Loss of Guf1 Impairs Sperm Mitochondrial Function and Leads to Male Infertility in Mice

Short title: Guf1 IS ESSENTIAL FOR MALE FERTILITY

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ABSTRACT

GTPase of unknown function 1 (Guf1) is a mitochondrial protein in eukaryotes. Although this protein was identified several years ago, its physiological role in mammals remains elusive. Here, we demonstrate that Guf1 is located specifically in sperm mitochondria. The physiological functions of Guf1 were investigated using a novel constructed knockout mouse model. Guf1Δ/Δ males were infertile. Sperm counts and motility were decreased markedly in Guf1Δ/Δ mice. Guf1Δ/Δ sperm in the epididymis was disorganized at the midpiece with poorly formed mitochondrial sheaths. Furthermore, levels of mitochondrial proteins, activities of mitochondrial respiratory chain complex IV, and ATP levels were significantly reduced in Guf1Δ/Δ sperm. These findings reveal that Guf1 is required for mitochondrial integrity in sperm, sperm motility and male fertility.

Key words: Guf1; sperm mitochondria; male infertility

INTRODUCTION

Spermatogenesis can be divided into three phases: the mitotic phase in which the spermatogonia undergo several mitotic divisions and give rise to spermatocytes; meiotic phase, in which spermatocytes divide by meiosis to form round spermatids; and the spermiogenesis phase, in which spermatozoa are released into the lumen of the seminiferous tubules [1]. The mature sperm tail has several accessory structures, including outer dense fibers (ODFs), a fibrous sheath (FS), and a mitochondrial sheath (MS). Based on these accessory structures, the sperm tail can be divided into three parts: the midpiece; principal piece; and endpiece [2]. The midpiece of mammalian sperm is characterized by a mitochondrial sheath that
surrounds the axonemal complex and the nine outer dense fibers [3]. Studies with gene knockout mice have proven that precisely regulated mitochondrial sheath formation is critical for sperm motility and fertility [4-7].

The mitochondrion is a complex sub-cellular organelle present in the cytoplasm of eukaryotes and is composed of a double membrane structure consisting of outer and inner membranes separated by an inter-membrane space. Mitochondria participate in various cellular functions, such as ATP production, calcium homoeostasis, generation of reactive oxygen species (ROS), the intrinsic apoptotic pathway and steroid hormone biosynthesis [8]. In mature mammalian spermatozoa, mitochondria are present in the midpiece of the flagella [9]. Mitochondria-encoded polypeptides consist of seven subunits of NADH CoQ reductase (complex I), cytochrome b (complex III), subunits I, II, and III of cytochrome C oxidase (complex IV), and subunits 6 and 8 of the H+ ATPase [10]. Mitochondria of spermatozoa differ from the corresponding organelles of somatic cells, in both their morphology and biochemistry [11]. Mitochondrial causes of infertility have triggered interest because of its location in the tail of the sperm and the immense energy requirement associated with sperm motility (reviewed in [12]).

GTPase of unknown function 1 (Guf1) is a gene encoding a novel evolutionarily conserved GTPase in budding yeast. Analysis of Guf1 deletion in budding yeast revealed that Guf1 is not essential; moreover, mutant cells did not reveal any marked phenotype [13]. However, another study demonstrated that Guf1 binds to mitochondrial ribosomes in a GTP-dependent manner and promotes mitochondrial protein synthesis under suboptimal conditions in budding yeast [14]. A recent study suggests that Guf1 deletion reduce mitochondrial translation and disrupts the assembly of respiratory chain supercomplexes containing complex IV in Caenorhabditis elegans [15]. To date, the importance of Guf1 in mammals is unknown. To elucidate the function of Guf1 in mammalian testis, we detected the expression of Guf1 and generated a germ cell-specific Guf1 knockout mouse model. We demonstrated that Guf1 was required for the correct midpiece structure of sperm tails. Additionally, our results revealed a role for Guf1 in mitochondrial function and support the importance of Guf1 in the organization and function of the mitochondria and male fertility.

