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# Bi-allelic truncating variants in *CFAP206* cause male infertility in human and mouse

Qunshan Shen<sup>1,2,3</sup> · Guillaume Martinez<sup>4,5</sup> · Hongbin Liu<sup>6</sup> · Julie Beurois<sup>4</sup> · Huan Wu<sup>1,2,3</sup> · Amir Amiri-Yekta<sup>7</sup> · Dan Liang<sup>1,2,3</sup> · Zine-Eddine Kherraf<sup>4,8</sup> · Marie Bidart<sup>4,9</sup> · Caroline Cazin<sup>4</sup> · Tristan Celse<sup>4,5</sup> · Véronique Satre<sup>4,5</sup> · Nicolas Thierry-Mieg<sup>10</sup> · Marjorie Whitfield<sup>4</sup> · Aminata Touré<sup>4</sup> · Bing Song<sup>1,2,3</sup> · Mingrong Lv<sup>1,2,3</sup> · Kuokuo Li<sup>1,2,3</sup> · Chunyu Liu<sup>11,12</sup> · Fangbiao Tao<sup>2,3</sup> · Xiaojin He<sup>1,2,3</sup> · Feng Zhang<sup>11,12</sup> · Christophe Arnoult<sup>4</sup> · Pierre F. Ray<sup>4,8</sup> · Yunxia Cao<sup>1,2,3</sup> · Charles Coutton<sup>4,5,13</sup>

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## Abstract

Spermatozoa are polarized cells with a head and a flagellum joined together by the connecting piece. Flagellum integrity is critical for normal sperm function, and flagellum defects consistently lead to male infertility. Multiple morphological abnormalities of the flagella (MMAF) is a distinct sperm phenotype consistently leading to male infertility due to a reduced or absent sperm motility associated with severe morphological and ultrastructural flagellum defects. Despite numerous genes recently described to be recurrently associated with MMAF, more than half of the cases analyzed remain unresolved, suggesting that many yet uncharacterized gene defects account for this phenotype. By performing a retrospective exome analysis of the unsolved cases from our initial cohort of 167 infertile men with a MMAF phenotype, we identified one individual carrying a homozygous frameshift variant in *CFAP206*, a gene encoding a microtubule-docking adapter for radial spoke and inner dynein arm. Immunostaining experiments in the patient's sperm cells demonstrated the absence of WDR66 and RSPH1 proteins suggesting severe radial spokes and calmodulin and spoke-associated complex defects. Using the CRISPR–Cas9 technique, we generated homozygous *Cfap206* knockout (KO) mice which presented with male infertility due to functional, structural and ultrastructural sperm flagellum defects associated with a very low rate of embryo development using ICSI. Overall, we showed that *CFAP206* is essential for normal sperm flagellum structure and function in human and mouse and that bi-allelic mutations in *CFAP206* cause male infertility in man and mouse by inducing morphological and functional defects of the sperm flagellum that may also cause ICSI failures.

## Introduction

Male infertility is a major and contemporary public health concern which regroups a wide range of sperm phenotypes often caused by severe gene defects. Recent approaches for genetic investigation allowed to significantly increase our knowledge about the genetic causes involved in the most

severe phenotypes of male infertility (Krausz and Riera-Escamilla 2018). Besides the mere identification of the genetic cause, these findings also allowed to better understand the physiopathological mechanisms associated with a specific sperm phenotype and to adapt the best clinical management. This assumption is particularly true for the “MMAF phenotype”, for Multiple Morphological Anomalies of the Flagella which is defined by a mosaic of sperm cells with absent, short, irregular and coiled flagellum. This condition is systematically associated with an extreme asthenozoospermia with near zero progressive sperm motility leading to male infertility (Touré et al. 2021). The sperm flagellum is a highly specialized organelle responsible for sperm motility and its migration in the female reproductive tract. The mammalian sperm has a central “9 + 2” conformation of microtubule doublets associated with hundreds of accessory proteins that together constitute an axoneme

Qunshan Shen and Guillaume Martinez contributed equally to this work.

Yunxia Cao and Charles Coutton contributed equally to this work.

