG16S stimulates Spag16L promoter activity
Relative luciferase activity, normalized to PGL3 control promoter plasmid co-transfected with pTarget control vector plasmid. Beas-2B cells were co-transfected with indicated promoter/PGL3 constructs (*Spag16L*, *Spag17*, and *Spag6*) and either pTarget or a SPAG16S/pTarget plasmid. Luciferase activity was measured after 48 hours to assess promoter function. (* $p < 0.05$ compared with pTarget control vector co-transfection;
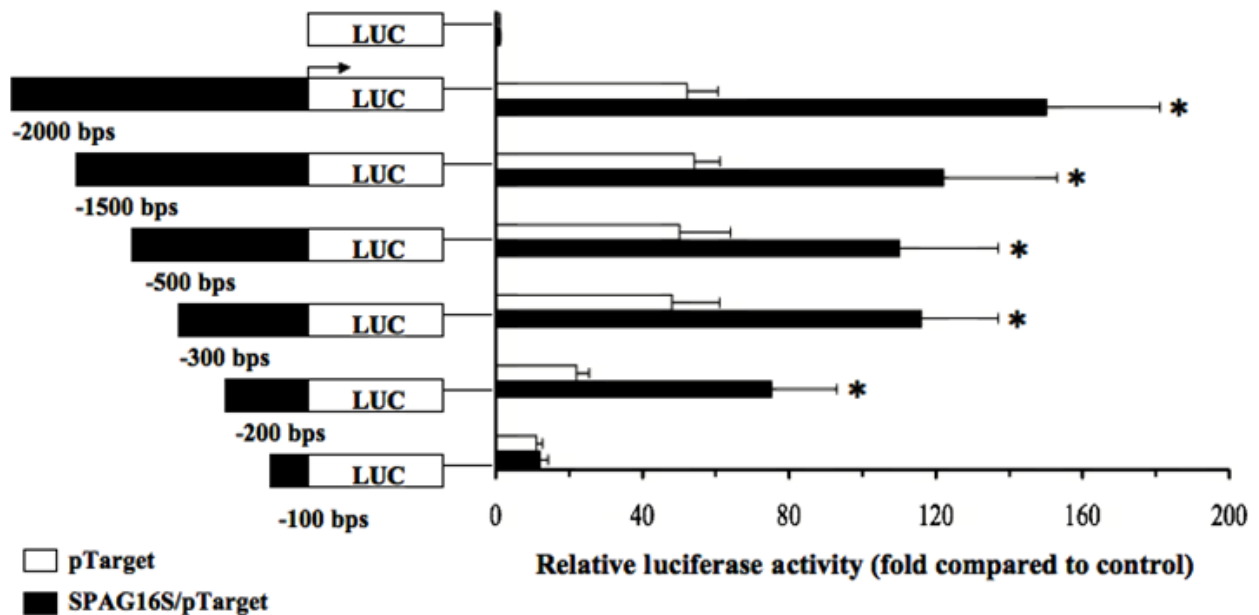
Figure 2-9. Identification of a Spag16L promoter region activated in the presence of SPAG16S

Relative luciferase activity, normalized to PGL3 control promoter plasmid co-transfected with pTarget control vector plasmid. Beas-2B cells were co-transfected with human *Spag16L* promoter plasmids (corresponding to the indicated regions upstream of the transcription start site) and either pTarget control or SPAG16S/pTarget. All promoter constructs except the -100 bp promoter demonstrated significantly increased activity in the presence of SPAG16S co-transfection. (* $p < 0.05$ compared with pTarget co-transfection for a given promoter)

```
                            20                        40                        60
Mouse  ATAAAATTCTTATGGAGTTCTGTACCTAAATATATAAATCATAGTGTTATTTTTTGCCAC
Rat    ATAAAATTCTTATGGAGTTCTGTACCTAAATATATAAATCATAGTGTTATTTTTTGCCAC

                            80                        100                       120
Mouse  TT-TATCTCGGAGGAAAGTGTTGACTATATTTCAGGAATGAAATATGTACAACACATTGA
Rat    TTCTATCTCGGAGGAAAGTGTTGACTATATTTCAAGAATGAAATATATACAGCACATTGA

                            140                       160                       180
Mouse  CTTGTGGGATGACTGTGGTTTCACCAAGTAGTCGTTACGGAGTGCCTATTATATATAAT
Rat    CTTGTGGGATGCCTATGCATTTACCAAGTGGTCGTTACAGAGTGCCTATTATATAT---

                            200                       220                       240
Mouse  ACTCTGTGCTGAAGAGACAGAAATGAATCACACGCTGTTGCATGCTGGTAGCTCTGACTT
Rat    --TCTATCCTGAAAAGACAGAAGTGAATAATAAGCTGTTGTTGCTGGTAGCTCATAGTT

                            260                       280                       300
Mouse  GAACTGGAGTAGACAGTGACCCAATTGTACTGTCCTCTATAACACGCAGTTCTACATTAA
Rat    GAACTGGAGTAGATAGTGACCCAATTGCATTGTTCTCCATAACACACAGTTCTACATTAA

                            320                       340                       360
Mouse  TAGCAGGGGTTGATGTAAACCAAGACAGC---GTGGCTTAGGATCGGTGTATGTTAAGGA
Rat    TAGCAGAGTTGATGTAAAACGAGACAGTACTGTGGCTTAGGGTTGGTTTATATTAAGAA

                            380                       400                       420
Mouse  TGAAGTGGTTCATTCCCCAAGAAGAAATCATAGATAAGTCTTAGAAGTGTAAACCTTACT
Rat    TGAAGTGATTCATTTCCCAAGAAGAAATCACAGACGAGTCTTAGAAATGTAAATCTTACT

                            440                       460                       480
Mouse  CTGCACTGTAGCATCCTCTGACAGTGCGCATAGAAAACAAGATAAGTGA----TTCTGGT
Rat    CTGTACTGTAGGGTCATCTGAGAGC-TGCATGGAAGACAAGATAAGTGAGTCTTTCTGTT

                            500                       520                       540
Mouse  AACCTGCTATGTAATTTTTTGAGAGGGGGTGGTCATTAAGCAGGGAGACACATGAGAGGCT
Rat    AACCTGCTATGTAATTTTGTGGGG----ATTCATTGAGCAGGGAGACACATCAGAGGCT

                            560                       580                       600
Mouse  GCAGCAGTGTCCTGTGGGGAACAGTGACAAAGTGATGAGAAGGGAGCCCATGTGA---AG
Rat    GCA-----CTCCTGTGGGAAACAATGGCAAAATGATGAGAAAGGAGCATACGTGATGAGC

                            620                       640                       660
Mouse  GAGGAAGTGGATCTCA-GGCCTAGCTTAGAGTGAGTCACTAGAAGAGGGCTGACAAGCAC
Rat    GAGGACATGGATCTCAAGGCTAGCTTAGAGTGAGTCACTAGATGATGATTGACAAGCAT

                            680                       700                       720
Mouse  CTCATCGCAACCGGGATGCGAAAAG--------------------------------TGTAG
Rat    CTCATCGTGACCATGAGGTGAAAAGAAGAATTTACGTAAACGTTTTTTAAAAAAATGTAG

                            740                       760                       780
Mouse  GCACAGTGTGA-TAGGGTCTTCATGGATTTTGTTGTTGTTCATTTGCTTTGCTTCAGTAG
Rat    GCACAGTGGGGGGTGGCTATTGGTGATTTTTATTGTTGTTCATTTGCTTTGCTTCAAAG

                            800                       820                       840
Mouse  TGTTTGGAACTGAGAAACCAAACTTGAATTCCAATCTGGATCAAACTGATCTGCTATTTG
Rat    TGTTTGAAACTGAGAAGTCAAAGTTGAGTTCCAATCTGGATCAAACTGGTCTGCTATTTG

                            860                       880                       900
Mouse  GGGGTTATCTTTGAGGCTATTCCAGCTTTGAGAACCACTTAACTTCTTTCCTTCCTTCT
Rat    GGAGTTATCTTCCAGGCTATTCCAGCTTTGAGGAACACCTAACTTCTTCCTTCCTTCC

                            920                       940                       960
Mouse  TTCCTTCCTTCTTTCCTTCCTTCTTTCCTTCCTTCCTTCCTTCCTTCTT-----CCTTCC
Rat    TTCCTTCCTTACTTCCTTCCTTCCTTCCTTCCTTCAGTCCTTCCTTCCTTCAGTCCTTCC

                            980                       1000                      1020
Mouse  TTTTTTTCTTCCTTCCTTCCTTCCTTCCTACCTCCCCTCTTTCTTTCCCTCCTTCCCCTTCCTT
Rat    TTCTTCCCTTCCTTCCTTCCTCCCCTCCCTCCCTCCTCCCTCCATGTTCTTNNNNNNNNN
```

60

Figure 2-10. Mouse and Rat Spag16S putative promoter and 5'-UTR regions
Sequences of mouse and rat transcription start sites and putative upstream promoter regions. Putative transcription start sites as noted in red correspond with GenBank sequences (mouse – NM_025728.3; rat – BC158602). Upstream genomic regions are also as noted in GenBank (mouse – AY742710.2; rat – NC_00508: 68851813 – 68853621). Alignment analysis performed using MacVector v10.6.

Chapter 3: Genetic Variation in SPAG16 Regions Encoding the WD40 Repeats is not Associated with Reduced Sperm Motility and Axonemal Defects in a Population of Infertile Males

Abstract

Background: SPAG16 is a critical structural component of motile cilia and flagella. In the eukaryotic unicellular algae *Chlamydomonas*, loss of gene function causes flagellar paralysis and prevents assembly of the "9 + 2" axoneme central pair. In mice, we have previously shown that loss of *Spag16* gene function causes male infertility and severe sperm motility defects. We have also reported that a heterozygous mutation of the human *SPAG16* gene reduces stability of the sperm axonemal central apparatus.

Results: In the present study, we analyzed DNA samples from 60 infertile male volunteers of Western European (Italian) origin, to search for novel *SPAG16* gene mutations, and to determine whether increased prevalence of *SPAG16* single nucleotide polymorphisms (SNPs) was associated with infertility phenotypes. Semen parameters were evaluated by light microscopy and sperm morphology was comprehensively analyzed by transmission electron microscopy (TEM). For gene analysis, sequences were generated covering exons encoding the conserved WD40 repeat region of the SPAG16 protein and the flanking splice junctions. No novel mutations were found, and

the four SNPs in the assessed gene region were present at expected frequencies. The minor alleles were not associated with any assessed sperm parameter in the sample population.

