Mechanistic insights into acephalic spermatozoa syndrome–associated mutations in the human SUN5 gene

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ABSTRACT

Acephalic spermatozoa syndrome has been reported for many decades; it is characterized by very few intact spermatozoa and tailless sperm heads in the semen and causes severe male infertility. The only gene whose mutations have been found to be associated with this syndrome encodes Sad1 and UNC84 domain-containing 5 (SUN5), a testis-specific nuclear envelope protein. The functional role of SUN5 has been well studied in mouse models, but the molecular basis for the pathogenic effects of mutations in the human SUN5 gene remain elusive. Here, we report on an intronic SUN5 mutation (c.475C>T, p.Arg159*) in an individual with acephalic spermatozoa syndrome. Using an artificial splicing system, we found that this mutation affects the splicing of SUN5 mRNA, yielding a premature stop codon that results in a truncated SUN5 protein. We also found that SUN5 interacts with the coupling-apparatus protein DnaJ heat shock protein family (Hsp40) member B13 (DNAJB13) during spermatogenesis and that substitutions in SUN5’s SUN domain impair its interaction with DNAJB13. Furthermore, we observed that many SUN5 mutations affect the secondary structure of the protein and influence its folding and cellular localization. In summary, our findings indicate an interaction of SUN5 with DNAJB13 during spermatogenesis, provide mechanistic insights into the functional role of this interaction in sperm head–tail integration, and elucidate the molecular etiology of acephalic spermatozoa syndrome–associated SUN5 mutations.

Introduction

Acephalic spermatozoa syndrome (MIM 617187) is characterized by decapitated flagella, very few intact spermatozoa and tailless sperm heads in the semen, which leads to severe male infertility (1-5). Acephalic spermatozoa result from impaired spermiogenesis in the testis. The sperm head-tail coupling apparatus (HTCA) is disrupted or detached from the sperm head, leading to the separation of the sperm head and the flagellum (6). In the epididymis and semen, over 90% of the spermatozoa are actually non-functional decapitated flagella, making acephalic spermatozoa syndrome one of the most severe forms of teratozoospermia.

Data from mouse models and acephalic spermatozoa syndrome patients indicate that acephalic spermatozoa syndrome is a result of a genetic disorder. Investigations in mice have found several genes involved in the production of acephalic spermatozoa, including Odf1 (outer dense fiber protein 1) (7), Hook1 (8), Oaz3 (ornithine decarboxylase antizyme 3) (9) and Spata6 (spermatogenesis associated 6) (10), but none of these mutations were found in acephalic spermatozoa syndrome patients. Sun5 (Sad1 and UNC84 domain containing 5) is the only gene whose presence and function was confirmed in a mouse model and also identified as pathogenic mutations in patients. Currently, 10 point mutations of SUN5 have been reported, including 3 nonsense mutations, 6 missense mutations and 1 intron mutation that may lead to splicing alterations (11). Recently, a homozygous deletion of 5090bp, including exon 8, in SUN5 was found in 3 acephalic spermatozoa syndrome patients, predicting the frameshift, p.(Leu143Serfs*30), and the inactivation of SUN5 (12).

Although the potential pathogenic mutations have been identified, their effects on SUN5 function remain unknown, and only one mutation c.340G>A was experimentally tested. The 340G>A mutation localizes to the last base on exon 5, which was predicted to abolish the donor splice site in intron 5. A study reported that
the SUN5 exonic variant c.340G>A (p.Gly114Arg) inhibits splicing of exon 5, causing a frameshift in the SUN5 protein (12).

The discovery of new SUN5 mutations is still underway, and we recently sequenced SUN5 exons from 3 unrelated acephalic spermatozoa syndrome patients and found that 2 patients carried biallelic SUN5 mutations, including an unreported variant. The new SUN5 mutation was identified as c.475C>T, (p.Arg159*), which resulted in a premature termination codon (PTC) in SUN5 mRNA and increased the number of acephalic spermatozoa syndrome-associated SUN5 mutations to 12.

Few studies have investigated the mechanism by which acephalic spermatozoa are produced, but the Spata6 knockout mouse model has provided some clues (10). SPATA6 was localized on HTCA, and the absence of SPATA6 resulted in the failure of HTCA assembly, which in turn destroyed the tight junction between sperm head and tail. But no SPATA6 mutations were found in patients with acephalic spermatozoa syndrome, so the abovementioned details might not be the faithful mechanism underlying human acephalic spermatozoa syndrome.

To clarify the mechanism in which SUN5 participates in acephalic spermatozoa syndrome, we identified DNAJB13 (DnaJ heat shock protein family (Hsp40) member B13) as a SUN5 interacting protein during spermiogenesis, and found that their dynamic interactions are required for the tight junction of the sperm head and tail. Based on these results, we systematically investigated the effects of SUN5 mutations on its function, including the localization, protein secondary structure, and interaction with DNAJB13. Because all the nonsense mutations resulted in truncated SUN5 lacking the SUN domain, which is required for SUN5-DNAJB13 interaction, we focused our analyses on the remaining six missense mutations, including the one intron mutation. We found that c.425+1G>A leads to a partially retained intron 7 in SUN5 mRNA, introducing a stop codon in protein translation. Mutations on the coiled coil domain and SUN domain altered the protein secondary structure and the protein conformation to different extents. Most of the mutations impaired the dynamic interactions between SUN5 and DNAJB13.

