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Phosphorylation of sperm axoneme central apparatus protein SPAG16L by a testis specific kinase, TSSK2*

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Abstract

Mammalian SPAG16L, the orthologue of *Chlamydomonas Pf*20, is an axoneme central apparatus protein necessary for flagellar motility. The SPAG16L protein sequence contains multiple potential phosphorylation sites and the protein was confirmed to be phosphorylated in vivo. A yeast-two-hybrid screen identified the testis-specific kinase, TSSK2, to be a potential SPAG16L binding partner. SPAG16L and TSSK2 interactions were confirmed by co-immunoprecipitation of both proteins from testis extracts and cell lysates expressing these proteins, and their co-localization was also noted by confocal microscopy in CHO cells where they were co-expressed. TSSK2 associates with SPAG16L via its C terminal domain bearing WD repeats. The N-terminal domain containing a coiled coil motif does not associate with TSSK2. SPAG16L can be phosphorylated by TSSK2 in vitro. Finally, TSSK2 is absent or markedly reduced from the testes in most of the SPAG16L null mice. These data support the conclusion that SPAG16L is a TSSK2 substrate.

Keywords

SPAG16L; kinase; phosphorylation; sperm motility

Introduction

The mammalian *Spag*16 gene is orthologous to the *Chlamydomonas PF*20 gene. In *Chlamydomonas*, a single PF20 protein is translated from the gene and this protein is localized to the flagellar axoneme microtubule doublets. *Pf20* mutants have paralyzed flagella and an associated absence of the entire central pair [1]. In mammals, two SPAG16 proteins, 71 kDa SPAG16L and 35 kDa SPAG16S, are translated from a single *Spag*16 gene [2]. SPAG16L is translated from a 2.5 kb mRNA, and both mRNA and protein are expressed in tissues with flagellated cells or cilia, such as testis, brain and trachea [3-5]. SPAG16S is translated from a 1.4 kb mRNA that is only present in the testis, indicating that the formation of this message is driven by a testis-specific promoter. The two SPAG16 isoforms play different roles: SPAG16L is incorporated into the axonemal central pair and is essential for flagellar motility [6], serving

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a homologous function to the *Chlamydomonas* PF20 protein. However, SPAG16S is localized in the nuclei of germ cells and is required for normal spermatogenesis [3]. Our recent studies suggest that SPAG16S is a transcription factor that can transactivate the *Spag*16L promoter (unpublished observation).

Both of the SPAG16 proteins contain seven WD repeats, a conserved motif known to be involved in protein-protein interactions [7,8]. WD repeats typically contain a GH dipeptide 11-24 residues from the N-terminus, and the WD dipeptide at the C-terminus. Proteins with WD repeats have diverse functions, such as signal transduction, RNA processing, transcription, cytoskeleton assembly and mitotic-spindle formation, regulation of vesicle formation and vesicular trafficking, control of various aspects of cell division, and the regulation of sulfur metabolism in fungi [9-18].

To search for potential binding partner(s) of SPAG16, a yeast-two-hybrid screen was performed previously with the WD repeat as the bait identifying several proteins, including SPAG6 and MEIG1, as potential binding partners [2,3]. The same screen identified another potential binding partner, TSSK2, a testis-specific serine/threonine kinase.

TSSK2 belongs to a testis-specific serine/threonine kinase family. Using degenerate oligonucleotides corresponding to two highly conserved motifs within the protein kinase catalytic domain and a PCR-based cloning strategy, TSSK1 was the first member identified [19]. By screening of a testis cDNA library using *Tssk*1 cDNA as probe under low stringency strategy, *Tssk*2 and *Tssk*3 were subsequently cloned [20-22]. TSSK4, also known as SSTK, was cloned by searching public databases with conserved amino acid sequence [23]. Later, TSSK5 was discovered [24] and added to this family of testis-specific kinases.

Little has been known about the functions of this kinase family. Mouse TSSK1 is abundant in germ cells during meiotic metaphase [25], while mouse TSSK2 is localized in the cytoplasm of male germ cells at late stage of spermatogenesis, and also in the flagella, acrosomal region of mature sperm, which is consistent with SPAG16L localization. In human sperm, TSSK2 was found in the neck region, equatorial region and midpiece of the flagella [20,25,26]. It has been shown that TSKS is a substrate for both TSSK1 and TSSK2 [19,21]. TSSK3 is expressed in Leydig cells of sexually mature mice [21], TSSK4 shares a similar expression pattern as TSSK2 [22], TSSK4 can phosphorylate histones and plays a role in post-meiotic chromatin remodeling and male fertility [23]. TSSK5 can phosphorylate CREB and stimulate the CRE/CREB responsive pathways [24]. In this study, we demonstrated that TSSK2 associates with SPAG16L, and SPAG16L can be phosphorylated by TSSK2. Thus, SPAG16L is a new substrate for TSSK2.

Materials and Methods

Ethics

The present study was approved by the Institutional Animal Use and Care Committee of the University of Pennsylvania and Virginia Commonwealth University. Procedures involving animals were conducted with the approval of the Institutional Animal Care and Use Committee of the Virginia Commonwealth University in accordance with the Guide for Care and Use of Laboratory Animals (protocol no. 06013417).

Yeast two-hybrid screening

A mouse testis cDNA library was screened previously using the C-terminus domain of SPAG16 as the bait [2].

Two dimensional Western blotting

Epididymal sperm were collected from male mice (CD1 retired breeders, Charles River Laboratories, Wilmington, MA) by mincing the caudae epididymides and allowing the sperm to swim out in PBS. The sperm were collected by centrifugation at 800 × *g* for 5 min at room temperature. The sample was dissolved in 2D sample buffer (40 m M Tris-HCl, pH 9, 8 M urea, 4% (w/v) CHAPS, 100 mM DTT, 1 × protease inhibitor mixture (Roche Applied Science), centrifuged at 10,000 × *g* for 5 min, and the pellet was discarded. The amount of protein was determined by the Bradford method, For phosphatase treatment, samples were pretreated with 1 IU of calf intestinal alkaline phosphatase (CIP) (New England Biolab) for 30 min at 37 °C. 100 μg of protein, treated with CIP or without treatment, was used for 2D gel electrophoresis following the same procedure as described by Cao et al [27,28].

Generation of an anti-mouse TSSK2 Antibody

A cDNA encoding the full length of mouse *Tssk*2 was amplified by RT-PCR with the following primers: forward 5'-GGGAATTCCATATGATGGACGATGCGGCGGTCC-3' (*Nde* I), and reverse 3'-CTGAAAGCTTCGGTACTTGCTTTCTCC-3' (*Hind* III). The PCR product was cloned into the pET28a vector for protein expression in bacteria. The fusion protein was purified and the rabbit polyclonal antibody was generated as described previously [2,3]. Antibodies against N-terminus and C-terminus of SPAG16 were generated in our laboratory previously [2,3].

