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Supplemental Data

Mutations in *PMFBP1* Cause Acephalic Spermatozoa Syndrome

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Supplemental Note: Case Reports

Case#1 and #2 (Familly 1_II-2 and II-3)

Individual1 and individual 2 are two primary infertile brothers. They came from a family of three children. Their brother with a heterozygous mutation (c.1462C>T/WT) had two children without fertility problems. Individual 1 and individual 2 had normal karyotype (46, XY) and negative results on Y chromosome microdeletion. Individual 1 and his partner were 34 and 31 years old, respectively. This couple presented with primary infertility over 10 years. Individual 2 and his partner were 31 and 27 years old, respectively. This couple presented with primary infertility over 6 years. All of them had a normal phenotype and karyotype, without a history of significant illness. The female partners of individual 1 and individual 2 underwent gynecological examination, hormone levels, hysterosalpingography and laparoscopy, which all demonstrated no abnormality.

Semen parameters

Individual 1

Semen analysis: 2.2 ml, 5.3 mil/ml, 0% motility A, 2.2% motility B, 2.7% motility C, 95.1% motility D.

Percentages of different morphologic spermatozoa: 0% normally formed, 2.8% abnormal head-tail junction, 0.5% decaudated, 96.7% acephalic.

Individual 2

Semen analysis: 3.3 ml, 5.6 mil/ml, 0% motility A, 1.6% motility B, 4.6% motility C, 93.8% motility D.

Percentages of different morphologic spermatozoa: 0% normally formed, 1.6% abnormal head-tail junction, 0.6% decaudated, 98.2% acephalic.

Case#3 (Familly 2_II-4)

Individual 3 and his partner were 29 and 26 years old respectively. This couple presented with primary infertility over 3 years. Both had a normal phenotype and karyotype, without a history of significant illness. Individual 3 had normal karyotype (46, XY) and negative for Y

chromosome microdeletion. The female partner underwent gynecological examination, hormone levels, hysterosalpingography and laparoscopy, which all demonstrated no abnormality. The man came from a family of four children, and his father and mother are first cousins. Both of his two sisters had two children and his elder brother had two children without fertility problems. His elder sister and brother had a heterozygous mutation (c.2725C>T/WT) .

Semen parameters

Semen analysis: 3.6 ml, 2.3 mil/ml, 0% motility A, 3.2% motility B, 11.4% motility C, 85.4% motility D.

Percentages of different morphologic spermatozoa: 0% normally formed, 2.4% abnormal head-tail junction, 0.8% decaudated, 96.8% acephalic.

Case#4 (Familly 3_II-2)

Individual 4 and his partner were 35 and 34 years old respectively. This couple presented with primary infertility over 5 years. Both had a normal phenotype and karyotype, without a history of significant illness. Individual 4 had normal karyotype (46, XY) and negative for Y chromosome microdeletion. The hormone levels, hysterosalpingography and laparoscopy of his female partner were no abnormality. The man came from a family of two children, and his father and mother are first cousins. His sisters had one child without fertility problems.

Semen parameters

Semen analysis: 2.7 ml, 3.3 mil/ml, 0% motility A, 0.9% motility B, 3.3% motility C, 95.8% motility D.

Percentages of different morphologic spermatozoa: 0% normally formed, 4.3% abnormal head-tail junction, 0.4% decaudated, 95.3% acephalic.

Case#5 (Familly 4_II-2)

Individual 5 and his partner were32 and 28 years old respectively. This couple presented with primary infertility over 6 years. Both had a normal phenotype and karyotype, without a history of significant illness. Individual 5 had normal karyotype (46, XY) and negative for Y

chromosome microdeletion. The hormone levels, hysterosalpingography and laparoscopy of his female partner were no abnormality. The man came from a family of two children. His sisters had one child without fertility problems. His father and sister had a heterozygous mutation (c.2092delG>T/WT) .

Semen parameters

Semen analysis: 3.1 ml, 3.4 mil/ml, 0% motility A, 6.2% motility B, 22.6% motility C, 71.2% motility D.

Percentages of different morphologic spermatozoa: 0% normally formed, 1.8% abnormal head-tail junction, 0.1% decaudated, 98.1% acephalic.