MATERIALS AND METHODS

Ethics Statement
Mice used in this study were generated on a C57BL6/129P2 mixed genetic background. Mice and New Zealand white rabbits (purchased from the Institute of Genetics of Chinese Academy of Sciences, Beijing, China) were housed in a 12-hour light/12-hour darkness cycle and provided food and water ad libitum. All animal procedures were in accordance with the Animal Care and Use Committee (IACUC) of Affiliated Hospital of YanBian University.

Generation of Guf1-Specific Antibody
For immunostaining characterization of Guf1, we designed a peptide sequence for antibody production with epitope specificity corresponding to amino acid positions 40-55 (DMSRFPVEDINRFSII) near the N-terminus. The Guf1 protein was expressed in Escherichia coli using the pGEX4T-1 vector and affinity purified with glutathione Sepharose (Sangon Biotech, Beijing, China). Anti-Guf1 antiserum was
produced in female New Zealand white rabbits by intradermal injection. Specific antibodies were affinity purified with the immunoblot method as previously described [16].

**Generation of a Guf1 Conditional Knockout Mouse Strain**
The Guf1 flox allele was generated using conditional gene targeting methods. A targeting vector containing Guf1 exons 2-8 flanked by a loxP site and a loxP-Neo cassette was constructed and introduced into mouse embryonic stem cells (AB1, 129/SvEv) by electroporation. Next, embryonic stem (ES) cell clones containing the targeted Guf1 construct were injected into C57BL/6 (B6) blastocysts to generate chimeras. Resultant male chimeras were identified by coat color and mated with wild-type females to obtain germline transmission. Tail biopsies of agouti-pigmented F1 animals were genotyped using a primer set specific to the neo cassette. Generation of the mouse model was performed by the Cyagen Company (Guangzhou, China). Primers for genotyping of the Guf1 flox allele were as follows: P1, CTGCAATACGTGAAGTTGTC; P2, TTAGTGATCTAGTATAGGCT; and P4, ACACCTTCACTTTTCAGAC. The use of primers P2 and P4 produced bands of 362 bp for the wild-type allele and 477 bp for the Guf1 flox Neo deleted allele. The use of primers P1 and P4 produced bands of 3,554 bp for the wild-type allele and 346 bp for the null allele. Guf1+/flox mice were mated with mice carrying Guf1+/flox and Stra8-Cre transgene mice. DNA isolated from tail biopsies was used for genotyping. The presence of the Guf1 flox allele was determined by PCR amplification, and genotyping for the Stra8-Cre transgene was performed as described [17]. A Western blot assay was conducted to confirm the deletion of the Guf1 protein.

**Immunohistochemical Analysis**
Testes and caudal epididymis from 3 month old mice were dissected in phosphate buffered saline (PBS) and fixed in 4% paraformaldehyde (PFA) for up to 24 hours, stored in 70% ethanol, and embedded in paraffin. Tissue sections (5 µm thick) were cut and mounted on glass slides. Sections were deparaffinized and rehydrated, followed by antigen retrieval in 10 mM sodium citrate buffer. After blocking with 5% BSA for 1 hour, the sections were incubated with primary antibody against GCNA1 (rabbit, 1:200; gift of Dr. George Enders) at 4°C overnight. After washing with PBS, biotin-labeled secondary antibody (goat anti-rabbit IgG; 1:200 dilution) was applied for 1 hour, followed by washing in PBS. Staining was visualized using a DAB substrate kit (Zhong Shan Technology, Beijing, China). All slides were counterstained with hematoxylin. Differential interference contrast images were obtained using a Leica DMRA2 microscope (Leica, Germany).