Constructs for mammalian cell expression

Full length *Tssk*2 cDNA was cloned into mammalian expression vectors, pTarget, DsRed-N_1, and pEGFP-N_2 respectively. The PCR primers are as follows. For pTarget, forward primer: 5'-GAATTCATGGACGATGCGGCGGTCCTA-3'(*EcoR* I), and reverse primer: 5'-GTCGACCTAGGTACTTGCTTTCTCCAC-3' (*Sal* I); for DsRed-N_1, forward primer: 5'-CGAGAATTCTGATGG ACGATGCGGCGGTCCTA-3' (*EcoR* I), and reverse primer 5'-CGCGGATCCCGGGTACTTGCTTTCTCCACCTC-3' (*BamH* I); for pEGFP-N_2, forward primer: the same as for pTarget, and reverse primer, 5'-CGCGGATCCCGGTACTTGCTTTCTCCACCTC-3' (*BamH* I). PCRs were performed with a mouse testis cDNA as template. The PCR products were cloned into pCR2.1 TOPO vector, after sequencing, the inserts were subcloned into the mammalian expression vectors. SPAG16L/pEGFP-N_2, SPAG16S/pEGFP-N_2, SPAG16L/pTarget and SPAG16S/pTarget were generated previously [2]. The DNA sequence encoding N-terminal SPAG16L was amplified with the same forward primer and diverse reverse primers to create N-SPAG16/pcDNA$_3$ and N-SPAG16/pEGFP-N_2. The forward primer: the same as for pTarget and reverse primers: 5'-CTCGAGTCAATCTACAGGAAATTCTGAATC-3' (*Xho* I, for pcDNA$_3$) and 5'-GGATCCCATCTACAGGAAATTCTGAATC-3' (*BamH* I, for pEGFP-N_2).

Western blot analysis

TSSK2/pTarget, TSSK2/DsRed-N_1, TSSK2/pEGFP-N_2, N-SPAG16/pcDNA$_3$ and N-SPAG16/pEGFP-N_2 plasmids were transfected into CHO cells. 48 h after transfection, the cells were lysed with RIPA buffer, and Western blotting was performed to verify TSSK2 and N-SPAG16 protein translation. A 1:2000 dilution of anti-TSSK2 antibody, and 1:1000 dilution of anti-GFP and N-SPAG16 antibodies were used in our studies.

Cell culture and transfection for confocal microscopy

Chinese hamster ovary (CHO) cells were cultured in two-well chamber slides. The cells were transfected with the SPAG16L/pEGFP-N_2, or TSSK2/DsRed-N_1, or combination of TSSK2/DsRed-N_1 and SPAG16L/pEGFP-N_2 using FuGENE (Roche). As controls, empty DsRed-

N_1 was co-transfected with empty pEGFP-N_2 or SPAG16/pEGFP-N_2 plasmid. Forty-eight hours after transfection, the cells were visualized by confocal microscopy.

Co-immunoprecipitation (Co-IP) from transfected cell lines and testicular extracts

CHO cells were co-transfected with indicated TSSK2 and SPAG16 plasmids. 48 h later, the cells were harvested into immunoprecipitation buffer (150 mM NaCl, 50 mM Tris·HCl, pH 8.0, 5 mM EDTA, 1% Triton X-100, 1 mM PMSF and proteinase inhibitor mixture), the lysates were passed through a 20-gauge needle. After centrifugation at 11,600 × *g* for 5 min, the supernatants were pre-cleared with protein A beads at 4°C for 30 min. The supernatants were then incubated with 1 μl (1μg/μl) of indicated antibodies or pre-immune serum at 4°C for 2 h, and protein A beads were added with a further incubation at 4°C overnight. The beads were washed with immunoprecipitation buffer three or four times, 1 × protein loading buffer was then added to the beads, which were boiled at 100°C for 10 min; the samples were then processed for Western blotting using monoclonal anti-GFP, or anti-TSSK2 and SPAG16 polyclonal antibodies.

Co-IP with testis extracts were performed with a Co-IP kit from Roche. Briefly, 1 mg of testis extract was pre-cleared with protein A beads, the pre-cleared extract was added with pre-immune serum or indicated antibody and the complex was incubated at 4°C with rotation for 3 h, followed by addition of protein A beads and the whole complex was incubated overnight at 4°C with rotation. After washing, the samples were subjected to 10% PAGE gel, and Western blotting was performed with indicated antibody.

In vitro kinase assays

COS-1 cells were co-transfected with TSSK2/pTarget and SPAG16/pTarget. 48 hours after transfection, the cells were harvested into immunoprecipitation buffer and co-immunoprecipitation was performed as previously with C or N-terminal (negative control) anti-SPAG16 polyclonal antibodies. After washing, the complexes were resuspended in 20 mM Tris-HCL, pH 7.5, 10 mM $MnCl_2$, 10 mM $MgCl_2$ containing 5 μCi [γ-^{32}P] ATP and incubated at 37°C for 15 min. Labeled proteins were resolved by SDS-PAGE and dried gels were exposed directly to x-ray films. To determine if N-SPAG16 and full length SPAG16 can be phosphorylated by purified recombinant TSSK2, N-SPAG16/pTarget and SPAG16/pTarget plasmids were transfected into COS-1 cells, and the two proteins were immunoprecipitated with an anti-N terminus SPAG16 antibody. Half of the sample was subjected to Western blotting for the confirmation of the presence of the two proteins, the rest was incubated with 2 μg of purified mouse TSSK2 protein (Invitrogen) at 37°C for 15 min in the same buffer as described above containing labeled ATP, and labeled proteins were resolved by SDS-PAGE and the dried gels were exposed to x-ray films.

Northern blot analysis

Total testicular RNA was isolated from sexually mature wild-type and *Spag*16L null mice. Northern blots were performed with specific mouse *Tssk2*, S*pag*16L and *Akap*4 probes.

Mice

*Spag*16L mutant mice were generated previously in our laboratory [6]. Five to six month old male mice were used for the study.

Results

Potential phosphorylation sites of SPAG16L proteins

Identification of TSSK2 in a yeast-two-hybrid screen with SPAG16 as bait suggested that SPAG16 proteins might be phosphorylated in vivo. To locate potential phosphorylation sites, the SPAG16L protein sequence (Fig 1, upper panel) was analyzed by NetPhos2.0 Server (http://www.cbs.dtu.dk/services/NetPhos/). SPAG16L potentially contains 30 serine, 11 threonine, and 7 tyrosine phosphorylation sites (Fig 1, lower panel). These potential phosphorylation sites are distributed throughout the protein. To predict potential kinases that might phosphorylate these sites, the SPAG16L protein sequence was analyzed using the NetPhosK1.0 Server (http://www.cbs.dtu.dk/services/NetPhosK/). Potential kinases are listed in Supplemental Table 1. Besides TSSK2, we also identified another kinase in our yeast 2-hybrid screen (Table 1), GSK-3β, but we failed to confirm the interaction of this kinase with SPAG16 in co-immunoprecipitation experiments (data not shown).

SPAG16L is phosphorylated in vivo

To investigate if SPAG16L protein undergoes post-translational modification, especially phosphorylation in vivo, epididymal sperm extracts were subjected to two-dimensional gel electrophoresis followed by Western blotting (2D Western) with an antibody against N-terminus of SPAG16L. A major signal was observed at a spot of 71 kDa and pI of 8.0, the predicted size for SPAG16L, while a more acidic 71 kDa immunoreactive species was also identified at around pI 4.0 (Fig 2, upper panel), indicating that SPAG16L undergoes post-translational modification. After the protein samples were treated with calf intestinal phosphatases (CIP), the 71 kDa signal at pI 4.0 disappeared (Fig 2, lower panel), suggesting that this acidic isoform of SPAG16L is phosphorylated in vivo.