Conclusions: Analysis of the *SPAG16* regions encoding the conserved WD repeats revealed no evidence for association of mutations or genetic variation with sperm motility and ultrastructural sperm characteristics in a cohort of Italian infertile males.

Introduction

Infertility impacts approximately 9% of couples globally, with reports ranging from 3.5% - 16.7% of couples (de Kretser and Baker 1999),   and it is estimated that male factor infertility plays a role in as many as 55% of cases (Jurewicz, Hanke et al. 2009; Chemes and Rawe 2010; de Kretser 2010). There are multiple causes of male infertility, including congenital factors and environmental exposures (Zariwala, Knowles et al. 1993; Zariwala, Knowles et al. 2007), as well as gene mutations which cause defects in spermatogenesis and sperm flagellar dysfunction.

The most studied mutations associated with abnormal sperm motility and male infertility cause the primary ciliary dyskinesias (PCD) (Ebersold, Levine et al. 1962; Pan, Wang et al. 2005). Genes involved in the structure and function of the "9 + 2" axoneme, the principle scaffold and regulator of motile cilia and flagella, have been extensively studied and characterized in the biflagellate eukaryotic algae *Chlamydomonas*, wherein loss of axonemal components has been shown to cause immotility (Bastin, Pullen et al. 2000; Inaba 2003). Most of the mutations identified to date that cause PCD in humans encode

proteins that are associated with the axoneme outer doublets. However, relatively little is known about the contribution of mutations or genetic variation in genes encoding the central apparatus of the axoneme to sperm motility defects. It is known that the "9 + 2" axoneme is strongly conserved in overall structure and function (Sapiro, Kostetskii et al. 2002).

We previously reported that mice lacking *Spag6*, which encodes the mammalian orthologue of the *Chlamydomonas* axonemal central apparatus protein PF16, exhibited a phenotype of both male infertility associated with severe sperm motility defects and axonemal structural abnormalities (Smith and Lefebvre 1997; Pennarun, Bridoux et al. 2002), demonstrating that loss of an axonemal central apparatus protein can have dramatic effects in mammals as well.

SPAG16, the mammalian orthologue of the essential *Chlamydomonas* central apparatus protein PF20 (Zhang, Kostetskii et al. 2006), is also required for male fertility and sperm motility in mice (Zhang, Kostetskii et al. 2006). The *Spag16* gene encodes two major transcripts in mice, which generate SPAG16L, a central apparatus protein, and SPAG16S, a protein localized to the male germ cell nucleus and cytoplasm. Male mice homozygous for a mutation ablating SPAG16L production have a sperm motility defect and are infertile (Lesich, Zhang et al. 2010). The axonemes of SPAG16L-deficient mice also show abnormal responses to calcium, indicating that loss of SPAG16L disrupts either the ability to process or the ability to respond to key molecular signaling (Zhang, Kostetskii et al. 2004). When the transcripts encoding SPAG16L and SPAG16S are both

disrupted in a transgenic male mouse, there is haploinsufficiency and abnormal spermatogenesis in the chimeric state, implicating SPAG16S in fundamental processes of sperm formation distinct from the structural role of SPAG16L (Zhang, Zariwala et al. 2007). Importantly, the *Spag16* mutant allele affecting both isoforms was not transmitted to offspring by chimeric males, demonstrating that heterozygous/non-homozygous *Spag16* mutation may cause a pronounced phenotype, and that the two murine SPAG16 isoforms each play different but essential roles.

We have also reported previously that sperm from human subjects heterozygous for a frameshift mutation in S*PAG16* exhibited instability of important central apparatus components SPAG16L, SPAG17, and SPAG6 (Yatsenko, Arias et al. 2009). While both subjects in this report were fertile, observed biochemical instability of the sperm axoneme suggests that even heterozygous disruption of *SPAG16* has phenotypic consequences that may reduce fecundity. Additionally, a preliminary study of oligozoospermic and oligoasthenozoospermic males has identified 29 previously unreported mutations in *SPAG16* transcripts isolated from ejaculated sperm, further suggesting a critical role for *SPAG16* in male fertility (Stirnimann, Petsalaki et al. 2010).

In the present study, 60 males of Western European (Italian) origin volunteered for semen and genomic analyses following diagnosis of male factor infertility. We evaluated the genomic region encoding the highly conserved WD domains of SPAG16 proteins (exons 10-16). WD regions are semi-conserved 20 amino acid sequences giving rise to a β-propeller tertiary structure. These domains are the only known functional domains

found in SPAG16 proteins, both isoforms of which contain seven such domains. These β-propeller structures are classically thought to mediate protein-protein interactions (reviewed in (Li and Roberts 2001)), playing crucial roles in macromolecular assembly relevant to a diverse array of cellular tasks, including cell growth and division, intracellular communication, apoptosis, and transcription regulation (Lau, Bachorik et al. 2009). Recent reports have also demonstrated that some WD domains interact directly with specific RNA sequences, further expanding the range of potential roles played by members of this class of proteins (World Health Organization 1999).

To better understand potential links between *SPAG16* gene variation and specific mechanisms of infertility, semen analysis was performed for each subject (Baccetti, Bernieri et al. 1995; Collodel and Moretti 2008). In addition, sperm ultrastructure was assessed by transmission electron microscopy (TEM) and described according to previously established standards (The International HapMap Consortium 2003).

Based on our previous observations of *Spag16* gene function in mice, and the influence of a heterozygous mutation affecting the WD repeat region in humans on central apparatus stability, we hypothesized that functional SNPs in the human *SPAG16* gene could cause defects in sperm motility and ultrastructure manifest in both the heterozygous and the homozygous states. The present study analyzed genetic variation in a population of infertile males, and their association with parameters that are potentially regulated by *SPAG16*.

Results

SPAG16 sequences are highly conserved

In order to establish whether SPAG16 amino acid mutations were likely to result in functional changes, amino acid sequences for several species were aligned to determine regions of conservation within the protein. Alignments are shaded and boxed to indicate identical amino acids relative to the human sequence or conservative substitution (Fig. 3-1). Quantitative assessment demonstrates greater similarity, as expected, between mammalian SPAG16 sequences as compared to the *Chlamydomonas* sequence (Table 3-1). However, the region most conserved across all species is the C-terminal end, which contains the WD40 repeat domains, the only known conserved domain within these proteins.

While SPAG16 proteins are highly conserved, phylogenetic analysis demonstrates especially strong relationships among mammalian forms, particularly rodents and primates (Fig. 3-2). Only mice have two verified SPAG16 isoforms encoded by two different transcripts: the flagellar form, found in all eukaryotic motile cilia and known in mice as SPAG16L, and a testis-specific SPAG16S, which consists of the WD repeat region. The only known rat SPAG16 is similar in structure to SPAG16S, and was isolated from testis. Furthermore, the nucleotide sequence of the 5'-UTR of rat SPAG16 mRNA [GenBank:NM_001134728.1] is similar to that found in mouse SPAG16S mRNA [GenBank:NM_025728.3]. It is reasonable to speculate that a rat SPAG16L protein exists containing both the conserved WD repeats on the C-terminal end and an approximately equally sized N-terminus, consistent with the protein's structure in all other species.

Analyzed *SPAG16* SNP allele frequencies are not altered in the sample population

For one SNP analyzed by Taqman Genotyping Assay, rs10167688, no individuals with the minor allele were detected. This result was further corroborated by exon region-specific sequencing. Heterozygosity for this SNP has been reported only in West African Yoruban populations (The International HapMap Consortium 2003), so its absence in our population was not unexpected. The probe set used to analyze this SNP was confirmed to be effective using a test set of West African female DNA samples obtained in an unrelated study (data not shown).

Two other SNPs were analyzed by TaqMan assay and confirmed by genomic sequencing, which also demonstrated two additional SNPs present within splice junctions. For all four of these SNPs, minor allele frequencies in our patient population did not differ significantly (rs2042791: p = 0.86; rs2042792: p = 0.49; rs12623569: p = 0.87; rs16851495: p = 0.20) from those reported in previous genomic studies of a reference population (Table 3-2) (Ng and Henikoff 2003). Each allele was found to be in Hardy-Weinberg equilibrium. No other alternate alleles corresponding to SNPs identified in previous genomic sequencing studies were found in the sample population. However, the minor allele frequencies for all of putative SNPs not found in the present study population are quite low or unconfirmed in the reference population (Table 3-3).

Of the four SNPs analyzed, two SNPs were found to be in linkage disequilibrium, rs2042791 and rs2042792 (Table 3-4).

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Analyzed *SPAG16* SNPs are not associated with alterations of axonemal or periaxonemal ultrastructure, fertility index, and sperm parameters

In the infertile male population studied, semen analysis revealed severe defects in sperm concentration, motility, and ultrastructure (full data in Table 3-5). No single parameter was uniformly perturbed amongst all patients carrying a particular SNP minor allele. Additionally, no mutation was found for which all homo- or heterozygous carriers displayed a similar phenotype beyond deficiencies common to the sample (infertile) population in general. There were nominally significant associations found between specific SNPs and sperm ultrastructural characteristics, but these were not significant after Bonferroni correction for multiple testing of 18 phenotypes with 7 genetic variations (4 SNPs and 3 haplotypes), which resulted in a Bonferroni corrected $p \leq 0.000397$ to indicate significance at the 5% level. Minor allele carriage at rs16851495 correlated negatively, but not significantly after Bonferroni correction for multiple testing, with normal axoneme structures (β = -0.16, nominal p = 0.039) and with normal accessory fiber structures (β = -0.12, nominal p = 0.041;). Minor allele carriage at rs12623569 showed a small, nominally significant, positive correlation with normal fibrous sheath structures (β = 0.09, nominal p = 0.14) (Table 3-6).

For two SNPs [dbSNP: rs2042791; dbSNP rs2042792] in LD, haplotype was not associated with studied sperm parameters or ultrastructural variation (Table 3-7).