✉ Yunxia Cao  
caoyunxia6@126.com

✉ Charles Coutton  
ccoutton@chu-grenoble.fr

Extended author information available on the last page of the article

(Lindemann and Lesich 2016). The sperm flagellum axoneme shares key structural similarities with the axoneme of other motile cilia (Brown and Witman 2014). However, the sperm flagellum is unique by its specific functions and mode of assembly (Inaba 2007) and differs from other cilia by the presence of numerous and specific peri-axonemal structures including the outer dense fibers (ODFs), and the fibrous and mitochondrial sheaths. At least twenty genes, encoding different proteins located the sperm flagellum, have so far been shown to be associated with the MMAF phenotype (Touré et al. 2021). A large majority of these genes encodes for axonemal protein highlighting the critical role of this structure in sperm flagellum integrity. Moreover, it seems that many of these MMAF-related proteins are closely located or related to the calmodulin- and spoke-associated complex (CSC) suggesting a key and specific role of this structure in the sperm flagellum assembly, stability and function (Touré et al. 2021). A large part of the genetic causes of MMAF remains to be identified and might further support this hypothesis. Here, we report that bi-allelic truncating mutations in *CFAP206*, a gene encoding an axonemal protein essential for the CSC stability, are associated with male infertility in human and mouse.

## 73 Materials and methods

### 74 Patients

75 In our previously established cohort of 167 MMAF patients  
76 (Coutton et al. 2019), we previously identified 77 patients  
77 with harmful variants in known MMAF-related genes (Lorès  
78 et al. 2021). To identify some additional causes associated  
79 with human asthenozoospermia due to MMAF, we retros-  
80 pectively analyzed the exomes of the remaining 90 unsolved  
81 cases. All patients of the cohort presented a typical MMAF  
82 phenotype characterized by severe asthenozoospermia (total  
83 sperm motility below 10%) with at least three of the follow-  
84 ing flagellar abnormalities present in > 5% of the spermato-  
85 zoa: short, absent, coiled, bent or irregular flagella (Coutton  
86 et al. 2019). All patients had a normal somatic karyotype  
87 (46, XY) with normal bilateral testicular size, normal hor-  
88 mone levels (FSH, testosterone and prolactin) and secondary  
89 sexual characteristics. All these patients presented only with  
90 non-syndromic infertility without any other clinical features.  
91 Sperm analysis was carried out in the source laboratories  
92 during routine biological examination of the patients accord-  
93 ing to World Health Organization (WHO) guidelines (Wang  
94 et al. 2014). One patient (P1), who was identified to carry  
95 a homozygous *CFAP206* variant, is described in this study.  
96 P1 sperm morphology assessed with Papanicolaou staining  
97 and detailed semen parameters were performed. The patient  
98 originated from the Middle East (Iran) and was recruited

at the Royan Institute (Reproductive Biomedicine Research  
Center) for primary infertility in 2016 and was born to first-  
cousin parents. Informed consent was obtained from the  
patient and controls participating in the study according  
to local protocols and the principles of the Declaration of  
Helsinki. The study was approved by local ethics commit-  
tees, and samples were stored in the Fertithèque collection  
declared to the French Ministry of health (DC-2015-2580)  
and the French Data Protection Authority (DR-2016-392).

### Exome sequencing and bioinformatic analysis

Data processing of the whole cohort of 167 MMAF patients  
was performed according to our previously described proto-  
col (Coutton et al. 2019). In brief, the enrichment of coding  
regions together with intron/exon boundaries was performed  
with the Exon V6 kit (Agilent Technologies, Wokingham,  
UK). Sequencing was performed with Illumina HiSeq X by  
a service provider (Novogene, Cambridge, UK). Exomes  
data were analyzed using a bioinformatics pipeline devel-  
oped in-house using two modules, both distributed under the  
GNU General Public License v3.0 and available on GitHub:  
<https://github.com/ntm/grexome-TIMC-Primary> and <https://github.com/ntm/grexome-TIMC-Secondary> and as described  
in part in Martinez et al. (2020). Variants with a minor allele  
frequency greater than 1% in gnomAD v2.0, 3% in 1000  
Genomes Project phase 3, or 5% in NHLBI ESP6500 were  
filtered out and only variants predicted to have high-impact  
(e.g., stop-gain or frameshift variants) by variant Effect Pre-  
dictor v92 (McLaren et al. 2016) were scrutinized.

### Sanger sequencing

The identified variant in *CFAP206* was validated by Sanger  
sequencing performed on ABI 3500XL (Applied Biosys-  
tems). Data analyses were performed using SeqScape soft-  
ware (Applied Biosystems). Sequences of primers used are  
reported in Table S1. Unfortunately, no parental DNA was  
available to perform the segregation analysis.