Semen parameters of F2:II4 F3:II2 F4:II2 have been shown in our previous research. [1]

Supplemental Figures

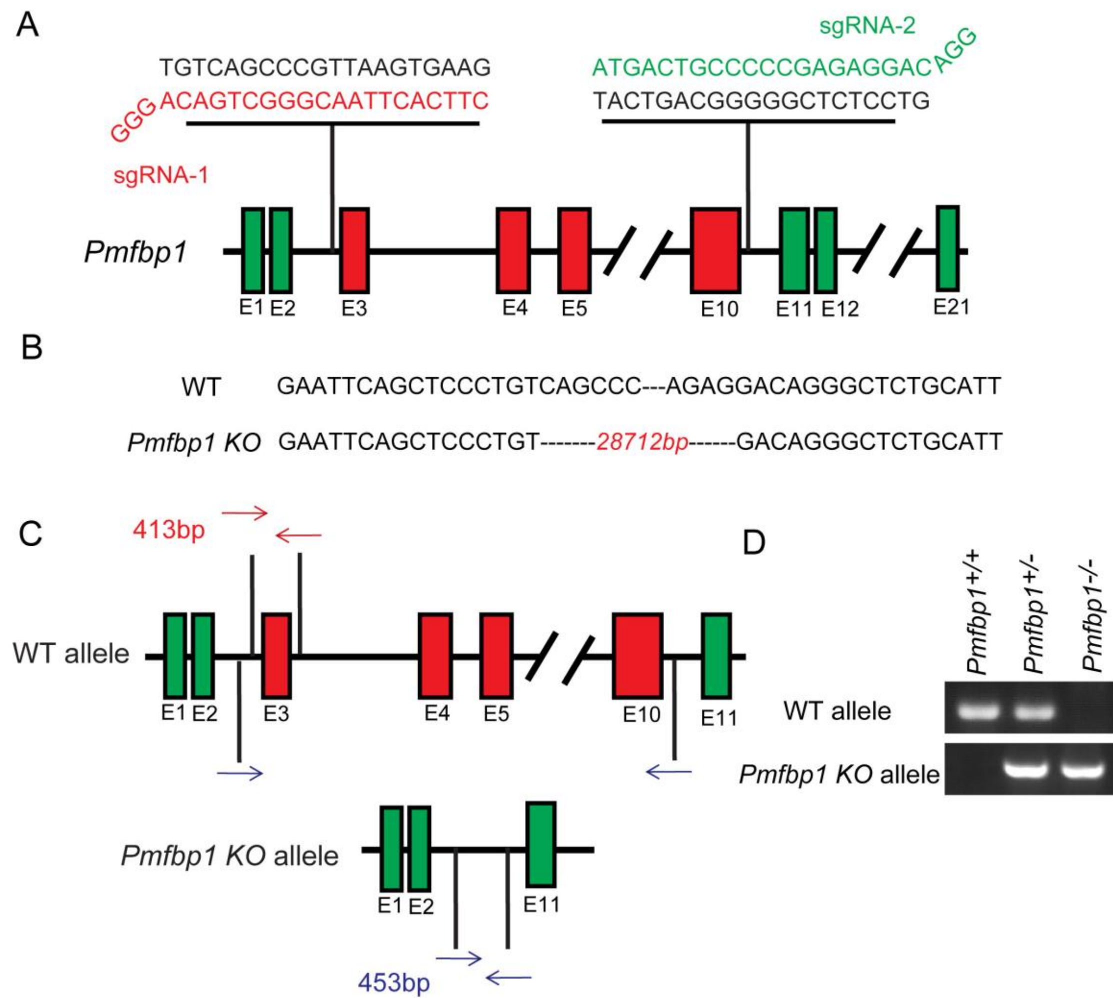

Figure S1 The generation of *Pmfbp1* knockout mice.

(A) The *Pmfbp1* knockout strategy in mice.

(B) Sequences of the WT and *Pmfbp1* mutant alleles in mice.

(C) and **(D)** Genotyping of founders to identify *Pmfbp1* knockouts.