Taken together, our study reveals mechanistic details of SUN5 mutations in acephalic spermatozoa, and provides new insights into the syndrome.

**Results**

**The phenotype and pedigree of the new SUN5 mutation c.475C>T (p.Arg159*)**

Three infertile men were diagnosed with acephalic spermatozoa syndrome in the Center for Reproductive Medicine in Peking University Third Hospital (P1-P3 in table 1). By sequencing all the SUN5 exons in the 3 patients and their parents, we found that 2 individuals had compound heterozygous SUN5 mutations [(P2: c.781G>A (p.Val261Met), c.1043A>T (p.Asn348Ile); P3: c.425+1G>A, c.475C>T, (p.Arg159*)]. The c.475C>T mutation is an unreported mutation that localizes to exon8 and leads to a premature stop codon and a truncated form of the SUN5 protein. The spermatozoa in the semen of P3 are typically acephalic and contain nuclei and intact acrosomes (Figure 1B), and no normal spermatozoa were found (Table 1). By analyzing the mutations in the family, we found the c.425+1G>A allele is maternally inherited, and the c.475C>T allele is paternally inherited.

**The protein status is altered by mutations at the splicing site and the transmembrane domain**
domain
Four mutations of human SUN5 were found in front of the conserved coiled-coil domain and the SUN domain, all of them were evolutionary conserved in mouse, and their corresponding locations in human and mouse SUN5 proteins were shown in Figure 2A. Among the 4 mutations, c.210G>A and c.375delA led to truncated SUN5 proteins; c.334G>A was computationally predicted to abolish the donor splice site in intron 5 and confirmed by splicing experiments in cells; and the c.425+1G>A splice-site variant was computationally predicted to abolish the donor splice site of intron 7 (11).

We first tested the splicing variant without a known function, c.425+1G>A, which localized to the first base of intron 7 next to exon 7 and was identified in P3 of our cohort. We amplified a fragment from exon 7 to exon 8 from the genomic DNA of P3, and subcloned the fragment into a pRK vector. The forward primer is designed on exon 7 and the reverse on exon 8. For the P3 contains heterozygous mutation of 425+1G>A, we obtained both the WT and mutant allele with the cloning. We then transfected the vectors carrying the WT or mutant allele to a mouse testicular teratoma cell line - F9 cells (ATCC, CRL-1720), and mRNA was extracted, reverse-transcribed to cDNA and amplified using the primers mentioned above. If intron 7 was properly spliced, the amplified fragment would be 144bp (exon 7 + exon 8), but if intron 7 was fully retained during the splicing, the amplified fragment would be 740bp (exon 7 + intron 7 + exon 8). Interestingly, we didn’t get the 740bp fragment with the mutant allele transcripts, but instead a fragment that was a bit larger than the WT allele (Figure 2B). By sequencing the amplified fragment, we detected that intron 7 was not completely spliced and retained an 18bp fragment within the mutant transcript. The inserted 18bp fragment introduced a premature stop codon in SUN5 mRNA and resulted in a truncated SUN5 protein, ending at Arg142 (Figure 2C). Mechanistically, we propose that when encountering the 425+1G>A mutation, the splicing machinery might skip to a similar splicing site near the 425+1G site (Figure 2E).

With c.334G being localized on the transmembrane domain (TM) of SUN5, if c.340G>A is not causing a splicing problem, the resulting missense change p.Gly112Arg is computationally predicted to severely impair the transmembrane helix of SUN5(11). To test this possibility, we cloned mouse SUN5 from testis tissues and fused a FLAG tag at the N terminus. We then introduced the mutation Gly112Arg (SUN5 G112R) and over-expressed the recombinant protein in HeLa cells because this kind of cell spreads well with a flattened morphology, making the protein more easily observed. Using immuno-staining of the FLAG tag, we found that WT SUN5 was restricted to the nuclear envelope (NE), while a majority of SUN5 G112R “escaped” to the cytosol with some evidence of protein aggregation near the NE (Figure 2F). These results indicate that both the truncated protein and mutations in the TM domain leads to a dysfunctional SUN5 protein.

SUN5 interacts with DNAJB13 at the coupling apparatus during spermiogenesis
Because the absence of SUN5 leads to a separation of the coupling apparatus from the sperm nucleus, we considered that an interactor or a partner of SUN5 might exist in the coupling apparatus. Using a GST fused mouse SUN5 protein (Glutathione S-transferase fused to the SUN domain of the SUN5 protein hereafter refers to
GST-SUN5), we employed a GST pull-down assay to identify SUN5 interactor(s) from mouse testis lysate. Two protein bands were resolved on gel electrophoresis (Figure 3A, lane 4), subjected to LC-MS analysis, and 15 and 37 proteins were identified by MS, respectively (Table 2, Supplementary file 1). Gene ontology analysis showed that only one protein, DNAJB13, from the bottom band, was annotated to be related to “sperm flagellum”, “axoneme”, or “motile cilium” (Supplementary file1). DNAJB13 was also the most abundant identified testis-specific protein according to absolute quantitation value by intensity based absolute quantification (iBAQ) (blank gels at corresponding molecular weight in lane 2 of Figure 3A were used as control). We then chose to analyze DNAJB13 using yeast two-hybrid system (Y2H) to validate the proteomic findings. As previously reported, we used SUN1C and KASH5 LR as positive controls (13) and found that both SUN5C (coiled-coil domain and the SUN domain of SUN5) and the SUN domain of SUN5 could interact with DNAJB13 but not the other candidates (Figure 3B). Full-length SUN5 did not show an interaction with DNAJB13 in the yeast two-hybrid assay, most likely because it contains a transmembrane domain. The domain may influence the protein’s binding capability, similar to the situation with SUN1 and SUN2 (13).

