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The expression characteristics of FAM71D and its association with sperm motility

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STUDY QUESTION: What are the features of FAM71D (Family with sequence similarity 71, member D) expression and is there an association between FAM71D expression and sperm motility?

SUMMARY ANSWER: FAM71D, a novel protein exclusively expressed in the testis, is located in sperm flagella and is functionally involved in sperm motility.

WHAT IS KNOWN ALREADY: Some testis-specific proteins have been reported as potential diagnostic biomarkers to evaluate the spermatogenesis process and sperm quality. We have identified a novel testis-specific protein, FAM71D, through microarray data analysis, yet little is known about its expression and function.

STUDY DESIGN, SIZE, DURATION: FAM71D mRNA and protein expression was quantified during mouse testis development. Its localization in germ cells was detected by dual-labeled immunostaining in testis sections and sperm smears. The clinical significance was assessed by comparing FAM71D expression in spermatozoa from normozoospermic controls and asthenozoospermic patients.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Testes were dissected from C57BL/6J male mice at postnatal ages of 1, 2, 3, 4, 6, 8 weeks and 6 months, and sperm was collected from cauda epididymides of adult mice by the swim-up method. Human spermatozoa were isolated from 100 human semen samples by density gradient Percoll centrifugation. RT-qPCR and western blot were performed to semi-quantify the expression of FAM71D in mouse testis, and in the ejaculated spermatozoa of normozoospermic controls and asthenozoospermic patients. Immunofluorescence staining was used to detect the localization of FAM71D. Co-immunoprecipitation assay was performed to evaluate the interaction between FAM71D and calmodulin. An antibody blocking assay was employed to assess the role of FAM71D in sperm motility.

MAIN RESULTS AND THE ROLE OF CHANCE: Our results showed that FAM71D was exclusively expressed in the testis in an age-dependent manner. FAM71D expression exhibited dynamic change in the cytoplasm of spermatids during spermiogenesis and was finally retained in sperm flagella. FAM71D could interact with calmodulin. Use of anti-FAM71D antibody on sperm significantly decreased sperm motility. Expression level of FAM71D was markedly reduced in the ejaculated spermataozoa of asthenozoospermic patients ($P < 0.05$), and this was correlated with sperm progressive motility ($r = 0.7435$, $P < 0.0001$).

LARGE SCALE DATA: N/A.

LIMITATIONS, REASONS FOR CAUTION: The sample size was limited and it is necessary to verify the correlation of FAM71D expression with sperm motility in larger cohorts. Furthermore, our results were descriptive and follow-up studies would be needed to elucidate the detailed role of FAM71D in sperm motility.

†The authors consider that the first two authors should be regarded as joint First Authors.

WIDER IMPLICATIONS OF THE FINDINGS: This is the first systematic study to document the expression of endogenous FAM71D and a function for FAM71D in sperm motility. It provides new insights into our understanding of sperm motility regulation and causes of male infertility.

STUDY FUNDING/COMPETING INTERESTS: This study was funded by the National Natural Science Foundation of China, Guangdong Natural Science Foundation and the Shenzhen Project of Science and Technology. The authors have no competing interests.

Key words: FAM71D / sperm flagella / calmodulin / sperm motility / asthenozoospermia

Introduction

In mammals, the generation of sperm capable of fertilization is a highly complex process including spermatogenesis in the testis and maturation in the epididymis (Neill, 2006). After spermatogenesis, germ cells enter epididymis as immotile cells and acquire motility during epididymal transit. It is accepted that sperm motility is a critical component of male fertility (Turner, 2006; Gianzo *et al.*, 2016). Individuals with poor sperm motility are usually infertile or sterile unless assisted reproductive techniques are employed. A more complete understanding of the molecular mechanisms of sperm motility will enable us to address the issue of poor sperm motility and male infertility.

With the development of high-throughput sequencing and gene targeting techniques during the last two decades, several genes critical for sperm motility have been identified (Escalier, 2006). Almost all proteins encoded by these genes are located in the sperm flagellum, and could be divided into different classes including: membrane ion channel proteins such as CatSper 1 (Ren *et al.*, 2001) and CatSper 2 (Quill *et al.*, 2001); cytoskeletal proteins such as Tektin-t (Tanaka *et al.*, 2004) and sperm-associated antigen 6 (Sapiro *et al.*, 2002); glycolytic enzymes such as phosphoglycerate kinase 2 (PGK2) (Liu *et al.*, 2016); and cell signaling proteins such as calmodulin (Ahmad *et al.*, 1995; Si and Olds-Clarke, 2000) and A-kinase anchor protein 4 (Miki *et al.*, 2002). It was also reported that in mouse male germ cells, the expression of 1652 genes is significantly up-regulated at the onset of meiosis, among which ~330 genes are exclusively expressed in the male germ line (Schultz *et al.*, 2003) and potentially involved in spermiogenesis or sperm motility. However, many of them, including unannotated genes, are still uncovered and need to be further studied.

Through analyzing high-throughput gene expression profiles of mouse tissues, organs and cell lines reported at BioGPS.org, as well as the EST profile in Unigene (Mm. 56514) (Nguyen *et al.*, 2014), we found that *Fam71d* (Family with sequence similarity 71, member D) was exclusively expressed in mouse testis. Human FAM71D was reported to form an in-frame MPP5-FAM71D fusion in a prostate cancer cell line (PC346C) and be important for the proliferation of PC346C (Teles Alves *et al.*, 2015). Other than that, there has been no reported study about the physiological function of FAM71D.