Interaction of SPAG16L and TSSK2

From our original yeast-two hybrid screen with the C-terminus of SPAG16 as bait, a number of candidates were identified, some have been confirmed by co-immunoprecipitation experiment, such as MEIG1 and SPAG6 (Table 1). TSSK2 was another potential molecule that might associate with SPAG16. To further study the interaction between the two proteins, TSSK2 mammalian expression plasmids were constructed. When TSSK2/DsRed-N_1 or TSSK2/pEGFP-N_2 were transfected into CHO cells, TSSK2 protein was expressed (Fig 3Aa) and the protein was localized in the cytoplasm (Fig 3Ab). To explore the interaction of TSSK2 and SPAG16L, SPAG16L/pTarget and TSSK2/pTarget plasmids were co-transfected into COS-1 cells, and co-immunoprecipitation was performed. The cell lysates were pulled down with an anti-TSSK2 antibody, and Western blotting was carried out with an anti-N-terminus SPAG16L antibody. SPAG16L was immunoprecipitated by the anti-TSSK2 antibody (Fig 3B).

The association of TSSK2 and SPAG16L in vivo was also investigated. Immunoprecipitates were isolated from testis extracts with anti-TSSK2 or pre-immune serum and analyzed on Western blots with anti-N-terminus SPAG16L antibody revealing that 71 kDa SPAG16L was present in the TSSK2 immune but not the pre-immune precipitates (Fig 3C). Even though 35 kDa SPAG16S also contains the same WD repeats as 71 kDa SPAG16L, the protein was not pulled down by anti-TSSK2 antibody (data not shown).

As described previously, SPAG16L protein was also localized in the cytoplasm of SPAG16L/pEGFP-N_2 plasmid transfected CHO cells [2], similar to the distribution pattern of TSSK2. To further study the association of the two proteins, TSSK2/DsRed-N_1 and SPAG16L/pEGFP-N_2 were co-transfected into CHO cells and the localization of each protein was examined by confocal microscopy. The two proteins co-localized in the cytoplasm (Fig 4, upper two panels). As controls, empty DsRed-N_1 plasmid was co-transfected with empty pEGFP-N_2 or

SPAG16L/pEGFP-N_2, plasmids. Both GFP and DsRed proteins were distributed throughout the cells (Fig 4, panel 3). Even though SPAG16L/GFP and DsRed are co-expressed in cells, they had different localizations. SPAG16L/GFP was present in the cytoplasm, while DsRed was in the cytoplasm and nuclei (Fig 4, panel 4).

C-terminal WD repeats mediate the association of TSSK2 and SPAG16L

To further investigate the domain that mediates the interaction, the cDNA encoding the 36 kDa N-terminus of SPAG16L was cloned into pEGFP-N_2 and pcDNA$_3$ to create expression plasmids. Both plasmids expressed the N-terminus SPAG16L protein in CHO cells as evaluated by Western blotting (Fig 5Aa).

CHO cells were co-transfected with TSSK2/pTarget and N-SPAG16/pEGFP-N_2 plasmids, and Co-IP was performed. The anti-TSSK2 antibody immunoprecipitated the TSSK2 protein (Fig 5Ab), but not N-SPAG16 protein (Fig 5Ac), suggesting that TSSK2 does not associate with the N-terminus of SPAG16L.

The C-terminus domain of SPAG16L contains 7 WD repeats, which mediate protein-protein interactions. To investigate if TSSK2 interacts with this domain, TSSK2/pEGFP-N_2 or TSSK2/pTarget and C-SPAG16/pTarget were co-transfected into CHO cells, and Co-IP experiments were carried out. The complex was pulled down with an anti-C-terminus SPAG16 antibody, and Western blot was performed with an anti-GFP antibody (Fig 5Ba) or anti-TSSK2 antibody (Fig 5Bb). In Fig 5Ba, 66 kDa TSSK2/pEGFP fusion protein, but not the 26 kDa GFP protein was selectively pulled down from the complex, indicating an interaction of TSSK2 with the C-terminus domain of SPAG16L. Similarly, as shown in Fig 5Bb, when the cells were co-transfected with TSSK2/pTarget and C-SPAG16/pTarget, the TSSK2 protein although lacking a tag can still be pulled down with anti-C-terminus SPAG16 antibody.

SPAG16 is phosphorylated by TSSK2 in vitro

To examine if SPAG16L can be phosphorylated by TSSK2, CHO cells were co-transfected with TSSK2/pTarget and SPAG16L/pTarget plasmids. After immunoprecipitation with anti-TSSK2 antibody, a Western blot was performed with half of the complex using anti-N-terminus of SPAG16L antibody (Fig 6, upper panel), another half of the complex was incubated with kinase buffer and [γ-^{32}P] ATP (Fig 6, lower panel). Immunoprecipitates of the labeled cells with anti-TSSK2 antibody revealed a major band corresponding to 71 kDa SPAG16L, indicating that SPAG16L is phosphorylated in this in vitro model (Fig 6).

The phosphorylation of SPAG16L and N-SPAG16L by recombinant TSSK2 protein was also investigated. CHO cells were transfected with SPAG16L/pTarget or N-SPAG16/pTarget plasmids. After immunoprecipitation with an anti-N-terminus SPAG16 antibody, a Western blot was performed with half of the complex using anti-N-terminus of SPAG16L antibody (Fig 7A, B), and half the complex was incubated with purified recombinant TSSK2 protein in the presence of kinase buffer and [γ-^{32}P] ATP (Fig 7C). SPAG16L but not N-SPAG16 was phosphorylated by TSSK2.

TSSK2 is reduced in the testis of most *Spag*16L null mice

*A Spag*16L knockout model previously generated in our laboratory was employed to study TSSK2 expression in *Spag*16L mutant testes. Of the 12 null mice analyzed, TSSK2 protein was undetectable or markedly diminished in 5 of them, reduced by approximately 50% in four, unaltered in three. However, TSSK2 was present in all 8 wild type mice analyzed (Fig 8A). Even though the TSSK2 protein was missing in some of the null mice, *Tssk2* mRNA was observed in all *Spag*16L null mice analyzed (Fig 8B).

Discussion

Protein phosphorylation is the most common posttranslational protein modification in eukaryotes and a fundamental mechanism for the direct or indirect control of all cellular processes. For example, protein phosphorylation is involved in the control of cell division, metabolic activity, cell adhesion and migration, cell to cell communication, and signal transduction [29,30]. During spermatogenesis, protein phosphorylation has been shown to play a role in several processes, including gene transcription [31], and chromatin remodeling [32]. During fertilization, spermatozoa undergo a series of changes before and during egg binding that are related to acquisition of the ability to fuse with the oocyte, including major changes in the phosphorylation of spermatozoa proteins. Increased protein phosphorylation is associated with capacitation, hyperactivated motility, zona pellucida binding, acrosome reaction and sperm-oocyte binding and fusion [33-37]. Several flagellar proteins are known to be phosphorylated during capacitation including the fibrous sheath proteins CABYR and AKAP 3 & 4 [38-41].

Phosphorylation of flagellar proteins is linked to hyperactivated motility in spermatozoa [35]. SPAG16L is localized in the central apparatus of the axoneme, and this protein could be one of the proteins phosphorylated in vivo in preparation for fertilization. Through searching the potential sites for phosphorylation, it was found that SPAG16L contains multiple phosphorylation sites and 2-dimention Western blotting confirmed that SPAG16L is phosphorylated in vivo.

Proteins are phosphorylated by protein kinases, which including serine/threonine or tyrosine kinases. From previous yeast 2-hybrid screening, TSSK2 was identified preliminarily as a potential SPAG16L binding partner and the present study confirmed that TSSK2 and SPAG16L are co-immunoprecipitated from the testis as well as from co-transfected cells in culture. Moreover, TSSK2 is able to phosphorylate SPAG16L in vitro. Taken together the yeast two hybrid results and direct biochemical co-isolation of these proteins in complex lead to the conclusion that, SPAG16L is a TSSK2 substrate.