**Breeding assays**
Three month old wild-type (n=10) and Guf1ΔΔ (n=23) males were used for the breeding assay. Each male mouse was caged with a wild-type female (7-8 weeks of age) and the vaginal plug was checked every morning. Once a vaginal plug was identified, another female was placed in the cage for another round of mating. Each male underwent three cycles of the breeding assay.

**Sperm motility assays**
The epididymides from 3 month old male mice (n=4) were dissected in 1 ml HTF media containing 0.075% penicillin G, 0.05% streptomycin sulfate, 0.001% phenol red, and 0.4% BSA; the pH was adjusted by gassing with 5% CO2 at 37°C. A reliable method to obtain spermatozoa from the
epididymides to use backflushing [18, 19], because sperm motility may be affected in certain gene knockout mice. At the appropriate pressure, spermatozoa will start to slowly come out of the broken tubule at the distal end of the cauda epididymis. Collected sperm sample was loaded onto a hemocytometer and the number of immotile sperm were counted. After this count, Kimura stain was added to another sample of the supernatant and the total sperm count determined. The percentage of motile sperm was then calculated as \((\text{total-immotile} \div \text{total}) \times 100\). A single observer that was blinded to the genotype of the animal performed all counts. Sperm samples were prepared as described above and analyzed by computer-assisted sperm analysis (CASA) using the Minitube Sperm-Vision Digital Semen Evaluation system (Minitube, MA) [20]. Briefly, sperm samples were diluted 1:10 with a modification of Whittingham’s PB1 media and then loaded into cannulae and analyzed (Minitube, MA). The following parameters were measured: progressive motility (%); path velocity (μm/sec); progressive velocity (μm/sec); track speed (μm/sec); lateral amplitude (μm); and beat cross frequency (MHz).

**Sperm Staining Assays**

Mouse sperm were collected from cauda epididymis using backflushing method and were fixed in 4% PFA for 30 min at room temperature and air dried on poly-L-lysine-treated slides. After blocking with 10% goat serum-PBS, Guf1 polyclonal antibody and goat anti-Cox1 (Santa Cruz, sc-48143) were used to detect co-localization at a 1:200 dilution overnight at 4°C. Sections were washed in 0.3% Triton X-100 in PBS and incubated with FITC- and TRITC-conjugated secondary antibodies (Jackson ImmunoResearch, PA) for one hour. The sections were washed in 0.3% Triton X-100 in PBS and images were captured by a Leica DMR Epifluorescence camera.

**Western Blotting**

Sperm were collected from the cauda epididymis, washed in PBS, and incubated in lysis buffer for 20 mins on ice. Similarly, testis or other tissue lysates were excised and homogenized in lysis buffer and then placed on ice for 1 hour. The extracts were centrifuged and the supernatants were collected. The proteins were denatured by boiling for 5 min in the presence of 1% SDS and 1% 2-mercaptoethanol, separated by 12% SDS-PAGE gels and transferred to nitrocellulose membranes (Millipore, MA). After blocking with 1% skim milk for 1 hour, blots were incubated with the primary antibody overnight at 4°C and then with the corresponding horseradish peroxidase-labelled secondary antibody for 1 hour at room temperature. Specific signals were detected using the enhanced chemiluminescence Western blotting detection system (Bio-Rad, MA). The following antibodies were used in this study: rabbit anti-Guf1 (1/1000, lab-made); goat anti-Cox1 (1/1000, Santa Cruz, sc-48143); goat anti-Cox4 (1/1000, Santa Cruz, sc-69359); mouse anti-Cyto C (1/1000, Santa Cruz, sc-13561); rabbit anti-Tom 20 (1/1000, Santa Cruz, sc-11415); goat anti-Tim 23 (1/1000, Santa Cruz, sc-13298); and rabbit anti-β-tubulin (1/1000, Abcam, ab6046).