In the present study, we performed a detailed analysis of the expression of endogenous FAM71D in mice and human. We reported on the developmental onset of expression of FAM71D, as well as its tissue and subcellular localization. Most importantly, we provide the first evidence documenting a role for FAM71D in sperm motility. This systematic study provides new clues for our understanding of sperm motility regulation as well as causes of male infertility.

Materials and Methods

Ethics statement

This study was approved by the ethics committee of Peking University Shenzhen Hospital. Written informed consent was signed by participants or their relatives.

Tissue collection

C57BL/6J mice were obtained from the Southern Medical University Animal Center, China. All animals were treated according to the Guide for the Care and Use of Laboratory Animals prepared by the Institute of Laboratory Animal Resources for the National Research Council. Testes were individually collected from mice aged 1, 2, 3, 4, 6, 8 weeks and 6 months. Other tissues including brain, heart, lung, liver, kidney, spleen, epididymis and bladder were from adult mice.

Human tissues including brain, heart, lung, liver, kidney, spleen, epididymis and bladder tissues were collected during surgical operations, whereas fertile human testis specimens were obtained from post-mortem studies and orchidectomies.

Sperm collection

Mouse sperm cells were collected from cauda epididymis of adult mice by the swim-up method as described before (Chen *et al.*, 2016). Briefly, cauda epididymides were dissected and placed in 2 ml Quinn's Advantage Fertilization (HTF) Medium (SAGE, Trumbull, CT, USA) at 37°C for 5–15 min after shearing to allow the dispersion of sperm. Then sperm samples were washed with fresh medium and resuspended to a final concentration of 1×10^7 cells/ml for subsequent experiments.

Human semen samples were obtained from 20 to 50-year-old men in the Department of Laboratory Medicine of Peking University Shenzhen Hospital. Semen was collected by masturbation after 3–5 days of sexual abstinence. After liquefaction, semen volume, concentration, motility, viability and morphology were analyzed by a computer-assisted semen analysis system (CASA, CASAS-QH-III, Qing Hua Tong Fang, Beijing, China) under 200× magnification (Lu *et al.*, 2014). According to World Health Organization Criteria (2010), semen samples with following parameters were classified as normal: semen volume ≥2 ml, pH ≥ 7.2, sperm concentration $\geq 15 \times 10^6$/ml, progressive motility (PR) ≥32%, morphology ≥4%. After excluding varicocele, reproduction tract infection, testicular injury, etc. (Zhou *et al.*, 2015), semen samples with normal semen volume and sperm concentration, but with progressive motility <32% were classified as athenozoospermic. Sperm samples were purified by Percoll gradient centrifugation (Pharmacia, USA) (Zhou *et al.*, 2015). The spermatozoa pellet was washed with PBS, and then visually inspected under the microscope for the absence of contaminating cells such as germ cells, leukocytes or bacteria. Then the sperm pellets were collected for further use.

Cell culture and plasmid construction

HEK293T, COS7 and TM4 cell lines were obtained from the American Type Culture Collection. The cells were maintained in Dulbecco modified Eagle medium (Life Technologies, Rockville, MD) supplemented with 10% fetal bovine serum at 37°C in a humidified atmosphere with 5% CO_2. To construct EGFP-FAM71D vector, the full length *Fam71d* cDNA was amplified by specific primers, and inserted into pEGFP-C1 via EcoR1 and BamH1 (New England Biolabs). Calmodulin cDNA was also inserted into pcDNA3.1-HA vector similarly. The PCR products were cloned and sequenced.

Reverse-transcription PCR (RT-PCR) and quantitative RT-PCR (RT-qPCR)

Total RNA was extracted from mouse and human tissues using TRIzol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. cDNA was synthesized using the PrimeScript™ RT Master kit (Takara, Dalian, Japan). PCR was performed with Takara Emerald Amp PCR Master Mix (Takara). Primers specific for mouse *Fam71d* were 5′-GCAATGAATAAGCAAGAA-3′ (forward) and 5′-CCAGTATAGGAGG AGATA-3′ (reverse). Mouse *Gapdh* was used as internal control; the primers for *Gapdh* were 5′-AGTGGCAAAGTGGAGATT-3′ (forward) and 5′-GTGGAGTCATACTGGAACA-3′ (reverse). The primers used for human *FAM71D* were: forward, 5′-GTTGGATGGAGGAGAGTAT-3′ and reverse, 5′-TCTTCTGTTGACCTGGATAA-3′; the primers used for human actin beta (*ACTB*) were: forward, 5′-CATGTACGTTGCTATCC AGGC-3′ and reverse, 5′-CTCCTTAATGTCACGCACGAT-3′. The annealing temperature for the above primers during PCR was 55°C.

RT-qPCR was carried out using the SYBR® Premix EX Taq™ II PCR Kit (Takara) following the manufacturer's instructions on the Roche Lightcycler 480 Real-Time PCR System. Data were calculated according to the Applied Biosystems comparative Ct method.

Western blot

Protein was extracted using Qproteome Mammalian Protein Prep Kit (QIAGEN, Hilden, Germany). Then samples were separated by 10% SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) and electrotransferred onto PVDF (polyvinylidene fluoride) membranes (Millipore, Bedford, MA, USA). The membranes were blocked with 5% (w/v) non-fat milk in TBST (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1% Tween 20). Anti-FAM71D antibody (ab90262, Abcam, Cambridge, UK, 1:500 dilution) and anti-ACTB antibody (ab179467, Abcam, 1:3000) were applied overnight at 4°C, followed by the incubation with corresponding HRP-labeled secondary antibody (ab6721/ab6789, Abcam) for 1 h at room temperature. Positive bands were detected using the ECL kit (Thermo Scientific, Waltham, MA, USA).