### Quantitative real-time RT-PCR (RT-qPCR) analysis

RT-qPCR was performed with cDNAs from a panel of 6  
different human tissues and one human cDNAs reference  
(pooled tissues) purchased from Life Technologies®. Each  
sample was assayed in triplicate for each gene on a StepO-  
nePlus (LifeTechnologies®) with Power SYBR®Green PCR  
Master Mix (Life Technologies®). The PCR cycle was as fol-  
lows: 10 min at 95 °C, 1 cycle for enzyme activation; 15 s at  
95 °C, 60 s at 58 °C with fluorescence acquisition, 40 cycles  
for the PCR. Primer sequences and RT-qPCR conditions are  
indicated in Table S2. The efficacy of primers was checked  
using a standard curve. Melting curve analysis was used to

confirm the presence of a single PCR product. RT-qPCR data were normalized using the reference housekeeping gene *GAPDH* for human with the  $-\Delta\Delta C_t$  method (Livak and Schmittgen 2001). The  $2^{-\Delta\Delta C_t}$  value was set at 0 in the cDNAs pooled tissues, resulting in an arbitrary expression of 1. Statistics were performed using a two-tailed *t*-test on Prism 4.0 software (GraphPad, San Diego, CA) to compare the relative expression of *CFAP206* transcripts in several organs. Statistical tests with a two-tailed *P* value  $\leq 0.05$  were considered significant.

### Immunostaining in human sperm cells

Immunofluorescence (IF) experiments were performed using sperm cells from control individuals and from the individual carrying the *CFAP206* variant. Sperm cells were fixed in phosphate-buffered saline (PBS)/4% paraformaldehyde for 1 min at room temperature. After washing in 1 ml PBS, the sperm suspension was spotted onto 0.1% poly L-lysine pre-coated slides (Thermo Scientific). After attachment, sperm were permeabilized with 0.1% (v/v) Triton X-100–DPBS (Triton X-100; Sigma-Aldrich) for 5 min at RT. Slides were then blocked in 5% normal serum–DPBS (normal goat or donkey serum; GIBCO, Invitrogen) and incubated overnight at 4 °C with the following primary antibodies: rabbit polyclonal anti-WDR66 (HPA040005, Sigma-Aldrich, rabbit, 1:50, green), rabbit polyclonal anti-RSPH1 (HPA017382, Sigma-Aldrich, 1:100) and monoclonal mouse anti-acetylated- $\alpha$ -tubulin (T7451, Sigma-Aldrich, 1:2000). Washes were performed with 0.1% (v/v) Tween-20–DPBS, followed by 1-h incubation at room temperature with secondary antibodies. Highly cross-adsorbed secondary antibodies (Dylight 488 and Dylight 549, 1:1000) were from Jackson ImmunoResearch®. Appropriate controls were performed, omitting the primary antibodies. Samples were counterstained with 5 mg/ml Hoechst 33,342 (Sigma-Aldrich) and mounted with DAKO mounting media (Life Technology). Fluorescence images were captured with a confocal microscope (Zeiss LSM 710). Two hundred sperm cells were manually analyzed by two different experienced operators and the IF staining intensity and pattern were compared with a fertile control. The specificity of the anti-WDR66 and anti-RSPH1 antibodies was previously validated (Kott et al. 2013; Auguste et al. 2018).

### CRISPR/Cas9 KO mice

#### *Cfap206* knockout mouse model

The *Cfap206* knockout mouse model (C57BL/6) was created by CRISPR/Cas-mediated genome engineering. Briefly, the single-guide RNAs (sgRNAs) were designed against *Cfap206* exon 3–11 (Table S3). Cas9 mRNA and

sgRNA were prepared according to the reference (Yang et al. 2014). One-cell-stage embryos were collected and injected with prepared Cas9 mRNA and sgRNA. Then the injected embryos were further cultured in KSOM medium (Millipore, Cat. #MR-106-D) at 37 °C under 5% CO<sub>2</sub> until blastocyst stage and transferred into pseudopregnant female mice. A frameshift variant in *Cfap206* was detected by Sanger sequencing in the founder mouse and its offspring, and the primer sequences are available in Table S4. Male mice (aged 8–12 weeks) were used for subsequent experiments in this study. All animal experiments were carried out in accordance with the recommendation in the Guide for the Care and Use of Laboratory Animals of the National Institutes of health. The study was approved by the animal ethics committee at Anhui Medical University.