TSSK2 appears to bind SPAG16L via the C-terminus domain, which contains WD repeats. Two lines of evidence support this conclusion. First, TSSK2 and SPAG16L were initially identified by a yeast-two hybrid screening with C terminus WD repeats domain as the bait. Second, TSSK2 associates with C-SPAG16 rather than N-SPAG16 in Co-IP experiments. The fact that TSSK2 interacts with the C-terminus of SPAG16L and is capable of phosphorylating it, at least under in vitro conditions, raises the possibility that TSSK2 also can phosphorylate SPAG16S. SPAG16S is localized to the nucleus of germ cells where it is thought to function as a transcription factor. If SPAG16S is phosphorylated by TSSK2, this event would likely occur in the cytoplasm. However, SPAG16S was not pulled down in the Co-IP experiment with testis extract using anti-TSSK2 antibody. Despite this negative result, future studies should investigate the possibility of TSSK2 phosphorylation of SPAG16S and its impact upon the distribution and function of SPAG16S.

Even though TSSK1 and TSSK2 share 83% identity in protein sequences, and TSSK2 also shares homologue with other testis-specific kinases, we did not identify other testis-specific kinases from initial yeast-two hybrid screen. This could reflect the fact that SPAG16L is not a substrate of those kinases, or the screen failed to detect them as potential binding partners. Thus, we cannot exclude the possibility at this juncture that other members of the TSSK family also phosphorylate SPAG16L.

Interestingly, in the testes of 75% of SPAG16L-deficient mice, TSSK2 protein was either absent or significantly reduced, although TSSK2 transcripts were present at normal levels. This may suggest that SPAG16L protein either feedbacks on TSSK2 translation by some yet

undiscovered mechanism, or that SPAG16L is required to sustain TSSK2 translation or protein integrity. The variation in this phenotype may reflect the mixed background (SV129 and C57) of the mutant mice. We have observed such variation in other knockout models that we have created targeting other central apparatus protein genes [42]. In any case, the absence of TSSK2 in more than half of these SPAG16 null animals indicates that SPAG16/TSSK2 co-expression is not a constant and that the association between these proteins may not be stable in vivo.

It has been shown a 65 kDa protein, TSKS is a substrate of TSSK2 [26]. TSKS localizes predominantly to the centrioles of human sperm, however, TSSK2 is localized to the sperm neck, mid-piece of sperm tail, and to the equatorial segment (personal communication from John C Herr). The broader localization of TSSK2 compared to TSKS implies that additional substrates for the enzyme may exist [43]. The fact that both TSSK2 and SPAG16L are present in the sperm tail is consistent with the notion that SPAG16L is another substrate for TSSK2 in vivo, and that TSSK2 might, through phosphorylation of SPAG16L, regulate sperm motility. *Tssk*1/2 mutant mice have been generated recently, in addition to defect in spermatogenesis, another major phenotype is severe sperm motility defects in the mutant mice (personal communication from John C. Herr).

Even though it is not clear which protein, TSSK1 or TSSK2, is responsible for these phenotypes, phosphorylation of some sperm proteins, such as SPAG16L might be impaired, which might be predicted to result in sperm motility defects.

In conclusion, We have identified a new substrate of TSSK2 in the sperm tail, leading to the conclusion that TSSK2 might regulate sperm motility through phosphorylation of sperm tail protein(s), one of which is SPAG16L.

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MAAPSGVPPLRVLEELGIGLSPTGEVTEAVTSEGAYYLEQVTITETSEDECEYEEIPDDNFSIPEGEEDLEKAIHIIGEQ 80
ARDIHILEQQTILPARNVMQEAIEDFLCNFLIKMGMTRTLDCFQAEWYELIQKKGSDFKGLGNVPDVYSQVMLLETENKN 160
LKKELKHFKQAAEKAKEDLLRTQKERDFHRMHHKRIVQEKNKLIADLKGLKLHYASYEPTIRVLHEKHHALLKEKMLTSL 240
ERDRAVGKISGLQATLKNIDMGHIQVPVIKGSYESASITRESGDRAGHSCEKENSSEGPTQKSLREAREEVGYKSKLKNE 320
KKDSEFPVDMQPDPNVTSCTENVSAAKFDYKLNNIFRLHELPVSCIVMHPCRDYLISCSEDRLWKMVGLPQGNVLLTGSG 400
HTDWLSGCCFHPSGSKLATSSGDSTIKLWDLNKGECTLTLEGHNHAVWSCTWHSCGDFVASASLDMTSKIWDVNSERCRY 480
TLYGHTDSVNSIEFFPFSNILLTASADKTLSVWDARTGKCEQSLYGHMHSVNDATFTPRGHIIASCDARGVTKLWDFRKL 560
IPIVSIDVGPSSGNEVNFDQSGRVLAQASANGIIHLLDLKSGQIHKLVGHESEVHSVVFSHLGENLYSGGSDGTIRLWI  640


.............................S...Y.....T...TS.....Y........S..................  80
..........T.............................................S.......................  160
.....................T................................S...T..................S.  240
.............................SY.S.S....S......S.....SS...T..S.........Y.S.....  320
...S.............S...........Y.......................Y..S.......................  400
...................SS..ST............................S........S....S............  480
T.........S...................S.....T.......Y....S......T.......................  560
..........SS.............................................S..................T.....  640

Phosphorylation sites predicted:     Ser: 30   Thr: 11   Tyr: 7
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Figure 1. Potential phosphorylation sites of SPAG16L protein

Mouse SPAG16L protein sequence (upper panel) and potential phosphorylation sites (lower panel) analyzed by NetPhos2.0 Server program. Potential serine (S), threonine (T), and tyrosine (Y) phosphorylation sites are indicated.