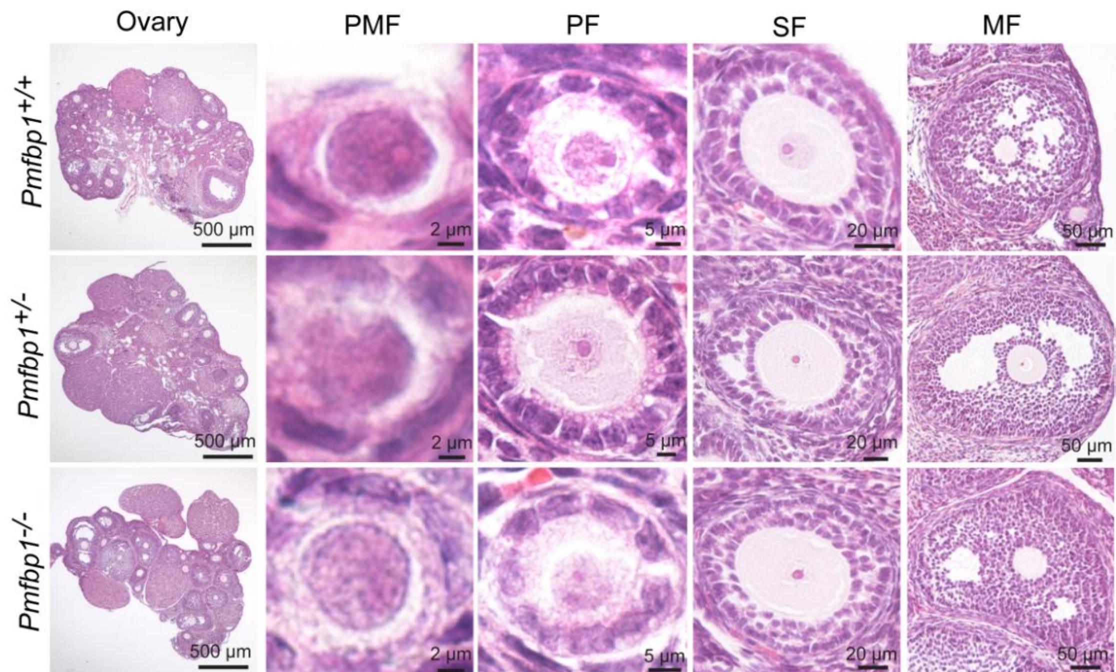

Figure S2 The disruption of *Pmfbp1* has no influence on follicle development in female mice.

The hematoxylin and eosin (H&E) staining of ovary in WT, *Pmfbp1*$^{+/-}$ and *Pmfbp1*$^{-/-}$ mice are shown. PMF: primordial follicles, PF: primary follicles, SF: secondary follicles, and MF: mature follicles.