### Immunoblotting of mouse testis

Mouse testis samples were homogenized in radioimmuno-precipitation assay (RIPA) buffer (Beyotime) via an ultrasonic homogenizer and then heated at 100 °C for 10 min. The lysates were separated on 10% polyacrylamide gel by SDS-PAGE and transferred to PVDF (polyvinylidene fluoride) membrane. Then, the membrane was sealed for 1 h at 25 °C with 5% milk diluted with TBST (TBS-0.1% Tween-20, Sangon Biotech). Anti-*CFAP206* antibody (HPA044891, Atlas Antibodies) was diluted in TBST at 1:1,000 and incubated with the membranes at 4 °C overnight. The membranes were then washed in TBST three times and incubated with HRP-conjugated anti-Rabbit IgG antibody (M21002, Abmart, 1:10,000) in blocking solution for 1 h at room temperature. Enhanced chemiluminescence (ECL) (BL520A, Biosharp) was used for visualization.  $\beta$ -tubulin was used as a loading control.

### Histological analysis of mouse tissues

Fresh mouse testes were gently fixed with modified Davidson's solution (50% diluted water, 30% formaldehyde, 15% ethanol, and 5% glacial acetic acid) for over 48 h. After fixation, the tissue was dehydrated in gradient ethanol (70% ethanol for 24 h, 80% ethanol for 2 h, 90% ethanol for 2 h, and 100% ethanol for 1 h). Then, tissues were placed in xylene for 1 h and embedded in paraffin wax. Finally, sections were cut at a 3  $\mu$ m thickness. For H&E staining, sections were deparaffinated in xylene at 65 °C overnight. After deparaffinating, slides were stained with H&E, dehydrated, and mounted.

### Mating test

Fertility was investigated in wild-type and *Cfap206* knockout adult male mice. At least three male mice that were

241 8–12 weeks of age were analyzed in each group. Each male  
242 mouse and three wild-type C57BL/6 females (8–12 weeks  
243 of age) were caged. Vaginal plugs were checked every morn-  
244 ing. Once a vaginal plug was identified, the male mouse  
245 was allowed to rest for 2 days before another two females  
246 were placed in the cage. After mating, the female mice were  
247 separated and fed in a single cage and the pregnancy results  
248 and number of pups were recorded.

## 249 Mouse semen parameters and sperm morphological 250 analysis

251 For sperm morphology and parameters analyses of the  
252 mouse, spermatozoa were extracted from the cauda  
253 epididymis through dissection of adult male mice and  
254 diluted in 1 mL human tubal fluid (HTF, 90,126, Millipore)  
255 for 15 min at 37 °C. Sperm count, progressive sperm rate  
256 and motile sperm rate were further analyzed by a computer-  
257 assisted analysis system (IVOSII, Hamilton). At least three  
258 C57BL/6 male mice aged 8–10 weeks were analyzed in each  
259 group. Sperm from cauda epididymis were fixed and stained  
260 by H&E staining for the morphology analysis.

## 261 Electron microscopy evaluation

262 For scanning electron microscope (SEM) analysis of mouse  
263 sperm cells, cauda epididymis samples were prepared as pre-  
264 viously described (Liu et al. 2019b). In brief, mouse sperm  
265 specimens were deposited on poly-L-lysine-coated cover-  
266 slips, fixed in 2.5% glutaraldehyde, washed in 0.1 mol/L  
267 phosphate buffer, and post-fixed in osmic acid. The speci-  
268 mens were then progressively dehydrated with ethanol and  
269 isoamyl acetate gradient, then dried with a CO<sub>2</sub> critical-point  
270 dryer (Eiko HCP-2, Hitachi). Next, the specimens were  
271 mounted on aluminum stubs, sputter-coated using of an ionic  
272 sprayer meter (Eiko E-1020, Hitachi), and analyzed via SEM  
273 (Stereoscan 260) under an accelerating voltage of 20 kV.

274 For transmission electron microscope (TEM) analysis of  
275 mouse sperm cells, mouse semen samples were rinsed and  
276 were progressively dehydrated with graded ethanol (50%,  
277 70%, 90%, and 100%) and 100% acetone, followed by infil-  
278 tration with 1:1 acetone and SPI-Chem resin overnight at  
279 37 °C. After being embedded in Epon 812, the specimens  
280 were sliced with ultra-microtome, stained with uranyl acetate  
281 and lead citrate, and observed and photographed via TEM  
282 (Tecnai G2, FEI) with an accelerating voltage of 120 kV.