Immunofluorescence staining (IF)

Mouse testes were fixed in Tissue TekH (Sakura, Torrance, CA, USA) and sectioned with a cryostat microtome (CM1850, Leica, Bensheim, Germany). After being blocked in 10% goat serum, the sections were incubated with anti-FAM71D antibody (1:50) overnight at 4°C. Then the sections were washed with PBS and incubated for 1 h at 37°C with the secondary antibodies (KIT-5001/KIT-5005, Maixin, Fuzhou, China).

Mouse and human mature sperm were collected as described previously (Zhou *et al.*, 2015; Chen *et al.*, 2016). The sperm were spotted onto slides, dried at room temperature, and fixed in 4% paraformaldehyde for 20 min. Paraffin sections were prepared as described previously. After being blocked in 10% goat serum, the slides were incubated with anti-FAM71D polyclonal antibody (1:50), anti-calmodulin antibody (ab2860, Abcam, 1:50), or Rhodamine-conjugated Arachis hypogaea lectin (peanut agglutinin, PNA) (RL-1072, QED Biologicals, 1:500) overnight at 4°C. The appropriate secondary antibodies including anti-rabbit-Cy3, anti-rabbit-Alexa Fluor 488, anti-mouse-Cy3, and anti-rat-Alexa Fluor 488 (Invitrogen) were used, and slides were counterstained with Hoechst 33342 (1 mg/ml, Invitrogen) and mounted with Prolong Gold Antifade Reagent (Invitrogen). Primary antibody pre-incubated with neutralizing peptide was used as a negative control. The results were observed by a laser scanning confocal microscopy (LSM710, Zeiss, Germany) and analyzed using the software Image-Pro Plus 5.1.

HEK293T, COS7 and TM4 cells were grown on coverslips in a 24-well plate. The cells were transfected with pEGFP-C1 or EGFP-FAM71D. Non-transfected cells were used as negative control. At 24 h after transfection, cells on coverslips were washed once with PBS and stained using FAM71D antibody as described above.

Co-immunoprecipitation (Co-IP)

EGFP-FAM71D was transfected into HEK293T cells together with calmodulin-HA using Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Cells were harvested 48 h after transfection. After washing with TBS (50 mM Tris-HCl (pH 7.4) and 150 mM NaCl), cells were lysed on ice for 30 min with lysis buffer (50 mM Tris-HCl (pH 7.4), 2 mM $CaCl_2$, 150 mM NaCl, 1% (v/v) NP-40, protease inhibitor cocktail, and 1 mM PMSF). The lysates were centrifuged at 16 100*g* at 4°C for 15 min. The supernatants were incubated with Dynabeads Protein G agrose (1003D, Thermo Fisher Scientific Inc., MA, USA) pre-bound with anti-GFP (sc-9996, Santa Cruz Biotechnology, California, USA) and anti-HA antibody (H3663, Sigma, St Louis, MO, USA) at 4°C overnight with gentle rotation. The beads were washed four times with lysis buffer, eluted with 0.1 M Glycine buffer (pH 3.0) and prepared with SDS-PAGE loading buffer for western blot analysis. For testing the endogenous interaction between FAM71D and calmodulin in protein extraction from mouse testis, the mouse testicular proteins were subjected to co-IP using antibody against FAM71D or calmodulin (ab45689, Abcam), respectively. The rest of the procedures were identical to the description stated above.

Antibody blocking assay

After preparation, the concentrations of mouse sperm and normal human sperm samples were adjusted to 2×10^7/ml. 20 μg/ml anti-FAM71D antibody was added to test samples and incubated at 37°C for 1 h in 5% (v/v) CO2. Normal rabbit IgG (sc-2027, Santa Cruz Biotechnology), and two other antibodies sold at the same concentration from the same manufacturer as anti-FAM71D antibody, i.e. anti-actin antibody (ab8227, Abcam) and anti-EGFP antibody (ab184601, Abcam), were used as controls. The samples were then placed in a counting chamber (0.01 mm, 10 μl deep) for CASA analysis.

Statistical analysis

All experiments were repeated at least three times. Statistical analysis was performed using GraphPad Prism Version 5.0. Data were expressed as the mean ± SD. Student's *t*-test was used to compare the difference between two groups. A Spearman correlation coefficient (*r*) was calculated to assess the significance of association between sperm motility and expression level of FAM71D. A *P* value less than 0.05 was considered to be statistically significant.