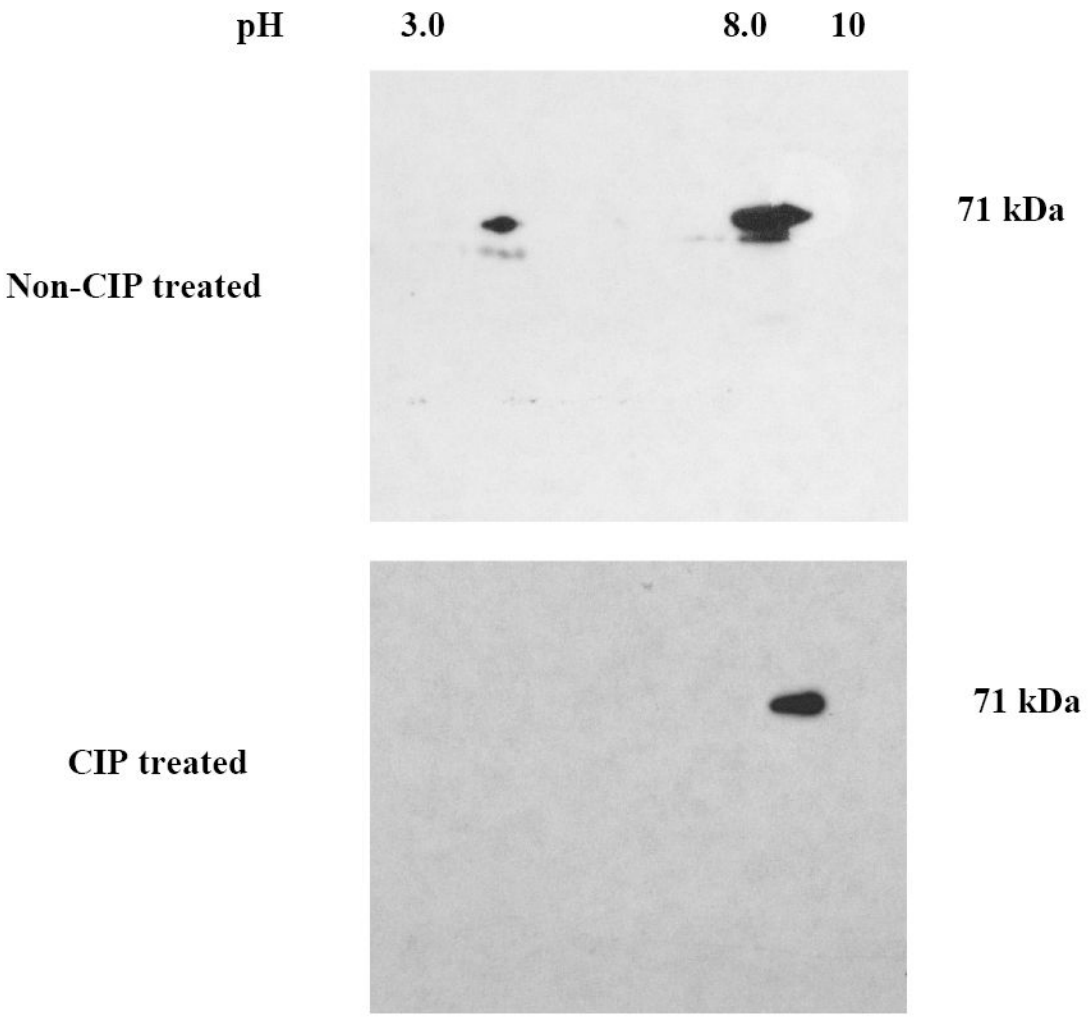

Figure 2. Mouse SPAG16L is phosphorylated in vivo as revealed by two dimensional Western blotting

Untreated control or calf intestinal phosphatase (CIP) treated mouse epididymal sperm protein extracts were subjected to two dimensional gel electrophoresis followed by Western blotting with an anti-N-terminus SPAG16 antibody. Upper panel: result from a non-CIP treated sample; lower panel: result from a CIP treated sample. The signal at pI 4. present in untreated samples disappeared after phosphatase treatment indicating the 71 kDa, pI 4.0 SPAG16 isoform is phosphorylated in sperm. Representative results are shown from three individual experiments.

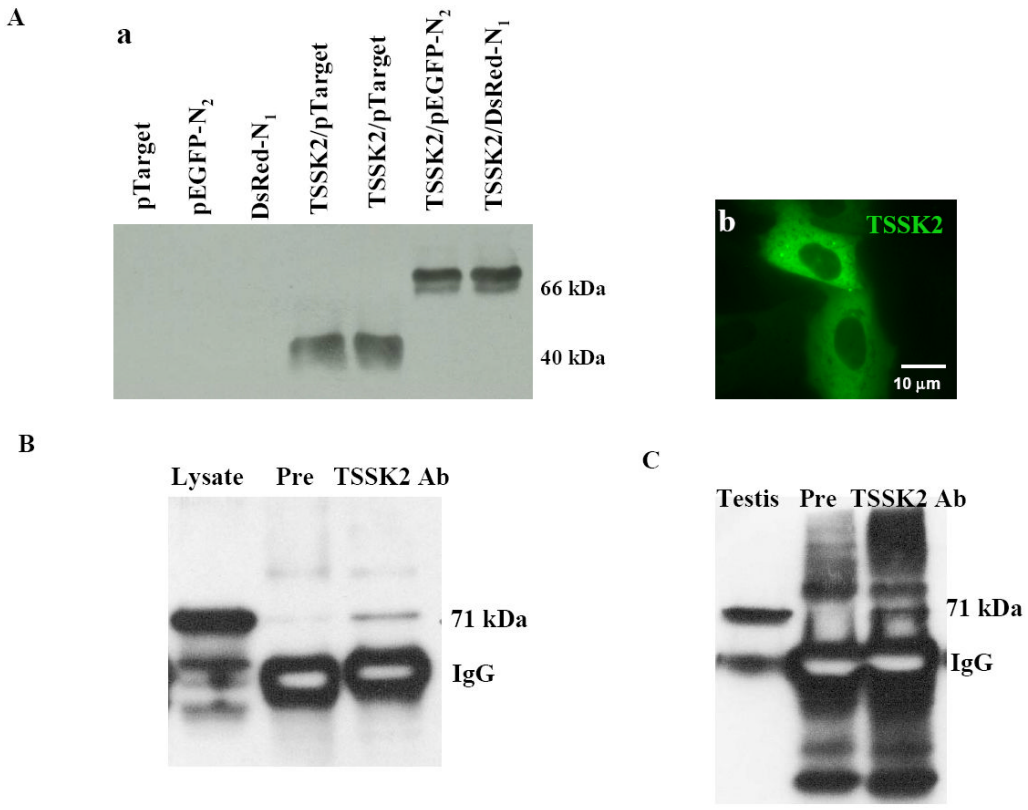

Figure 3. Association of TSSK2 and SPAG16L

A. Analysis of expression of TSSK2 protein in CHO cells by Western blotting (a). CHO cells were transfected with empty pTarget, pEGFP-N_2 or Ds-Red-N_1 plasmids, or these plasmids containing full length mouse TSSK2 cDNA, the cells lysates were subjected to a Western blot analysis with an anti-TSSK2 antibody. b. Localization of TSSK2 in the cytoplasm of CHO cells. CHO cells were transfected with TSSK2/pEGFP-N_2 plasmids, images were taken with a fluorescent microscopy. B. Co-IP with lysate from CHO cells co-transfected with TSSK2/pTarget and SPAG16L/pTarget. The lysates were immunoprecipitated with an anti-TSSK2 antibody or pre-immune serum, and Western blot was performed with an anti-N-terminus SPAG16L antibody. C. Co-IP with testis extracts. The testis extracts were immunoprecipitated with an anti-TSSK2 antibody or pre-immune serum, Western blot was performed with an anti-N-terminus SPAG16L antibody. Representative results are shown from at least three individual experiments.