The interaction between SUN5 and DNAJB13 was further confirmed with immunoblotting after GST pulldown (Figure 3C). To investigate the physiological interaction between SUN5 and DNAJB13, we generated murine DNAJB13-specific polyclonal antibody and its specificity was validated (Figure 3D-E). Next, the interaction between SUN5 and DNAJB13 was confirmed with immunoprecipitation of endogenous SUN5 by DNAJB13 antibody in mouse testis lysate (Figure 3F).

Using immunofluorescence assays, we determined that overexpressed FLAG-SUN5 could recruit MYC-DNAJB13 to the nuclear envelope, while MYC-DNAJB13 itself could not be recruited to the nuclear envelope (Figure 4A). Next, we set out to characterize the dynamic interaction between SUN5 and DNAJB13 during spermiogenesis, using immunofluorescence. DNAJB13 is a member of the type II HSP40 (heat shock protein 40) family, is defined as an axoneme-associated component in spermatozoa, and subsequent studies found that DNAJB13 is localized on the sperm head-tail coupling apparatus during spermiogenesis (14-16). Additionally, biallelic Dnajb13 mutant mice have been shown to produce abnormal spermatozoa, including headless tails and tailless heads (17). These data support the idea that the interaction between SUN5 and DNAJB13 might be required for the sperm head-to-tail tight junction. We further examined the dynamic distribution of DNAJB13 in developing spermatids. In WT spermatids, DNAJB13 was rapidly enriched in the coupling apparatus with the elongation of the spermatid, and the protein was tightly attached to the implantation fossa during the maturation of the spermatid (Figure 4B, Top, arrows), which is similar to the distribution of SUN5. In addition, in Sun5-null spermatids, although DNAJB13 was enriched to the coupling apparatus, its tight association with the nucleus was never observed. Instead, there was a gap between the nucleus and DNAJB13 (indicated by the red segment in Sun5-null spermatid). Finally, in the late-stage spermatid, DNAJB13 was only found in the headless tail spermatozoa (Figure 4B, Bottom). In WT spermatids,
co-localization of SUN5 and DNAJB13 in the sperm head-to-tail coupling apparatus was observed, but in Sun5-null late spermatids (6), DNAJB13 signal could only be found at the top of headless tails (Figure 4C). A previous investigation reported that DNAJB13 was localized to the neck region of developing spermatids in testis, but the protein migrated to the flagellum in the mature spermatozoa (16). To test this idea, we performed similar immunofluorescence staining of DNAJB13 together with SUN5 in mature spermatozoa from epididymis. We found that in WT sperm, SUN5 was localized to the neck region, and DNAJB13 was evenly distributed along the flagellum; the DNAJB13 signals were not enriched in the neck region. In Sun5-null sperms, SUN5 was absent from both head and tail, and DNAJB13 was only found in the headless tails (Figure 4D). These observations suggested that, although SUN5 is associated with DNAJB13 during the development of spermatids inside the testis, DNAJB13 might not be the last partner of SUN5 to anchor the sperm head to the tail, because DNAJB13 migrated from the neck region to the tail in mature sperms. Considering the fact that DNAJB13 is a chaperone for protein folding, the dynamic associations between SUN5 and DNAJB13 might help to facilitate the proper folding of the SUN5 protein and promote its binding to the right partner in the HTCA in mature sperms.

**Mutations in the coiled-coil domain affect the secondary structure of the SUN5 protein**

The last 5 mutations and the newly found c.475C>T were all localized to the conserved coiled-coil domain and the SUN domain. All were evolutionary conserved in mouse (Figure 5A). Two mutations were found in the coiled-coil domain of the human SUN5 protein, c.475C>T(p.Arg159*) and c.485T>A (p.Met162Lys), which corresponded to mouse c.469C>T(p.Arg157*) and c.479 T>A (Met160Lys). c.469C>T(p. Arg157*) led to a truncated protein, and disrupted the coiled-coil SUN domain. We first tested the mutant proteins’ ability to bind DNAJB13 in the Y2H assay and found that the truncation at R157* abolished the protein’s interaction with DNAJB13 (Figure 5B) as predicted. On the other hand, the M160K mutation didn’t influence the SUN5C-DNAJB13 interaction. On the contrary, this mutation seems to enhance the interaction. To reveal the differences in the protein properties between the mutant and WT SUN5C, we purified GST tagged WT mouse SUN5C (coiled coil and SUN domain) and the mutant (SUN5C M160K) (Figure 5C), then examined their secondary structure by circular dichroism (CD). We found that the secondary structure of SUN5C is altered by the mutation M160, particularly in the α-helix region (Figure 5D).

Finally, we co-expressed the full length FLAG-SUN5 and MYC-DNAJB13 in mammalian cells to examine their properties in vivo. Our studies revealed that WT SUN5 was localized on the NE and it could partially recruit DNAJB13 to the NE, but the distribution of SUN5 R160K was not as restricted as WT SUN5. Many of the SUN5 R160K signals were found in the cytosol and localized to protein aggregate. In addition, the mutant lost its ability to recruit DNAJB13 to the NE (Figure 5E).