## 283 Intracytoplasmic sperm injection with the sperm 284 of wild-type and *Cfap206* knockout mice

285 Mice intracytoplasmic sperm injection (ICSI) was conducted  
286 as previously described (Liu et al. 2019a). Briefly, MII  
287 oocytes were collected from the oviduct of superovulated

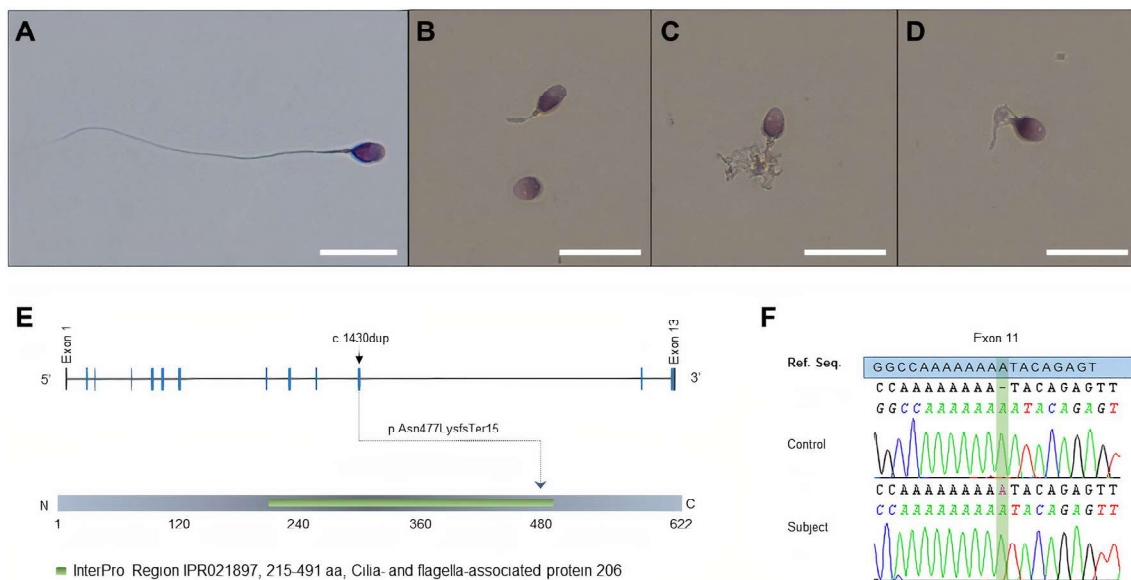
wild-type C57BL/6 female mice. Sperm were collected from  
wild-type and *Cfap206* knockout mice. Sperm heads only  
were injected into mouse oocytes by a Piezo driven pipette  
according to the previously reference (Ron-El et al. 1995).  
Then, the injected oocytes were cultured in KSOM medium  
at 37 °C under 5% CO<sub>2</sub>. Cleavage and blastocyst rates were  
further recorded around 24 h and 96 h, respectively.

## Results

### Exome sequencing identified bi-allelic variants in *CFAP206* in an MMAF patient

In the whole cohort of 167 MMAF patients, we previ-  
ously identified 77 patients (46%) with deleterious vari-  
ants in known MMAF-related genes (Lorès et al. 2021).  
After reanalysis of the remaining exomes, we identi-  
fied one additional subject with a homozygous variant in  
*CFAP206* (MIM \*609910), a gene not previously associ-  
ated with any pathology in human. Patient's sperm mor-  
phology and his detailed semen parameters are presented  
in Fig. 1A–D and Table 1, respectively. The patient had  
a frameshift variant c.1430dupA; p.Asn477LysfsTer15  
(NM\_001031743.2) (Fig. 1E; Table 1). The variant  
c.1430dupA is a single-nucleotide duplication predicted  
to induce a translational frameshift and a premature stop  
codon (p.Asn477LysfsTer15) expected to lead to the com-  
plete absence of the protein or the production of a truncated  
protein. The variant is present in the Genome Aggregation  
Database (gnomADv3.1) with a minor allele frequency  
(MAF) of  $6.63 \times 10^{-6}$ . The presence of this homozygous  
variant in the patient was also consistent with the known  
consanguinity of his parents (cousins). The variant identified  
by exome sequencing was validated by Sanger sequencing as  
illustrated in Fig. 1F. This *CFAP206* variant is deposited  
in ClinVar under reference SUB9294549. No other candidate  
variants reported to be associated with cilia, flagella or male  
fertility were otherwise detected.