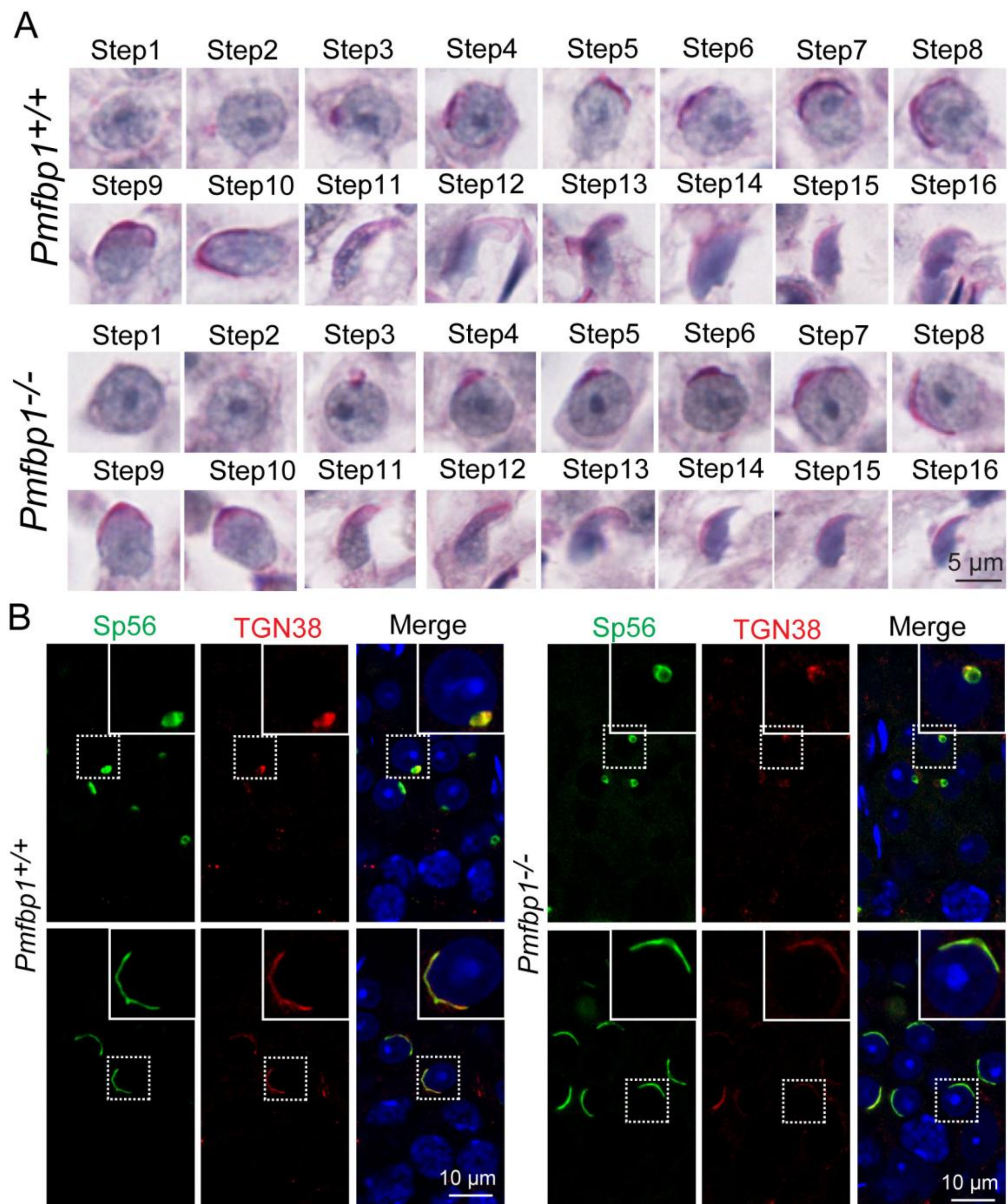

Figure S3 The *Pmfbp1* knockout has no effect on acrosome biogenesis.

(A) Acrosome morphology in different steps of spermatid development was normal in *Pmfbp1*-deficient mice. The Periodic Acid-Schiff (PAS) and hematoxylin staining was performed in WT and *Pmfbp1*$^{-/-}$ mouse. All steps of spermatid development in WT and *Pmfbp1*$^{-/-}$ testis are shown to compare acrosome morphology.

(B) Proacrosomal vesicle transportation and fusion were not affected in *Pmfbp1*$^{-/-}$ mice. Immunodetection of the co-localization of sp56 (green) and TGN38 (red) in the seminiferous tubules of WT and *Pmfbp1*$^{-/-}$ mice are shown. Nuclei were stained with DAPI (blue).