**TUNEL assay**

TUNEL assays were conducted with the In Situ Cell Death Detection Kit, Fluorescein (Promega, America) as recommended by the manufacturer’s instructions. Slides were counterstained with 4’,6-diamidino-2-phenylindole (DAPI) (Sigma, St. Louis, MO) to identify the nuclei. Images were acquired with a Nikon DMR Epifluorescence Microscope, and images were captured by a Hamamatsu CCD camera.
Scanning, Transmission, and Immunogold Electronic Microscopy
Caudal epididymides were collected from WT and Guf1Δ/Δ mice and fixed overnight in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4), to allow the release of sperm. The sperm were collected and subsequently fixed in 2.5% (w/v) glutaraldehyde solution in phosphate buffer (pH 7.4) at 4°C for 3 hours. Then, the sperm were washed in phosphate buffer, collected on poly-L-lysine-coated glass coverslips, postfixed with 1.0% osmium tetroxide for 1 hour, dehydrated in a graded series of ethanol, and embedded in EPON/Araldite resin. Thin sections were cut, mounted on 200-mesh grids, and stained with uranyl acetate and lead citrate. The samples were then examined with a JEOL JSM-6360 LV scanning electron microscope. For the immunogold electron microscopy, the sperm was fixed in 4% PFA, infused in 10% gelatin, dehydrated in sucrose and then frozen in liquid nitrogen. Cryosections were prepared and incubated at room temperature with 0.1% cold water fish skin gelatin (CWFS gelatin, AURION) and 5% BSA for 30 min, then incubated with Guf1 polyclonal antibody (1:50) overnight. After extensive washing in PBS, cryosections were then incubated with colloidal gold-conjugated secondary antibody (Goat anti-Rabbit, AURION). Sections were then stained with uranyl acetate and methylcellulose, and observed by transmission electronic microscopy.

Characterization of WT and Guf1Δ/Δ Spermatozoa
A simple and reliable assessment of the mitochondrial respiratory chain enzymatic function (Complex IV) for minute quantities of sperm samples using a single-wave length spectrophotometer was performed similar to previously described procedures [21]. The ATP values of spermatozoa were determined using the ATP Bioluminescence Assay Kit CLS II (Roche, Mannheim, Germany). The results were normalized to the protein concentration of the samples.

Statistical Analysis
Statistical analyses were performed with SPSS 11.5. Experiments in duplicate were repeated a minimum of three times. Statistical analysis of data derived from immunoblotting was performed with two-way ANOVA followed by Student’s t test. Each bar represents the mean±SEM of n=3. * p < 0.05. Other statistical analyses were done using unpaired two-tailed t-test.

RESULTS
Expression of Guf1 in Sperm Mitochondria
To investigate the expression and localization of Guf1, we generated specific antibodies against the N-terminal fragments of mouse Guf1. In the present study, various tissue distributions of the Guf1 protein in adult mice were detected by western blotting. Endogenous Guf1 expression was detected in brain, lung, testis and epididymis, while lower levels of Guf1 were detected in spleen and kidney (Figure 1A). Immunostaining results indicated that Guf1 protein was specifically localized to the sperm tail during late spermatogenesis and sperm storage in epididymis (Figure 1B). Additionally, high-magnification immunofluorescence analysis showed that Guf1 co-localized with mitochondrial marker cytochrome c oxidase subunit 1 (Cox1) to the midpiece of spermatozoon (Figure 1C). To determine the sub-cellular localization of Guf1 in spermatozoon in detail, mouse spermatozoa were processed for pre-embedding immuno-electron microscopy using the anti-Guf1 antibody and gold-conjugated, goat anti-rabbit IgG. The majority of gold particles was found to be associated with
mitochondria (Figure 1D).