*CFAP206* (also known as *c6orf165*) is located on chro-  
mosome 6 and contains 14 exons encoding cilia- and  
flagella-associated protein 206 (CFAP206), a predicted  
622-amino acid protein (Q8IYR0). *CFAP206* is predomi-  
nantly expressed in the testis according to data from GTEx  
(<https://gtexportal.org>) and described to be associated with  
cilia and flagella (Vasudevan et al. 2015; Beckers et al.  
2020). In addition, the encoded CFAP206 protein was  
detected in human sperm proteome (Wang et al. 2013)  
whereas it was found at a low level in human airway cilia  
(Blackburn et al. 2017). RT-qPCR experiments performed  
with a panel of six human tissues including other ciliated  
tissues such as trachea confirmed these results, showing



**Fig. 1** Morphology of normal and *CFAP206* mutant spermatozoa and presentation of the patient's variant. **A–D** Light microscopy analysis of spermatozoa from fertile control individuals (**A**) and the *CFAP206* patient (**B–D**). Most spermatozoa from the *CFAP206* patient have flagella that are short, absent, coiled or of irregular caliber. Scale bars: 10  $\mu$ m. **E** Structure of the canonical transcript of *CFAP206* show-

ing the position of the observed variant. The functional structure of the encoded protein is shown in the lower panel. *CFAP206*-domain (IPR021897) is highlighted in green. **F** Electropherograms from Sanger sequencing indicating the homozygous state of the identified variant c.1430dupA; p.Asn477LysfsTer15 (NM\_001031743.2) in *CFAP206*. Variants are annotated following HGVS recommendations

336 that *CFAP206* is largely overexpressed in the testis compared to all the other tested tissues (Fig. S1).

337  
338 To explore the ultrastructural defects induced by  
339 the c.1430dupA *CFAP206* variant in human sperm, we  
340 subsequently studied the presence of different proteins  
341 belonging to various axonemal substructures by immuno-  
342 fluorescence (IF). Taking into account the expected role  
343 of *CFAP206* in RS and CSC stability (Vasudevan et al.  
344 2015; Beckers et al. 2020), the presence of the follow-  
345 ing proteins was first investigated: RSPH1 as a marker of  
346 the radial spokes (RS) and WDR66, a protein localized  
347 in the CSC at the base of radial spoke 3 in *Tetrahymena*  
348 and *Chlamydomonas* (Urbanska et al. 2015). Due to the  
349 limited amount of sperm cells, no further IF experiments  
350 could be performed. In the patient's sperm cells, RSPH1  
351 staining was totally absent, dramatically reduced or dis-  
352 played an abnormal dotted and irregular pattern (Fig. 2A).  
353 In control sperm, WDR66 immunostaining decorated the  
354 full-length flagella, but in the *CFAP206* patient, WDR66  
355 staining was completely absent whereas tubulin staining  
356 remained detectable (Fig. 2B). These results demonstrate  
357 that RS and the CSC are strongly disorganized and support  
358 that *CFAP206* is a key axonemal component ensuring the  
359 stability of the CSC and the anchoring of the RS. Unfortu-  
360 nately, Transmission Electron Microscopy (TEM), which  
361 might provide evidence of the resulting defect, could not  
362 be performed due to the very low number of sperm cells  
363 available.

