The testis specific LINC component SUN3 is essential for sperm head shaping during mouse spermiogenesis

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Running Title: SUN3 and spermiogenesis

Abstract
Sperm head shaping is a key event in spermiogenesis and is tightly controlled via the acrosome–manchette network. Linker of nucleoskeleton and cytoskeleton (LINC) complexes consist of Sad1 and UNC84 domain–containing (SUN) and Klarsicht/ANC-1/Syne-1 homology (KASH) domain proteins and form conserved nuclear envelope bridges implicated in transducing mechanical forces from manchette to sculpt the sperm nuclei into a hook-like shape. However, the role of LINC complexes in sperm head shaping is still poorly understood. Here, we assessed the role of SUN3, a testis-specific LINC component harboring a conserved SUN domain, in spermiogenesis. We show that CRISPR/Cas9-generated *Sun3*-knockout male mice are infertile, displaying drastically reduced sperm counts and a globozoospermia-like phenotype, including a missing, mislocalized, or fragmented acrosome, as well as multiple defects in sperm flagella. Further examinations revealed that the sperm head abnormalities are apparent at step 9 and that the sperm nuclei fail to elongate because of the absence of manchette microtubules and perinuclear rings. These observations indicated that *Sun3* deletion likely impairs the ability of the LINC complex to transduce the cytoskeletal force to the nuclear envelope required for sperm head elongation. We also found that SUN3 interacts with SUN4 in mouse testes and that the level of SUN4 proteins is drastically reduced in *Sun3*-null mice. Altogether, our results indicate that SUN3 is essential for sperm head shaping and male fertility, providing molecular clues to the underlying pathology of the globozoospermia-like phenotype.

Keywords: Sad1 and UNC84 domain-containing 3 (SUN3); LINC complex; Manchette; Sperm shaping; Male infertility; Spermiogenesis

Introduction
Spermiogenesis involves drastic morphological changes of spermatids, including acrosome formation, elongation and condensation of nucleus, and disposal of residual cytoplasm, to differentiate into mature spermatozoa consisting of a head and a tail (1,2). During this complex process, a transient cytoskeletal structure, termed as manchette, appears concurrently with the spermatid nucleus elongation. The manchette,
which was first evident in step 8 spermatids in mice (3), is composed of parallel arrays of microtubules aligned with the long axis of the nucleus and a belt-like perinuclear ring made of actins where the microtubules anchored, forming a sleeve-like structure that encircles the spermatid nucleus (4-6). It’s believed that the manchette microtubules were emanated from perinuclear rings as these microtubules appear from the post-acrosomal region in mouse and bovine spermatids (7). As spermatids differentiate, the microtubular structure and the perinuclear ring move caudally to the posterior pole of spermatid nucleus, thus sculpting the nucleus into a hook-like morphology.

The important role of manchette in sperm head shaping has been demonstrated in several studies, in which disruptions of manchette-related proteins result in various forms of sperm head abnormalities and eventually impairs male fertility. For example, loss of functional HOOK1 causes ectopic positioning of manchette microtubular structure within the spermatids, resulting in abnormal sperm head morphologies, such as being club-shaped and crescent forms (8). Similarly, disruption of RIM-BP3, a HOOK1-interacting protein located in the manchette, also caused ectopic positioning of manchette and sperm head abnormalities similar to those of Hook1 mutant mice (9). The microtubule “plus-end-tracking protein” CLIP-170, localizes prominently to the manchette rings during manchette formation and male mice knockout of Clip-170 were subfertile due to defective sperm head shaping and abnormal elongation of the manchette tubules (2,10). Despite the essential role of the manchette in spermiogenesis, how the microtubule manchette are assembled and regulated remain poorly understood.