Figure S4

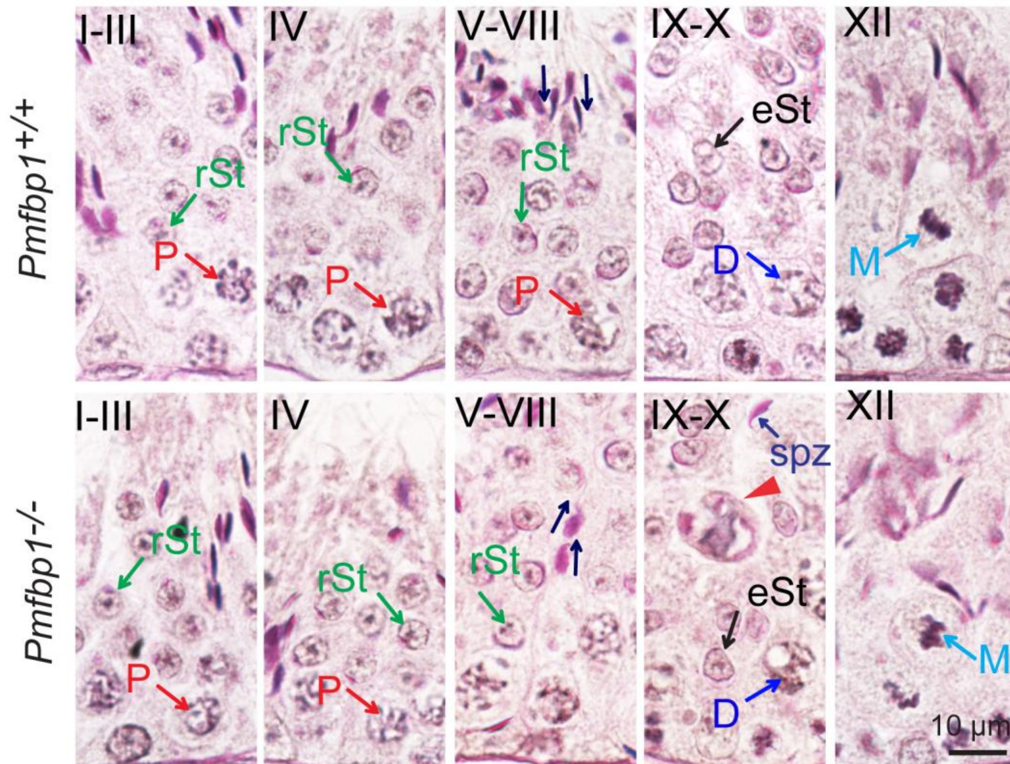

Figure S4 Spermiation defects in *Pmfbp1*-deficient mice.

PAS and hematoxylin staining was performed in WT and *Pmfbp1*$^{-/-}$ mouse. The mature sperm head could still be detected at stages IX-X in *Pmfbp1*-deficient testes. The arrowhead indicates the destroyed sperm head, which was surrounded by the globular membrane at stages IX-X in *Pmfbp1*$^{-/-}$ testes. *Pmfbp1*-null spermatids have lost their orientation toward the basement membrane during spermiation in stage V-VIII seminiferous epithelia. The arrows indicate the orientation of the sperm heads. P: pachytene spermatocyte, D: diplonema spermatocyte, rSt: round spermatid, eSt: elongating spermatid, M: meiotic spermatocyte, spz:

spermatozoa.