Results

Expression of FAM71D in mouse testes

FAM71D expression in various tissues of mice and human was detected via RT-PCR (Fig. 1A) and western blot (Fig. 1B). The results

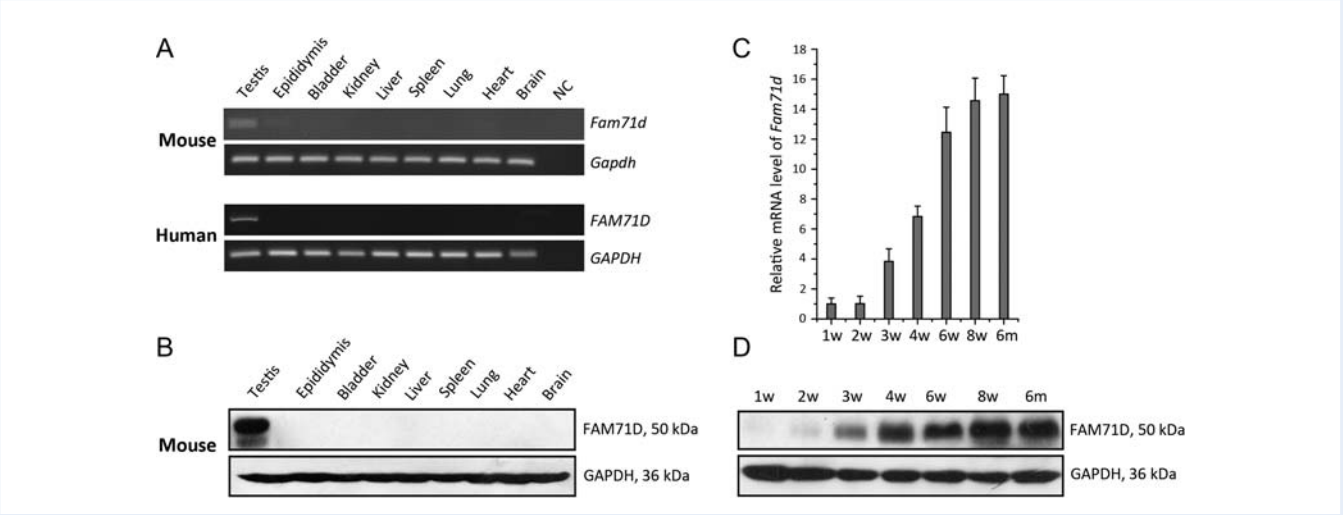

Figure 1 Distribution of FAM71D in mouse and human tissues. The presence of *Fam71d*/*FAM71D* mRNA (**A**) and protein (**B**) in various tissues of adult mouse and human was detected by RT-PCR and western blot, respectively. NC: negative control. The expression profile of testicular *Fam71d* mRNA (**C**) and its protein (**D**) from mice at indicated ages was quantified by RT-qPCR and western blot, respectively. The relative *Fam71d* mRNA level was normalized to *Gapdh*, and then evaluated relative to that of 1-week old mouse. Data were shown as mean ± SD ($n = 5$).

showed that the gene was exclusively expressed in the testis, which was consistent with the expression profile reported at BioGPS.org. As seen in Fig. S1, a thick 50-kDa band corresponding to FAM71D in mouse testis was detected by western blot, with the same molecular weight as that theoretically expected. Although several potential non-specific bands also could be observed, they were too weak compared with the main 50-kDa band. The timing of FAM71D expression during postnatal testis development at both mRNA and protein levels was determined by RT-qPCR (Fig. 1C) and western blot (Fig. 1D), respectively. In Fig. 1C, *Fam71d* mRNA level was increased from 3 weeks to 6 months, while no change could be observed between that in the testis at postnatal 1 week and 2 weeks. The protein level also increased in an age-dependent manner during testis development (Fig. 1D), which was consistent with the expression of *Fam71d* mRNA.

Immunofluorescence staining was performed to investigate the subcellular localization of FAM71D during testis development (Fig. 2). The specificity of the FAM71D antibody in IF was detected using cells (without endogenous expression of FAM71D) transfected with EGFP-FAM71D vector. Signals of FAM71D antibody staining merged well with GFP signals in the cytoplasm of HEK293T, COS7 and TM4 cells, showing the specificity of FAM71D antibody in IF experiments (Fig. S2). PNA, Peanut agglutinin, a commonly used marker to visualize the outer membrane of the acrosome, was used to indicate different phases of spermiogenesis to observe the localization of FAM71D protein in mouse testis sections. In mouse testis, FAM71D was mainly localized in the cytoplasm of round spermatids and elongated spermatids. Interestingly, we found that in the cap phase FAM71D remained close to the acrosomes but began to migrate to other regions around the nuclei. As spermiogenesis progressed, FAM71D further separated from the acrosomes. In the acrosome phase, while the acrosomes formed hook-like structures and moved toward one end of the nuclei, FAM71D moved to the opposite end. In the maturation phase, FAM71D moved completley to the end opposite to the acrosomes, and, at the end of spermiogenesis, FAM71D was removed to the residue body together with most of the other cytosolic components. Finally, caudal sperm was isolated to examine whether FAM71D was discarded, and the results showed that FAM71D was retained in sperm flagella. In mature sperm, FAM71D was colocalized with MTCO1, a marker of mitochondria, which indicated that FAM71D was localized in the midpiece of flagella (Fig. S3).

FAM71D Interaction with calmodulin

Sequence analysis through bioinformatics suggested that mouse FAM71D contained potential calmodulin binding motifs (http://calcium.uhnres.utoronto.ca/, accessed 1 September 2017).The results of immunofluorescence staining showed that FAM71D (green) was colocalized with calmodulin (red) in round spermatids and elongated spermatids (Fig. 3A) in mouse testis and sperm flagella (Fig. 3B). In Fig. 3C, a band located at ~77 kD could be detected with both anti-GFP antibody and anti-FAM71D antibody in HEK293T cells transfected with EGFP-FAM71D plasmid, which was absent from non-transfected cells or cells transfected with pEGFP-C1 empty vector. The data suggested the antibody against FAM71D was specific. To confirm the interaction between FAM71D and calmodulin, FAM71D expression vector tagged with EGFP was co-transfected into HEK293T cells with calmodulin-HA. In the Co-IP experiment using anti-HA antibody, co-expressed EGFP-FAM71D came down in the precipitate (Fig. 3D, upper panel). When the Co-IP experiment was performed reciprocally using anti-GFP antibody for immunoprecipitation, calmodulin-HA was observed to co-precipitate with EGFP-FAM71D (Fig. 3D, lower panel). Moreover, endogenous immunoprecipitation using mouse testicular protein also cofirmed the association between FAM71D and calmodulin (Fig. 3E). Taken together, these results above indicated that FAM71D physically interacts with calmodulin.

