The American Journal of Human Genetics, Volume *107*

Supplemental Data

Bi-allelic *DNAH8* Variants Lead to Multiple Morphological Abnormalities of the Sperm Flagella and Primary Male Infertility

Chunyu Liu, Haruhiko Miyata, Yang Gao, Yanwei Sha, Shuyan Tang, Zoulan Xu, Marjorie Whitfield, Catherine Patrat, Huan Wu, Emmanuel Dulioust, Shixiong Tian, Keisuke Shimada, Jiangshan Cong, Taichi Noda, Hang Li, Akane Morohoshi, Caroline Cazin, Zine-Eddine Kherraf, Christophe Arnoult, Li Jin, Xiaojin He, Pierre F. Ray, Yunxia Cao, Aminata Touré, Feng Zhang, and Masahito Ikawa

Supplemental Note: Case Reports

All the cases of this study were examined carefully to exclude primary ciliary dyskinesia associated symptoms such as sinusitis, bronchitis, pneumonia and otitis media.[1] These men had normal development of male external genitalia, bilateral testicular sizes, hormone levels, and secondary sexual characteristics. Chromosomal karyotypes of these subjects were also normal, and no microdeletions were found in the human Y chromosome. Furthermore, three healthy men with normal fertility served as control subjects. Informed consents were obtained from all subjects participating in the study. This study was approved by the institutional review boards at all the participating institutes.

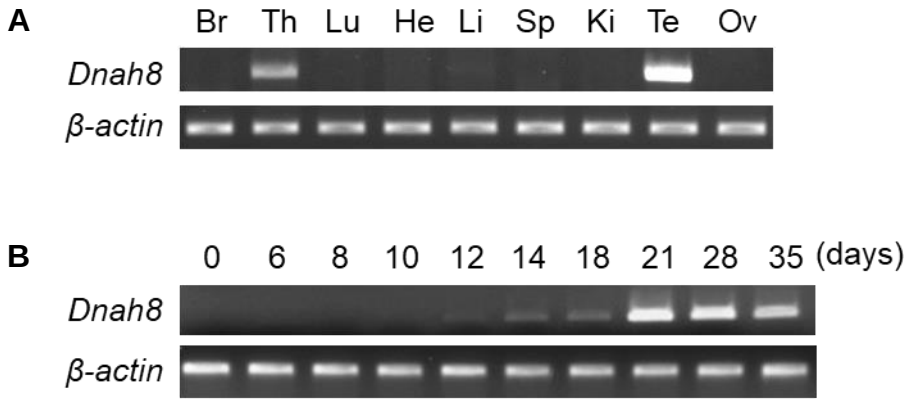

Figure S1. Expressions of Mouse *Dnah8* in Different Tissues and Postnatal Testes.

(A) Expressions of *Dnah8* were investigated by RT-PCR in various tissues from adult male mice. *β-actin* was used as an internal control locus. Br, brain; Th, thymus; Lu, lung; He, heart; Li, liver; Sp, spleen; Ki, kidney; Te, testis; Ov, ovary. (B) Expressions of mouse *Dnah8* were analyzed by RT-PCR using testicular tissues from male mice of different days after birth.

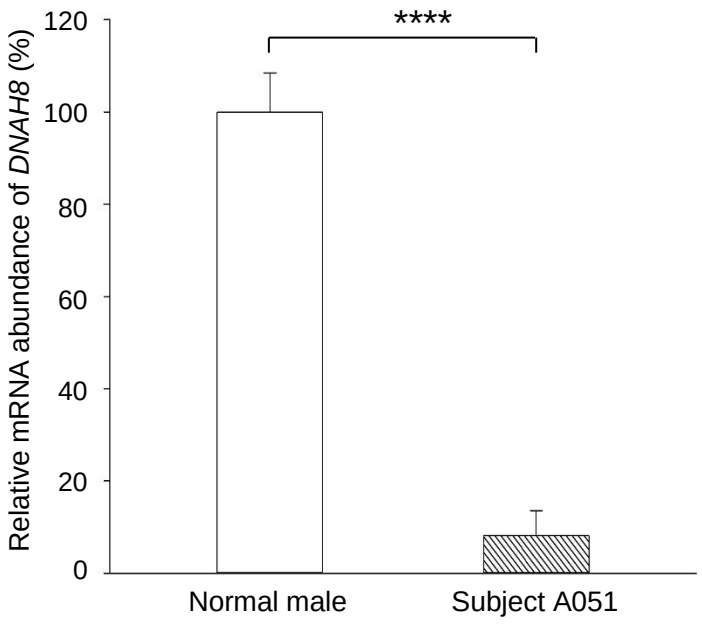

Figure S2. Measure of *DNAH8* mRNA Abundance in the Spermatozoa from a Normal Male Control and Subject A051 Harboring Bi-allelic *DNAH8* Variants.