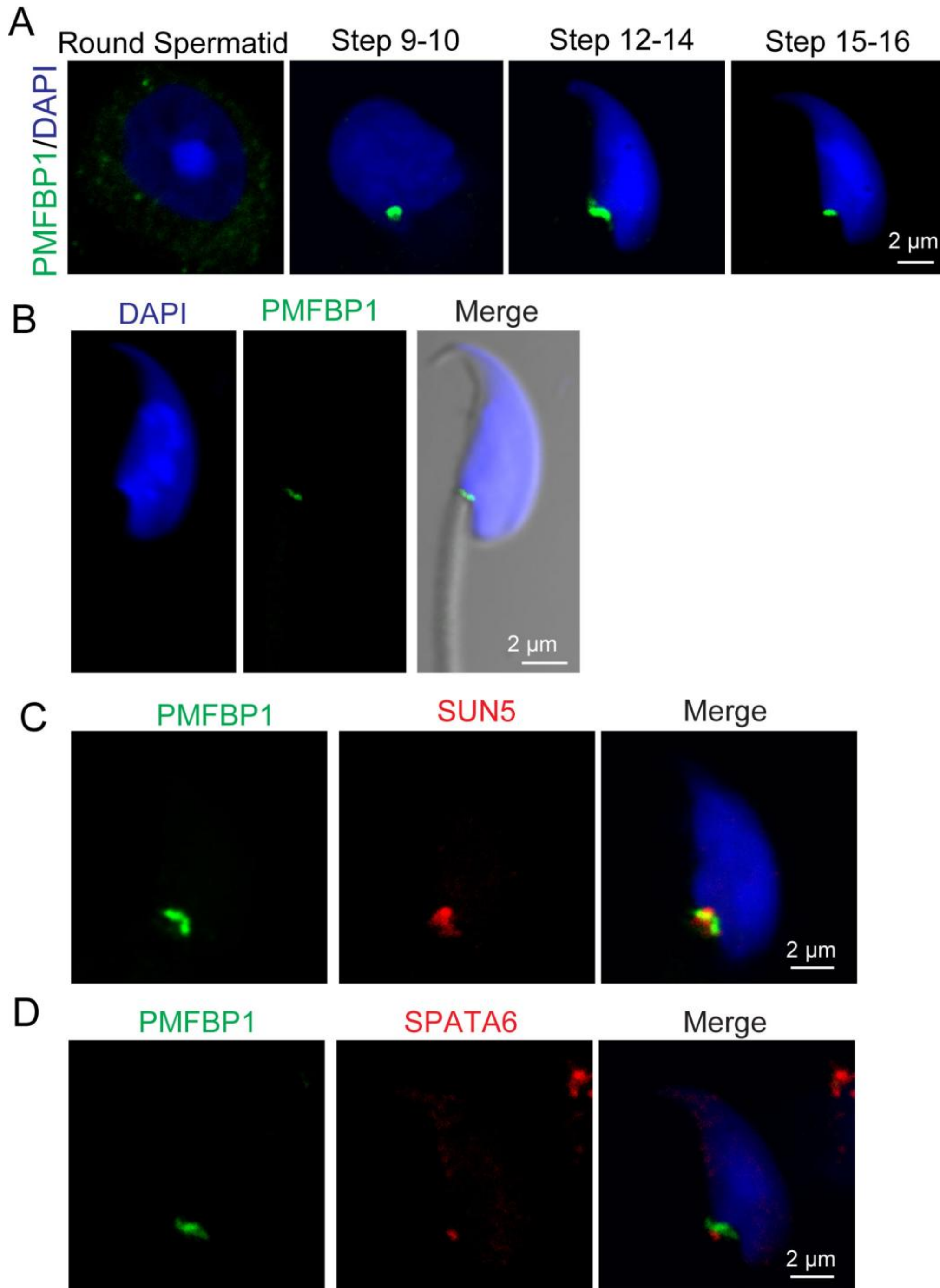

Figure S5 PMFBP1 is localized on the coupling apparatus of the mouse spermatozoa.

(A) The localization of PMFBP1 at different developmental stages. The immunofluorescence analysis for PMFBP1 (green) was performed in a testis smear. Nuclei were stained with DAPI

(blue).

(B) PMFBP1 is localized in the sperm head-to-tail connecting piece in mature spermatozoa. Single-sperm immunofluorescence analysis for PMFBP1 (green) is shown. Nuclei were stained with DAPI (blue).

(C) Immunofluorescence analysis for PMFBP1 (green) and SUN5 (red) was performed in the testis smear. Nuclei were stained with DAPI (blue).

(D) The immunofluorescence analysis for the PMFBP1 (green) and SPATA6 (red) was performed in a testis smear. Nuclei were stained with DAPI (blue).