**Mutations in the SUN domain impaired SUN5 protein folding and SUN5-DNAJB13 interactions**

The results of the M160K mutation show that the in vivo status might reflect the protein property faithfully, so we introduced the 4 missense and 1 nonsense mutations identified in the SUN domain into full
length SUN5 and transfected them into HeLa cells with DNAJB13, respectively. We found each mutation could cause abnormal distribution of SUN5 inside the cells, and their ability to recruit DNAJB13 was also impaired (Figure 6A). These results suggested that the protein folding of these mutants may also be impaired, so we attempted to purify E. coli-expressed GST-tagged SUN domain proteins carrying each of the mutations. Most of the mutants could be purified successfully (Figure 6B), but to our surprise, the Y259M and S282* mutations led to protein precipitation, suggesting these mutations brought striking changes to protein conformation and yielded miss-folded proteins (Figure 6C).

For the purified mutants, we examined their secondary structure by CD and found that each mutation caused changes in secondary structure at different levels, especially in the β-sheet region compared to the GST-SUN5 WT protein (Figure 6D-F). The changes are presumably due to changes in the amino acid properties, such as from non-polar to polar or from charged to uncharged (Figure 6G). We then introduced the mutants into the Y2H system and monitored their interaction with DNAJB13. As expected, most SUN mutants showed reduced interaction with DNAJB13 (Figure 6H), with only one exception. Importantly, the c.1037A>T, p.Asn346Ile (N346I) mutant showed an enhanced interaction with DNAJB13. These results suggest that the dynamic interaction between SUN5 and DNAJB13 is required for spermiogenesis, the disruption of any step of the binding-releasing process might be harmful to sperm head-tail tight junction.

**Discussion**

Most of our knowledge about acephalic spermatozoa is obtained from animal models. SUN5 is the first gene whose function had been validated in both an animal model and acephalic spermatozoa syndrome patients. Because species variation exists for many genes and proteins, we have aligned the protein sequences of SUN5 and its homologues in other mammals and found they are evolutionary conserved from mouse to human (6). A previous patient cohort found that 47.06% patients have SUN5 mutations, and we found that 2 of 3 patients carried biallelic SUN5 mutations and 1 patient showed a novel SUN5 mutation. These results suggest that acephalic spermatozoa syndrome is associated with a high frequency of SUN5 mutations. Among the 11 known point mutations, 6 of them led to premature stop codons and truncated proteins, among which 5 localized upstream of the conserved coiled-coil domain, and only 1 mutation was found inside the SUN domain. In summary, these nonsense mutations are highly harmful to the protein function. In the current study, we investigated the effects of missense mutations on the function of SUN5 and found that the substituted amino acids changed either the protein conformation or its distribution in cells, influencing the protein’s dynamic interactions with DNAJB13. Since all these mutations in the patients are evolutionary conserved in mouse, we believe that the mouse results also apply to human spermatid development.

SUN5 itself cannot bring the sperm head and tail together to form a tight junction, it must interact with other partner(s). We identify the novel SUN5 interactor as DNAJB13. From the beginning of the spermatid elongation, we observe that SUN5 and DNAJB13 are co-localized to the neck region of spermatids. DNAJB13 knockout has been shown to result in
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acephalic spermatozoa in mice (17), and mutations of DNAJB13 have been implicated in human ciliary dyskinesia and male infertility (18). These findings suggest DNAJB13 plays a vital role in spermiogenesis, and the protein’s direct interaction with SUN5 may be essential to sperm head-tail integration. We find that when the elongation is completed in mature spermatozoa, DNAJB13 is no longer enriched in the neck region, but the tight junction between sperm head and tail has already been established based on SUN5. This result suggests that there must be another interactor of SUN5 in the mature spermatozoa, and mutations of this interactor would be predicted to be associated with acephalic spermatozoa syndrome.

We noticed that most of the mutations reduced the interaction between SUN5 and DNAJB13, but two mutations M160K and N346I strengthened the binding of SUN5 to DNAJB13, which also led to acephalic spermatozoa. This implies that overly weak or overly strong interactions between SUN5 and DNAJB13 are both harmful to protein function. A weak interaction between SUN5 and DNAJB13 may not be sufficient to provide enough force to pull the nuclear envelope and HTCA together during elongation, whereas a strong binding between the two proteins might inhibit the release of DNAJB13 after the elongation of the spermatids, thus preventing the continuous functioning of DNAJB13 in mature spermatozoa. We speculate that during the complete process of spermiogenesis, DNAJB13 might serve as a chaperone as well as an interactor in the sperm head-to-tail anchoring mechanism. Considering the dynamic interaction between SUN5 and DNAJB13 during spermiogenesis, we suggest that DNAJB13 might be a protein chaperone that assures that SUN5 folds properly and binds to another protein in the HTCA. In a working model for SUN5-DNAJB13 and the potential SUN5 interactor, we propose that SUN5, localized to the nuclear envelope, needs DNAJB13 to help its folding and to facilitate its binding to a “protein X” during spermatid elongation. In mature spermatozoa, the well-folded SUN5 will build a solid interaction with protein X to anchor the sperm head to the tail. Once DNAJB13 finishes its mission, it is released from the neck region (Figure 7).