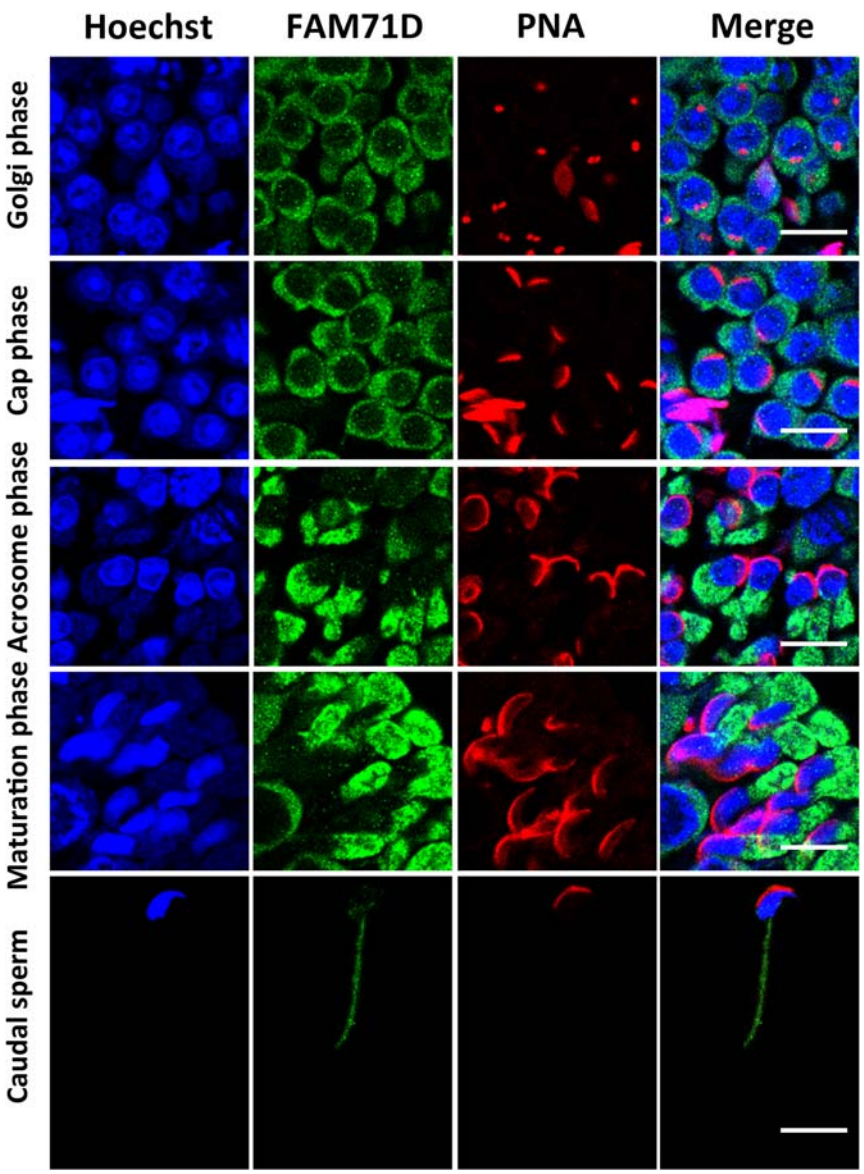

Figure 2 Dynamic expression of FAM71D during spermiogenesis in mice. The intracellular distribution patterns of FAM71D (green) were determined using immunofluorescence during four phases of acrosome formation. PNA (red) was used to label acrosomes. Hoechst 33342 (blue) was used to stain nuclei. Bar: 5 μm.

Marked inhibition of sperm motility by anti-FAM71D antibody

Fig. 4 A shows that human FAM71D was localized in the midpiece of sperm flagella, which was coincided with that of mouse FAM71D. To investigate whether FAM71D was involved in sperm motility, an antibody blocking assay was carried out to assess the effect of anti-FAM71D antibody on sperm motility. In Fig. 4B, mouse sperm motility was ~56% without treatment. It then decreased to 23.2% after anti-FAM71D antibody incubation, while no significant change was observed after incubation with normal rabbit IgG, anti-actin antibody and anti-EGFP antibody. Anti-FAM71D antibody treatment also significantly damaged human sperm motility, compared with both the negative control and irrelevant IgG controls in Fig. 4C. These results suggested a role of FAM71D in sperm motility.