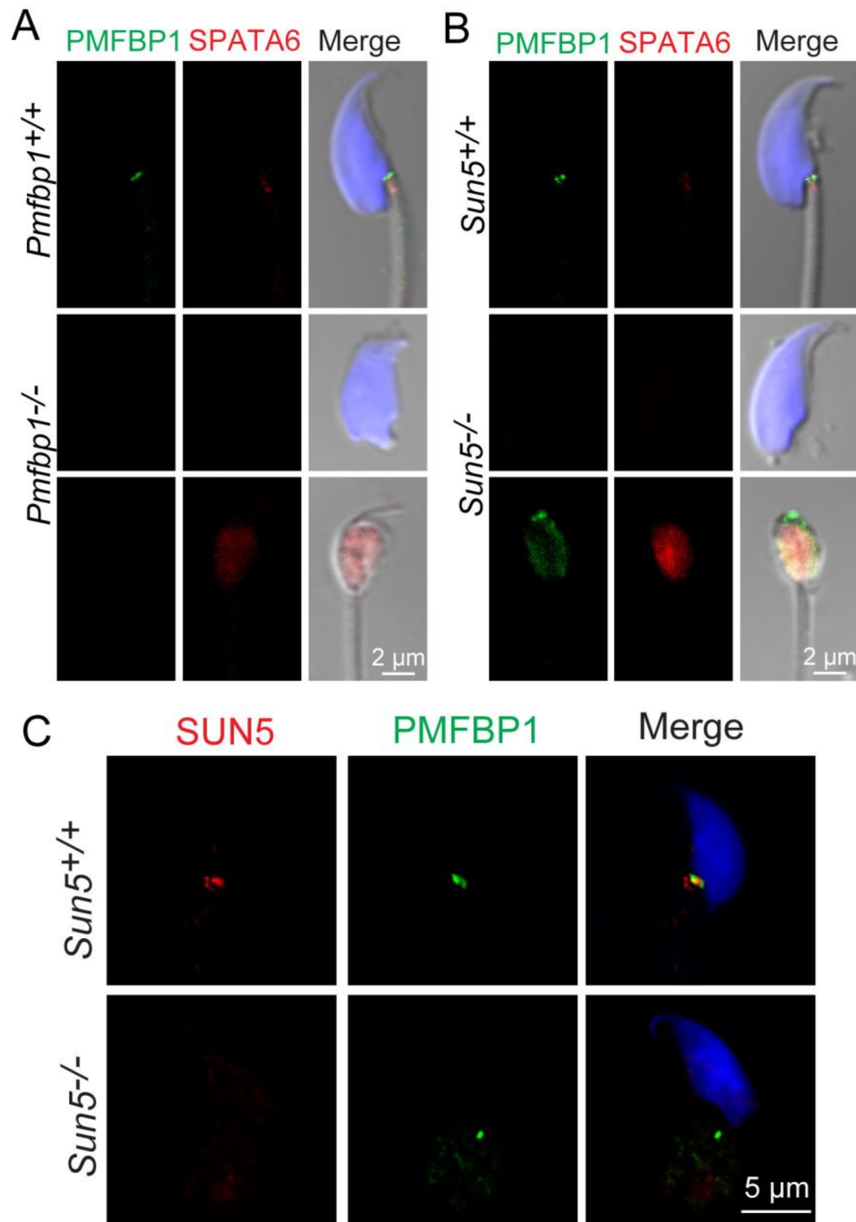

Figure S6 PMFBP1 cooperates with SUN5 and SPATA6 to maintain the sperm head and tail integrity.

(A) and **(B)** PMFBP1 and SUN5 work in the same way to connect the coupling apparatus to the sperm nuclear envelope. Single-sperm immunofluorescence analysis for PMFBP1 (green) and SPATA6 (red) was performed in WT and *Pmfbp1*$^{-/-}$ spermatozoa (A). A similar immunofluorescence analysis of PMFBP1 (green) and SPATA6 (red) was performed in WT and *SUN5*$^{-/-}$ spermatozoa. Nuclei were stained with DAPI (blue)

(C) The disruption of *Sun5* impairs the localization of PMFBP1 to the coupling apparatus. Immunofluorescence analysis for PMFBP1 (green) and SUN5 (red) was performed in a WT and *Sun5*$^{-/-}$ testis smear. Nuclei were stained with DAPI (blue).

Figure S7

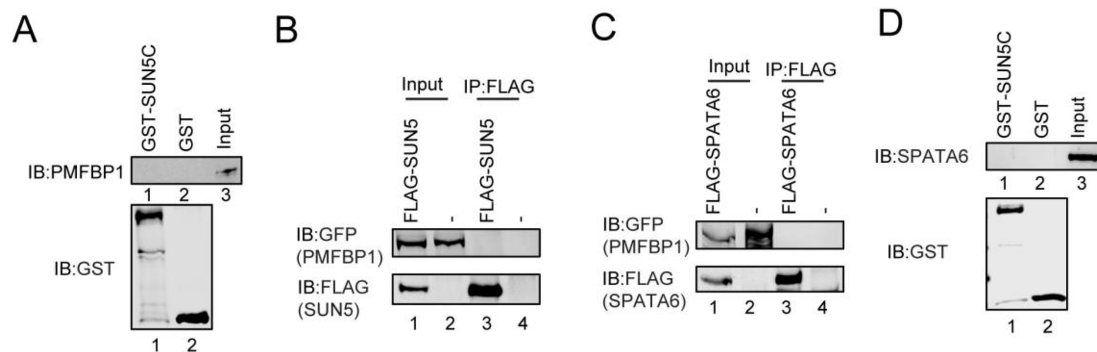

Figure S7 There is no direct interaction among SUN5, PMFBP1 and SPATA6.

(A) and **(B)** PMFBP1 could not bind SUN5 as shown with GST-pulldown and coimmunoprecipitation in HEK293T cells. GST-SUN5C was purified and used to pull down MBP-PMFBP1. GST was used as a control in (A). *pRK-FLAG-Sun5* and *pEGFP-Pmfbp1* were co-transfected into HEK293T cells. 24 h after transfection, cells were collected for immunoprecipitation (IP) with anti-FLAG antibody and analyzed with anti-GFP antibodies, respectively.

(C) PMFBP1 could not interact with SPATA6. *pRK-FLAG-Spata6* and *pEGFP-Pmfbp1* were co-transfected into HEK293T cells. 24 h after transfection, cells were collected for immunoprecipitation (IP) with anti-FLAG antibody and analyzed with anti-GFP antibodies, respectively.