In summary, our study identified the functional interactor of SUN5 during spermatogenesis - DNAJB13 - and explored the pathogenesis of SUN5 mutations recently found in acephalic spermatozoa syndrome patients. The splicing variant leads to a premature stop codon, yielding a truncated SUN5 protein that lacks the essential SUN domain. We also characterized the missense mutations that affected either SUN5’s protein folding, localization or interaction with DNAJB13. Together, our studies provide mechanistic insights into the functional role of SUN5 in connecting the sperm head to the tail and the etiology of acephalic spermatozoa syndrome-associated SUN5 mutations.

Experimental procedures

Patients

This study conforms to the principles of the Declaration of Helsinki and is approved by the Reproductive Study Ethics Committee of Peking University Third Hospital (Reference NO.201552-008 ). Three patients gave their informed consent for their samples to be used for research and to identify the genetic cause of their infertility. The data are treated confidentially and anonymised. Related examinations of their samples are listed in Table 1.
Amplification and sequencing of SUN5 exons for the patients were performed as described (11).

Animals
The Sun5-null mice and WT mice were kept as described (6), and all of the animal experiments were performed with the approval of Institutional Animal Care and Use Committee (IACUC) (#08-133) of the Institute of Zoology, Chinese Academy of Sciences.

Plasmids
Full length mouse Sun5 was amplified and subcloned into pRK using SalI and NotI. Mouse SUN5 (full length), SUN5C (residue 133-374), the SUN domain (residue 226-374), and SUNIC (residues 458–913) were subcloned into pGBT9 using EcoRI and SalI. SUN5C and SUN domain were also cloned into pGEX-4T-1 for protein purification using EcoRI and SalI. Mouse Dnajb13 was cloned into pCS2+ using EcoRI and AscI. Mutations of SUN5 were generated by site-directed mutagenesis.

Antibodies.
The rabbit DNAJB13 antibody has been described previously (16). The mouse DNAJB13 antibody was generated in the lab. Briefly, FLAG-tagged full length Dnajb13 was inserted into pET28a+, and the His-FLAG tagged protein was purified using Ni-beads (GE Healthcare) in purification buffer (50mM Tris-HCl pH8.0, 150mM NaCl, 2mM MgCl₂, 5% Glycerol). The recombinant protein was dialyzed in PBS and used to immunize mice for 6 weeks. The anti-sera against DNAJB13 was recovered from mouse blood and used in the following experiments. The rabbit anti-MYC antibody (ab1027t) was purchased from Boaoruijing (Beijing, China), the mouse anti-FLAG antibody (M20008) was purchased from Abmart (Berkeley Heights, NJ), and the rabbit anti-FLAG antibody (PM020) was purchased from MBL international (Aichi, Japan).

Testis smear.
The indicated mice (8-week-old) were euthanized by cervical dislocation. The testes were surgically removed and the tunica albuginea was removed from the testes. Then, the testes were digested with 1 mg/ml collagenase and 1 mg/ml hyaluronidase. Cells were dissociated by gentle pipetting, filtered through a 70-μm filter and then pelleted by centrifugation at 500 x g for 10 min. Cells were suspended in 1 ml of phosphate-buffered saline (PBS; Gibco, C14190500BT) and fixed with 4% paraformaldehyde (PFA) solution, washed with PBS and finally spread onto polylysine-coated slides for staining.

Immunofluorescence (IF).
The immunofluorescence and immunohistochemical assays were performed as previously described (19). For IF staining of human sperm, semen was spread on polylysine-coated slides and dried in air, then fixed with 4% PFA and stained with FITC-PNA for acrosomes and DAPI for DNA. For cellular immunofluorescence, 1×10⁶ HeLa cells were plated on glass cover slips in a 35-mm dish and cultured in DMEM with 10% fetal bovine serum for 24 h. The cells were transfected with the corresponding plasmids for 24 h and were rinsed 3 times with PBS, fixed, and stained with corresponding primary and secondary antibodies. To test the functions of SUN5 mutations, WT FLAG-SUN5 was transfected as a control group. For IF staining of the testis smear, the slides was rinsed 3 times with PBS, treated with 0.5% Triton-X100, blocked with 5% BSA and incubated with corresponding antibodies overnight. The signals were detected using corresponding secondary antibodies. The
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testis smears from the WT mice were used as a control group in these experiments. The IF images were taken immediately using an LSM 780/710 microscope (Zeiss, Oberkochen, Germany) or SP8 microscope (Leica, Wetzlar, Germany).

**mRNA splicing assay**
The fragment containing exon 7-intron 7-exon 8 from genomic DNA of P3 was subcloned into a pRK vector, the forward primer was designed on exon 7 (5’GCGTCGACCAGATGCAGCATAATTGGT3’) and reverse primer on exon 8 (5’TTCGCCGGCTCTACGGACATGGCTTCCA3’). For the 425+1G>A heterozygous mutation in P3, so we could get both WT and mutant allele during cloning. The vectors carrying the WT and mutant allele were then transfected to 293T cells, and mRNA was extracted and reverse-transcribed to cDNA. The spliced cDNA was amplified using the primer designed above, and PCR product were analyzed by 2% agarose gel and subsequent sequencing.