The SUN proteins are a family of nuclear membrane proteins that share a conserved C-terminus, the SUN (Sad1p/Unc84 homology) domain. SUN proteins and Klarsicht/ANC-1/Syne-1 homology (KASH) proteins form a linker of nucleoskeleton and cytoskeleton (LINC)complex that functions like a bridge across the inner and outer nuclear membranes to physically connect nucleus to cytoskeleton (11). This complex is responsible for various important cellular functions such as mechanotransduction, cellular signaling, nuclear anchorage and positioning. Till now, 5 SUN proteins have been described in mammals and have been demonstrated or suggested to play roles in germ cell development. SUN1 and SUN2 are ubiquitously expressed in various tissues (12-17) and mediate the meiotic telomere attachment to the nuclear envelope (18-22). SUN3, SUN4 and SUN5 are specifically expressed in testes (12-17). SUN5 localization is restricted to the head-tail junctions of sperm and is essential for anchoring sperm head to the tail (23). SUN4 localizes to the spermatid nuclear envelope, in close association with manchette microtubules (15,16). Knockout of Sun4 in mice disrupted the lateral interactions of manchette to the nucleus and the nucleus thus failed to elongate, eventually resulting in a globozoospermia-like phenotype and male infertility (15,16). SUN3 protein expression begins at postnatal day 15 and, similar to SUN4, its localization is closely associated with manchette in developing spermatids (14). However, due to the lack of animal models, whether Sun3 indeed plays a role in spermiogenesis is not clear.

To investigate the physiological functions of Sun3 during mammalian spermatogenesis, we generated Sun3 knockout mouse model by the CRISPR/Cas9 genome editing technology. We found that Sun3−/− male mice are infertile, displaying abnormal sperm head morphology and irregular acrosome localization likely resulting from disruption of manchette assembly. These findings
demonstrated that Sun3 is essential for the sperm head shaping during spermiogenesis.

**Results**

**Generation of Sun3<sup>−/−</sup> mice by CRISPR/Cas9-mediated genome editing**

Consistent with previous findings (14,16), SUN3 is testis-specifically expressed (Fig.S1A) and was detected in spermatids, localizing to the nuclear pole distal to acrosome, and overlapping with α-Tubulin and SUN4 (Fig. S1B). In order to investigate the biological function of SUN3, we generated Sun3 null mice using CRISPR/Cas9 genome editing technology (Fig. 1A). Sanger sequencing of genomic DNA from the mutant mice revealed that a Thymidine (T) was inserted between nucleotide position 184 and 185 (c.184_185insT) in exon 4 of Sun3, predicted to result in a pre-mature translation termination (p.Pro62Leufs*2) (Fig. 1B). Western blotting analyses further confirmed that full-length SUN3 proteins were absent in the testes of Sun3<sup>−/−</sup> mice (Fig. 1C and Fig. S2).

**Deletion of Sun3 impairs spermatogenesis**

Mice lacking Sun3 appeared normal, displaying no obvious abnormalities in development and behavior. Given the testis-restricted expression pattern of SUN3, we studied fertility in Sun3<sup>−/−</sup> male mice. Mating attempts of Sun3<sup>−/−</sup> males with wild-type females did not produce any offspring, indicating that Sun3<sup>−/−</sup> males are infertile. Besides, Sun3<sup>−/−</sup> females showed no overt abnormalities in fertility.

Sun3<sup>−/−</sup> mice had smaller testes (Fig. 2A and B) and sharply declined epididymal sperm number (0.17 ± 0.03 million/ml) as compared with those in wild-type mice (12.80 ± 0.36 million/ml) (Fig. 2C). To further characterize the spermatogenic defects in Sun3<sup>−/−</sup> mice, hematoxylin and eosin (H&E) staining of testis and epididymis sections was performed. All types of spermatogenic cells were present in an orderly way in Sun3<sup>+/+</sup> seminiferous tubules and mature spermatozoa with a canonical hook-shaped head could be seen in the lumen of tubules (Fig. 2D (a-b)). However, in Sun3<sup>−/−</sup> mice, all the elongating and elongated spermatids, as well as spermatozoa, were observed with a non-canonical round head (Fig. 2D (c-d)). Consistent with the result of sperm counting per epididymis, the numbers of these non-canonical spermatids and spermatozoa were apparently less in seminiferous tubules and rarely seen in cauda epididymides of Sun3<sup>−/−</sup> mice, comparing to those in Sun3<sup>+/+</sup> mice (Fig. 2D (e-f)). Furthermore, TUNEL assay in combination with germ cell specific marker (MVH) was performed and the results indicated apoptosis in cells with a small amorphous nuclear shape which corresponds to the non-canonical spermatids in Sun3<sup>−/−</sup> mouse (Fig. 2E). The frequency of TUNEL<sup>+</sup> tubules (11.99 ± 0.75% v.s. 32.00 ± 3.17%) and number of TUNEL<sup>+</sup> cells per TUNEL<sup>+</sup> tubule (1.70 ± 0.15% v.s. 2.52 ± 0.15%) in Sun3<sup>−/−</sup> mice were significantly increased than in Sun3<sup>+/+</sup> mice (Fig. 2F), suggesting that spermatids of Sun3<sup>−/−</sup> mice underwent apoptosis. These results indicated that Sun3 is essential for spermatogenesis and particularly for the development of spermatids.