Amino acid-altering *SPAG16* SNPs are predicted to be functionally tolerated

Two of the identified SNPs cause point mutations in SPAG16 proteins. These amino acid substitutions were assessed for predicted effects on protein structure and function. Both amino acid substitutions were predicted to be tolerated using SIFT (Schwede, Kopp et al. 2003), which analyzes amino acid properties and conserved identities.

The crystallographic structures of other WD repeat proteins have been determined and these structures can be used to model the structure of the SPAG16 WD repeat region. Using SWISS-MODEL (Sherry, Ward et al. 2001), SPAG16L was evaluated for homology with proteins of known structure, and the amino acid sequence was aligned with that of WDR5Delta23, a member of the WD repeat protein family. The locations of the amino acid residues affected by the SNPs evaluated are labeled in the 3-D structure model shown in Figure 3-3. K425 is predicted to be located on the periphery of the molecule, which is not a known binding site of the structural homologue WDR5Delta23. Q361 extends into the predicted binding pocket.

Discussion

In the *SPAG16* regions analyzed, both major and minor alleles were present in the study group for four known polymorphisms, and no previously unreported mutations were detected. At rs2042791 (The International HapMap Consortium 2003), the minor allele replacement of an adenosine by a cytosine residue causes the replacement of a glutamine at position 361 [GenBank: NP_078808.3] with a histidine. Previous genotyping studies have reported a minor allele frequency of 0.39 (Ng and Henikoff 2003) in

individuals of Caucasian ancestry for this SNP. Fisher's exact test demonstrated no significant difference between the measured minor allele frequency of 0.408 in our sample population and that established in control populations ($p > 0.05$). While the present study lacks sufficient power to detect small effects of these alleles on variation in observed parameters of sperm characterization, among patients carrying this SNP no single factor was uniformly deficient within the distribution of sub-normal semen analysis of the study participants. Full genotype and semen analysis results are included for reference (Supplemental Table 2).

The lack of association between this SNP and sperm dysfunction is not surprising, given that the glutamine replacement by histidine is predicted to be tolerated in the protein structure (The International HapMap Consortium 2003). Further, the amino acid is not conserved in mammals – *Mus musculus* [GenBank:AAI20669.1, NP_080004.1, NP_083436.2] and *Rattus norvegicus* [GenBank:NP_001128200.1, AAI58603.1] reference sequences for the orthologous proteins in fact report a histidine at this position, consistent with the human minor allele. The same amino acid replacement is predicted in *Pan troglodytes* [GenBank:XP_001148592.1, XP_526016.2, XP_001148393.1]. These data are consistent with the hypothesis that structural rather than sequence homology is essential to maintenance of axoneme function.

The rs2042792 minor allele was not found at a significantly different frequency than in the reference population ($p > 0.05$), and did not correlate with assessed sperm phenotypes.

Rs2042791 exists in LD with rs2042792 in our population, but no phenotypic consequences were associated with any of the observed haplotypes.

Minor allele replacement of an adenosine with a cytosine in rs12623569 causes an amino acid replacement at position 425 [GenBank:NP_078808.3], with a threonine taking the place of a lysine. Previous genotyping studies have shown a minor allele frequency for this SNP in individuals of Caucasian ancestry of 0.30 (Nagarkatti-Gude, Jaimez et al. 2011). The present sample population exhibited a minor allele frequency of 0.333, which was not significantly different than that in control populations (p > 0.05). While the lysine at position 425 encoded by the major allele is present in other mammals (*Mus musculus* – GenBank:NP_080004.1, NP_083436.2; *Rattus norvegicus* – GenBank:NP_001128200.1, AAI58603.1), replacement by threonine is predicted to be tolerated by the protein structure. Interestingly, minor allele carriage was found to be nominally associated with a higher percentage of normal accessory fibers of the axoneme. Although the statistical significance of this association was lost upon correction for multiple testing, further studies on a larger study population may be warranted to identify contributions of the SNP to sperm flagellar structure.

At rs16851495, minor allele replacement of guanine with an adenine residue does not affect protein translation directly, as the position lies outside the translated exon region. However, minor allele carriage was nominally associated with the reduced presence of normal axoneme structure in the study population. The statistical significance of this

association did not withstand correction for multiple testing. However, the limited power of our study may have contributed to the absence of a robust association.

For all SNPs discussed, both heterozygous and homozygous individuals were present in the sample population, and statistical analysis demonstrated no difference in genotype frequency between the sample and control populations (p > 0.05). While the study did not have sufficient power to analyze possible contributions to complex traits, the results negate the hypothesis that amino acid modification at the tested positions may result in a loss of protein function that would mimic the severe phenotypes observed in transgenic mouse studies.

Our results suggest that non-synonymous amino acid substitutions at residues 361 and 425 in the SPAG16 protein are not sufficient to explain a reduction of sperm motility and fertility index, the presence of axonemal/periaxonemal alterations, or an increased percentage of sperm pathologies in the assessed patient population. Based on the strong homology between members of the WD protein family, it is tempting to suspect that these resides may be non-essential in other WD repeat proteins as well.

The profound defects observed in studies of *Spag16* gene effects in other species suggest that a functional mutation would significantly perturb sperm function, and would be observed even in a small sample population. Our observations do not, however, preclude the possibility that alternative variations in the *SPAG16* gene cause an increase in sperm ultrastructural alterations, and a reduction in sperm motility. Indeed, our

previous studies suggest that more significant SPAG16 mutations, such as a frameshift, can reduce sperm central apparatus stability. Further studies of *SPAG16* gene variation are warranted to offer a comprehensive understanding of the gene's contributions to male fertility. The sensitivity of the present study to detect variation with the sample is limited by the lack of similarly extensive sperm analysis data, in particular TEM ultrastructural analysis, from a control population. Future development of this area of research should include control samples from men with known fertility and/or sperm count, motility, and structure in the normal range.

Future studies in mammals are necessary as well to explore the nature of *Spag16* gene evolution, and the possibility that male germ cell-specific isoforms exist in multiple species. The only identified rat SPAG16 protein is similar to SPAG16S in size, and is derived from mRNA similar to murine *SPAG16S*, with a 5'UTR from the region upstream of the first exon suggestive of independent transcription, rather than splice variation. A putative *SPAG16S* promoter and 5'UTR have been identified in humans as well [GenBank: EF591776.1], raising the possibility that SPAG16S maybe be common to mammalian male germ cells. We have recently shown that murine SPAG16S is enriched within nuclear speckles of male germ cells (Sherry, Ward et al. 2001; The International HapMap Consortium 2003). This unique localization, combined with phenotypic results from various *Spag16* transgenic mice, strongly suggests a unique processing role for the SPAG16S protein, and thus a distinct role for the *Spag16* gene apart from its structural role in the axoneme.

Conclusions

In a sample population of 60 infertile males of Western European origin, mutations in *SPAG16* were not significantly associated with a single phenotype of sperm alteration. The findings suggest that mutations and or genetic variation in the *SPAG16* regions encoding the protein's WD repeats are not likely to be major causes of sperm motility and sperm flagellar defects.

Materials and Methods

DNA sample acquisition

DNA used in the present study was isolated from peripheral blood lymphocytes submitted by 60 male volunteers of Western European (Italian) ancestry diagnosed clinically as sub- or in- fertile. Inclusion criteria for patients were: sperm progressive motility $\leq$ 10%, normal lymphocyte karyotype, normal hormone levels, absence of anatomical pathologies, genitourinary infections. Patients with known ultrastructural sperm defects of possible genetic origin (dysplasia of the fibrous sheath, cilia immotile syndrome, round headed sperm, etc.) were also excluded.

Ethics

Research was carried out in full compliance with the Helsinki Declaration of ethical principles for medical research involving human subjects. All patients signed a declaration of informed consent to participate in the research. The work was approved of and performed in full accordance with policies governing human subject research at the by the Ethics Committee for the University of Siena (CEL-AOUS) for specimens collected

by non-invasive methods for clinical analysis, and the Virginia Commonwealth University Institutional Review Board.

Genotyping

Allelic genotyping was performed using Applied Biosystems TaqMan SNP Genotyping Assays according to manufacturer's instructions. Specific allelic discrimination probes and primer sets were ordered for the following 3 SNPs: dbSNP: rs2042791, rs12623569, rs10167688. Results were compared to allele frequency detected in NCBI and HapMap studies (Purcell, Neale et al. 2007).

Genotype was confirmed by direct sequencing. Briefly, exon plus flanking regions were amplified by PCR and sequenced using tested primers. Primer suitability for sequencing was tested by comparing results to sample PCR products digested and amplified in a Topo TA Vector (Invitrogen).

PCR and Sequencing Primers

Ex10 – Forward: 5' - TTCATGTAAATTCTGGGCAAA - 3'; Reverse: 5' - GCAAACCATTTCAACCATGA - 3'

Ex11 – Forward: 5' - TGGGGCCAGTACTCTCAAAA - 3'; Reverse: 5' - TTCAGTGCAGGGTGTGTTGT - 3'

Ex12 – Forward: 5' - GCAATTCAAGTTAGCAATTGTG - 3'; Reverse: 5' - CCTGGGGTAGCATCAAGG - 3'

Ex13 – Forward: 5' - TTATTTCATGCCTCAGTTCCTT - 3'; Reverse: 5' - GCCCTTGCACAATCACTTTT - 3'

Ex14 – Forward: 5' - GGGAGGAGGGGCTAAAAATTA - 3'; Reverse: 5' - CCTAAAGTTGTTCTTCTCACCTCA - 3'

Ex15 – Forward: 5' - AGAGGAATGTAATCTTATGGCTGT - 3'; Reverse: 5' - TTCATATGACATGCTATACGTAATGA - 3'

Ex16 – Forward: 5' - CTGACCCCTAACACAGAATGA - 3'; Reverse: 5' - CCAGGTTTTCCTGCAGTTT - 3'

Statistical analysis

Fisher's exact tests implemented in the open-source *R* software package were used to test for differences between allele frequencies observed in the sample population and those reported in HapMap for a population of Western European origin (Purcell, Neale et al. 2007). Hardy-Weinberg equilibrium tests and inter-SNP linkage disequilibrium calculations were performed using PLINK (World Health Organization 1999). Haplotypes were assigned to subject based on the most likely phase reconstructed haplotype generated by the expectation-maximization algorithm implemented in PLINK. Haplotypes were then imported to *R* and an additive term for the haplotype of interested was coded as 0, 1, or 2 based on copy number present. Single SNP and haplotype associations with all phenotypes were carried out using multiple logistic regression in *R*. Phenotypes measured as percentages were transformed with arcsine of the square root of the percentage to stabilize the differences in variance between samples. The Fertility Index is a standard measure ranging from 0 to 100+. All values greater than 100 were coded as 100. To achieve a more normal distribution and minimize residual errors, sperm/mL was

analyzed using a log transformation. A Bonferroni p value was calculated to correct for multiple testing error.