Low expression of FAM71D in patients with asthenozoospermia

To further confirm the role of FAM71D in sperm motility, we analyzed the association between FAM71D expression and asthenozoospermia via RT-qPCR and western blot. Fig. S1 shows that anti-FAM71D antibody recognized a single band representing human FAM71D at ~47 kD. Compared to normozoospermic males, both *FAM71D* mRNA (Fig. 5A) and FAM71D protein (Fig. 5B) levels were significantly decreased in patients with asthenozoospermia, which indicated that reduced FAM71D

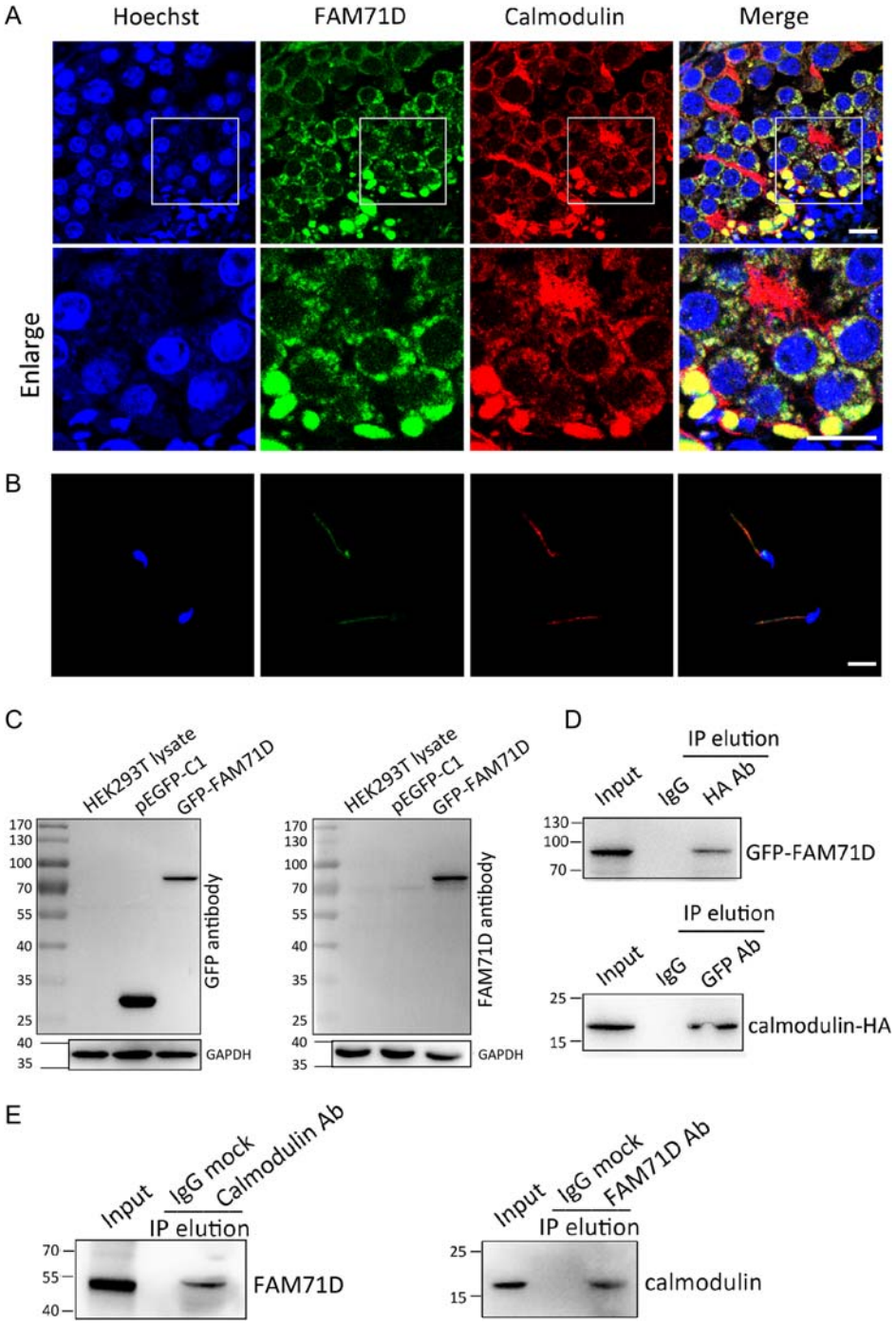

Figure 3 FAM71D interaction with calmodulin. Localization of FAM71D (green) and calmodulin (red) in mouse testis (**A**) and mature sperm (**B**) was detected by immunofluorescence. Hoechst was used to stain nuclei (blue). Bar: 10 μm. (**C**) The specificity of anti-FAM71D antibody tested by western blot in HEK293T cells. Co-immunoprecipitation of FAM71D and calmodulin in HEK293T cells (**D**) or mouse testis (**E**). Cell lysates or testicular proteins were immunoprecipitated with Dynabeads bound with specific antibodies. The eluted immunoprecipitates were then detected by western blot with antibodies as indicated. Normal IgG was used as control.

expression is associated with asthenozoospermia. Further Spearman correlation analysis of FAM71D protein expression with sperm progressive motility showed that the FAM71D protein level was positively correlated with sperm progressive motility ($r = 0.7435$, $P < 0.0001$) (Fig. 5C). These data suggested that FAM71D is clinically involved in asthenozoospermia probably by affecting sperm progressive motility.