**Sun3<sup>−/−</sup> mice produce round-headed spermatozoa**

We further analyzed the sperm morphology in the epididymis and found that, in contrast to the typical hook-shaped appearance of sperm heads in Sun3<sup>+/+</sup> mice, all the sperm heads in Sun3<sup>−/−</sup> mice were amorphous with a smaller and more rounded shape (Fig. 3A and B). Additionally, approximately 89.67 ± 2.02% of spermatozoa from Sun3<sup>−/−</sup> mice also displayed various midpiece defects, such as irregular-caliber, bent, coiled and/or cracked (Fig. 3A and C, Fig. S3). Immunofluorescence staining of Peanut agglutinin (PNA), a marker of acrosome, were performed on sperm smears and
seminiferous tubules. In Sun3+/+ mice, acrosomes with a typical crescent-shape were found on top of the nucleus in the anterior-dorsal part of the sperm head. However, the acrosomes of spermatooza from Sun3−/− mice were missing, mislocalized or fragmented (Fig. 3D). Because failure of sperm head elongation usually leads to a rounded head appearance, to understand the specific step at which head abnormalities occur in Sun3−/− mice, we compared the spermatids between Sun3+/+ and Sun3−/− mice at different steps of spermiogenesis. PAS staining revealed that the morphology of spermatids till step 7-8 were comparable between Sun3+/+ and Sun3−/− mice, indicating that the development of round spermatids was normal in Sun3−/− mice (Fig. 4). At step 9, the nuclei of spermatids became flattened and initiated to elongate along with condensation of chromatin in Sun3+/+ mice, while in Sun3−/− mice, spermatids still have a round nucleus though the chromatin has been condensed. At step 10, spermatid nuclei in the Sun3+/+ mice become thinner and more elongated; however, the nuclei of spermatids were small, deformed and remained round in Sun3−/− mice (Fig. 4). Besides, we also noted that the number of spermatids were markedly reduced from step 10. Altogether, these findings demonstrated that abnormal morphology of Sun3−/− spermatids occurred when the spermatids started to elongate.

**Manchette assembly is disrupted in Sun3−/− spermatids**

Because microtubule manchette is essential in the elongation of spermatids and SUN3 localization is closely associated with the manchette (4,24), we next detected whether the structure of manchette was disrupted after Sun3 deletion. Co-immunofluorescence staining of testis sections with antibodies against α-Tubulin, a marker of manchette microtubules, and PNA was conducted. In Sun3+/+ mice, manchette microtubules were found tightly attached to the nuclear periphery at the caudal region opposite the acrosome of spermatids, and these structures, however, were not detected in Sun3−/− mice despite some weak and diffuse labelling that were ectopically positioned (Fig. 5A). To confirm the loss of manchette microtubules in Sun3 knockout spermatids, immunofluorescence staining on testis cell smears was subsequently performed. In Sun3+/+ mice, spermatids from step 1 to step 16 could be distinguished based on the morphology of the acrosome, and the acrosome covering the anterior side of the nucleus as well as the microtubules of the manchette tightly surrounding the caudal region, could be seen from step 7/8 to step 16 in the Sun3+/+ spermatids. In Sun3−/− mice, we did not detect any morphological abnormalities in spermatids till step 7/8 and acrosomes covering the anterior side of the nucleus were found to appear normal in round spermatids. However, the typical microtubule arrays of manchette were not observed in all the spermatids from Sun3−/− mice. Aberrantly polymerized microtubule bundles that were dissociated from the nucleus were observed in about 5% of spermatids (Fig. 5B). Moreover, the perinuclear ring, a belt-like structure surrounding the nucleus where manchette microtubules inserted, was also absent in spermatids from Sun3−/− mice (Fig. S4). Transmission electron microscopy (TEM) further revealed the presence of manchette microtubule bundles, which are closely associated with nuclear envelope in elongating spermatids from Sun3+/+ testes; however, these recognizable structures were not seen in elongating spermatids from Sun3−/− testes, although some disorganized microtubule bundles that had lost their interaction with nucleus were observed (Fig. 5C). Thus, we conclude that SUN3 is not only indispensable for manchette formation but also likely required for organization of...
manchette during sperm head shaping in mice.