RT-qPCR assays suggested that the level of *DNAH8* mRNA was dramatically reduced in the sperm from subject A051 when compared to that of a normal male control. The experiments were performed three times and statistical analysis was performed using two-tailed Student's paired or unpaired *t* tests. Data represent the means ± standard error of the means (S.E.M.). **** $P < 0.0001$.

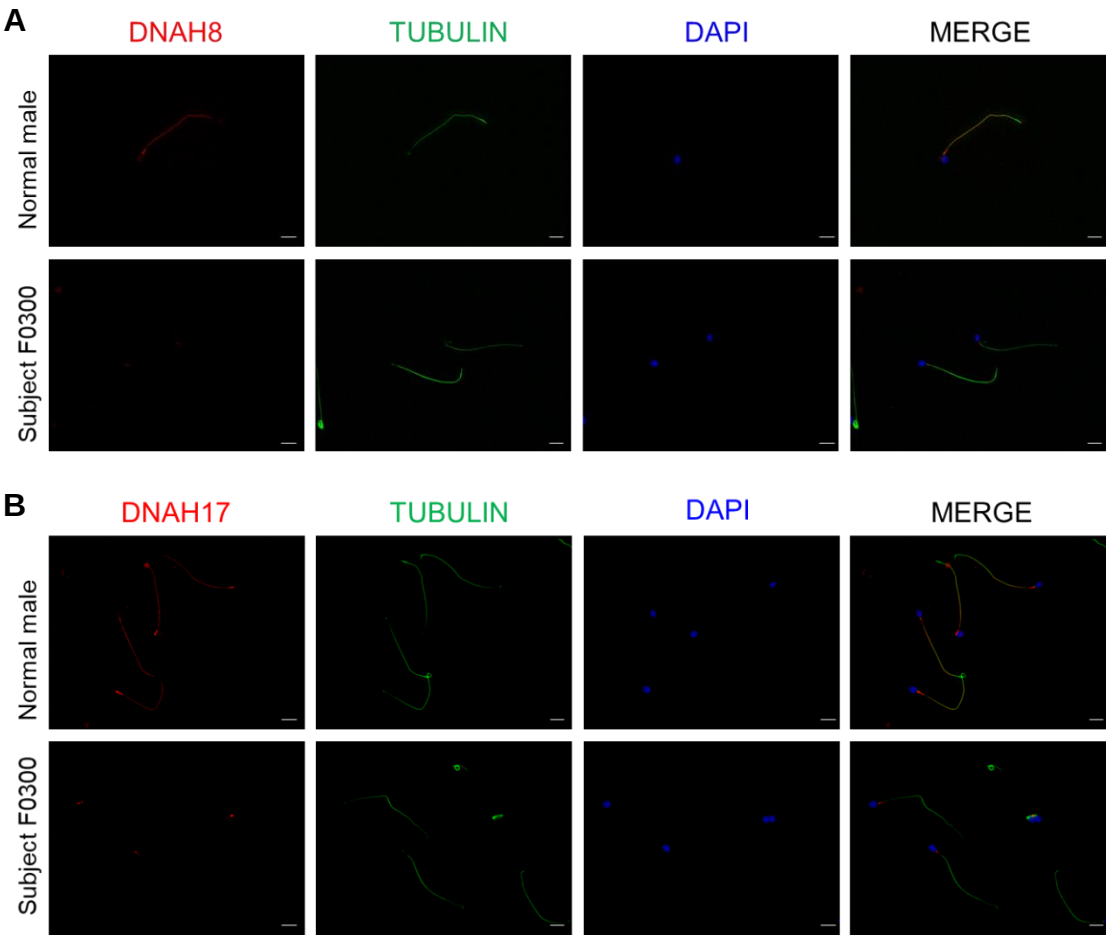

Figure S3. Immunostaining of DNAH8 and DNAH17 in the Spermatozoa from a Normal Male Control and Subject F0300 Harboring a Homozygous *DNAH8* Frameshift Variant.

(A) Immunofluorescence staining of the spermatozoa from a control and subject F0300 with anti-DNAH8 (red) and anti-α-tubulin (green) antibodies. (B) Immunofluorescence of the spermatozoa from a control and subject F0300 with anti-DNAH17 (red) and anti-α-tubulin (green) antibodies. Spermatozoa were counterstained with DAPI (blue) as a nuclei marker. The scale bars represent 5 μm.