**Guf1 Is Essential for Male Fertility**

To determine the requirement of Guf1 for spermiogenesis, we generated Guf1 knockout mice by homologous recombination. Mouse Guf1 encoding a protein of 651 aa is a 17-exon gene located on Chr. 5. By homologous recombination, a Guf1 allele in which exons 2-8 are flanked by loxP sites (Figure 2A) was introduced into the mouse germ line. Successful removal of the Neo cassette by crossing offspring that inherited the targeted allele with the flippase mouse was assessed by PCR using primers 2 and 4 (P2 and P4) (Figure 2B). Recombination of the loxP sites using Stra8-cre resulted in the removal of exons 2-8 (Figure 2C). The deletion of Guf1 was further verified by western blotting using our self-made anti-Guf1 polyclonal antibody (Figure 2D). As expected, the Guf1 protein was not detected in Guf1\(^{ΔΔ}\) testes.

The similar frequencies of vaginal plugs noted in mated females supported the normal mating behavior of wild-type and Guf1\(^{ΔΔ}\) male mice. However, the Guf1\(^{ΔΔ}\) males engendered no pregnancies over a period up to 3 months (n=23 males and 46 females). In contrast, the wild-type littermate males were almost 100% fertile (n=10 males and 20 females; Figure 3A). Body and paired testis weights of the Guf1\(^{ΔΔ}\) male mice were not obviously different from those of their wild-type counterparts (Figure 3B, C). Sperm counts from Guf1\(^{ΔΔ}\) male caudal epididymis were significantly reduced compared with the control group (Figure 3D).

To elucidate the reason for the infertile phenotype of Guf1\(^{ΔΔ}\) males, sperm motility was tested through computer-assisted sperm analysis (CASA). When measured in noncapacitated media, total motility of Guf1\(^{ΔΔ}\) male mice was decreased to approximately 15% of that of the wild-type male mice (Figure 3E). Additionally, the absence of Guf1 drastically affected other parameters of sperm motility, including progressive motility, path velocity (VAP), progressive velocity (VSL), track speed (VCL) and lateral amplitude (ALH) (Figure 3E). Therefore, these results demonstrate that the infertility phenotype caused by Guf1 disruption was associated with a severe reduction in sperm count and motility.

**Histological Changes in the Testes and Epididymis of Guf1\(^{ΔΔ}\) Male**

Multiple layers of GCNA1-positive germ cells were noted in the seminiferous tubules of both wild-type (Figure 4A) and Guf1\(^{ΔΔ}\) (Figure 4B) male testes from 3 month old mice. Compared to wild-type testes, some seminiferous tubules of Guf1\(^{ΔΔ}\) male testes exhibited vacuolization (Figure 4B; Star; compared to the use of IgG as a negative control in Figure 4C). The wild-type caudal epididymis contained a full dose of mature sperm without GCNA1-positive germ cells (Figure 4D). However, we observed premature release of GCNA1-positive germ cells from the seminiferous epithelium with sloughing into the caudal epididymis from 3 month old Guf1\(^{ΔΔ}\) mice (Figure 4E; Arrow; compared to the IgG as negative control in Figure 4F). TUNEL staining further showed that the majority of these prematurely released germ cells in the epididymal lumen were apoptotic (Figure 4H; Arrow). No apoptotic signals were observed in the wild-type caudal epididymis (Figure 4G) and negative controls (Figure 4I).

**Guf1 Is Essential for the Assembly of Mitochondrial Sheaths in the Midpiece of the Flagella**

An intact sperm structure is essential for sperm motility [6]. The reduced sperm motility of Guf1\(^{ΔΔ}\) sperm suggested that they might exhibit morphological defects. To analyze this possibility, caudal epididymal sperm from wild-type (Figure 5A) and Guf1\(^{ΔΔ}\) (Figure 5B-E) mice were examined using differential
interference contrast (DIC) microscopy. The head of Guf1Δ/Δ sperm appeared normal in morphology. However, the photomicrographs showed variations in abnormalities in the Guf1Δ/Δ sperm tail in the midpiece of the flagella. Compared with the integrated mitochondrial sheaths of wild-type sperm (Figure 5A), the mitochondrial sheaths of Guf1Δ/Δ sperm displayed various defects, including abnormal sperm with incompletely and rough mitochondrial sheaths (Figure 5B, E), curving and rough mitochondrial sheaths (Figure 5C), and incomplete, curving and rough mitochondrial sheaths (Figure 5D). The percentages of the three defects were 63%, 22% and 15%, respectively, determined by separate observations of more than 500 sperm counted from 3 knockout mice (Figure 5F).