**SUN3 interacts with SUN4 in mouse testes**

Deletion of SUN4, another SUN protein that associates with manchette, also led to defects in acrosome and manchette formation in mice (25), which are similar to the findings in Sun3 null mice. In order to explore whether SUN3 interacts with SUN4 in testis, we performed immunoprecipitation using anti-SUN3 antibody with testis lysates from Sun3+/+ mice. Western blotting detected both SUN3 and SUN4 in the lysates immunoprecipitated by the anti-SUN3 antibody, but not in IgG-immunoprecipitated lysates (Fig. 6A and B). Immunofluorescence staining in Sun3+/+ and Sun3-/- testicular sections revealed clear signals of SUN4 proteins surrounding the caudal region of nucleus opposite the acrosome where manchette locates in step 7/8 spermatids, elongating spermatids and elongated spermatids in Sun3+/+ mice, while only unspecific diffuse signals were observed in the cytoplasm of spermatids from Sun3-/- mice (Fig. 6C). Besides, Western blotting of testicular lysates revealed that the level of SUN4 proteins was drastically reduced in adult Sun3-/- mice compared to that in Sun3+/+ mice (Fig. 6D). These findings demonstrated that SUN3 and SUN4 interact and that SUN3 is required for maintain the level of SUN4 proteins in vivo.

**Discussion**

Sperm head shaping is a key event of spermiogenesis and misshaping of sperm heads often leads to male infertility (26). In the present study, we generated mice lacking Sun3 through CRISPR/Cas9 technology and investigated the function of SUN3 during spermiogenesis. We found that loss of SUN3 leads to a drastic reduction of sperm numbers, globozoospermia-like phenotype accompanied with multiple sperm tail defect resulting from failure of manchette formation during sperm head shaping, and ultimately male infertility. Additionally, we also demonstrated that SUN3 interacts with SUN4 in vivo and is required to maintain the level of SUN4 proteins in testes. Hence, we reported for the first time that Sun3 is indispensable for sperm head shaping and particularly required for the formation of manchette.

A typical manchette is characterized by highly organized microtubule bundles attached to the perinuclear ring, forming a sleeve-like structure surrounding the posterior part of spermatid nucleus (5,27). This transient structure appears when the spermatids are going to elongate and disappears when the sperm heads are properly shaped. Several genes have been reported to be implicated in manchette function in mice, including Hook1, Katnb1, Lrguk1, Meig1, Pacrg, Spef2, etc. (8,28-31). To be noted, the manchettes in these mutant mice displayed highly disorganized structure and/or perturbed disassembly, resulting in spermatozoa with a deformed shape (8,28-31). However, we found that, in most cases, a clear manchette structure was not detected in the spermatids after Sun3 knocked out, and some aberrantly-polymerized straight microtubule bundles were observed in only a few step 8-16 spermatids (approximately 5%) but these bundles were completely dissociated from the nucleus. Besides, the nuclear shape of the epididymal sperm from Sun3 knockout mice was generally rounder than those observed in other models of disrupted manchette. Thus, it is suggested that SUN3 is essentially required for the formation and coupling of the manchette. SUN3 and SUN4 are exclusively expressed in spermatids and co-localize in close association with the manchette (14,32), suggesting a cooperative function in coupling of the manchette to the nuclear periphery so that the cytoplasmic forces could be transduced to shape the sperm nuclei. In Sun4-/- mice, manchette microtubules were
disorganized, lost their lateral interaction with nucleus, and in some cases were even completely missing, thus resulting in a globozoospermia-like phenotype (25). Deletion of Sun4 caused SUN3 mislocalization with a tendency forming aggregates in the cytoplasm, suggesting that SUN3 localization depends on SUN4 (15). In the present study, we found that manchette tubules were not observed in majority of spermatids in Sun3−/− mice, though a few spermatids showed some straight microtubule bundles but these structures were dissociated with nuclei, which closely resembled the phenotype of Sun4−/− mice. Interestingly, the level of SUN4 proteins in testes was drastically reduced in Sun3−/− testes, indicating that SUN3 is also required to maintain the level of SUN4 proteins in vivo. Thus, it indicates that the functions of SUN3 and SUN4 are likely interdependent. It is worthy to mention that we have tried many times to optimize the experimental protocols of co-immunoprecipitation using the anti-SUN3 antibody and finally we were able to detect SUN4 proteins in the testicular lysates immunoprecipitated by the anti-SUN3 antibodies. However, we could not detect SUN3 in the lysates immunoprecipitated by anti-SUN4 antibodies, which might be explained by that the anti-SUN4 antibody might not be suitable for co-immunoprecipitation experiments, or that the interaction of SUN4 with the anti-SUN4 antibody may interfere the binding of SUN3 with SUN4. It is interesting to conduct further studies to confirm the interaction between SUN3 and SUN4, and to investigate how SUN3 interacts with SUN4 and particularly, how they coordinate and functions in assembly or stabilization of the manchette cytoskeletal structures.