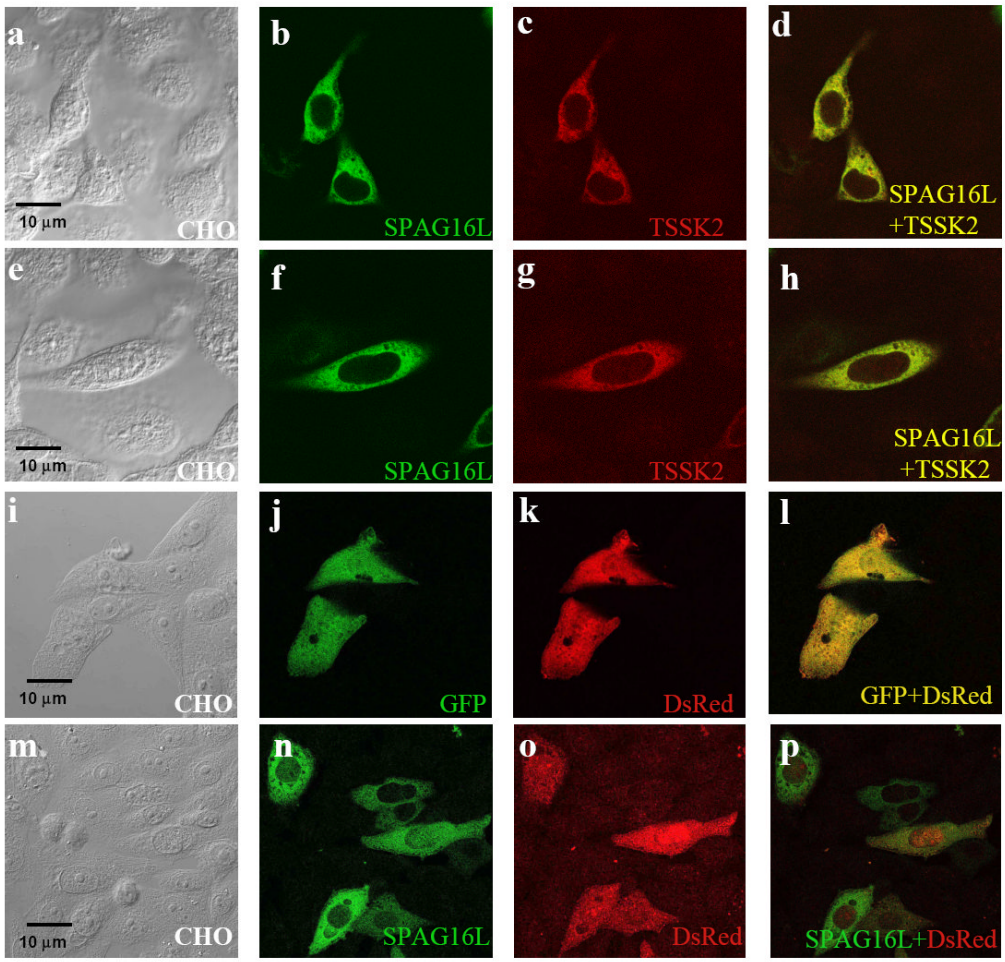

Figure 4. Co-localization of TSSK2 and SPAG16L in CHO cells

CHO cells were co-transfected with TSSK2/Dsred-N_1 and SPAG16L/pEGFP-N_2 plasmids (Panels 1 and 2); empty pEGFP-N_2 and Dsred-N_1 plasmids (panel 3); SPAG16L/pEGFP-N_2 and empty Dsred-N_1 plasmids (panel 4). Images were captured by confocal microscopy. SPAG16L/GFP is co-localized with TSSK2/DsRed but not DsRed only. a, e, i, m: phase contrast; b, f, n: SPAG16L/GFP; c, g: TSSK2/DsRed; d: merged image from b and c, h: merged image from f and g; j: GFP; K, o: DsRed; l: merged image from j and k; p: merged image from n and o.

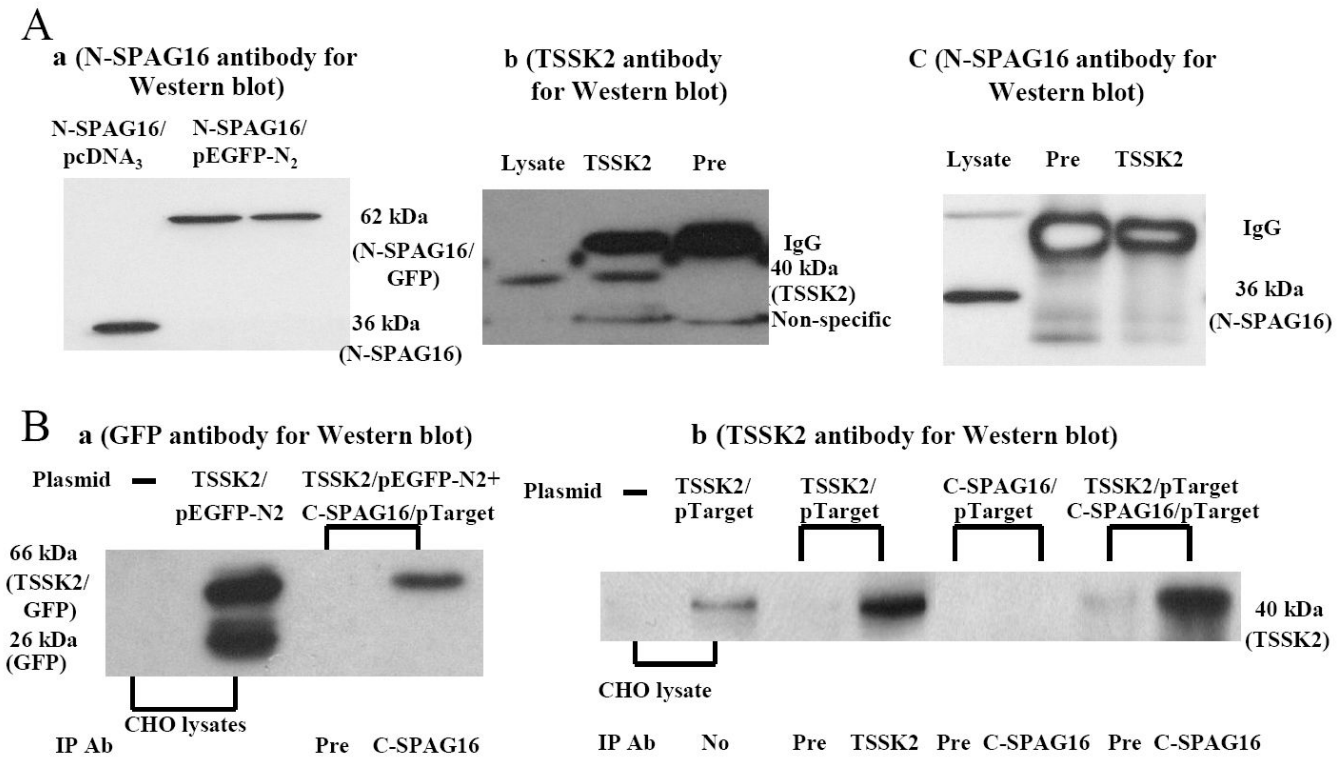

Figure 5. C-terminus WD repeats domain mediates the association of SPAG16L and TSSK2

A. TSSK2 does not associate with N-terminus of SPAG16. a. Western blot analysis of N-SPAG16 protein expression in CHO cells: CHO cells were transfected with N-SPAG16/ $pcDNA_3$ or N-SPAG16/pEGFP-N_2 plasmids, the cells lysates were subjected to a Western blot analysis with an anti-N-terminus SPAG16 antibody, identifying the 36 kDa N-SPAG16 protein and 62 kDa N-SPAG16/GFP fusion protein; b, c: TSSK2 but not N-SPAG16 can be pulled down by an anti-TSSK2 antibody. CHO cells were transfected with TSSK2/pTarget and N-SPAG16/pTarget plasmids. Immunoprecipitation was performed with either pre-immune serum or anti-TSSK2 antibody and immunoprecipitated proteins were subjected to Western blotting with an anti-TSSK2 antibody (b) or an anti N-terminus SPAG16 antibody (c). TSSK2 was immunoprecipitated (b) and SPAG16 was readily identified in the starting lysate (c), however no SPAG16 appeared in the immunoprecipitate (c).