During spermatogenesis, mitochondria become organized in a highly ordered fashion along the axoneme surrounding the midpiece of the sperm. To exclude fixation artifacts on isolated sperm, we performed in situ transmission electron microscope (TEM) of the cauda epididymis. TEM analysis of the cauda epididymis revealed a high degree of homogeneity in the size and arrangements of mitochondria in wild-type sperm along the axoneme in the midpiece region (Figure 6A, C). In contrast, mitochondria in Guf1Δ/Δ sperm had variable size and irregular appearance, and they were completely absent in some region (asterisks) (Figure 6B, D). This suggests that mitochondrial Guf1 proteins are required for mitochondrial architecture and integrity in sperm midpiece.

Abnormal Sperm Mitochondrial Function in Guf1Δ/Δ Male Mice
Sperm motility was positively correlated with mitochondrial respiration [22]. To further evaluate mitochondria function, we detected the expression of mitochondrial complex proteins (Cox1, Cox4, Cyto C, Tom20 and Tim23) and the activities of the mitochondrial respiratory chain in the sperm samples from wild-type and Guf1Δ/Δ mice. The protein levels of Cox1 and Cox4 were decreased approximately 4-fold in Guf1Δ/Δ sperm samples compared with wild-type sperm samples (Figure 7 A, B).

Compared with wild-type control littermates, loss of Guf1 resulted in a 69% decrease in the relative activities of mitochondrial respiratory chain complex IV in Guf1Δ/Δ sperm samples (Figure 7C). The activities of the other electron transport chain (ETC) complexes were not affected. A major function of the ETC is production of ATP [23]. Because Guf1Δ/Δ mice have a deficit in COX activity, we hypothesized that this might result in a decrease in ATP production. Next, we measured ATP levels of isolated control and Guf1Δ/Δ spermatozoa from caudal epididymis, and detected significantly reduced ATP levels in Guf1Δ/Δ sperm cells (0.21±0.14 μM/μg protein) compared to their wild-type litter mates (0.83±0.17 μM/μg protein; Figure 7D). We further determined sperm motility incubated in noncapacitated medium with glucose (10 mM), to test whether the insufficient levels of cellular ATP are the cause for poor motility in Guf1Δ/Δ sperm or not. Glucose, which provides ATP via glycolysis, could significantly restore the general motility of Guf1Δ/Δ sperm (Figure 7E). However, the progressive motility of Guf1Δ/Δ sperm were not altered by addition of glucose (Figure 7F). These suggest that the poor motility in Guf1Δ/Δ sperm is largely caused by structural destruction of mitochondria.

DISCUSSION
Loss-of-function studies to identify new genes required for mammalian sperm flagella structures leading to motility disorders have enriched our knowledge of male infertility (reviewed in [24]). These models revealed genetic factors that are required for the proper assembly of the axoneme, the annulus, the mitochondrial sheath and the fibrous sheath. Our current study investigates a possible role for novel
candidate gene Guf1 in infertile men with specific sperm flagellum anomalies. Guf1 is a mitochondrial protein with unknown function [14]. A previous study showed that Guf1 binds to mitochondrial ribosomes in a GTP-dependent manner and promotes mitochondrial protein synthesis under suboptimal conditions in budding yeast [14]. Prior to our current study, the in vivo importance of Guf1 in mammals was unknown. To determine the requirement of Guf1 for spermiogenesis, for the first time we generated a Guf1 specific antibody and knockout mice. The Guf1 protein is localized to sperm mitochondria, and Guf1 knockout males exhibited a severe reduction in sperm count and motility with structural defects of the mitochondrial sheath in the midpiece of the sperm flagellum.