**GST pull-down assay.**
The SUN domain (residue 226-374) of the SUN5 protein was cloned into a pET42a vector and expressed in E. coli. A 20-μg quantity of GST-SUN5 or GST protein was immobilized on Glutathione Sepharose 4B (GE Healthcare, Marlborough, MA) in IP buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 2 mM MgCl2, and 0.1% Triton X-100) with 0.1% BSA and 1 mM phenylmethylsulphonyl fluoride (Amresco, M145, Solon, OH). WT mouse testes were lysed in RIPA buffer with 1 mM phenylmethylsulphonyl fluoride (Amresco, M145, Solon, OH) and a proteinase inhibitor cocktail (Roche Diagnostics, 04693132001, Basel, Switzerland) for 30 minutes on ice and cleared twice at 13,200 x g for 15 min at 4°C. Then, the supernatant was diluted with an equal volume of IP buffer and then cleaned by incubating with GST beads to remove endogenous GST proteins. The cleaned testes extract was incubated with immobilized GST or GST-SUN5 overnight, and then, the beads were washed with wash buffer (25 mM Tris-HCl pH 7.5, 2 mM EGTA, and 0.1% Nonidet P-40) 3 times. After the addition of SDS loading buffer, the samples were separated by SDS-PAGE, and stained using a Colloidal Blue Staining Kit (Thermo Fisher, LC6025, Waltham, MA).

**Liquid Chromatography-Mass Spectrometry analysis.**
The specific bands from Colloidal Blue-stained gel were excised, denatured, alkylated, trypsin-digested using previously described methods (20). The digested peptides were desalted with StageTip (Thermofisher Scientific, Eugene, OR), and analyzed by LTQ Orbitrap Velos mass spectrometer (Thermo Finnigan, San Jose, CA). The MS/MS spectra were searched against UniProt Protein database using MaxQuant (version 1.3.0.5) for protein identification with a false discovery rate of 1% for both peptides and proteins(21). Three replicates were performed, and for label-free quantification, protein expression levels were estimated using the Intensity Based Absolute Quantification (iBAQ) algorithm embedded in MaxQuant (22). A protein with over 2 unique peptide reads, uniquely identified in the GST pull-down lane with statistical difference (Student’s t-test, p<0.01) was considered for analysis. Gene ontology annotation was performed by DAVID Bioinformatics Resources 6.8 (23). Testis-specific gene annotation was from previous published data (24). The mass spectrometry proteomics data have been deposited to the ProteomeXchange
To determine the secondary structure of GST-SUN5C, GST-SUN5 and their variants, the CD spectra of the proteins were taken at 0.1 mg/ml in 50mM Tris-HCl pH8.0, 150mM NaCl, 2mM MgCl₂, 5% Glycerol, using a Chirascan Plus CD spectrometer (Applied Photophysics, Surrey, UK) and a 1 mm quartz sample cell equilibrated at 20°C. Scans between 200 and 260 nm were performed at a scan rate of 50 nm per min.

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**Conflict of interest**

The authors declare that they have no conflict of interest.

**Author contribution**

Y.S., J.Y. and W.T. collected the materials, performed most of the experiments and wrote the paper. C.L. and S.X. purified the proteins and performed CD analysis. Y.G. performed the MS analysis of GST pulldown result. L.Y. designed the detection of DNAJB13. L.C. and H.J. analyzed some of the patient samples. X.G. designed the MS analysis and collected the data. J.Q. and W.L. designed the project, supervised the whole research and wrote the paper. All authors reviewed the results and approved the final version of the manuscript.

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The abbreviations used
PTC: premature termination codon, HTCA: head-tail coupling apparatus, TM: transmembrane domain, NE: nuclear envelope, iBAQ: intensity based absolute quantification, CD: circular dichroism.
Mechanism of SUN5-related acephalic spermatozoa syndrome

**Table 1. Clinical information of the acephalic spermatozoa syndrome patients**

| Patient | Age | Sperm volume (ml) | Concentration (10^6/ml) | Motility PR/NP/IM (%) | Percentage of spermatozoa morphology (%) |
|---------|-----|-------------------|-------------------------|-----------------------|------------------------------------------|
| P1      | 29  | 3.40              | 2.22                    | 0/0/11.1/88.89        | Normally formed 0.00  Abnormal head-tail junction 3.03  Decadated 1.51  Acephalic 95.45 |
| P2      | 27  | 3.40              | 1.69                    | 8.33/8.33/83.33       | Normally formed 0.00  Abnormal head-tail junction 2.74  Decadated 17.48  Acephalic 79.78 |
| P3      | 34  | 2.0               | 41.3                    | 19.93/0.80/79.28      | Normally formed 0.00  Abnormal head-tail junction 0.00  Decadated 21.05  Acephalic 78.95 |

The sperm motility and the percentages of morphologically normal and abnormal spermatozoa were evaluated according to the World Health Organization guidelines (WHO, 2010). Most of the SUN5-mutation associated spermatozoa were acephalic sperm with low motility. PR: Progressive motility, NP: Non-progressive motility, IM: Immotility.

**Table 2. Proteins identified by MS analysis in GST-SUN5 pulldown assay**

| Band 1 | UBXN4, DLD, PTPN9, NCL, PDHA2, NPLOC4, STXBP3, STIP1, CLPX, CDKN2AIP, PDHX, CKAP4, NARS, KLHL10, VNN1 |
|--------|--------------------------------------------------------------------------------------------------|
| Band 2 | NDUFA9, SYNCRIP, 4931406C07RIK, RALY, HNRPDL, COPS6, CLU, GM5428, CSNK1A1, AKR1CL, DLD, DSCR3, SRSF5, COPS5, HMOX2, MDH2, PDHA1, DBT, RPS6, RPS3, FRZB, PPP4C, NUDT18, CRYZL2, EIF2S1, DNAJB13, PDHX, DLAT, TXNL1, GTF2H3, PTGR1, PDK3, MARC2, DHRS1, PRPS2, BLVRA, AGPAT3 |

iBAQ and Gene ontology analysis of the listed proteins were available in Supplementary file 1.
Figures and Figure legends

Figure 1. Morphology and pedigree of P3 and newly identified SUN5 mutation.