Anchorage and positioning of cell nucleus plays an important role during diverse developmental processes such as fertilization, cell migration, establishment of polarity (33-35). In mammalian somatic cells, the LINC complex, composed of SUN domain and KASH domain proteins, forms a protein bridge within the perinuclear space that connects the nucleus with the cytoskeleton (36,37). Two spermiogenesis specific LINC complex have been described, one is SUN1η/NESPRIN3 localized to the anterior acrosomal side, the other is SUN3/NESPRIN1 situated to the posterior pole of spermatid nucleus (14). However, unlike the various functions of SUN proteins in spermatogenesis, the known KASH domain proteins appear not that important. Mice mutant for KASH domain proteins, Nesprin1 or Nesprin3, were fully fertile and disruptions of genes encoding for other important KASH proteins, Nesprin2, Nesprin4 and Lrmp also displayed no overt impact on fertility (38,39). So far only KASH5 has been shown to be essential for spermatogenesis. KASH5 was predominantly expressed in meiotic stages and aggregated at one nuclear pole in round and elongated spermatids (18). Mice lacking KASH5 manifested meiotic arrest, and thus, we currently do not know whether KASH5 plays a role in spermiogenesis as well. However, given the localization pattern of KASH5 in spermatids, it is a poor candidate as a LINC complex partner for SUN3. Thus, we assumed that NESPRIN1 is not essentially required for spermiogenesis and SUN3 may form links with other alternative KASH proteins to physically couple the manchette to the nucleus.

Altogether, our study indicates that Sun3 is required for sperm head shaping during mammalian spermiogenesis. Disruption of Sun3 in mice caused a drastic reduction of sperm numbers and severe sperm head defects because of manchette formation failure, ultimately resulting in male infertility. Thus, these results will deepen the understanding of the role of LINC complexes in sperm head shaping and provide new
molecular cue underlying the pathology of globozoospermia.

**Experimental procedures**

**Mice**

Sun3^{−/−} mice were generated using CRISPR/Cas9 genome editing as previously described (40). To generate Sun3^{−/−} mice, we used optimized CRISPR Design tool (https://crispr.mit.edu) to design guide RNA (gRNA) sequence (GGCGTTGCTTAAAGACATGA) targeting exon 4 5’-GACTCCGCATGTCTAAGGA-3’ of Sun3 gene, co-injected with Cas9 mRNAs into B6D2F1 (C57BL/6×DBA/2) zygotes, which were subsequently transferred to the oviducts of pseudo pregnant ICR female mice. Sanger sequencing of tail genomic DNA confirmed a female founder mouse carrying a homozygous one-nucleotide insertion in exon 4 (5’-GACTCCGCATGTCTAAGGA-3’, the inserted T nucleotide is underlined) was obtained. The female founder mouse was crossed to wild-type C57BL/6 mice. The resulting heterozygous mice were crossed to obtain homozygous mutants. Animal experiments were approved by the Institutional Animal Care Committee of the University of Science and Technology of China. Following sequences of primers that were used to confirm the genotype of Sun3 mice:

5’-GGCAGGCTGAGAAAGACACATG-3’

and

5’-GGCTTCACAGCTGACAATGGCAT-3’.

**Western blotting**

Testes from adult mice (8- to 12-week-old) were homogenized in Radio-Immunoprecipitation Assay (RIPA) buffer (25 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate) supplemented with protease inhibitors using a Tissuelyzer, and then cleared by centrifugation. Western blotting was carried out as previously described (41,42). Antibodies included rabbit polyclonal anti-SUN3 (Proteintech, 26434-AP-1; 1:1000), guinea pig polyclonal anti-SUN3 (1:3000) (16), rabbit polyclonal anti-SUN4 (Proteintech, 19721-1-AP; 1:3000) and anti-β-Actin (Abcam, ab8227; 1:100).