The midpiece of mammalian sperm is characterized by a mitochondrial sheath that surrounds the axonemal complex and the nine outer dense fibers [3]. Studies with gene knockout mice have proven that precisely regulated mitochondrial sheath formation is critical for sperm motility and fertility [4-7]. The mitochondrial sheaths of Guf1Δ/Δ sperm displayed various defects, including incomplete mitochondrial sheaths, curving mitochondrial sheaths or rough mitochondrial sheaths. Mitochondria in Guf1Δ/Δ sperm had variable size and irregular appearance, and they were completely absent in some region. Spermatozoa from Guf1Δ/Δ testes displayed abnormal morphology in the tail, leading to a severe reduction in sperm motility and infertility of Guf1Δ/Δ males. In addition, we show that Guf1 regulates the expression of Cox1 and Cox4, activities of the mitochondrial respiratory chain complex IV, and ATP levels in sperm. However, glucose, which provides ATP via glycolysis, could not significantly restore the progressive motility of Guf1Δ/Δ sperm. These suggest that the poor motility in Guf1Δ/Δ sperm is largely caused by structural destruction of mitochondria. Because Cox1 and Cox4 are key factors for the biogenesis of complex IV [25], we suggest that Guf1 plays an important role in the formation of cytochrome c oxidase via regulation of the expression of Cox1 and Cox4 either directly or indirectly. Therefore, the question of whether Guf1 can bind to mitochondrial ribosomes and promote mitochondrial protein synthesis in mammals is of great interest. Given the fact that Guf1 is a GTPase and GTPase defective mutants resulted in obstacle of mitochondrial fusion, fission or trafficking [6, 26], special attention should be paid to determine whether Guf1 is involved in microtubule associated mitochondrial movement.

Analysis of Guf1 conventional knockout mice (Guf1floxflox, Zp3-Cre) showed that loss of Guf1 resulted in no overt defects other than male infertility. Because the structural changes in the mitochondria that occur during spermiogenesis are unique, it is possible that Guf1 function is not required in other tissues. Ongoing studies should reveal specific cellular and molecular roles for Guf1 in sperm mitochondrial function and spermiogenesis.

Recently, it has been demonstrated that mtDNA substitutions can influence semen quality. Several mutations in mitochondrial complex genes including Cox1 have been identified in asthenozoospermic infertile men [27] [28, 29]. So it is significant to detect whether Guf1 mutant gene is also present in human patients with non-obstructive azoospermia in the further investigation of Guf1 functions.

In conclusion, for the first time our findings demonstrate the biological role of Guf1 in male fertility. The disruption of Guf1 leads to a reduced sperm count, sperm motility and severe morphological defects of the mitochondrial sheath in the midpiece of the sperm flagellum. Furthermore, we provided evidence that Guf1 regulates the expression of Cox1 and Cox4, activities of the mitochondrial respiratory chain complex IV, and ATP levels in sperm.
REFERENCES


FIGURE LEGENDS

Figure 1 Guf1 is expressed in the sperm mitochondria. (A) In mice, Guf1 was predominantly expressed in brain, lung, testis and epididymis, with lower levels detected in spleen and kidney by Western blot analysis. (B) Immunofluorescence staining of Guf1 in mouse testis (left) and cauda epididymis (right). Scale bars: 50 μm. (C) High-magnification immunofluorescence analysis of isolated sperm from cauda epididymis showed that Guf1 (red) co-localized with Cox1 (green) to the midpiece of the spermatozoa. mp, midpiece; pp, principal piece. Scale bars: 200 μm. (D) Immunogold-labeled electron microscopic detection shows that gold particles are stained at sperm mitochondria. Mi, mitochondria. Scale bars: 300 nm.