(A) Morphology of the spermatozoa from ejaculated semen smear, showing that most of the sperm are headless tails, nuclear DNA was stained with DAPI (Blue) and acrosomes were stained with FITC-labeled PNA (Green). (B) Single sperm showing that decapitated tail contains no DNA and acrosome, while decaudated sperm head contains DNA and acrosome. (C) Pedigree of the family of P3 with new SUN5 mutation (c.475C>T; p. Arg159*) is shown. The individuals I-1, I-2, II-1 were Sanger sequenced. The mutation c.425+G>A was a previously reported mutation.
Figure 2. The characteristics of mutations in the splicing site and the transmembrane domain.

(A) The distribution of SUN5 mutations in the TM domain and splicing sites were shown in the corresponding sites of human SUN5 and mouse SUN5 proteins. (B) The effect of SUN5 425+1G>A mutation on mRNA splicing in F9 cells, showing that SUN5 425+1G>A mutation yielded spliced transcripts that were a bit larger than that of the WT. The asterisk indicates the
mutant band. (C) The sequence of spliced SUN5 mRNA in F9 cells, showing that 18bp of intron 7 was retained in the 425+1G>A mutant transcripts, which introduced a stop codon in SUN5 mRNA. (D) The proposed splicing mechanism of the c.425+1G>A mutant. When encountering c.425+1G>A, the splicing machinery might skip to a similar splicing site near the 425+1G site. (E) The G112R mutation leads to an altered distribution of full-length SUN5 in mammalian cells. FLAG tagged full length WT or G112R SUN5 were transfected into HeLa cells. After 24 h incubation, the immunofluorescence staining was performed using an anti-FLAG antibody (Red) to examine the distribution of the overexpressed proteins. DNA was stained with DAPI (Blue). The WT FLAG-SUN5 was used as a control.
Figure 3. SUN5 directly interacts with DNAJB13.

(A) GST pulldown of SUN5-interacting proteins in testes. The SUN domain of mouse SUN5 was purified and immobilized on GST beads and then incubated with testis lysate. The eluates were resolved by SDS–PAGE and stained using a Colloidal Blue Staining Kit. The asterisks indicate the bands that were specifically pulled down by GST-SUN5 beads and retrieved for analysis by mass spectrometry. GST protein was used as a negative control.

(B) SUN5 could interact with DNAJB13 in the yeast two-hybrid assay. The SUN5C (coiled-coil domain and the SUN domain of SUN5) and SUN domain, instead of the full length SUN5, could interact with DNAJB13 in the yeast two-hybrid assay. SUN1C and KASH5 LR were used as a positive control. Empty vector was used as a negative control.

(C) GST-SUN5 pulldown results could be confirmed with immunoblotting of endogenous DNAJB13. GST pulldown was repeated as in (A), but the eluates were analyzed with immunoblotting against DNAJB13 following SDS-PAGE. The analysis showed that DNAJB13 could be detected in the GST-SUN5 pulldown lane but not the GST alone lane.

(D) Mouse polyclonal DNAJB13 antibody could detect overexpressed MYC-DNAJB13 and co-localized with MYC signals in HeLa cells. MYC-DNAJB13 was overexpressed in HeLa cells and immunofluorescence staining was performed using rabbit anti-MYC antibody (Green), mouse anti DNAJB13 antibody (Red) or a combination of the two antibodies, DNA was stained with DAPI (Blue).
DNAJB13 signal could only be detected in the overexpressed cells (middle panel) and overlapped with the MYC signal, indicating the specificity of murine polyclonal DNAJB13 antibody. (E) Murine polyclonal DNAJB13 antibody could detect purified His-FLAG-DNAJB13 and endogenous DNAJB13 in testis. 500ng, 100ng, 10ng purified His-FLAG-DNAJB13 and 20μg adult testis and ovary extracts were loaded and detected by murine DNAJB13 antibody and rabbit FLAG antibody. Ovary extract was used as a negative control. FLAG antibody was used as a positive control for immunoblotting. (F) SUN5 could bind DNAJB13 in testis. Endogenous co-immunoprecipitation of SUN5 and DNAJB13 from testis lysate was performed with murine DNAJB13 antibody, and SUN5 was detected with rabbit anti-SUN5 antibody.
Figure 4. The dynamic interaction between SUN5 and DNAJB13 during spermiogenesis

(A) FLAG-SUN5 could partially recruit MYC-DNAJB13 to the nuclear envelope when overexpressed in HeLa cells. FLAG-SUN5 localized to the nuclear envelope (upper panel, Red). MYC-DNAJB13 itself randomly distributed throughout the cell when overexpressed in HeLa cells (middle panel, Green). When FLAG-SUN5 and MYC-DNAJB13 were co-transfected in HeLa cells, MYC-DNAJB13 could be partially recruited to the nuclear envelope (lower panel). DNA in the cells was stained with DAPI (Blue).