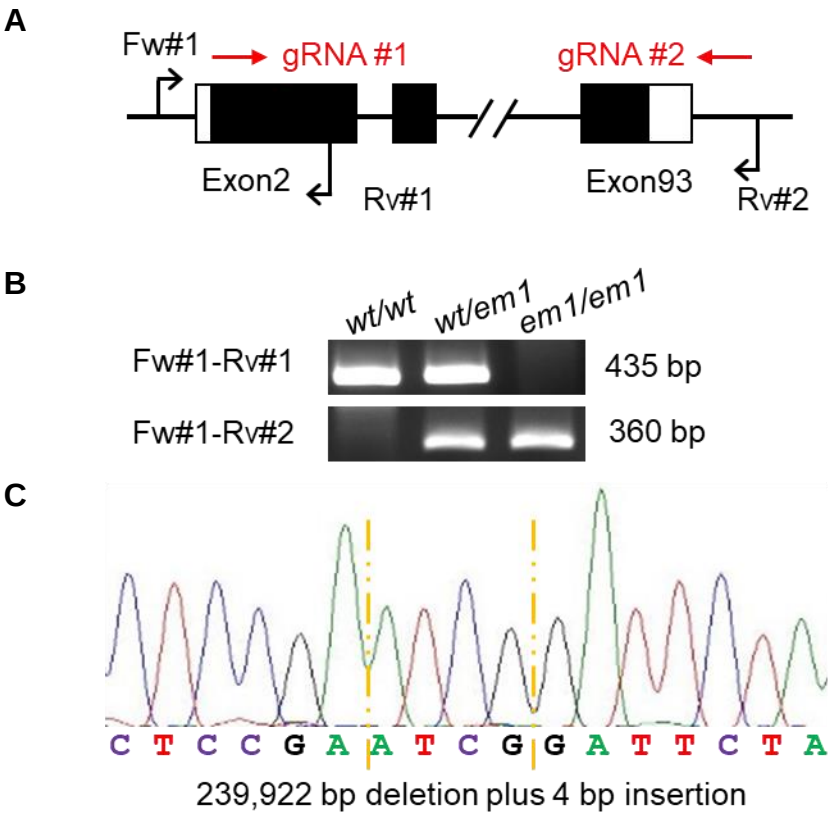

Figure S4. CRISPR/Cas9 Targeting Scheme for Generating *Dnah8*-KO (*em1/em1*) Mice.

(A) The gRNAs were targeted in exons 2 and 93 of mouse *Dnah8* to delete the entire coding region. (B) Mouse genotyping with specific primers. Primers Fw#1 and Rv#1 were used for the wild-type allele (*wt*), and primers Fw#1 and Rv#2 were used for the *Dnah8* mutated allele (*em1*). (C) Sanger sequencing result of *Dnah8*$^{em1/em1}$ mice showed a 239922-bp deletion plus a 4-bp insertion.

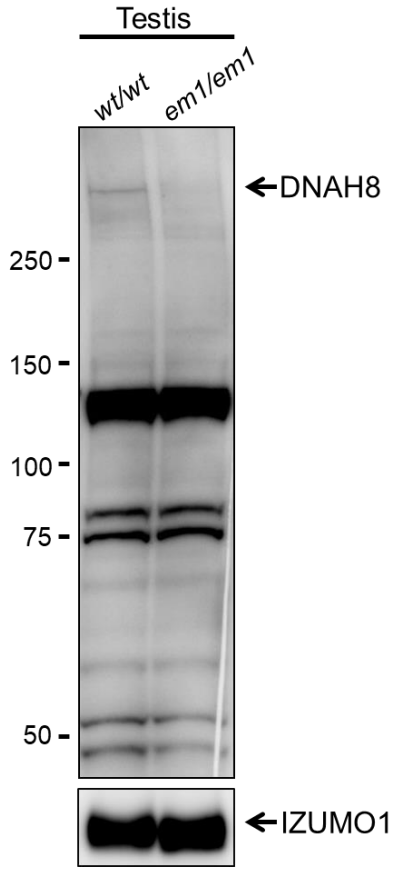

Figure S5. Expression Analysis of DNAH8 Protein in the Testes from Wild-type (*wt/wt*) and *Dnah8*-KO (*em1/em1*) Male Mice.

Western blotting analysis revealed the almost absence of DNAH8 protein in the spermatozoa from *Dnah8*-KO male mice. IZUMO1 was used as a loading control.