**Sperm counting**

Adult male mice (8- to 12-week-old) were sacrificed by cervical dislocation. Their epididymides were dissected, minced into small pieces in 1 mL Dulbecco’s Modified Eagle Medium, and incubated at 37°C in a 5% CO2 humidified incubator for 1 hour to allow sperm release. Sperm counts were determined using a haemocytometer under microscope (Nikon Eclipse E200).

**Histological examination, TUNEL assay and immunofluorescence staining**

Mice were euthanized by cervical dislocation. Testes were removed and fixed overnight in Bouin’s solution (for H&E and PAS) or 4% paraformaldehyde (for immunofluorescence staining and TUNEL assay). Samples were dehydrated through a graded series of ethanol, embedded in paraffin and sectioned at 5 μm for subsequent H&E and PAS staining. Sperm smears were prepared from epididymis as we described (43). To prepare testis smears, testes were peeled of the testicular capsule, cut into pieces in PBS and filtered through a 200-mesh cell strainer. The filtered cell suspensions were spread onto a glass slide. H&E and PAS stainings of tissue sections or sperm smears were performed as described (43). TUNEL assay was performed using *In Situ* Cell Death Detection Kit (Roche, 11684795910) according to the manufacturer’s instructions. For immunofluorescence, tissue sections, testis smears or sperm smears were blocked in antibody dilution buffer (ADB) (10% normal donkey serum, 3% bovine serum albumin, 0.05% Triton X-100 in PBS) for 30 minutes, followed by an overnight incubation at 4°C.
with primary antibodies against α-Tubulin (Sigma, F2168; 1:200), rabbit polyclonal anti-SUN4 (1:3000) and Lectin PNA (Invitrogen, L32458; 1:200). After washing with PBST for four times, sections were incubated with secondary antibodies (Invitrogen, Alexa Fluor 488 Goat anti-Mouse IgG and Alexa Fluor 555 Donkey anti-Rabbit IgG, 1:200) at 37°C for 1 hour. Finally, sections were mounted with VECTASHIELD mounting medium (Vector Laboratories, H-1000) containing Hoechst 33342 (Invitrogen, H21492). Staining of epididymal sperm mitochondria was performed using MitoTracker probes (Molecular Probes, M-7512). Images were captured using a Nikon ECLIPSE 80i microscope (Nikon) equipped with a CCD camera (Hamamatsu) and analyzed using NIS-Element Microscope imaging software (Nikon).

Co-immunoprecipitation
Tissue extracts were prepared using a Dounce homogenizer in cold IP buffer with 1% Triton X-100, 0.5% sodiumdeoxycholate, 0.1% SDS, 1 mM Na3VO4, 1 mM EDTA, 1 mM EGTA, 50 mM Tris (pH 7.5) and 150 mM NaCl dissolved in 500 ml PBS supplemented with 1 mM phenylmethylsulfonyl fluoride and protein inhibitor cocktail (Roche, 04693116001). All the reagents were analytical grade. Lysates were then centrifuged at 15,000 g at 4°C for 15 minutes and the supernatant was divided into two aliquots. Each aliquot was then incubated with either 1.5 μg rabbit anti-SUN3 antibody (Proteintech, 26434-AP-1) or rabbit IgG unspecific antibody (CST, 2729S). After incubation at 4°C overnight with rotation, beads were washed by RIPA buffer for 5 times. Finally, the beads were resuspended in 3xSDS-sample buffer (120 mM Tris/HCl, 10% SDS, 20% Glycerol, 20% 2-mercaptoethanol, bromphenol blue, pH 6.8), boiled at 100°C for 15 minutes and subsequently conducted with Western blotting.

Transmission electron microscopy
Ultrastructural analysis of Sun3+/− mice testis were performed as described previously (44). Briefly, testes from Sun3+/+ and Sun3−/− mice were fixed in 4% paraformaldehyde containing 0.05% glutaraldehyde in 0.1 M phosphate buffer, and then post-fixed in 1% osmium tetroxide. Dehydration was carried out in ethanol and the samples were embedded in Epon 812. Ultrathin sections were obtained, counterstained with uranyl acetate and lead citrate, and examined with a JEOL JEM-1230 transmission electron microscope.

Statistical analysis
Testis/body weight ratio, sperm number, sperm morphology and TUNEL positive cells between Sun3+/+ and Sun3−/− mice were analyzed statistically by using the Student’s t-test. Results are presented as mean ± SD. Statistically significance is defined as p<0.05.