Sperm analysis

Semen samples of patients were collected by masturbation after 4 days of sexual abstinence and examined after liquefaction for 30 min at 37°C. Volume, pH, concentration (sperm x 10^6/mL) and total motility (a, rapid + b, slow, + *in situ*) were evaluated according to World Health Organization guidelines (Baccetti, Bernieri et al. 1995; Collodel and Moretti 2008). For electron microscopy, sperm samples were prepared as previously described (Baccetti, Bernieri et al. 1995).

In each sample the percentage of normal axonemal pattern, of coiled axonemes, of well-assembled fibrous sheaths and accessory fibers were estimated by TEM. Moreover, all TEM data were elaborated using the statistical mathematical formula by Baccetti et al. (Collodel and Moretti 2008), based on the Bayesian method, which calculates the number of spermatozoa probably free of structural defects (fertility index, FI) and the percentages of three main phenotypic sperm pathologies: immaturity, necrosis and apoptosis (Schwede, Kopp et al. 2003).

Molecular structure prediction

Using SWISS-MODEL (Ng and Henikoff 2003), mouse SPAG16L was evaluated for homology with proteins of known structure, and the results mapped on the 3-D structure of the most closely related homologue.

The molecular analysis tool SIFT (Thompson, Higgins et al. 1994) was used to predict whether the analyzed amino acid substitutions would be tolerated, based on sequence homology and amino acid physical properties.

Amino Acid Alignment

Alignment analysis, including scoring of conservative versus semi-conservative or radical amino acid substitutions, was performed using recommended default settings in MacVector 11.6 (MacVector. Inc.; Cary, NC) for the following isoforms:

Homo sapiens – GenBank:NP_078808.3

Mus musculus – GenBank:NP_083436.2

Bos taurus - GenBank:DAA32462.1

Gallus gallus - GenBank:XP_421865.2 (predicted)

Pan troglodytes - GenBank: XP_001148592.1 (predicted)

Chlamydomonas reinhardtii - GenBank:AAB41727.1

Micromonas sp. RCC299 - GenBank:ACO61299

Maximum Likelihood Tree

Amino acids sequences were aligned by ClustalW (CLUSTALW) using default settings, hosted by GenomeNet at the Kyoto University Bioinformatics Center (Felsenstein 2005). Aligned sequences were analyzed for maximum likelihood tree and formatted for figure output using programs in the Phylip 3.69 software package (Li and Roberts 2001). Isoforms used were as follows:

C. reinhardtii – GenBank:AAB41727.1

M. pusilla CCMP1545 – GenBank:EEH57835.1

E. siliculosis – GenBank:CBN79843.1

P. patens – GenBank:EDQ58898.1

T. brucei – GenBank:AAC83819.1

T. gondii ME49 – GenBank:EEA99732.1

(Rooster) *G. gallus* – GenBank:XP_421865.3 (Predicted)

(Opossum) *M. domestica* - GenBank:XP_003341903.1 (Predicted)

(Macaque f.) *M. fascicularis* – GenBank:BAE01109.1

(Rhesus macaque) *M. mulatta* – GenBank:XP_001082826.2 (Predicted)

(Gibbon) *N. leucogenys* - GenBank:XP_003254083.1 (Predicted)

(Chimpanzee) *P. troglodytes* - GenBank:XP_001148592.1 (Predicted)

(Human) *H. sapiens* N-terminal variant – GenBank:NP_001020607.1

(Human) *H. sapiens* SPAG16L – GenBank:NP_078808.3

(Bull) *B. taurus* - GenBank:DAA32462.1

(Rat) *R. norvegicus* (discovered in testis; SPAG16S homologue) – GenBank:AAI58603.1

(Mouse) *M. musculus* SPAG16S – GenBank:NP_080004.1

(Mouse) *M. musculus* SPAG16L – GenBank:NP_083436.2

Figure 3-1. Alignment of SPAG16 amino acid sequences
Aligned amino acid sequences are given for full-length proteins in human, mouse, bull, rooster, chimpanzee, *Chlamydomonas*, and *Micromonas*.

Table 3- 1. SPAG16 amino acid sequence conservation analysis
Quantitative assessment of identity scores (starting from left column) and similarity scores (starting from top row), as calculated by MacVector alignment using default settings.

	Homo sapiens	Mus musculus	Bos taurus	(Predicted) Gallus gallus	(Predicted) Pan troglodytes	Chlamydomonas reinhardtii	Micromonas	Similarity Scores (%)
Homo sapiens	100	74	85.1	49.2	99	35.7	33.6	
Mus musculus	85.1	100	74.6	46.7	74	34.8	34.6	
Bos taurus	92.2	86	100	49.4	85.4	34.9	34.1	
(Predicted) Gallus gallus	64.5	62.9	65.7	100	49.1	33.1	33.9	
(Predicted) Pan troglodytes	99.5	85.1	92.4	64.5	100	35.7	33.6	
Chlamydomonas reinhardtii	52	51.7	52.9	48.5	52.3	100	52.1	
Micromonas	53.8	52.6	54.4	51.5	54	67.6	100	
Identity Scores (%)								
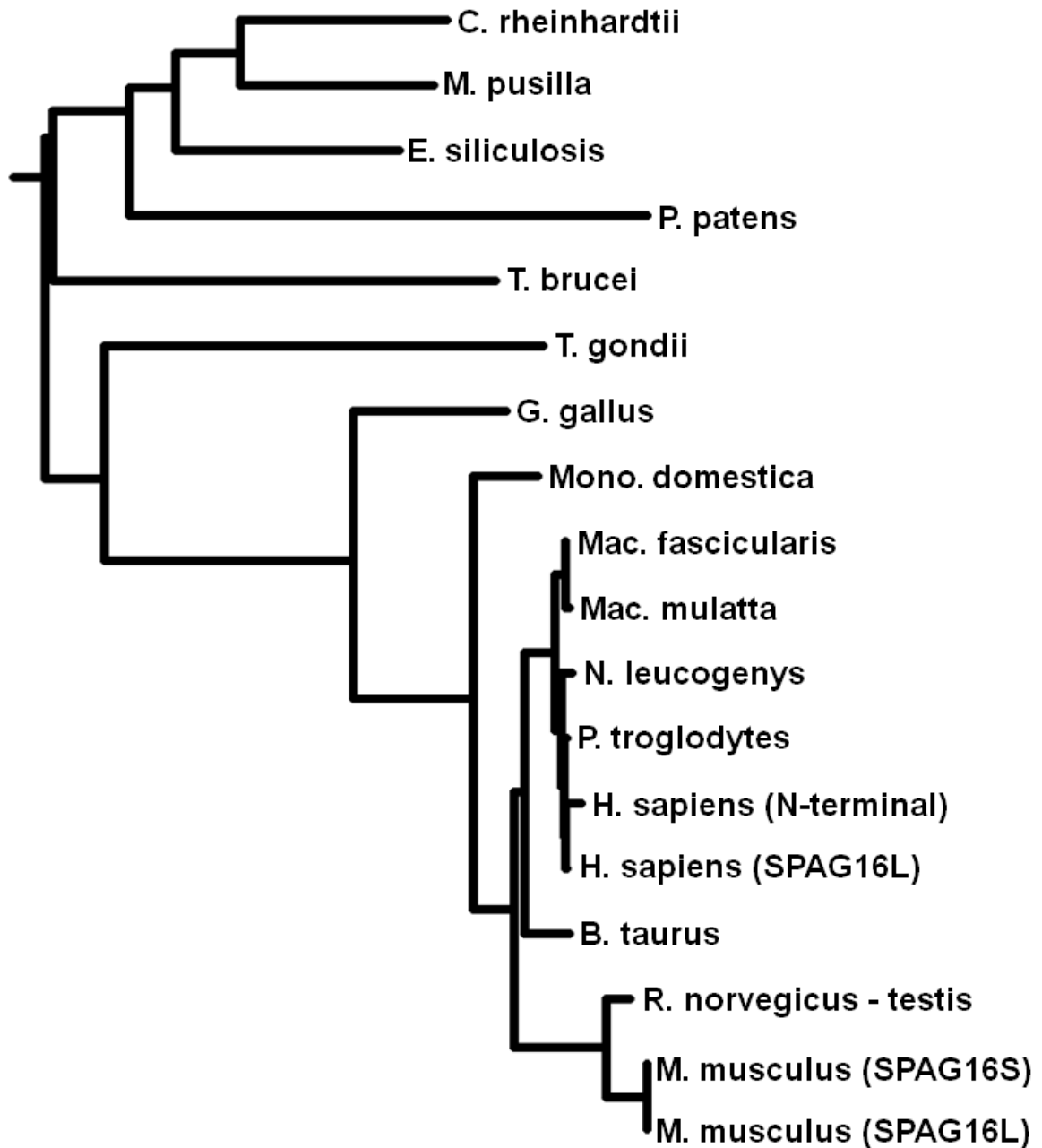
Figure 3-2. Sequence analysis of SPAG16 proteins
Maximum likelihood tree of SPAG16 and SPAG16-orthologous proteins (e.g. PF20). The accession numbers for each entry are listed in the methods section.

Table 3- 2. SPAG16 SNP distributions in sample population
Major and minor allele representation in the sample population. SNPs rs2042792
and rs16851495 are located in non-coding exon splice regions, while SNPs
rs2042791 and rs12623569 are in coding regions of SPAG16 mRNA, and alter
amino acid translation. The nucleic acid and amino acid positions indicated refer,
respectively, to mRNA GenBank:NM_024532.3 and to protein
GenBank:NP_078808.3. Expected frequencies refer to the HapMap CEU
(European) study (The International HapMap Consortium 2003).