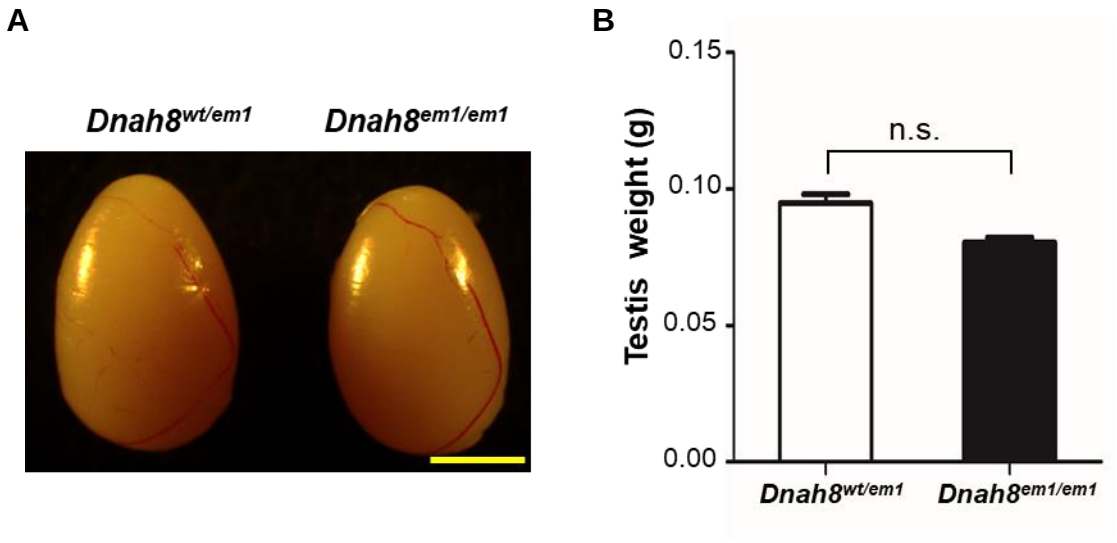

Figure S6. Testis Sizes and Weights in *Dnah8*-KO (*Dnah8*$^{em1/em1}$) Male Mice and Heterozygous Mutated (*Dnah8*$^{wt/em1}$) Male Mice.

(A) Testis Sizes of the Mouse Model. The scale bar represents 2 mm. (B) No significant difference was observed in testis weights between *Dnah8*$^{em1/em1}$ and *Dnah8*$^{wt/em1}$ male mice. Data represent the means ± SEM; n.s., not significant.

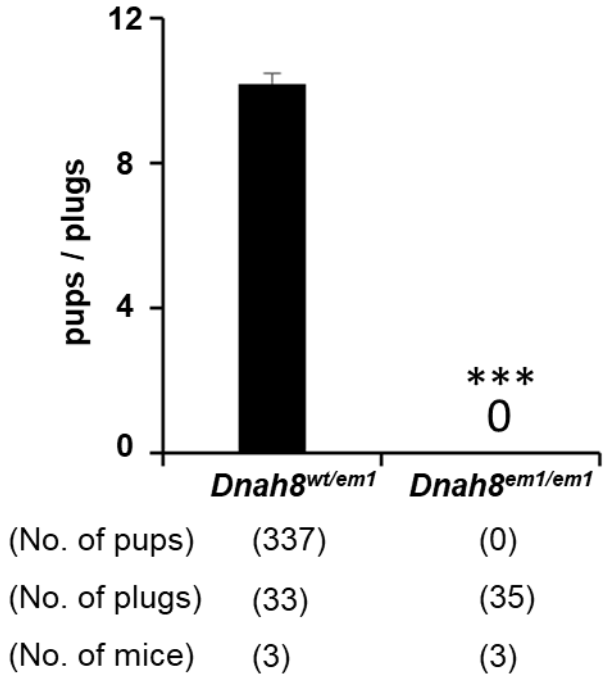

Figure S7. Fertility of *Dnah8*-KO (*Dnah8*$^{em1/em1}$) Male Mice and Heterozygous Mutated (*Dnah8*$^{wt/em1}$) Male Mice.

Dnah8$^{wt/em1}$ (3 males) and *Dnah8*$^{em1/em1}$ (3 males) mice were examined in this assay. Each male mouse was bred with three wild-type female mice, and the plugs & pups were measured. *Dnah8*$^{em1/em1}$ males were sterile. Unpaired *t* test: ***$P < 0.001$; the error bar represents the standard deviation.

Table S1. Primers Used for Verification of Human *DNAH8* Variants.

Primer Name	Primer Sequence (5'-3')	Tm
M1-F	TAGTCTGTCAGCAGGAATG	61 ℃
M1-R	AAAGAGTAGAAATACAAGCA	
M2-F	ACTGTTGCTATGATGGTTC	49 ℃
M2-R	ACTATTGCTAGGAGGAAAG	
M3-F	TGGACTTTGGCAGTGAATTG	58 ℃
M3-R	GTGGAGCTGGACGAGGTTAC	
M4-F	TTGCCCCCACTTTATGTACC	61 ℃
M4-R	ACCACTGGCCATCTCCTAAA	
M5-F	GCACATCCCAGCTAGCTGAT	60 ℃
M5-R	AGACATCTGCGTTGTTCCGT	

Table S2. Rates of Ultrastructural Abnormalities in the Spermatozoa from Men Carrying Bi-allelic *DNAH8* Variants.

	Control[a] (n=50)	Subject A051 (n=40)	Subject X003 (n=40)	Subject F0300 (n=34)
Missing CP	1 (2.0%)	11 (27.5%)	16 (40.0%)	3 (8.8%)
Missing MT	6 (12.0%)	15 (37.5%)	10 (25.0%)	5 (14.7%)
Global disorganization	1 (2.0%)	11 (27.5%)	12 (30.0%)	13 (38.2%)
Normal sections	42 (84.0%)	3 (7.5%)	2 (5.0%)	13 (38.2%)

[a]Values represent the mean of three normal males; n, number of cross sections for analysis.