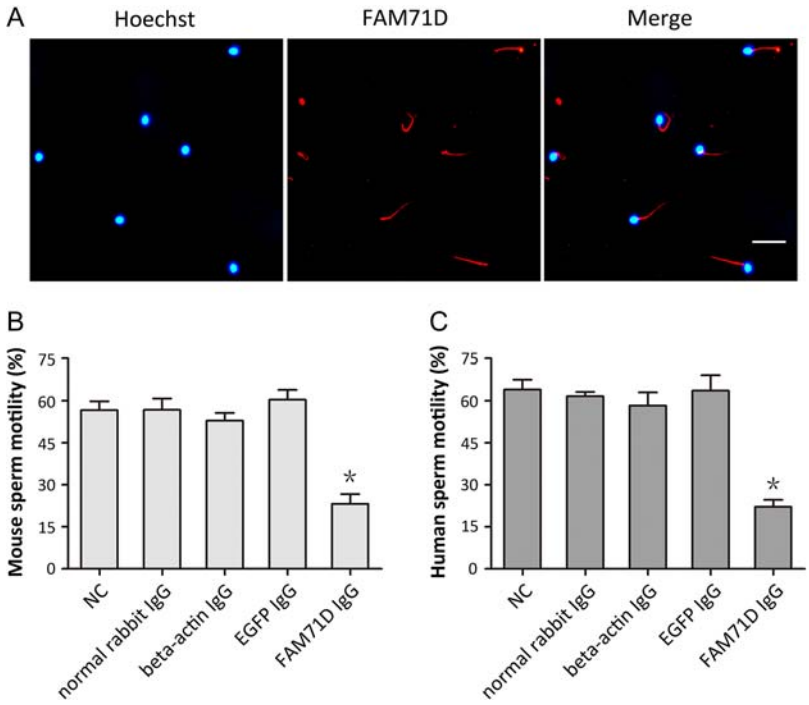

Figure 4 Inhibitory effects of anti-FAM71D antibody on mouse and human sperm motility. (**A**) Healthy human spermatozoa were subjected to immunofluorescence for FAM71D (red) and Hoechst (blue). Bar: 20 μm. Motility of mouse sperm (**B**) and human sperm (**C**) was detected using CASA before and after incubation with 20 μg/ml anti-FAM71D antibody or irrelevant IgGs (as control) for 1 h. Data were presented as mean ± SD ($n = 3$), compared by Student's t test. * $P < 0.05$.

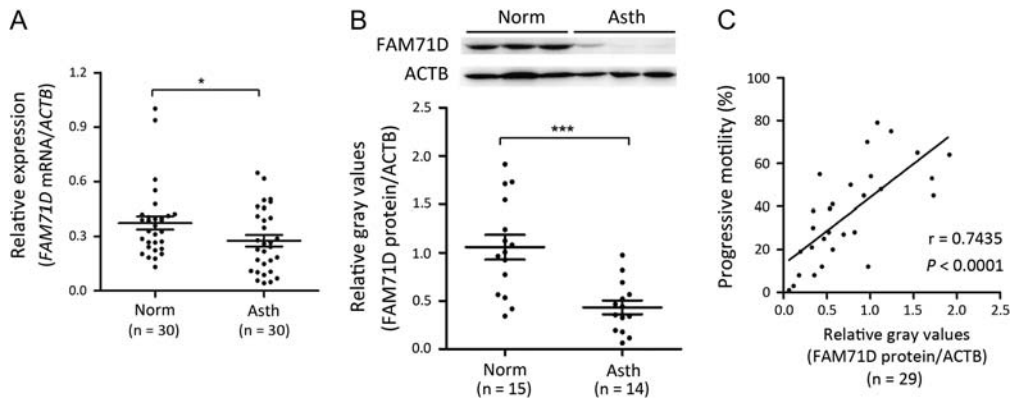

Figure 5 Low expression of both *FAM71D* mRNA and its protein in ejaculated spermatozoa of patients with asthenozoospermia. (**A**) The expression level of *FAM71D* mRNA in the ejaculated spermatozoa of normozoospermic males and asthenozoospermic patients was detected by RT-qPCR, with *ACTB* as a control. (**B**) The expression level of FAM71D protein in the ejaculated spermatozoa of the normal group and asthenozoospermic group was detected by western blot, and then relative gray values of bands were analyzed using Image Pro software. (**C**) The significance of the association between FAM71D protein level and sperm progressive motility was assessed by Spearman correlation analysis in 29 samples. Data were shown as mean ± SEM, *$P < 0.05$, ***$P < 0.001$. Norm, normal group; Asth, patients with asthenozoospermia.

Discussion

Sperm motility is coordinated by a myriad of proteins and various signaling pathways, and is considered to be a key parameter in sperm quality examination (Escalier, 2006; Turner, 2006). However, the underlying molecular mechanisms have been too complex to be fully understood so far. Previous mouse model studies and clinical reports

have shown that some testis-specific proteins such as lactate dehydrogenase C (Odet *et al.*, 2011) and PGK2 (Liu *et al.*, 2016) could be used as potential diagnostic biomarkers to evaluate the spermatogenesis process as well as sperm quality. Thus identification and functional studies of novel testis-specific genes are of great value not only for study of mechanisms but also for clinical diagnosis of male infertility. In the present study, we identified a novel abundantly expressed testis protein, FAM71D, which was located in the flagella of mature sperm and functionally involved in sperm motility.

As revealed by RT-PCR and western blot, the expression of *Fam71d* mRNA and protein began in mouse testis at postnatal 3 weeks, the time when the first wave of spermatogenesis occurs, suggesting that FAM71D expression is mainly expressed in postmeiotic germ cells. This was further confirmed by immunofluorescence that observed the dynamic change of FAM71D expression from cytoplasm of round spermatids to the residual body of elongated spermatids during spermiogenesis in testis sections. In addition, FAM71D was localized in the flagellum in mature sperm, which provides the motile force for the sperm to reach oocyte. The flagellum localization of FAM71D in mature sperm coincides with reports for other proteins including sperm-associated antigens (Fitzgerald *et al.*, 2006), Tektins (Murayama *et al.*, 2008) and Kinesin light chain 3 (Zhang *et al.*, 2012), which are also involved in sperm motility, suggesting a potential role of FAM71D in sperm motility.