Data availability
It is stated that all the data related to this manuscript is contained within the manuscript.

Author Contributions: Q Gao, R Khan and C Yu performed the experiments; M Alsheimer provided the SUN3 and SUN4 antibodies; Q Gao, H Ma, R Khan wrote the paper; Q Shi, X Jiang and M Alsheimer revised the manuscript; Q Shi, H Ma and X Jiang conceived and designed the experiments.

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Figure legends

**Figure 1. Generation of Sun3<sup>−/−</sup> mice.** (A) Diagram illustrating the CRISPR/Cas9 targeting strategy to generate Sun3 knockout mice. A thymine deoxyribotide was inserted in exon 4 between nucleotide position 184 and 185, predicted to cause a frameshift at amino acid position 63 that introduces a pre-mature stop codon at position 64 (p. Pro62Leufs<sup>+</sup>). (B) Representative Sanger sequencing chromatograms showing the thymine deoxyribotide inserted between nucleotide position 184 and 185, as indicated by red arrow. (C) Western blotting using the rabbit anti-SUN3 antibody confirms the lack of full-length SUN3 proteins in testis lysates from 8-week-old Sun3<sup>−/−</sup> mice.

**Figure 2. Deletion of Sun3 impairs spermatogenesis.** (A) Representative images of testes from 8-week-old Sun3<sup>+/+</sup> and Sun3<sup>−/−</sup> mice. Each grid represents 1 mm. (B) Average testes/body weight ratio of 8-week-old Sun3<sup>+/+</sup> and Sun3<sup>−/−</sup> mice (n = 5). (C) Epididymal sperm number of 8-week-old Sun3<sup>+/+</sup> and Sun3<sup>−/−</sup> mice (n = 3). (D) Representative images of H&E stained testicular sections (a-d) and epididymides (e-f) from 8-week-old Sun3<sup>+/+</sup> and Sun3<sup>−/−</sup> mice. b and d showed the higher magnification images of the rectangular area outlined with white boxes in a and c respectively. Scale bars = 50 μm. (E) TUNEL assay on testicular sections from 8-week-old Sun3<sup>+/+</sup> and Sun3<sup>−/−</sup> mice. c and d showed the higher magnification image of the rectangular area outlined with white boxes in a and b respectively. Scale bars = 50 μm. (F) Percentage of TUNEL-positive tubules in testis sections from 8-week-old Sun3<sup>+/+</sup> and Sun3<sup>−/−</sup> mice (n = 3). (G) Quantification of TUNEL-positive cells per TUNEL-positive tubule. 298 tubules examined from three Sun3<sup>+/+</sup> mice. 375 tubules were examined from three Sun3<sup>−/−</sup> mice. Data are presented as mean ± SD; Student’s t-test; * p<0.05, ** p < 0.01.

**Figure 3. Morphological abnormalities of spermatozoa from Sun3<sup>−/−</sup> mice.** (A) Representative images of spermatozoa from 8-week-old Sun3<sup>+/+</sup> and Sun3<sup>−/−</sup> mice. Spermatozoa from Sun3<sup>+/+</sup> mice present with typical morphology, while spermatozoa from mice presents an amorphous head accompanied with various midpiece defects, such as thickened (i, ii), coiled (ii, v), thinned (iii-v) and cracked (iv). Scale bars = 10 μm. (B) Quantification of spermatozoa with an amorphous head from 8-week-old Sun3<sup>+/+</sup> and Sun3<sup>−/−</sup> mice (n = 3). Student’s t-test; **** p<0.0001. (C) Quantification of spermatozoa with a defective midpiece from 8-week-old Sun3<sup>+/+</sup> and Sun3<sup>−/−</sup> mice (n = 3). Student’s t-test; *** p<0.001 (D) Representative images of spermatozoa from 8-week-old Sun3<sup>+/+</sup> and Sun3<sup>−/−</sup> mice stained for PNA (red) and α-Tubulin (green). DNA was counterstained with Hoechst. Scale bars = 10 μm.

**Figure 4. Abnormalities of spermatids occur when spermatids start to elongate in Sun3<sup>−/−</sup> mice.** PAS staining of testis sections from 8-week-old Sun3<sup>+/+</sup> and Sun3<sup>−/−</sup> mice revealed abnormal sperm nucleus at stage IX and X-XI in Sun3<sup>−/−</sup> mice. Scale bars = 50 μm.