The sequenced region area also covered the following SNPs, for which no minor
alleles were found in the sample population: rs10167688, rs115473269,
rs61752199, rs2248214, rs28606463, rs114135655, rs113852644, rs117619722,
rs6746741, rs71855401, rs12988372, rs12988374, rs80016542. Location and
expected frequencies for SNPs not present in the sample population are given in
Table 3-3.

	rs2042791	rs2042792	rs12623569	rs16851495
mRNA	A1175C	C [exon 11 3'+15] G	A1366C	G [exon 14 3' +25] A
Protein	Q361H	–	K425T	–
Homozygous Major allele	19	26	29	43
Heterozygous	33	28	25	17
Homozygous Minor allele	8	6	6	0
Sample population Minor allele frequency	.408	.333	.308	.142
HapMap minor allele frequency	.39	.39	.30	.08

Table 3- 3. Minor allele frequencies of all SNPs in the tested SPAG16 region
Expected minor allele frequencies refer to HapMap CEU European population (Baccetti, Bernieri et al. 1995) where available. Unknown = no data available for suitable reference population; * = Pilot 1 HapMap CEU panel, 60 individuals

Exon	SNP	Sample minor allele freq.	Expected minor allele freq.	SNP	Sample minor allele freq.	Expected minor allele freq.	SNP	Sample minor allele freq.	Expected minor allele freq.
10	rs10167688 [C1062A; P324T]	0.00	0.00	rs115473269 [T11116A ; Y342N]	0.00	unknown	rs61752199 [G11159A; S356N]	0.00	.033*
11	rs2248214 [5′ -3 G/T]	0	unknown	rs2042791 [A1175C ; Q361H]	.408	0.39	rs2042792 [3′ +15 C/G]	.333	0.39
12	rs114135655 [5′ -26 G/C]	0	unknown	rs28606463 [5′ -25 C/T]	0.00	unknown	rs12623569 [A1366C; K425T]	.308	0.28
13	no known/detected SNPs								
14	rs113852644 [A1663G; N524S]	0.00	unknown	rs117619722 [3′ +12 C/T]	0.00	unknown	rs16851495 [3′ +25 G/A]	.142	.08
15	rs71855401 [5′ -31 2bp delet]	0.00	unknown	rs6746741 [T1715C; no mutation]	0	0			
16	rs12988372 [C1946T; no mutation]	0.00	unknown	rs12988374 [C1951T; S620F]	0.00	unknown	rs80016542 [G1975A; R628Q]	0.00	unknown

Table 3- 4. Analysis of Linkage Disequilibrium in Exon 11 SNPs
Linkage equilibrium analysis of two SNPs found in the sample population and present in the genomic proximity: in order, rs2042791 and rs2042792. '2' indicates minor allele, '1' indicates major allele.

Haplotype	Observed	Expected
22	0.333	0.136
12	0	0.197
21	0.075	0.272
11	0.592	0.394
	r^2	.724
	D'	1.000

Table 3- 5. Tests for association between all SNPs in sample population and sperm phenotype
Association tests for each SNP found in the sample population and studied indicators of sperm structure and function. * = data assessed using arcsine of the square root of the phenotype; ^ = data assessed using log transformation of the phenotype; c = Bonferroni correction results in p > 0.1

SNP	MAF	normal acc. fibers*		coiled axonemes*		normal 9+2 axoneme*		normal fibrous sheath*		% immaturity*		fertility index (FI)		in situ motility		motility 'a'		motility 'b'	
		β (SE)	p	β (SE)	p	β (SE)	p	β (SE)	p	β (SE)	p	β (SE)	p	β (SE)	p	β (SE)	p	β (SE)	p
rs2042791	0.408	0.03 (0.04)	0.499	0.00 (0.04)	0.964	0.00 (0.06)	0.998	0.01 (0.04)	0.879	-0.02 (0.03)	0.442	2.10 (12.56)	0.868	-0.48 (0.83)	0.563	-0.14 (0.50)	0.785	0.30 (0.59)	0.615
rs2042792	0.333	0.05 (0.04)	0.207	-0.01 (0.04)	0.774	0.04 (0.06)	0.479	0.01 (0.04)	0.835	-0.01 (0.03)	0.829	-16.83 (13.14)	0.209	0.05 (0.84)	0.955	0.00 (0.50)	0.994	0.25 (0.59)	0.673
rs12623569	0.282	0.01 (0.04)	0.702	0.02 (0.03)	0.601	0.00 (0.05)	0.978	0.09 (0.04)	**0.014c**	0.04 (0.03)	0.1	20.80 (10.72)	0.06	1.13 (0.74)	0.132	0.31 (0.45)	0.5	-0.65 (0.53)	0.221
rs16851495	0.142	-0.12 (0.06)	**0.041c**	0.01 (0.05)	0.873	-0.16 (0.08)	**0.039c**	-0.03 (0.06)	0.674	-0.03 (0.04)	0.515	-10.07 (16.19)	0.538	-0.90 (1.18)	0.453	-0.71 (0.70)	0.32	0.10 (0.85)	0.905

SNP	MAF	motility 'a+b'		total motility		necrosis		% necrosis*		sperm/mL^		volume/mL		total sperm		apoptosis		% apoptosis*	
		β (SE)	p	β (SE)	p	β (SE)	p	β (SE)	p	β (SE)	p	β (SE)	p	β (SE)	p	β (SE)	p	β (SE)	p
rs2042791	0.408	0.16 (0.76)	0.831	-0.32 (1.25)	0.798	-0.78 (5.1)	0.878	0.00 (0.06)	0.991	-0.13 (0.34)	0.715	-0.21 (0.47)	0.66	4.45 (20.26)	0.827	-1.94 (2.28)	0.399	-0.04 (0.03)	0.292
rs2042792	0.333	0.26 (0.76)	0.739	0.30 (1.25)	0.81	-6.21 (5.04)	0.224	-0.06 (0.06)	0.303	-0.55 (0.35)	0.128	0.04 (0.47)	0.939	18.60 (20.25)	0.363	-1.75 (2.30)	0.45	-0.03 (0.03)	0.305
rs12623569	0.282	-0.35 (0.69)	0.616	0.78 (1.13)	0.49	-2.73 (4.60)	0.556	-0.04 (0.05)	0.471	0.44 (0.32)	0.178	-0.25 (0.42)	0.55	12.83 (18.33)	0.487	-1.93 (2.07)	0.356	-0.03 (0.03)	0.361
rs16851495	0.142	-0.61 (1.08)	0.579	-1.50 (1.77)	0.401	11.55 (7.10)	0.11	0.14 (0.08)	0.084	0.46 (0.53)	0.382	0.24 (0.70)	0.723	47.74 (29.11)	0.107	1.68 (3.28)	0.611	0.02 (0.05)	0.637

Table 3- 6. Tests for association between haplotypes and sperm phenotype
Association tests for rs2042791-rs2042792 haplotypes and studied indicators of sperm structure and function. 2' indicates minor allele, '1' indicates major allele. Transformations as in Supplemental Table 2, * = data assessed using arcsine transformation; ^ = data assessed using log transformation.

Haplotype	Freq	normal acc. fibers β (SE)	p	coiled axonemes β(SE)	p	normal 9+2 axoneme β (SE)	p	normal fibrous sheath β (SE)	p	% immaturity β (SE)	p	fertility index (FI) β (SE)	p	in situ motility β (SE)	p	motility 'a' β (SE)	p	motility 'b' β (SE)	p
22	0.333	0.05 (0.04)	0.207	-0.01 (0.04)	0.774	0.04 (0.06)	0.479	0.01 (0.04)	0.835	-0.01 (0.03)	0.829	-16.83 (13.14)	0.209	-0.13 (1.08)	0.904	-0.14 (0.84)	0.863	0.04 (0.92)	0.966
21	0.075	-0.05 (0.06)	0.386	0.03 (0.06)	0.618	-0.09 (0.08)	0.283	-0.01 (0.06)	0.933	-0.04 (0.04)	0.402	29.30 (15.83)	0.073	-1.04 (1.75)	0.556	-0.23 (1.36)	0.864	0.17 (1.49)	0.912
11	0.592	-0.03 (0.04)	0.499	0.00 (0.04)	0.964	0.00 (0.06)	0.998	-0.01 (0.04)	0.879	0.02 (0.03)	0.442	-2.10 (12.55)	0.868	0.53 (1.09)	0.626	0.24 (0.84)	0.78	-0.10 (0.93)	0.911

Haplotype	Freq	motility 'a+b' β (SE)	p	total motility β(SE)	p	necrosis β (SE)	p	% necrosis β (SE)	p	sperm/mL β (SE)	p	volume/mL β(SE)	p	total sperm β (SE)	p	apoptosis β(SE)	p	% apoptosis β (SE)	p
22	0.333	0.01 (1.12)	0.991	0.00 (0.00)	1.000	-6.21 (5.04)	0.224	-0.06 (0.06)	0.303	-0.55 (0.35)	0.128	-0.13 (0.89)	0.881	-16.16 (17.16)	0.35	-1.75 (2.30)	0.45	-0.03 (0.03)	0.305
21	0.075	-0.11 (1.81)	0.95	-1.20 (2.54)	0.639	12.25 (7.50)	0.108	0.14 (0.09)	0.123	0.93 (0.53)	0.083	-0.44 (1.45)	0.761	48.62 (27.28)	0.08	-0.48 (3.48)	0.89	0.00 (0.05)	0.959
11	0.592	0.03 (1.13)	0.978	0.46 (1.58)	0.771	0.78 (5.08)	0.878	0.00 (0.06)	0.991	0.13 (0.36)	0.715	0.31 (0.90)	0.734	-2.41 (17.41)	0.891	1.94 (2.28)	0.399	0.04 (0.03)	0.292

Table 3- 7. Genotype and phenotype analysis of each study participant
Semen parameters and TEM sperm analysis results for each patient. For SNPs, 0-2 indicate number of minor alleles at the given location, hence 0 = homozygous major allele, 1 = heterozygous, 2 = homozygous minor allele. *Ax norm* = Normal axoneme structure 9+2 by TEM examination; *Ax coiled* = Coiled axoneme by TEM examination; *Accfibers norm* = Normal accessory fibers structure by TEM examination; *Fibrous sheath norm* = normal fibrous sheath structure by TEM examination; *FI* = Fertility Index; *Imm* = % Immaturity (normal < 55%); *Apopt* = % Apoptosis (normal < 5%); *Necr* = % Necrosis (normal <21%). FI and sperm pathologies (immaturity, apoptosis, necrosis) were assessed by TEM . *VaLf* = Left varicocele; *CripRg* = Right Cryptorchidism; *ExVaLf* = Operated Left Varicocele; *VaRg* = Right Varicocele; *VaBil* = Bilateral Varicocele