B. C-terminus of SPAG16 mediates the association of TSSK2 and SPAG16L. a. CHO cells were co-transfected with TSSK2/pEGFP-N_2 and C-SPAG16/pTarget plasmids, the cell lysates were immunoprecipitated with pre-immune serum or an anti-C-terminus SPAG16 antibody, Western blot was performed with an anti-GFP antibody. Note the antibody recognized the 66kDa TSSK2/GFP fusion protein and the 26 kDa free GFP protein truncated from the fusion protein in the CHO lysates expressing TSSK2/GFP; b. CHO cells were transfected with TSSK2/pTarget and C-SPAG16/pTarget plasmids. The cell lysates were immunoprecipitated with indicated antibodies, and Western blot was performed with an anti-TSSK2 antibody. All are representative results from at least three individual experiments.

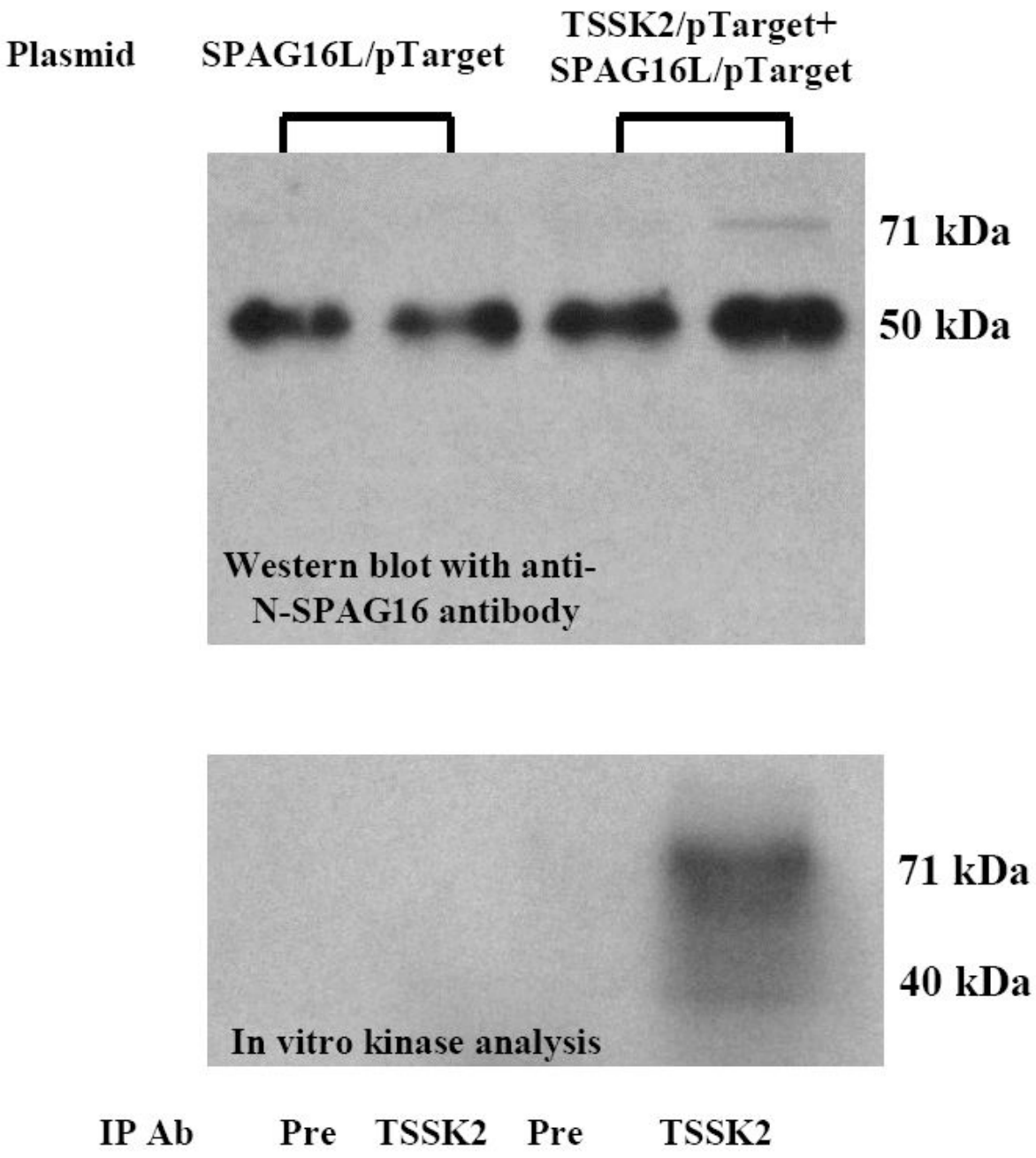

Figure 6. SPAG16L is phosphorylated in vitro

CHO-1 cells were transfected with TSSK2/pTarget, SPAG16L/pTarget or a combination of the two plasmids. The lysates were immunoprecipitated with pre-immune rabbit serum or an anti-TSSK2 antibody. Western blot was performed with half of the immunoprecipitates (upper panel), another half were washed and incubated in kinase buffer at 37°C for 15 min with [γ-^{32}P] ATP and then resolved by SDS-PAGE (lower panel). The dried gel was exposed to an X-ray film. In the co-transfected cells, the 71 kDa signal is suggestive of phosphorylation of the SPAG16L.