(D) SUN5C could not directly bind with SPATA6. GST-SUN5C was purified and used to pull down MBP-SPATA6. GST was used as a control.

Supplemental Table

Table S1. Semen parameters of individuals with acephalic spermatozoa syndrome due to *PMFBP1* mutations

Individuals	**F1:II2**	**F1:II3**	**F2:II4**	**F3:II2**	**F4:II2**
volume (ml)	2.2	3.3	3.6	2.7	3.1
Concentration [c]	5.3	5.6	2.3	3.3	3.4
Motility A (%)	0	0	0	0	0
Motility B (%) [c]	2.2	1.6	3.2	0.9	6.2
Motility C (%) [c]	2.7	4.6	11.4	3.3	22.6
Motility D (%)	95.1	93.8	85.4	95.8	71.2
Percentages of different morphologic spermatozoa (%)					
Normally formed	0	0	0	0	0
Abnormal head-tail junction	2.8	1.6	2.4	4.3	1.8
Decaudated	0.5	0.6	0.8	0.4	0.1
Acephalic	96.7	98.2	96.8	95.3	98.1

a. Values are means of semen parameters calculated from more than two ejaculated semen analyses.

b. The unit of concentration is "$\times 10^6$ / ml". Sperm concentration was based on normally formed spermatozoa, abnormal head-tail junction spermatozoa, and decaudated spermatozoa.

c. Mobility (%) represents the total motility of normally formed spermatozoa, abnormal head-tail junction spermatozoa and acephalic spermatozoa. No rapid progressive mobility sperm (grade A) was observed in all patients.

d. Semen parameters of F2:II4 F3:II2 F4:II2 have been shown in our previous research.[1]

Note: All groups showed oligozoospermia with less than 15 $\times 10^6$/ml sperm (or 39 $\times 10^6$ per ejaculate) The normally formed spermatozoa, the abnormal head-tail junction spermatozoa and the decaudated heads observed in fresh semen were counted as sperm.[1] Asthenospermia (the motility of the normally formed spermatozoa, the abnormal head-tail junction spermatozoa and the acephalic spermatozoa) was less than 40%.[1] No sperm showed rapid progressive motility (grade A).

Table S2. Effects of novel PMFBP1 mutations predicted using in silico tools.

Chromosome 16 coordinates[a]	cDNA alteration	Amino acid alteration	Exon	Mutation	ExAC allele frequency	ExAC homozygotes frequency	Mutation Taster
72164607G>A	c.1462C>T	p.Gln488*	11	Nonsense	1/106548	0/106548	1.000 (D)[b]
72159154G>A	c. 2404C>T	p.Gln802*	16	Nonsense	Not found	Not found	1.000 (D)
72156856G>A[*]	c. 2725C>T	p.Arg909*	19	Nonsense	25/121280	1/121280	1.000 (D)
72160028_72160028delC	c.2092delG[c]	p.Ala698Profs*7	15	Frameshift	Not found	Not found	1.000 (D)

a. All data are based on GRCh37/hg19.

b. D: deleterious.

c. The mutation c.2092delG was predicted to cause a frameshift

[*]In this study, we found a recurrent nonsense mutation c.2725C>T;p.Arg909* in two individuals ,which has an allele frequency of 25/121280 and homozygous alleles frequency of 1/121280 in ExAC browser. It may due to the fact that female with homozygous mutations or carriers with heterozygous mutatons are fertile and can transmit the mutation to their offsprings.

Table S3. Outcomes of ICSI cycles in the three individuals

Variables	P1	P2	P3
Male age (years)	34	31	29
Female age (years)	31	30	26
MII oocytes(n)	4	19	14
2PN(n)	3	13	11
Blastocyst (n)	2	6	9
Frozen-all	no	yes	yes
Transferred embyos(n)	2	2	2
Clinical pregnancy	yes	yes	yes
Delivery(n)	Boy (1)	Boy (1)	Ongoing(twin)

Supplemental Reference

1. Zhu, F.X., Wang, F.S., Yang, X.Y., Zhang, J.J., Wu, H., Zhang, Z., Zhang, Z.G., He, X.J., Zhou, P., Wei, Z.L., et al. (2016). Biallelic SUN5 Mutations Cause Autosomal-Recessive Acephalic Spermatozoa Syndrome. Am J Hum Genet 99, 942-949.