ID	rs2042791	rs2042792	rs12623569	rs16851495	Ax nor (9+2)	Ax coiled	Acc fibers nor	Fibrous Sheath nor	Spm/mL 10^6	Vol/mL	Motility a+b	*in situ*	FI	Imm	Apopt	Necr	Path	Hormones	Semen Infections
101	1	1	0	0	11/24	7/28	12/20	1/18	0.975	4	3+5	5	60	52.86	15.7	67.9	ExVaLf		*Strp. Anginosus*
102	2	2	0	0	0/23	19/23	1/23	0/21	74	2.5	0+1	1	0	94.61	21.9	98.7	ExVaLf		
103	2	2	0	1	0/18	16/30	3/22	3/27	0.48	3	0	0	0	65.75	12.5	98.4			*E.Faecalis*
104	1	1	1	0	6/31	9/26	6/22	6/24	4.88	5.5	3+5	10	193	86.58	4.1	61.3			
105	1	1	0	0	8/13	2/13	4/8	2/11	0.8	3.5	0+10	2	479	70.68	14.6	51.7	ExVaLf		
106	1	1	0	1	4/27	13/32	5/23	3/22	1,925	1.9	0+10	0	0	63.77	43	93	VaLf		*E.Coli*
107	1	1	1	0	0/16	9/27	4/12	2/14	47.25	1	0+1	3	0	70.63	6.8	88.7	ExVaLf		*Strp. Agalactiae*
108	1	1	1	1	3/13	9/13	3/9	5/10	4	8	2+5	4	609	87.6	18	72.6	VaBil		*E. Coli*
109	0	0	2	0	7/20	10/27	3/11	11/16	19.5	3.8	0+1	10	11,796	78	6.8	79.3			
110	1	1	1	1	6/25	10/26	3/19	3/22	92.5	3.6	0+6	12	34,366	58.94	0.9	54.3			
111	1	1	0	0	10/24	5/25	3/15	13/23	4	5	0+3	13	1,073	63.03	11.2	53.7	VaLfidRg		*E.Faecalis/Faecium*
112	0	0	1	1	2/21	8/25	2/16	5/20	2.2	4.7	4+4	2	98	72.37	9.2	47.5			

113	2	2	0	0	6/22	8/23	8/23	7/22	21.2	2	3+1	9	3,920	68.55	13.67	20.05	VaLf		*UUrealyt icum. G.Vagin alis*
114	1	1	1	0	13/27	8/27	9/20	7/23	36.5	2	7+2	9	23,838	74.53	21.88	16.36			
115	1	1	1	0	4/25	19/30	1/21	6/26	0.325	2.5	0	0	0	72.85	11.2	87.5	ExVaLf	LH ?	
116	0	0	0	0	9/22	7/23	4/17	6/21	99	2.5	3+1	5	1,099	71.57	6.8	58.8			*E. Faecalis*
117	0	0	1	1	8/24	10/24	7/18	7/22	1.3	2	3	1	243	74.5	6.72	41.23			
118	1	1	1	0	5/20	4/22	2/16	6/20	3.6	2	6+1	0	1	59.03	43.07	36.42	VaBil		
119	1	0	2	1	5/25	9/34	4/26	14/30	28	3	2+0	5	2,593	55.52	4.7	83.2	VaLf		*Strp. Agalactia e.Bovis I*
120	1	0	1	0	7/26	9/26	5/25	9/25	5,375	5	0+9	0	641	52.36	37.4	85.1	ExVaLf		
121	1	1	0	0	7/25	5/25	2/23	5/24	15	1.5	0+3	3	0	48.03	37.3	77.5	VaLf		
122	1	1	1	1	9/26	8/28	7/22	10/23	13.5	2.5	7+3	8	14429	58.24	9.2	67.38	VaLf		
123	1	0	0	0	3/18	9/16	4/18	5/16	0.9	3	2	0	0	75.5	12.4	88.67	Exvabil		
124	0	0	0	0	2/21	12/25	5/20	0/19	8.75	5	3+6	0	2	68.88	19.23	53.31			
125	1	1	1	1	5/23	6/23	3/21	3/22	h	2.5	1+2	1	7	77.3	24.9	62.14			
126	1	1	0	0	0/16	3/19	8/14	2/15	4.5	1	1+1	0	1	58.17	0.69	62.23			
127	1	1	1	1	14/30	9/32	4/26	13/26	19.8	4	5+5	2	152,796	67.77	9.4	25.9			
128	1	1	1	0	4/30	19/35	5/30	4/29	8.75	1.5	2+7	4	25	85.45	12.8	43			
129	1	0	0	0	3/27	13/28	1/22	5/27	6.5	2	4+4	6	8	78.07	18.3	37.5	VaLf		*E.Faecali s*
130	0	0	0	0	14/30	10/31	4/26	8/25	8.7	2	3+2	1	100	91.54	17.49	36.51	VaLf		
131	0	0	1	0	14/22	7/27	5/16	10/21	3.75	2.5	7+3	11	50,746	58.82	28.48	12.94			
132	1	1	1	0	15/21	14/28	8/18	4/16	13.5	0.5	8+2	7	2,293	85.62	8	45.17			
133	0	0	0	0	8/19	8/18	4/17	2/16	3.75	5	3+4	1	3	76.64	23.21	68.24			
134	1	1	1	1	9/18	9/18	5/14	4/14	4.4	5	6+2	4	48	73.95	13.68	68.66	VaLf		
135	2	2	0	0	12/22	10/29	6/20	5/18	173	1.2	2+3	2	66,814	66.65	0.79	42.51			
136	1	1	0	0	2/32	25/32	1/24	2/26	14.87	3	2+0	0	89	86.06	7.9	85.44	VaLf		
137	2	2	0	0	1/20	9/20	3/18	4/19	287	2	0+1	1	30367	44.2	4.2	90.22			
138	0	0	1	1	4/24	4/24	16/19	5/16	125	5	5+1	1	1E+06	50.74	8.06	51.41			*U Urealytic um*

|																			
139	2	2	0	0	13/23	8/25	6/21	7/19	7.6	5	5+3	1	17,635	69.91	7.59	22.62	VaLf		
140	0	0	2	0	11/22	13/26	10/20	10/21	7.25	2.5	4+3	2	10,265	84.23	9.3	32.4			
141	0	0	0	0	6/21	16/23	5/23	1/28	1.75	2.5	1+2	0	2	85.07	16.2	66.2	ExVaLf	LH ↓	
142	0	0	1	0	3/26	8/28	7/25	4/23	50.25	1.2	2+0	7	5,733	40.98	2.7	90			*E.Faecalis*
143	1	1	0	0	3/20	10/20	2/15	7/20	0.32	5	4+4	2	41	77.8	3.6	61.7			*U Urealyticum*
144	1	1	1	0	9/25	15/27	10/25	8/21	1.93	3	4+1	5	431	83.47	26.2	60	VaLf	FSH ↑	
145	0	0	1	1	6/29	10/32	6/26	4/28	1	3	4+2	2	12	80.47	5.5	91.4			*E.Faecalis*
146	0	0	1	0	9/25	12/26	6/23	9/24	3.4	6	2+2	4	47	77.8	17.8	72.6	VaLf	FSH ↑	
147	1	1	0	0	5/13	2/13	2/12	6/13	0.3	8.5	0	0	1	64.43	20	38.6	VaBil		
148	0	0	1	1	2/24	12/26	0/23	3/25	9.5	1.8	1+3	5	0	78.11	24.7	67.8	VaLf		*LS E.Faecalis*
149	1	1	0	0	4/16	5/17	4/12	5/13	0.95	1.4	0+8	11	0	49.91	14.5	69		FSH ↑	
150	1	0	0	0	8/22	5/22	4/16	6/20	12.88	5.1	6+4	4	4,567	55.97	16.3	79	VaLf		*Strp. Agalactiae*
151	2	0	0	0	2/20	12/31	7/23	4/21	177.5	1.9	1+0	0	995	61.7	6.2	92.2	VaLfCripRg		
152	0	0	2	0	4/22	9/23	4/20	5/20	8.37	4.8	3+4	9	334	78.5	8.9	41.5			
153	1	1	1	0	10/26	8/26	11/20	9/23	6.3	8	3+4	7	15,664	77.7	0.8	55.1	ExVaLf		
154	1	1	0	0	1/24	16/25	1/23	2/23	0.325	1.5	0	0	0	73.17	13.5	96.5			*E.Faecalis*
155	0	0	1	1	4/21	11/31	4/27	5/23	57.75	1.2	0+1	1	57	58.44	11.8	92	VaLf		
156	0	0	0	1	0/34	16/35	3/33	3/31	69	6	1+2	5	0	57.3	25.8	96.2			
157	2	1	0	0	2/28	16/28	3/27	2/26	4.5	2	1+7	3	28	81.14	10.1	61.5	ExVaLf		
158	0	0	2	1	0/24	8/27	5/24	2/22	10.25	1.5	0	0	0	78.5	6.8	58	VaRg		
159	1	0	2	0	4/35	25/34	4/31	5/32	9.2	1.2	0+1	2	142	86.95	12.8	73.2		FSH ↑	
160	1	1	0	0	6/23	12/24	3/22	2/23	6.05	9	0+2	4	1	71.34	10.9	84.5	VaLf		
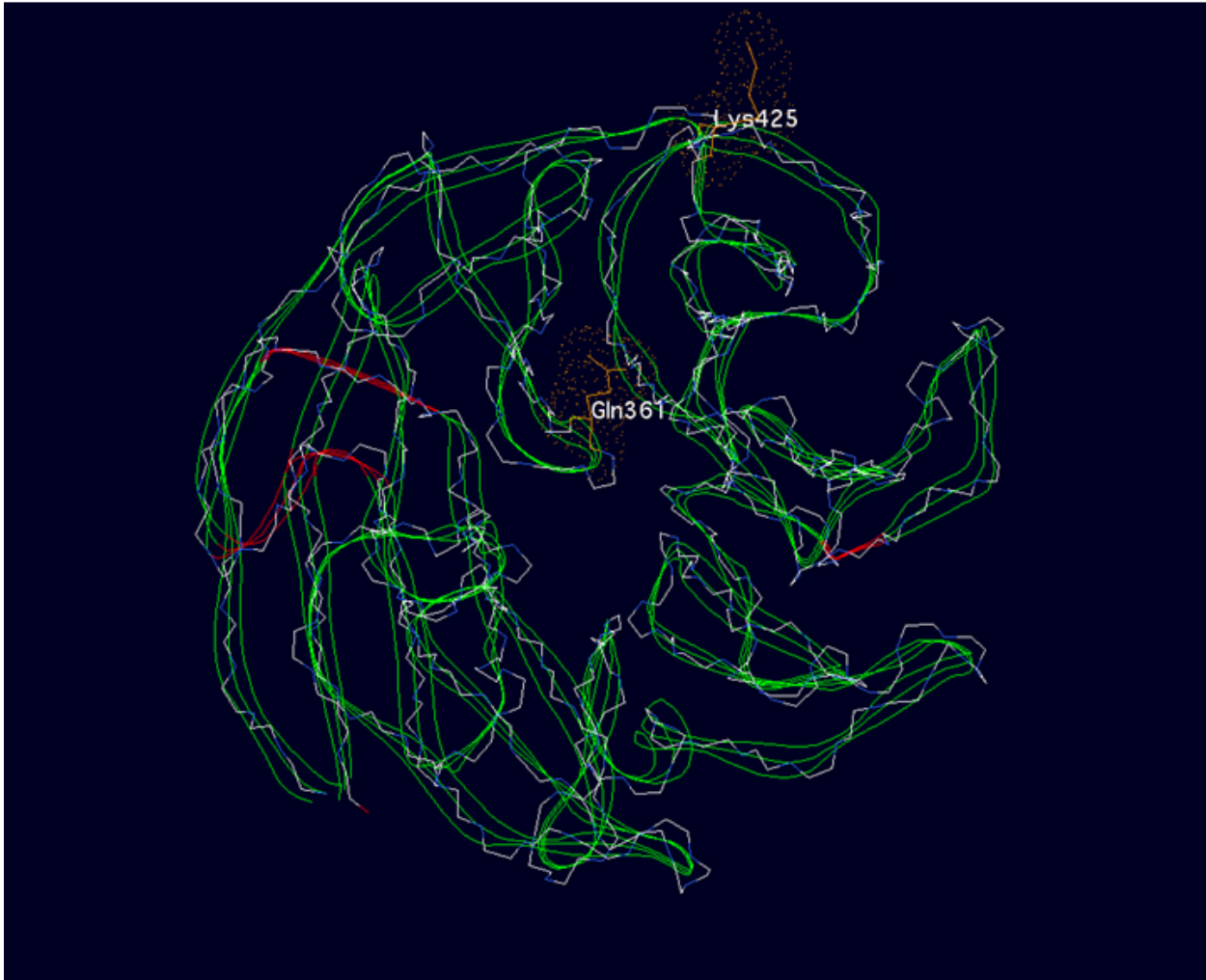
Figure 3-3. Predicted approximate 3-D structure of SPAG16
Predicted 3-D structure of human SPAG16L as determined by overlay on the known structure of WDR5Delta23, the closest related protein with a known crystal structure. Location of SNP-affected amino acids is indicated by shaded sections.