## CRISPR/Cas 9 KO mice

364  
365 Then, we assessed the impact of the absence of *CFAP206* on  
366 mouse by generating KO mice using the CRISPR-Cas9 tech-  
367 nology. We obtained a strain with a deletion of nine exons  
368 (exons 3–11) (Fig. S2A). RT-PCR and Western-blot analyses  
369 performed on testes from KO mouse *Cfap206*<sup>-/-</sup> confirmed  
370 the deleterious effect of the CRISPR/Cas9 induced deletion  
371 with the production of abnormal transcripts leading to the  
372 total absence of the protein (Fig. S2B, C). Reproductive phe-  
373 notypes were then studied. KO females were fully fertile  
374 and gave litters of normal size, contrary to KO males, which  
375 exhibited complete infertility when mated with females  
376 (Fig. 3A). The testis to body weight ratio was comparable  
377 between *Cfap206*<sup>+/+</sup> and *Cfap206*<sup>-/-</sup> male mice (Fig. 3B,  
378 C). As well, hematoxylin and eosin staining of testicular  
379 tissues showed no significant inter-group difference in the  
380 overall morphology of germ cells (Fig. S3). Total sperm  
381 counts obtained from the epididymes of *Cfap206*<sup>-/-</sup> male  
382 mice were significantly lower than those from *Cfap206*<sup>+/+</sup>  
383 male mice ( $3.92 \times 10^6 \pm 0.64$  versus  $22.63 \times 10^6 \pm 2.47$ ,  
384 respectively; \*\*\**P* < 0.001) (Fig. 3D; Table S5), and sperm  
385 from *Cfap206*<sup>-/-</sup> animals showed a significant decrease in  
386 total and progressive motility (Fig. 3E, F; Table S5). Con-  
387 sistent, these functional flagellum defects were associ-  
388 ated with morphological defects (Figs. 3G and S4). Sperm  
389 from *Cfap206*<sup>-/-</sup> males mainly had a flagellum of normal  
390 length (Table S5) but most of them showed abnormal forms,

**Table 1** Detailed semen parameters for the *CFAP206* patient

Individual	<i>CFAP206</i> variant (NM_001031743.2)	Semen parameters																
		Sperm volume (ml)	Sperm conc. (10 <sup>6</sup> /ml)	Total motility 1 h	Vitality	Normal spermatozoa	Absent flagella	Short flagella	Coiled flagella	Bent flagella	Flagella of irregular caliber	Tapered head	Thin head	Microcephalic	Macrocephalic	Multiple heads	Abnormal base	Abnormal acrosomal region
CFAP206_1	c.1430dupA	6	0.2	0	76	0	0	83	2	0	0	15	0	0	0	2	8	0
Reference limits <sup>a</sup>		1.5 (1.4–1.7)	15 (12–16)	40 (38–42)	58 (55–63)	23 (20–26)	5 (4–6)	17 (15–19)	13 (11–15)	2 (1–3)	3 (2–4)	14 (12–16)	7 (5–9)	1 (0–2)	2 (1–3)	42 (39–45)	60 (57–63)	

Values are percentages unless specified otherwise

NA not available

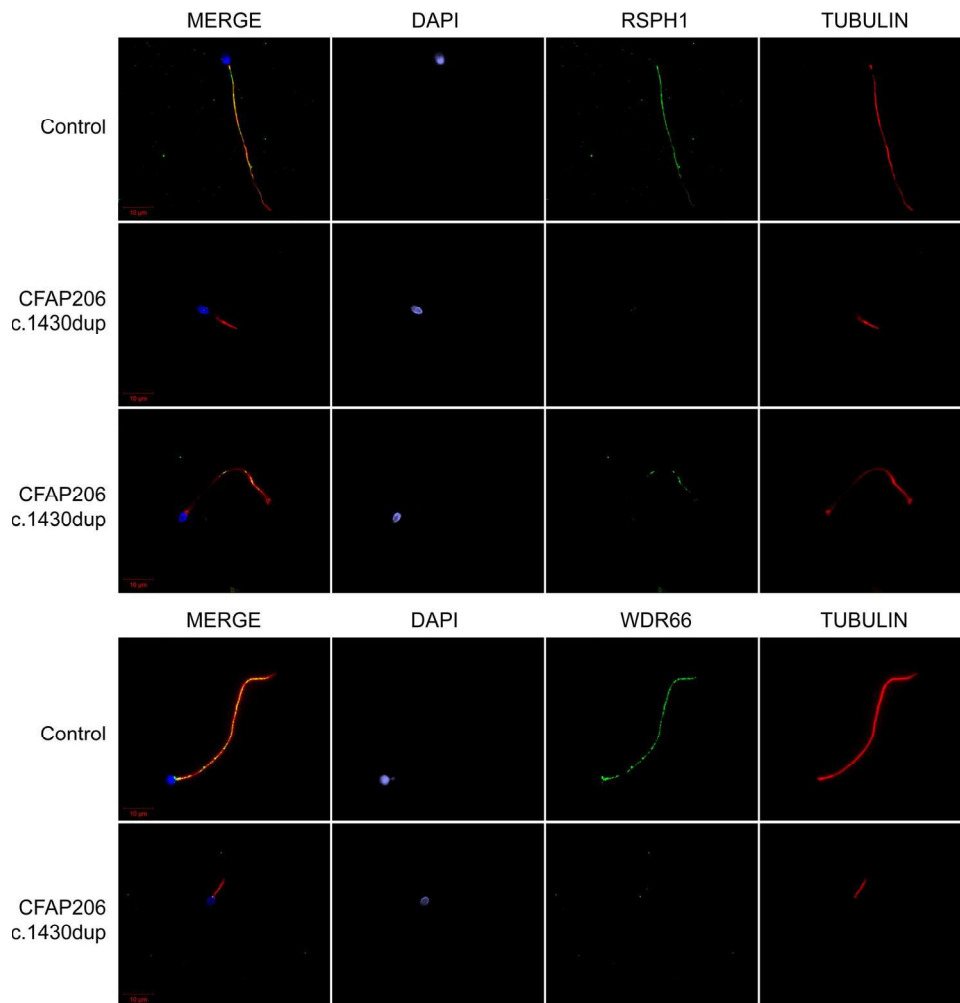
<sup>a</sup>Reference limits (5th centiles and their 95% confidence intervals) according to the World Health Organization<sup>18</sup>