(B) Dynamic distribution of DNAJB13 in developing WT and Sun5-null spermatids was revealed by IF. In WT spermatids, DNAJB13 signals (Green) were tightly attached to the implantation fossa during the maturation of the spermatid. The red arrow in WT spermatids indicate the implantation fossa (upper two panels). In Sun5-null spermatids, although DNAJB13 (Green) was enriched to the coupling apparatus, DNAJB13 signals were absent from the spermatid
nucleus (lower two panels). The red segment in the Sun5-null spermatid indicated a gap between nucleus and DNAJB13. The spermatid nuclear DNA was stained with DAPI (Blue). 

(C) Co-localization of SUN5 and DNAJB13 in WT and Sun5-null spermatids from testis. In WT spermatids, co-localization of SUN5 (Green) and DNAJB13 (Red) in the sperm head-to-tail coupling apparatus was observed (upper panel), but in Sun5-null late spermatids, DNAJB13 signal could only be found at the top of headless tails (lower panel). The spermatid nuclear DNA was stained with DAPI (Blue). 

(D) The distribution of SUN5 and DNAJB13 in mature spermatozoa from epididymis. In WT mature spermatozoa, DNAJB13 (Red) migrated to the flagella and SUN5 (Green) stayed in the HTCA (upper panel). In Sun5-null mature spermatozoa, DNAJB13 could only be found in the tip of the acephalic flagella (lower two panels). The sperm nuclear DNA was stained with DAPI (Blue).
Figure 5. The secondary structure of the SUN5 protein was altered by mutations in the coiled-coil domain

(A) The mutations in the coiled coil domain and SUN domain were observed in human and mouse SUN5, showing that these mutations are highly conserved. Due to the codon degeneracy, human Ser284 is coded by TCA, while mouse Ser282 is coded by TCG. Consistent with the human nonsense mutation 851C>G (TCA-TGA), the corresponding nucleotide (845C) in mouse was mutated to A (TCG-TAG). Other mutations in mouse were consistent with those found in human. (B) The influence of two mutations in the coiled-coil domain on the SUN5-DNAJB13 interaction as revealed by Y2H assay. WT SUN5C or its variants were co-transformed with DNAJB13 in the Y2H assay, and yeast cells were diluted by 10^1, 10^2, 10^3. Aliquots were spotted onto control (SD-Leu-Trp) and selective (SD-Leu-Trp-His) plates. (C) Protein purification of GST-SUN5C and its variant, the samples were separated by SDS-PAGE and stained with coomassie blue. (D) The secondary structure of SUN5C was altered by M160K as revealed by CD analysis. (E) The nuclear envelope (NE) localization of full length SUN5 in HeLa cells was impaired by the M160K mutation. WT-FLAG–SUN5 or FLAG–SUN5 M160K, together with MYC-DNAJB13, were co-transfected into HeLa cells. After 24 h incubation, the transfected cells were immunostained with mouse anti-FLAG (Red) and rabbit anti-MYC (Green) antibodies. The nuclear DNA was stained with DAPI (Blue). WT-FLAG–SUN5 localized to the NE, and
DNAJB13 was partially recruited to the NE (upper panels), while FLAG–SUN5 M160K showed abnormal localization, and could not recruit DNAJB13 to the NE (lower panels).
Figure 6. Mutations in the SUN domain affected protein secondary structures, localization and interaction with DNAJB13.

(A) Mutations in SUN domain affected the distribution of full-length SUN5 in mammalian cells, and recruitment of DNAJB13. WT-FLAG-SUN5 or its variants, together with MYC-DNAJB13, were co-transfected into HeLa cells. After 24 h incubation, the transfected cells were immunostained with anti-FLAG (Red) and Anti-MYC (Green) antibodies. The nuclear DNA was stained with DAPI (Blue). WT-FLAG-SUN5 localized to the NE, and DNAJB13 was partially recruited to the NE. FLAG-SUN5 V259M, T273M, S282*, N346I and R354C showed abnormal localization, and could not recruit DNAJB13 to the NE. (B) GST-SUN5 and its variants. The GST-SUN5 and its variant proteins were expressed in E. Coli and purified by GST beads. They were separated using SDS-PAGE and stained with coomassie blue. (C) The mutations V259M and S282* led to protein precipitation during in vitro purification. The samples were separated by SDS-PAGE and stained with coomassie blue. (D) - (F) The secondary structure of the GST-SUN5 protein was affected by incorporated mutations T273M, N346I and R354C, as revealed by CD analysis. (G) All the SUN domain mutations caused striking changes to amino acid properties. (H) Most mutations in the SUN domain impaired SUN5-DNAJB13 interaction as characterized by the Y2H
system. WT SUN (SUN domain of SUN5) or its variants were co-transformed with DNAJB13 in the Y2H assay, and yeast cells were diluted by $10^1$, $10^2$, $10^3$ and then spotted onto control (SD-Leu-Trp) and selective (SD-Leu-Trp-His) plates.
Figure 7. Potential functional roles of DNAJB13 and SUN5 during spermiogenesis.
SUN5 interacts with DNAJB13 in the elongating spermatids, and DNAJB13 could facilitate SUN5 protein folding to promote its binding to an unknown “protein X” during spermatid elongation. In mature spermatozoa, the well-folded SUN5 will build a solid interaction with protein X to anchor the sperm head to the tail. Meanwhile DNAJB13 is released from the neck region. NE, nuclear envelope.
Mechanistic insights into acephalic spermatozoa syndrome–associated mutations in the human SUN5 gene
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