Through bioinformatics analysis, it was predicted that mouse FAM71D contained possible calmodulin binding motifs (http://calcium.uhnres.utoronto.ca/, accessed 1 September 2017). Calmodulin has been reported to be localized in the acrosome, post-acrosomal sheath, the perinuclear ring of developing spermatids and flagellum (Jones *et al.*, 1980; Weinman *et al.*, 1986; Kann *et al.*, 1991; Li *et al.*, 2014). The localization of FAM71D overlapped with calmodulin in the flagellum. It is also commonly known that calmodulin is a classical intracellular calcium receptor, and it was reported to be involved in sperm motility possibly via regulation of its downstream targets, such as different isoforms of calmodulin kinases (Turner, 2006). Co-immunoprecipitation assays confirmed that FAM71D did interact with calmodulin. These results are consistent with previous reports that Fam71 in African trypanosomes may function as a calcium-binding protein through its EF-hand calcium-binding domain (Jackson *et al.*, 2012). The interaction between FAM71D and calmodulin again raised the possibility of the involvement of FAM71D in sperm motility. However, the underlying mechanism is still unknown.

Mature spermatozoa have been always considered to be carriers of only genetic messages. However, several studies have reported that a complex repertoire of mRNA exists in mature spermatozoa, which plays an important role in sperm motility, acrosomal reaction and early zygotic and embryonic development (Ostermeier *et al.*, 2002, 2004; Lambard *et al.*, 2004). In this study, we also found *FAM71D* mRNA in total spermatozoan RNA through RT-PCR (data not shown). As reported for other sperm transcripts, although only some RNAs are retained in spermatozoa, the *FAM71D* transcript might be selectively maintained in spermatozoa to be subsequently translated to protein during the aforementioned activities (Ostermeier *et al.*, 2004). Western blot analysis and immunofluorescence staining assays further confirmed the presence of FAM71D protein in the ejaculated spermatozoa.

When anti-FAM71D antibody was incubated with motile spermatozoa, both mouse and human sperm motility was decreased to a rather low state, ~20%. The same antibody blocking effects have also been observed in other sperm motility proteins, such as PGK2 (Liu *et al.*, 2016) and β-defensin 1 (Diao *et al.*, 2014). According to WHO standards (2010), sperm progressive motility less than 32% is defined as asthenozoospermia, which is the most common semen abnormality, and one of major causes of male infertility (Chemes *et al.*, 1998). We therefore wondered whether the abundance of FAM71D in sperm was associated with asthenozoospermia. So we compared FAM71D expression in normozoospermic samples and asthenozoospermic semen samples. Compared with the normozoospermic group, *FAM71D* mRNA and its protein expression were significantly reduced in ejaculated spermatozoa of patients with asthenozoospermia. All the above data indicate that FAM71D is involved in sperm motility.

Unlike membrane or surface proteins, such as CD147 (Chen *et al.*, 2012) and β-defensin 1, FAM71D was located in the cytoplasm of germ cells as revealed by the immunofluorescence results. In addition, *in-silico* analysis (http://www.cbs.dtu.dk/services/TMHMM, http://mendel.imp.ac.at/sat/DAS, accessed 1 September 2017) shows that there is no transmembrane motif in FAM71D protein. Thus FAM71D is a cytoplasmic protein. There have been reports that antibodies of intracellular proteins influence sperm functions. It was reported that antibodies to intra-acrosomal protein SP-10 (Coonrod *et al.*, 1996) and SNARE proteins (Ramalho-Santos *et al.*, 2000) inhibited acrosome reaction of bovine sperm in vitro. Additionally anti-actin antibody inhibited the zona-pellucida-induced acrosome reaction in human sperm (Liu *et al.*, 2002) while blocking experiments using an antibody against PGK2 significantly decreased sperm motility of human spermatozoa. How did these antibodies work? There have been reports that antibodies enter live cells. It was reported that anti-ribonuleoprotein IgG could penetrate live human T gamma lymphocytes (Alarcon-Segovia *et al.*, 1979), while anti-actin monoclonal antibody could enter human sperm cells during capacitation (Liu *et al.*, 2002).Therefore it is possible that anti-FAM71D antibody entered sperm and exerted a deleterious effect on FAM71D.

Sperm motility is dependent upon sophisticated coordination of a myriad of proteins and signaling pathways. The present study reveals that FAM71D, a novel abundantly expressed testis protein, is located in the flagellum of mature sperm and is involved in sperm motility in both mice and human. In addition, expression of FAM17D is lower in asthenozoospermic spermatozoa compared with normozoospermic control samples and correlates with progressive sperm motility. This study is the first to document the expression of endogenous FAM71D and a function for this novel flagellum protein. These results will provide new insights into the mechanisms of sperm motility and may contribute to the diagnosis and treatment for asthenozoospermia.

Supplementary data

Supplementary data are available at *Human Reproduction* online.

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Authors' roles

Q.M., Y.L. and Y.G. designed the study and wrote the manuscript with feedback from the other authors; Q.M., Y.L. and J.C. were responsible for tissue collection, sperm fraction, RNA extraction, PCR and western blot; M.L., H.G. and S.L. were responsible for immunostaining experiments; Y.D. and Z.J. participated in data analysis; all authors read and approved the final version of the manuscript.

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Conflict of interest

None declared.

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