Abbreviations: CP, central pair of microtubules; MT, peripheral microtubule doublet.

Table S3. Primers Used for RT-qPCR Analysis.

Primer Name	Primer Sequence (5'-3')	Tm
H-*DNAH8*-F	CAGCAGCTGAGGTAAGTGAA	60 ℃
H-*DNAH8*-R	CTCTTTTGAGGTAGTGGTGA	
H-*GAPDH*-F	GGAGCGAGATCCCTCCAAAAT	60 ℃
H-*GAPDH*-R	GGCTGTTGTCATACTTCTCATGG	

Table S4. Primers Used for RT-PCR Analysis.

Primer Name	Primer Sequence (5'-3')	Tm
M-*Dnah8*-F	CAGGTCCACTATGACTTTGG	65 ℃
M-*Dnah8*-R	AGATCCCATCTGTCCAGTCG	
M-*β-actin*-F	CATCCGTAAAGACCTCTATGCCAAC	65 ℃
M-*β-actin*-R	ATGGAGCCACCGATCCACA	

Supplemental Material and Methods

Whole-Exome Sequencing (WES) and Bioinformatic Analysis

WES was performed on the 90 Chinese MMAF-affected men according to our previously described protocol.[2,3] Briefly, genomic DNAs were isolated from peripheral blood samples of human subjects using the DNeasy Blood and Tissue Kit (QIAGEN, Germany). The human exome was enriched by SureSelect XT Human All Exon Kit (Agilent, USA), and then sequenced with the Illumina HiSeq X-TEN platform at Cloud Health Genomics (Shanghai, China). The obtained data were mapped to the human genome assembly hg19 by the Burrows-Wheeler Aligner (BWA) software. The Picard software was employed to remove PCR duplicates and evaluate the quality of variants by attaining effective reads, effective base, average coverage depth, and 90× – 120× coverage ratio.[4] For the 167 MMAF-affected men in the second cohort, WES and bioinformatic analyses were performed with a previously described protocol using the human genome assembly GRCh38.[5] The ANNOVAR software was used for functional annotation with OMIM, Gene Ontology, KEGG Pathway, SIFT, PolyPhen-2, MutationTaster, 1000 Genomes Project, and the gnomAD database.[6-11] Sanger sequencing was employed for variant validation using the primers listed in Table S1.

Animals

All animal experiments were approved by the Animal Care and Use Committee of the Research Institute for Microbial Diseases, Osaka University. Mice were purchased from Japan SLC or CLEA Japan.

Y Chromosome Microdeletion Analysis

The analysis of Y chromosome microdeletion was performed using the Y Chromosome Microdeletions Detection Kit (Tellgen, Shanghai, China) according to the manufacturer's

protocol as described in previous study.[12] Briefly, genomic DNA was extracted from peripheral blood lymphocytes and multiplex polymerase chain reaction (PCR) was performed to detect the deletions of following sequence-tagged sites: AZFa, sY84 and sY86; AZFb, sY127 and sY134; and AZFc, sY254 and sY255. *ZFX/ZFY* and *SRY* were used as internal control loci, and positive control DNA samples were obtained from men with normal spermatogenesis.

Semen Characteristics Analysis

Semen sample of men harboring bi-allelic *DNAH8* variants were obtained by masturbation after a period of 3 to 7 days of sexual abstinence and examined after liquefaction for 30 min at 37℃. Semen volume, sperm concentration and motility were evaluated by the Sperm Class Analyzer CASA System (Spain) in the source hospitals during routine examination. Morphological analysis of the sperm cells was performed with hematoxylin and eosin (H&E) staining and scanning electron microscopy (SEM). Morphological abnormalities of the sperm flagella were classified into five categories as absent, short, bent, coiled flagella and flagella of irregular caliber according to World Health Organization guidelines.[13] For each subject, at least 200 spermatozoa were counted to evaluate the percentages of morphologically abnormal spermatozoa.

To examine sperm morphology and motility of mice, spermatozoa were extracted from the cauda epididymes, and then dispersed in TYH medium for 10 min.[14] Sperm morphology and motility were observed under an Olympus BX53 differential interference contrast microscopy equipped with an Olympus DP74 color camera (Olympus, Japan).

Real-time Quantitative PCR (RT-qPCR) and Reverse Transcription PCR (RT-PCR)

For RT-qPCR**,** total RNAs were extracted from human spermatozoa using the Allprep DNA/RNA/Protein Mini Kit (QIAGEN). Approximately 0.5 ug of obtained RNAs were

converted into cDNAs with Hiscript ll Q RT SuperMix for qPCR (Vazyme). Then the RT-qPCR was performed using AceQ qPCR SYBR Green Master Mix (Vazyme) on a CFX Connect™ Real–Time PCR Detection System. Each assay was performed in triplicate for each sample, and the human *GAPDH* gene was used as an internal control locus. The $2^{-\Delta\Delta Ct}$ method was used for data analysis. The primers for RT-qPCR are listed in Table S3.