Figure 2 Targeted disruption of Guf1 by homologous recombination. (A) Partial genomic structure of mouse Guf1 and targeting vector. Exons 2-8 are flanked by loxP sites. (B) Successful flox insertion was confirmed using PCR analysis to detect a 362 bp WT band and a 477 bp Guf1flNeo deleted band using primers P2 and P4. (C) Cre recombination was validated using PCR analysis to detect a 3,554 bp WT band and a 346 bp null band using primers P1 and P4. (D) Immunoblot showing that Guf1 was deleted in Guf1Δ/Δ testes.

Figure 3 Male infertility caused by Guf1 disruption. (A) Fertility of Guf1Δ/Δ and wild-type males (n = 10 WT and 23 Guf1Δ/Δ). Mouse body (B) and paired testis weight (C) were not significantly different (n = 7 WT and 10 Guf1Δ/Δ). (D) The sperm count was significantly reduced in Guf1Δ/Δ testes (n = 4). (E) Sperm motility was determined using CASA and different parameters of sperm movement, such as progressive motility, path velocity (VAP), progressive velocity (VSL), track speed (VCL), lateral amplitude (ALH) and beat cross frequency (BCF). *p<0.05.

Figure 4 Analysis of wild-type and Guf1Δ/Δ testes and epididymides. GCNA1-stained testis sections of wild-type (A) and Guf1Δ/Δ testes (B) at 3 months of age. GCNA1-stained epididymal sections of control littermate (D) and Guf1Δ/Δ male mice (E). Arrows indicated GCNA1-positive germ cells. (C, F) IgG served as a negative control for the immunohistochemistry assay. TUNEL stain showing apoptotic germ cells in epididymis sections from 3-month-old wild-type (G) and Guf1Δ/Δ (H) mice with DAPI counter staining of the nucleus. Green fluorescence labels the apoptotic cells. Arrows pointed to randomly selected apoptotic germ cells. I indicates the negative control for the TUNEL assay. Scale bars = 50 μm.

Figure 5 Spermatozoa from Guf1Δ/Δ testes display abnormal morphology in the tail. Sperm from wild-type (A) and Guf1Δ/Δ epididymis (B-E) were plated onto glass coverslips and fixed using 4% PFA. Images were taken under bright field. Arrows in B, D and E indicate the region where mitochondrial sheaths were incompletely. Asterisks in C and D indicate the curving sperm. Scale bars in A-E: 10 μm. (F) The percentage of the three defects was shown by analyzing more than 500 sperm counts from 3 knockout mice.

Figure 6 Defects in mitochondrial architecture in Guf1Δ/Δ sperm. Electron microscopy of sperm cross-sectional view (A, B) and longitudinal view (C, D) in wild-type and Guf1Δ/Δ mice. Arrows indicate mitochondria in sperm midpiece. Asterisks indicate the region where mitochondria were completely
absent. The annulus is assembled in both wild-type and \textit{Guf1}^{Δ/Δ} spermatozoa (E, F). Mi, mitochondria; An, annulus; FS, fibrous sheath; Ax, axoneme. Scale bar in A for all panels: 200 nm.

\textbf{Figure 7} Expression and activity of mitochondrial complex IV in sperm. (A) Immunoblot showing steady-state levels of Cox4, Cox7, Cyto C, Tom20 and Tim23 in sperm samples with β-tubulin serving as a loading control. (B) Histogram summarizing selected immunoblotting results in A and normalized against β-tubulin. (C) The relative activities of mitochondrial respiratory chain complex IV were measured in sperm samples. (D) ATP production was measured in sperm samples. Each bar, n=3 experiments (E, F) Effect of glucose (Glu) on \textit{Guf1}^{Δ/Δ} sperm motility. Fresh sperm were incubated in PB1 medium with and without glucose (10 mM). Motility data for both general (E) and progressive (F) motility were performed using CASA. The values represent the mean±SD of six experiments. *p<0.05.
FIGURE 1, Piao et al.
FIGURE 2, Piao et al.
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