**Figure 5. Manchette formation is disrupted in Sun3<sup>−/−</sup> mice.** (A) Representative images of testis sections from 8-week-old Sun3<sup>+/+</sup> and Sun3<sup>−/−</sup> mice stained by anti-PNA (red) and anti-α-tubulin (green) antibodies. DNA was counterstained with Hoechst. Scale bars = 20 μm. (B) Representative images of spermatids from 8-week-old Sun3<sup>+/+</sup> and Sun3<sup>−/−</sup> mice stained by anti-PNA (red) and anti-α-Tubulin (green) antibodies. DNA was counterstained with Hoechst. Type 1-5 show various abnormal spermatids observed in Sun3<sup>−/−</sup> mice. Scale bars = 2 μm. (C) Manchette microtubule bundles were not observed in Sun3<sup>−/−</sup> mice by transmission electron microscopy. The red rectangles show the presence of manchette microtubule bundles in Sun3<sup>+/+</sup> mice, while yellow rectangles indicate the absence of such microtubule bundles in Sun3<sup>−/−</sup> mice. Scale bars = 2 μm.
Figure 6. SUN3 interacts with SUN4 in mouse testes and SUN4 protein level is drastically reduced in Sun3−/− mice. (A) Western blotting of immunoprecipitated testis extract showing the presence of SUN3 proteins in lysates immunoprecipitated by the guinea pig anti-SUN3 antibodies. Rabbit IgG was used as a negative control. (B) Western blotting showing the presence of SUN4 proteins in testis lysates immunoprecipitated by the guinea pig anti-SUN3 antibodies. Rabbit IgG was used as a negative control. (C) Immunofluorescence staining of SUN4 (red), SP56 (green) and Hoechst (blue) on testis cell spreads from 8-week-old Sun3+/+ and Sun3−/− mice showing mis-localization of SUN4 in Sun3−/− spermatids. Scale bars = 20 μm. (D) Western blotting showing the levels of SUN4 proteins in Sun3+/+ and Sun3−/− testes. β-Actin was used as the loading control. For (A), (B) and (D), the bands corresponding to the sizes of proteins of interest are indicated by arrows.
Figure 1

A

Sun3 gene

Sun3 mRNA

5'UTR 3'UTR

p.Pro62Leufs*2

Transmembrane Coiled coil SUN domain

Nonmutant protein

B

Sun3+/+

Sun3−/−

35 kDa

45 kDa

35 kDa

C

SUN3

β-Actin

Sun3+/+  Sun3−/−
Figure 3

A

Sun3\textsuperscript{+/+}  

Sun3\textsuperscript{−/−}

B

Spermatocytes with round head (%)

C

Spermatocytes with defective midpiece (%)

D

DNA/PNA/\alpha\textsuperscript{-}Tubulin
Figure 4

Sun3^{+/+}  Sun3^{-/-}

Stage VII-VIII

Stage IX

Stage X-XI
Figure 5

A

Stage VIII

Stage IX

Stage X

Sun3\textsuperscript{+/+}

Sun3\textsuperscript{-/−}

DNA/PNA/α-Tubulin

B

\begin{tabular}{cccc}

\textit{Sun3\textsuperscript{+/+}} & Step 1 & Step 2 & Step 3 & Step 4 \\
Step 5 & Step 6 & Step 7/8 & Step 9 \\
Step 10/11 & Step 12/13 & Step 14/15 & Step 16 \\

\textit{Sun3\textsuperscript{-/−}} & Step 1 & Step 2 & Step 3 & Step 4 \\
Step 5 & Step 6 & Step 7/8 & Type 1 \\
Type 2 & Type 3 & Type 4 & Type 5 \\
\end{tabular}

C

\begin{tabular}{cc}

\textit{Sun3\textsuperscript{+/+}} & \textit{Sun3\textsuperscript{-/−}} \\
\end{tabular}
Figure 6

A

B

C

D

Input
IP: IgG
IP: SUN3

Input
IP: IgG
IP: SUN3

DNA/SUN4/SP56

Sun3+/+

Sun3−/−

WB: SUN3

WB: SUN4

WB: SUN4

WB: β-Actin

45 kDa

45 kDa

45 kDa

35 kDa

35 kDa

45 kDa

35 kDa
The testis specific LINC component SUN3 is essential for sperm head shaping during mouse spermiogenesis
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