For RT-PCR**,** total RNAs were extracted from various tissues of adult C57BL/6N mice or testes from 1- to 5-week-old C57BL/6N males, and then converted to cDNA with SuperScript III First Strand Synthesis System (Thermo Fisher) using an oligo (dT) primer. PCR was performed using 10 ng of cDNA, and the primers were listed in Table S4. The amplification conditions were 3 min at 94 ℃, followed by 35 cycles of 94 ℃ for 30 s, 65 ℃ for 30 s, and 72 ℃ for 30 s, with a final two-minute extension at 72 ℃.

Immunofluorescence Analysis

Immunofluorescence (IF) experiments were conducted using the sperm cells from case and control subjects. After washes in phosphate-buffered saline (PBS), the sperm cells were fixed in 4% paraformaldehyde for 30 min at room temperature and coated on the slides treated with 0.1% poly L-lysine pre-coated slides (Thermo Fisher). Then the slides were blocked in 10% donkey serum for 1h and incubated overnight at 4 ℃ with the following primary antibodies: rabbit polyclonal anti-DNAH8 (bs-14367R, Bioss, 1:100 and HPA 028447, Sigma, 1/2000), rabbit polyclonal anti-DNAH17 (24488-1-AP, Proteintech, 1:100 and HPA 024354, Sigma, 1:200), and monoclonal mouse anti-α-tubulin (T9026, Sigma, 1:500). Next, washes were performed using PBS with 0.1% (v/v) Tween20, followed by an hour incubation at room temperature with highly cross-absorbed secondary antibodies Alexa Fluor 488 anti-Mouse IgG (1:1000, 34106ES60, Yeasen) and Cy3-conjugated AffiniPure Goat Anti-Rabbit IgG (1:4000, 111-165-003, Jackson). Fluorescence images were captured with a confocal microscope

(Zeiss LSM 880).

Electron Microscopy Analysis

For SEM assay, the sperm samples were fixed in 2.5% glutaraldehyde, rinsed in 0.1 mol/L phosphate buffer for 30 min, and post-fixed in osmic acid. Next, the specimens were rinsed thoroughly again in 0.1 mol/L phosphate buffer for 30 min, progressively dehydrated with an ethanol and isoamyl acetate gradient, and dried by a CO_2 critical-point dryer (Eiko HCP-2, Hitachi). Afterward, the specimens were mounted on aluminum stubs, sputter coated by an ionic sprayer meter (Eiko E-1020, Hitachi), and analyzed by SEM (Stereoscan 260) under an accelerating voltage of 20 kV.

Transmission electron microscopy (TEM) assay for human sperm samples was performed according to a previously described protocol.[15] Briefly, semen samples were rinsed and fixed in glutaraldehyde. Dehydration was performed using graded ethanol (50%, 70%, 90%, and 100%) and 100% acetone followed by infiltration with 1:1 acetone and SPI-Chem resin overnight at 37 ℃. After infiltration and being embedded in Epon 812, ultrathin sections were stained with uranyl acetate and lead citrate, and then observed and photographed by TEM (TECNAI-10, Philips) with an accelerating voltage of 80 kV.

For TEM analysis of mouse sperm, cauda epididymis samples were prepared as described previously.[16] The sections were observed using a JEM-1400 plus electron microscope (JEOL, Japan) at 80 kV with a CCD Veleta 2K×2K camera (Olympus).

Generation of *Dnah8*-knockout (*Dnah8*-KO) Mice by CRISPR-Cas9

The *Dnah8*-KO mouse model was generated using the CRISPR-Cas9 technology based on the B6D2F1 mouse strains. The guide RNAs were designed against the regions near the start codon (gRNA #1: 5′-GCCACCCCCTCCGAGTGAAG-3′) and stop codon (gRNA #2: 5′-TAGAATCAGAAGCGTGAATT-3′) to delete the entire coding region. To

check gRNAs/Cas9 cleavage efficiency, plasmids expressing hCas9 and gRNAs were prepared by ligating oligonucleotides into the *Bbs*I site of pX459.[17] The pCAG-EGxxFP reporter plasmids were prepared as previously described[18] using 5′-AGTTGAGATCCCCTCTACCC-3′ (plus *Nhe*I site) (Fw #1) and 5′-GTCAGTCACGGAAGTAGCAGG-3′ (plus *Sal*I site) (Rv #1) to clone the genomic region near the start codon and primers 5′-CCTCACCTTCATCACCGTGG-3′ (plus *Nhe*I site) and 5′-CTGGGGTCCTTTTAGGAGGG-3′ (plus *Sal*I site) (Rv #2) to clone the genomic region near the stop codon. Confirmation of gRNAs/Cas9 cleavage efficiency was performed by transfecting HEK293T cells with pCAG-EGxxFP and pX459 plasmids, as previously reported.[18]