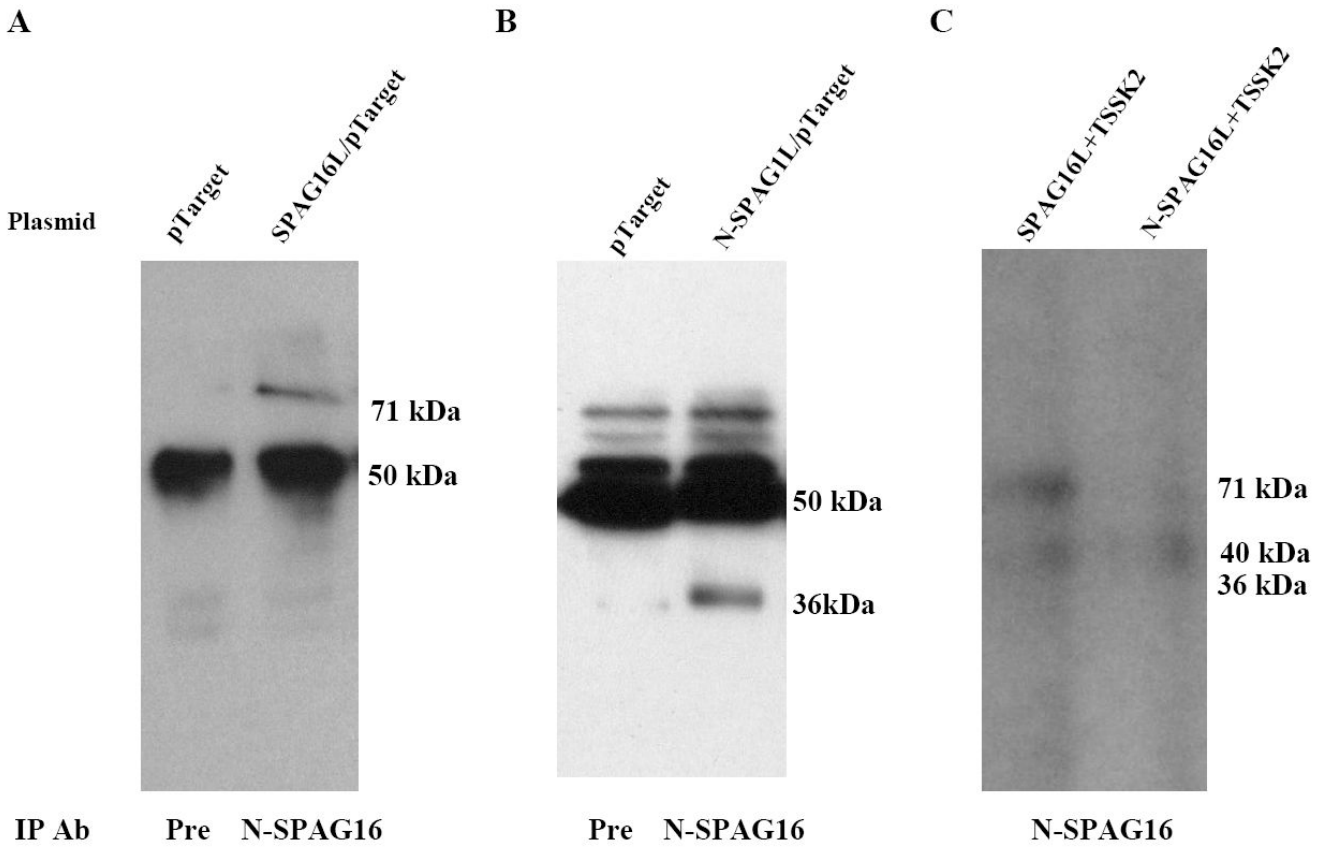

Figure 7. Phosphorylation of SPAG16L by purified recombinant TSSK2 protein

CHO-1 cells were transfected with SPAG16L/pTarget, or N-SPAG16L/pTarget. The lysates were immunoprecipitated with pre-immuno rabbit serum or anti-N-terminus SPAG16 antibody. Western blots were performed with half of the immunoprecipitates using the anti-N-terminus SPAG16 antibody to confirm the presence of SPAG16L (A) and N-SPAG16 (B) in the complex. C: The immunoprecipitated SPAG16L and N-SPAG16 were incubated with recombinant mouse TSSK2 protein for in vitro phosphorylation analysis. The 71 kDa signal is suggestive of phosphorylation of the SPAG16L, the 40kDa signal presumably represents autophosphorylation of TSSK2 protein. No 36 kDa signal was detected in the complex from cells transfeted with N-SPAG16L/pTarget. All experiments were repeated three times.

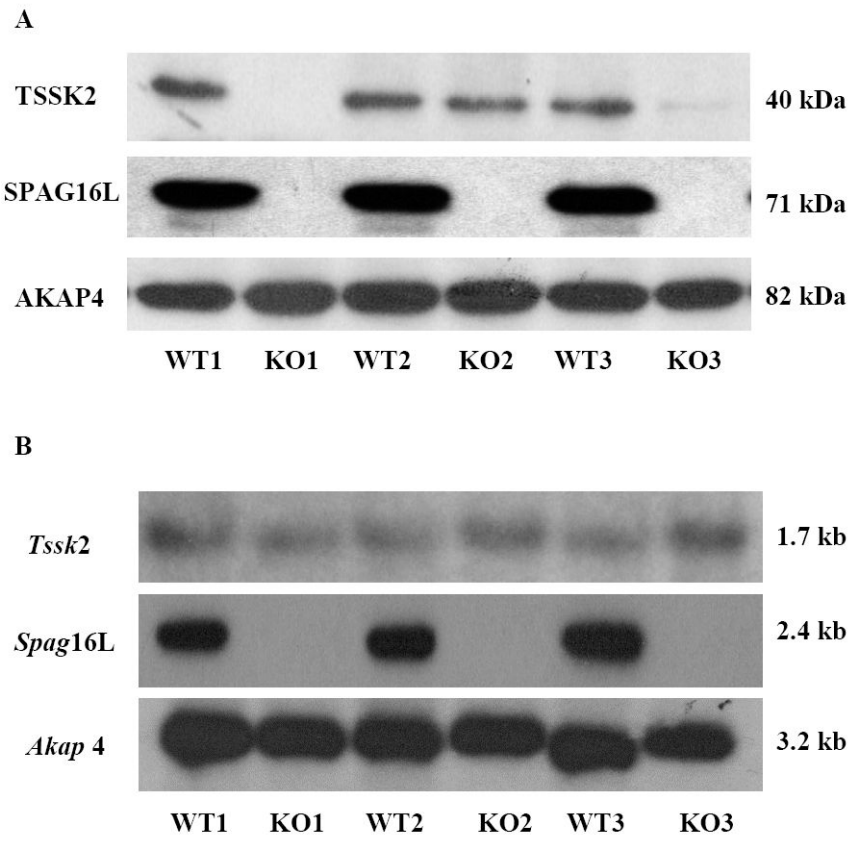

Figure 8. TSSK2 protein but not mRNA is absent or reduced in the testes of most *Spag*16L null mice

Testicular proteins and total RNAs were isolated from wild type and *Spag*16L null mice, the protein and mRNA expression of indicated genes were analyzed by Western (A) and Northern blot (B) with specific antibodies and probes. A representative analysis is shown.

Table 1

Potential proteins that associate with SPAG16

A mouse testis cDNA library was screened using the C-terminus domain of SPAG16 as the bait [2]. Plasmid DNA from the positive yeast clones was sequenced, and the candidate interacting genes were identified by searches of public databases. ND: not determined. To confirm protein-protein interactions of selected candidates, we performed co-immunoprecipitation studies (Co-IP). Some of the full length candidate cDNAs were cloned to the pEGFP-N_2 vector. Each of the plasmid DNAs and SPAG16S/pTarget were co-transfected into Chinese hamster ovary cells. The cell lysates were immunoprecipitated with anti-C-terminus SPAG16 antibody, and Western blots were carried out with an anti-GFP antibody.

GeneBank accession no.	Gene	Confirmed with Co-IP
NM_015773	Sperm associated antigen 6 (Spag6)	Yes
BC049538	Meiosis expressed gene 1(Meig I)	Yes
NM_009436	Testis-specific serine kinase 2 (Tssk2)	Yes
BC003224	Mus musculus regulator of chromosome condensation (RCC1) and BTB (POZ) domain containing protein 2	Yes
AK012254	RNA Polymerase II transcription elongation factor B (Elongin B)	Yes
NM_178254	Transcription factor-like 5 (basic helix-loop-helix) (Tcfl5)	ND
AF156099	Glycogen synthase kinase 3 beta	Negative
NM_013637	Protomine 1	ND
NM_021318	Four and a half LIM (Fh15)	ND
NM_013498	cAmp responsive element modulator (Crem)	ND
NP_839985	Transcription factor-like 5 protein	ND
NM_008303	Heat shock protein 1 (chaperonin 10) (Hspe1)	ND
BC038083	Prostaglandin D2 synthase	ND
AF126294	ICR/Hsd phosphoinositide-dependent protein kinase 1 mRNA	ND
NM_010294	Glycerol kinase 2 (Gk2)	ND
AF247846	PN10-like protein (Cpn10-rs1) gene	ND
NM_030237	Spermatogenic Zip 1 (Spz1)	ND
AK006048	Adult male testis cDNA, RIKEN full-length enriched library, clone: 1700016M24 product:hypothetical ARM repeat structure containing protein, full insert sequence	ND
AK005884	Adult male testis cDNA, RIKEN full-length enriched library, clone:1700012A03 product:hypothetical protein, full insert sequence.	ND
NM_008133	Glutamate dehydrogenase (Glud)	ND
AK015666	Adult male testis cDNA, RIKEN full-length enriched library, clone:4930500J03 product:hypothetical Uncharacterized protein family UPF0005 protein, full insert sequence	ND