Chapter 4: Perspectives

Throughout the course of this dissertation, I have presented data that describe the roles of *Spag16*, a bifunctional gene playing a highly conserved role in eukaryotic motile cilia as well as an important role in mammalian male germ cell development. The involvement of *Spag16* in spermatogenesis has been suggested by previous work, predominantly published by our lab, and is a particular focus of the work presented here.

In Chapter 1, I presented background information for the dissertation, beginning with the twin clinical goals that have prompted these studies: understanding male infertility, and developing male contraceptives. Previous studies by our lab have strongly suggested a novel role for the mammalian *Spag16* gene aside from its conserved structural role, leading to the central hypothesis of this work that *Spag16* has evolved an additional function unique to mammalian male germ cells. This prior work from our lab has shown that mice have two distinct isoforms of SPAG16: a longer SPAG16L that fulfills the conserved structural role in all eukaryotic motile cilia, and a shorter SPAG16S, found only in male germ cells and present in the nucleus as well as the cytoplasm. While transgenic mice lacking SPAG16L are infertile and show defects in sperm motility, mice with both SPAG16S and SPAG16L disrupted fail to transmit the mutant allele, and exhibit a phenotype of spermatogenesis arrest at the round spermatid stage, leading us to hypothesize that nuclear SPAG16S plays an important role in sperm maturation.

In Chapter 2, I presented new information on the localization of murine SPAG16S, demonstrating that the protein concentrates in sub-nuclear structures known as nuclear speckles. The appearance of these SPAG16S-enriched nuclear speckles is concurrent with haploid sperm maturation at the round spermatid stage, the point of spermatogenesis at which sperm development was disrupted in a highly chimeric transgenic mouse with SPAG16S (as well as SPAG16L) disrupted. I also demonstrated that SPAG16S is capable of eliciting SPAG16L expression and of binding to the *Spag16L* promoter. While these data do not explain the phenotype seen with disruption of SPAG16S, they provide the first evidence of a specific SPAG16S function, consistent with the gene regulatory role suggested by SPAG16S localization in nuclear speckles.

In Chapter 3, I examined a patient population of 60 infertile males with low sperm motility to see if *SPAG16* gene mutations might be a contributory factor to their infertility. I designed primers to fully sequence exonic regions of *SPAG16* corresponding to the shared SPAG16L and SPAG16S functional domains. No novel mutations were found, and previously reported SNPs were found at a frequency not significantly different from that found in a control population, Europeans enrolled in the International HapMap study. Extensive EM and semen analyses were performed by our collaborators, and we tested these sperm parameters for association with SNPs and haplotypes in our patient population. After correction for multiple testing, no significant associations were found. These data suggest that identified *SPAG16* polymorphisms are not functionally

detrimental to either SPAG16L or SPAG16S, as we have seen that loss of these proteins carries significant phenotypic consequences in mice.

Related Projects

This section will discuss related work to evaluate the full implications of the presented data, suggest insights into functional mechanisms, and outline directions of future study. Given that the only identified functional domains of both SPAG16S and SPAG16L are WD repeats, which are known to mediate protein-protein interactions, it is highly likely that both proteins function via interaction partners. Three yeast two-hybrid screens have been performed (two within the context of this dissertation, one previously) using the mouse SPAG16S/C-terminal SPAG16L sequence as bait against libraries of normalized mouse and mouse testis cDNA (Clontech).

The results of the these interaction studies have varied, perhaps suggesting that SPAG16 proteins are promiscuous but unenthusiastic interactors outside the presence of defined macromolecular structure components, such as the conditions found surrounding SPAG16L in the axoneme central apparatus. Despite ongoing efforts, thus far it has not been possible to reliably predict WD interactors based on analysis of any putative epitope-like specific sequences within WD repeats (Zhang, Kostetskii et al. 2004). The putative interactors presented below suggest possible SPAG16 functions by association.

Meig1

Our lab published the first studies suggesting an interaction between SPAG16 and MEIG1 (Don, Winer et al. 1994; Ever, Steiner et al. 1999), a 15-kDa protein expressed abundantly in murine testes during and immediately after meiosis (Zhang, Kostetskii et al. 2004). Based on yeast two-hybrid screens and supported by co-immunoprecipitation, an important interaction was hypothesized between the WD region of SPAG16 and Meig1 (Steiner, Ever et al. 1999).

Initial results in the literature theorized that MEIG1 played an important role in the early stage of meiosis, when it was observed in the nucleus interacting with spermatocyte chromosomes (Zhang, Shen et al. 2009). The nuclear localization of MEIG1 suggested an interaction with SPAG16S, rather than SPAG16L, yet the spermatogenesis arrest in the chimeric SPAG16 mouse at the round spermatid stage led to a hypothesis that the crucial role for SPAG16S occurred post-meiotically.

In order to better understand the germ cell-specific roles of the MEIG1 protein, and shed light on the importance of SPAG16S interaction, a conditional *Meig1* knockout mouse was generated (Clermont, Oko et al. 1990; Zhang, Shen et al. 2009)(http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2746124/). Homozygous *Meig1*-null mice were found to be viable, but completely sterile, with very few, morphologically abnormal sperm. There were no other observed defects that would suggest ciliary immotility. Importantly, spermatogenesis in *Meig1*-null males progressed normally through meiosis, refuting the hypothesis that MEIG1 plays a critical role at this juncture.

However, the final stages in sperm development, those of elongation and condensation, failed to progress. A failure of spermiogenesis after the round spermatid stage strongly resembles the phenotype of the *Spag16* chimeric mouse ascribed to lack of SPAG16S, and provides interesting anecdotal support for the hypothesis that MEIG1 and SPAG16S interact at the round spermatid stage to regulate the final stages of sperm development.

Spermiogenesis disruption in *Meig1*-null males was linked to a specific ultrastructural defect, failure of manchette formation. The manchette is a transient microtubular structure that assembles during spermatid elongation, and is believed to play a role in transport of proteins within the developing sperm tail (Salzberg, Eldar et al. 2010). In sperm from *Meig1*-null males, components of this structure, such as actin and tubulin, were observed to be present in the cytoplasm, yet disorganized.

A similar, independent study noted the same phenotype of sterility and lack of sperm development in the elongation and condensation stage. Scanning EM revealed the presence of gross sperm head malformations and misconnections to the tail, yet did not address sub-cellular structures such as the misformed manchette components we reported (Zhang, Kostetskii et al. 2004).

Both of the above-referenced studies were conducted in animals with a global inactivation of *Meig1*. In order to establish the timing of MEIG1 activity and to confirm the germ cell specificity of the phenotype, the conditional knockout mouse was crossed with mice expressing Cre recombinase under the control of several specific testis-related

promoters: *Hsp2a* (heat shock protein 2; spermatocytes), *Prm* (Protamine 1; post-meiotic spermatids), and *Amh* (anti-Mullerian hormone; Sertoli cells) (Appendix 1).