For generating KO mice, superovulation-induced B6D2F1 female mice were mated with B6D2F1 males, and fertilized eggs were collected from the oviduct. Two-pronuclear zygotes were electroporated with crRNA/tracrRNA/Cas9 ribonucleoprotein complexes using a NEPA21 Super Electroporator (NEPAGENE, Japan).[19] The treated zygotes were cultured in KSOM medium[20] to the two-cell stage and transplanted into the oviducts of pseudopregnant ICR females at 0.5 day after mating with vasectomized males. The *Dnah8*-mutated F0 mice carrying large deletions were identified by genomic PCR using the same primer sets as those used for the construction of pCAG-EGxxFP (Fw#1 and Rv#1 for the WT allele, Fw#1 and Rv#2 for the KO allele). The DNA sequence of the mutant alleles was further confirmed by Sanger sequencing. After genotype validation, *Dnah8* mutated F0 mice underwent serial mating to generate homozygous offspring.

Western Blotting Analysis

Whole protein extracts from mouse testes were obtained in lysis buffer (6M urea, 2M thiourea, 2% sodium deoxycholate). The protein samples were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride membrane (Immobilon-P, Merck Millipore, USA). Then the

membranes were blocked with 10% skimmed milk (Nacalai tesque, Japan) and incubated with rabbit anti-DNAH8 (#ab121989, Abcam, UK) or rat anti-IZUMO1 (#KS64-125)[21] antibodies overnight at 4℃, followed by horseradish peroxidase (HRP)-conjugated secondary antibodies (#111-036-045 or #112-035-167, Jackson ImmunoResearch, USA) for 2 hours at room temperature. Detection was carried out with Chemi-Lumi One Super (Nacalai tesque).

Periodic Acid-Schiff (PAS) Staining

Epididymal tissue samples from male mice were fixed in Bouin's solution (Polysciences, USA) and further processed for paraffin embedding. Paraffin sections were cut at a thickness of 5-μm on a Microm HM325 microtome (Microm, Germany), stained with 1% periodic acid (Nacalai Tesque) and Schiff's reagent (Wako, Japan), followed by counterstaining with Mayer hematoxylin solution (FUJIFILM WakoPure Chemical, Japan).

H&E Staining

Fresh testicular tissue samples from male mice were fixed in Bouin's solution (Polysciences) and embedded in paraffin. Sections were cut at a 5-μm thickness on a Microm HM325 microtome (Microm) and stained with Mayer hematoxylin solution and 1% eosin Y solution (FUJIFILM WakoPure Chemical). For staining mouse spermatozoa, samples were collected from cauda epididymes, washed in PBS, fixed in 4% PFA and stained in the same way as the testes.

Intracytoplasmic Sperm Injection (ICSI)

B6D2F1 female mice were superovulated by injecting equine chorionic gonadotropin (eCG, ASKA Animal Health, Japan) and human chorionic gonadotropin (hCG, ASKA Animal Health) with 48 hours interval. Mature oocytes were collected 14 hours after the

hCG injection. After treatment with 1 mg/mL of hyaluronidase (FUJIFILM Wako Pure Chemical) for 5 min to remove the cumulus cells, oocytes were placed in KSOM medium at 37℃ under 5% CO_2 in air until subjected to ICSI. For wild-type male mice, sperm heads were separated from the tails by applying a few piezo pulses. For *Dnah8*-KO male mice, the whole sperm was used for ICSI due to the difficulty for separating the head and tail. The sperm head or the whole sperm was injected into a mature oocyte using a piezo manipulator (PrimeTech, Japan). Then, 2-cell embryos were transferred to pseudopregnant ICR females on the next day.