Germ cell-specific disruption of *Meig1* at the spermatocyte stage, in the presence of *Hsp2a-Cre*, resulted in a phenotype with no observable difference from that seen with global *Meig1* disruption under the control of *Cmv-Cre*: male mice were infertile and exhibited failure of spermiogenesis. Male mice with a later germ cell-specific disruption of *Meig1*, under the control of *Prm-Cre*, exhibited dramatic reductions in *Meig1* mRNA and MEIG1 protein, yet were completely fertile and displayed normal sperm count and sperm motility. Sertoli cell-specific disruption of *Meig1* due to the action of *Amh-Cre* had no effect on sperm function, testis histology, or fertility.

These data further highlight the existence of a critical point in sperm development near the end of meiosis as elongation and condensation are initiated, and suggest that *Meig1* plays a role at this same juncture disrupted when SPAG16S is not present. While a role for SPAG16S-MEIG1 interaction in manchette assembly at the round spermatid stage cannot be firmly established, we hypothesize this highly plausible mechanism on the basis of these collected data. We speculate that the *Spag16* gene has evolved to regulate its own expression, with nuclear SPAG16S coordinating the assembly and trafficking of cytoskeletal components with which cytoplasmic SPAG16L will interact in the axoneme. This hypothesis is consistent with the observation the SPAG16S regulates SPAG16L expression (Chapter 2), and is consistent with the relatively moderate,

"downstream" phenotype observed in mice lacking SPAG16L compared to the spermiogenesis arrest seen with concurrent disruption of SPAG16S.

RC/BTB2

The same study that identified MEIG1 as a SPAG16 binding partner (Tang, Lalli et al. 1982; Berruti and Paiardi 2011) also elicited RC/BTB2 as a candidate SPAG16 interactor following a yeast two-hybrid screen. Like *Spag16*, murine *Rc/btb* encodes both a somatic isoform and a testis-specific isoform. The testis-specific transcript, Rc-btb2, appears in male mice at day 8 after birth but reaches high levels of expression at day 20 (Wang, Teves et al. 2012)(http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3387240/), concurrent with the first wave of spermatogenesis and slightly preceding expression of Spag16S at day 20 (Chapter 2). The RC-BTB2 protein is not heavily expressed in testis until day 30, at which point the first cohort of round and elongated spermatids has been produced.

A full-length GFP-tagged RC/BTB2 construct and a GFP-tagged BTB domain alone were present asymmetrically along the nuclear membrane of transfected CHO cells. In mouse testis sections, RC-BTB2 was observed in round spermatids, where it co-localized significantly with a marker for the acrosome, peanut lectin. The acrosome is a highly conserved structure in male germ cells present across one side of the nucleus at the beginning of spermiogenesis. This organelle is believed to derive from the Golgi and function as a spermiogenesis-specific secretory vesicle (Yu, Ng et al. 2008). While SPAG16 interaction data remain limited to the yeast two-hybrid screen, the timing of RC-BTB2 condensation and its specific nuclear location are consistent with the observation

that SPAG16S-related proteins localize discretely at the beginning of spermiogenesis, and seem to play crucial roles in trafficking and assembly of spermiogenesis-specific cellular components.

SOX5

Given the strong evolutionary conservation of axoneme genes, many of them are subject to common regulatory mechanisms. One transcription factor gene, *Foxj1*, has been established as a master regulator of ciliogenesis (Okada, Ohta et al. 2004). Loss of this gene has been shown to fully disrupt motile cilia formation in a variety of tissues, including mammalian oviduct (Brody, Hackett et al. 1997), airway (Blatt, Yan et al. 1999), and embryo (Hiraoka, Ogawa et al. 1998).

The evolution of novel isoforms (e.g. SPAG16S) from conserved axoneme genes in mammals supports the notion that new regulatory mechanisms must also exist to control the more complex array of genes activated in mammalian ciliogenesis. We examined *Sox5*, a transcription factor gene that expresses both a somatic isoform in mice and an isoform first discovered in testis, known as S-SOX5 (Zhang, Sapiro et al. 2002).

Spag6, a known central apparatus gene and an important interaction partner of SPAG16L (Kiselak, Shen et al. 2010), contains several predicted SOX5 binding sites in its promoter region (Kiselak, Shen et al. 2010; O'Toole, Giddings et al. 2012)(http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2945543). The S-SOX5 transcript and protein are present in murine testis, lungs, and brain – all tissues with motile cilia.

We demonstrated that SOX5 was able to elicit *Spag6* promoter function both independently and synergistically with FOXJ1 in model epithelial cell lines (BEAS-2B = respiratory; MDCK = kidney). While data from this work focuses on the *Spag6* promoter, *Spag16L* and *Spag17* promoters are also responsive to SOX5, suggesting that S-SOX5 may play a general role in cilia formation in mammals. Interestingly, the putative *Spag16S* promoter region was not responsive to S-SOX5 (data not shown), which may indicate a temporally different activation and/or an inability to function in ciliated cell types other than male germ cells. Similar to the work discussed here on SPAG16, we hypothesize that elucidating the roles of the S-SOX5 protein may help clarify the unique aspects of mammalian ciliogenesis.

Future Directions

Spermatogenesis has been extensively studied in both mice and humans, and to a somewhat lesser extent in rats. Critical genes have been suggested by *Chlamydomonas* work on the axoneme, and the mammalian functions of these genes explored largely through the use of transgenic mouse models. Yet a lack of comprehensive gene expression studies across species limits the insights of comparative biology with respect to spermatogenesis.

Recent work in the field has utilized advanced imaging techniques to compare human and *Chlamydomonas* cilia, and thus highlight the fact that important differences exist in both the structure and composition of these related structures; *Chlamydomonas* and *Homo sapiens* are separated evolutionarily by over a billion years, and there have been

design tweaks along the way (Hoops and Witman 1983). Prior studies have pointed out *Chlamydomonas* structures lacking in human cilia, such as certain bridges between outer doublets and projections from the outer doublets (Wanner, Salathe et al. 1996; Silflow and Lefebvre 2001). Additionally, it is known that *Chlamydomonas* flagella can express two waveforms in their flagella, while human cilia are capable of only one waveform (Silflow and Lefebvre 2001).

Our work is particularly interested in the evolution of the central pair, the axoneme region where SPAG16L enables assembly and coordination of the core structure of the cytoskeleton. Interestingly, it has been observed that the central pair rotates in *Chlamydomonas*, causing a gradual twist within the flagellum, whereas the central pair is rotationally static in humans (O'Toole, Giddings et al. 2012). Instead, connections between the central pair (specifically C2) and the outer spokes are enhanced in humans (Lechtreck, Delmotte et al. 2008). This same phenomenon has been observed in mice (The International HapMap Consortium 2003), which supports the hypothesis that mammalian axonemes express important differences from *Chlamydomonas* standards as a result of shared evolutionary history. Speculatively, we hypothesize that the presence of SPAG16 in the center of the axoneme may have conferred built-in evolutionary flexibility, as WD protein promiscuity may allow for the introduction and interaction of new polypeptides.

In Chapter 3 I discussed the conservation of *Spag16* in eukaryotes, yet analysis of isoform evolution is somewhat speculative in light of current predictive, rather than

established, data from other mammalian species. We speculate that the evolution of SPAG16S and its testis-specific interaction partners may reflect a branch in spermatogenic strategies; however, at present it is not possible to conclusively state which species express this isoform and which do not, and thus we remain insufficiently informed to describe the molecular bases of differing spermatogenesis mechanisms.

When both SPAG16 isoforms are disrupted, but not when SPAG16L alone is disrupted, murine spermatogenesis is arrested at the junction between meiosis and spermiogenesis. When the SPAG16S interaction partner MEIG1 is not present in mouse male germ cells at the late stages of meiosis, males are infertile, and an important structure responsible for intracellular trafficking during spermiogenesis fails to assemble. RC/BTB2, another potential interaction partner of SPAG16S, assembles at the sperm nuclear head during the same stage of sperm development that appears to require both SPAG16S and MEIG1. Just prior to condensation and elongation, as haploid round spermatids begin the last stage of specialized development, SPAG16S becomes highly concentrated in transient sub-nuclear structures known as speckles, splicing factor-enriched structures which are only somewhat understood in somatic cells and virtually unexplored in male germ cells.

I have presented new information on a gene that plays two highly distinct roles in male fertility, yet I have also demonstrated that the two isoforms of this gene are related in their expression and actions. Evidence from studies of *Spag16* interaction partners suggests that the germ cell-specific SPAG16S may play a role in the nucleus to enable

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formation of the specialized structures in developing sperm that guide cytoplasmic SPAG16L to its established location with the central apparatus of the "9 + 2" axoneme. Future studies to define expression of non-canonical SPAG16 isoforms and further elucidate mechanisms by which haploid male germ cells coordinate nuclear condensation and elongation will continue to clarify the numerous roles of the *Spag16* gene.

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Appendices

Appendix 1   Teves ME, Jha KN, Song J, Nagarkatti-Gude DR, Herr JC,
                  Foster JA, Strauss JF 3rd, Zhang Z. "Germ Cell-Specific
                  Disruption of the *Meig1* Gene Causes Impaired
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Vita

Davíd Roberto de Vela Nagarkatti-Gude, née Davíd Roberto Gude, was born on September 20, 1982, in San Antonio, TX. He graduated from Keystone School in 2000 and attended Harvard University for his undergraduate education. In 2004, Davíd earned his Bachelor of Arts in Folklore and Mythology, focusing on Mexican America. He was also awarded a citation for study of Spanish language and literature. His undergraduate work culminated in the production of an honor's thesis, entitled "Sin Acento: Dynamics of Cultural Identity in a Modern Tejano Family." Following graduation, Davíd studied a science curriculum at Virginia Commonwealth University, earning Dean's List recognition. He subsequently worked in the laboratory of Sarah Spiegel, PhD, during which time he published several papers. He was accepted into the Virginia Commonwealth University Combined MD-PhD program commencing the 2006-2007 academic year, at which point he studied two years of the medical curriculum before joining the laboratory of Jerome F. Strauss, III, MD, PhD to study evolutionary gene roles in mammalian spermatogenesis. Over this period, he has authored several articles published in peer-reviewed journals, been continuously funded by NIH-NICHD predoctoral fellowship, and been recognized by the Phi Kappa Phi national graduate honor society. During the 2011-2012 academic year he engaged in additional work as a visiting researcher at Leiden University, Netherlands, in the area of genetic contributions to depression. In the autumn of 2012, Davíd will rejoin the medical curriculum and complete his clinical rotations with plans to earn his medical degree in May 2014.

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