Supplemental References

1. Knowles, M.R., Zariwala, M., and Leigh, M. (2016). Primary Ciliary Dyskinesia. Clin Chest Med *37*, 449-461.
2. Tang, S., Wang, X., Li, W., Yang, X., Li, Z., Liu, W., Li, C., Zhu, Z., Wang, L., Wang, J., et al. (2017). Biallelic Mutations in CFAP43 and CFAP44 Cause Male Infertility with Multiple Morphological Abnormalities of the Sperm Flagella. Am J Hum Genet *100*, 854-864.
3. Liu, W., He, X., Yang, S., Zouari, R., Wang, J., Wu, H., Kherraf, Z.E., Liu, C., Coutton, C., Zhao, R., et al. (2019). Bi-allelic Mutations in TTC21A Induce Asthenoteratospermia in Humans and Mice. Am J Hum Genet *104*, 738-748.
4. Li, H., and Durbin, R. (2010). Fast and accurate long-read alignment with Burrows-Wheeler transform. Bioinformatics *26*, 589-595.
5. Coutton, C., Martinez, G., Kherraf, Z.E., Amiri-Yekta, A., Boguenet, M., Saut, A., He, X., Zhang, F., Cristou-Kent, M., Escoffier, J., et al. (2019). Bi-allelic Mutations in ARMC2 Lead to Severe Astheno-Teratozoospermia Due to Sperm Flagellum Malformations in Humans and Mice. Am J Hum Genet *104*, 331-340.
6. Wang, K., Li, M., and Hakonarson, H. (2010). ANNOVAR: functional annotation of genetic variants from high-throughput sequencing data. Nucleic Acids Res *38*, e164.
7. Ashburner, M., Ball, C.A., Blake, J.A., Botstein, D., Butler, H., Cherry, J.M., Davis, A.P., Dolinski, K., Dwight, S.S., Eppig, J.T., et al. (2000). Gene ontology: tool for the unification of biology. The Gene Ontology Consortium. Nat Genet *25*, 25-29.
8. Kanehisa, M., Furumichi, M., Tanabe, M., Sato, Y., and Morishima, K. (2017). KEGG: new perspectives on genomes, pathways, diseases and drugs. Nucleic Acids Res *45*, D353-D361.
9. Kumar, P., Henikoff, S., and Ng, P.C. (2009). Predicting the effects of coding non-synonymous variants on protein function using the SIFT algorithm. Nat

Protoc *4*, 1073-1081.

10. Adzhubei, I.A., Schmidt, S., Peshkin, L., Ramensky, V.E., Gerasimova, A., Bork, P., Kondrashov, A.S., and Sunyaev, S.R. (2010). A method and server for predicting damaging missense mutations. Nat Methods *7*, 248-249.
11. Schwarz, J.M., Cooper, D.N., Schuelke, M., and Seelow, D. (2014). MutationTaster2: mutation prediction for the deep-sequencing age. Nat Methods *11*, 361-362.
12. Tang, D., Liu, W., Li, G., He, X., Zhang, Z., Zhang, X., and Cao, Y. (2020). Normal fertility with deletion of sY84 and sY86 in AZFa region. Andrology *8*, 332-336.
13. Wang, Y., Yang, J., Jia, Y., Xiong, C., Meng, T., Guan, H., Xia, W., Ding, M., and Yuchi, M. (2014). Variability in the morphologic assessment of human sperm: use of the strict criteria recommended by the World Health Organization in 2010. Fertil Steril *101*, 945-949.
14. Toyoda, Y., Yokoyama, M., and Hosi, T. (1971). Studies on the fertilization of mouse eggs in vitro I: In vitro fertilization of eggs by fresh epididymal sperm. Jap J Anim Reprod *16*, 147-151.
15. Liu, C., Lv, M., He, X., Zhu, Y., Amiri-Yekta, A., Li, W., Wu, H., Kherraf, Z.E., Liu, W., Zhang, J., et al. (2020). Homozygous mutations in SPEF2 induce multiple morphological abnormalities of the sperm flagella and male infertility. J Med Genet *57*, 31-37.
16. Shimada, K., Kato, H., Miyata, H., and Ikawa, M. (2019). Glycerol kinase 2 is essential for proper arrangement of crescent-like mitochondria to form the mitochondrial sheath during mouse spermatogenesis. J Reprod Dev *65*, 155-162.
17. Cong, L., Ran, F.A., Cox, D., Lin, S., Barretto, R., Habib, N., Hsu, P.D., Wu, X., Jiang, W., Marraffini, L.A., et al. (2013). Multiplex genome engineering using CRISPR/Cas systems. Science *339*, 819-823.
18. Mashiko, D., Fujihara, Y., Satouh, Y., Miyata, H., Isotani, A., and Ikawa, M. (2013). Generation of mutant mice by pronuclear injection of circular plasmid expressing

Cas9 and single guided RNA. Sci Rep *3*, 3355.

19. Abbasi, F., Miyata, H., Shimada, K., Morohoshi, A., Nozawa, K., Matsumura, T., Xu, Z., Pratiwi, P., and Ikawa, M. (2018). RSPH6A is required for sperm flagellum formation and male fertility in mice. J Cell Sci *131*, jcs.221648.
20. Ho, Y., Wigglesworth, K., Eppig, J.J., and Schultz, R.M. (1995). Preimplantation development of mouse embryos in KSOM: augmentation by amino acids and analysis of gene expression. Mol Reprod Dev *41*, 232-238.
21. Ikawa, M., Tokuhiro, K., Yamaguchi, R., Benham, A.M., Tamura, T., Wada, I., Satouh, Y., Inoue, N., and Okabe, M. (2011). Calsperin is a testis-specific chaperone required for sperm fertility. J Biol Chem *286*, 5639-5646.