sperm exhibiting bent and coiled flagella (Fig. 3G and S4; Table S5). We also observed a significant increase of sperm cells with absent or short flagella compared to the WT mice (Table S5). To visualize the impact of the absence of the CFAP206 protein on the flagellum ultrastructure, sperm from *Cfap206*<sup>-/-</sup> males were analyzed by TEM (Fig. S5). Some longitudinal sections of *Cfap206*<sup>-/-</sup> sperm showed a distorted axoneme (Fig. S5) which may be the cause or the consequence of the bent and coiled flagella. In addition, observations of transversal sections often revealed a deep disorganization with several defects such as lack of peripheral doublets, absence of the central pair or abnormal distribution of the DMTs or/and of the ODFs (Fig. S5E and F). No extra-reproductive features (including hydrocephaly) were observed in *Cfap206*<sup>-/-</sup> mice.

ICSI experiments performed with CFAP206 deficient sperm cells showed a significant lower rate of 2-cell embryos (56.18% vs 91.11%, respectively) and a dramatic decrease of blastocyst rate (7% vs 31.7%, respectively) compared to WT mice (Fig. 4; Table S6).

## Discussion

In the present work, we show that the presence of bi-allelic truncating *CFAP206* variants induces male infertility in human and mouse associated with morphological and motility defects demonstrating that CFAP206 is critical for sperm flagellum structure and function. Ultrastructure of the sperm flagellum was found to be deeply affected by the absence of CFAP206's protein. In *Cfap206*<sup>-/-</sup> mouse, TEM experiments revealed severe axoneme disorganization and periaxonemal defects (Fig. S5). In human, immunofluorescent analyses showed that the CFAP251/WDR66 was totally absent from sperm cells from our patient, supporting a role of CFAP206 in the assembly and/or the stability the CSC in mammalian sperm flagella (Fig. 2B). The CSC is a complex multi-protein structure located within the region that interconnects the bases of RS2, RS3, and the N-DRC (Urbanska et al. 2015) and is expected to mediate the regulatory signals between the radial spokes and the dynein arms, therefore regulating flagellar motility (Urbanska et al. 2015). Based on the localization of the CSC complex, at the base of the radial spokes, it was expected that the absence of CSC's members would result in radial spoke assembly defects (Dymek and Smith 2007). This assumption was confirmed by IF analyses which showed that the RS were severely and consistently impacted, although few sperm cells showed residual RSPH1 staining (Fig. 2A). Such results are consistent with the known localization of CFAP206 in the axoneme. Inactivation of the conserved ciliary protein FAP206 in the ciliate *Tetrahymena* resulted in slow cell motility. In addition, cryo-electron tomography showed that the 96 nm repeats



**Fig. 2** Radial spokes and the calmodulin- and spoke-associated complex are affected in the *CFAP206* patient. **A** Sperm cells from a fertile control individual and the *CFAP206* patient stained with anti-RSPH1 (HPA017382, Sigma-Aldrich, rabbit, 1:100, green), a protein located at the head of the radial spokes, and anti-acetylated tubulin (32–2500, ThermoFisher, mouse, 1: 1000, red) antibodies. DNA was counterstained with DAPI II. RSPH1 immunostaining is present throughout the flagellum in control sperm cells but is mainly absent or strongly reduced in *CFAP206* patients. In a few sperm cells the RSPH1 stain-

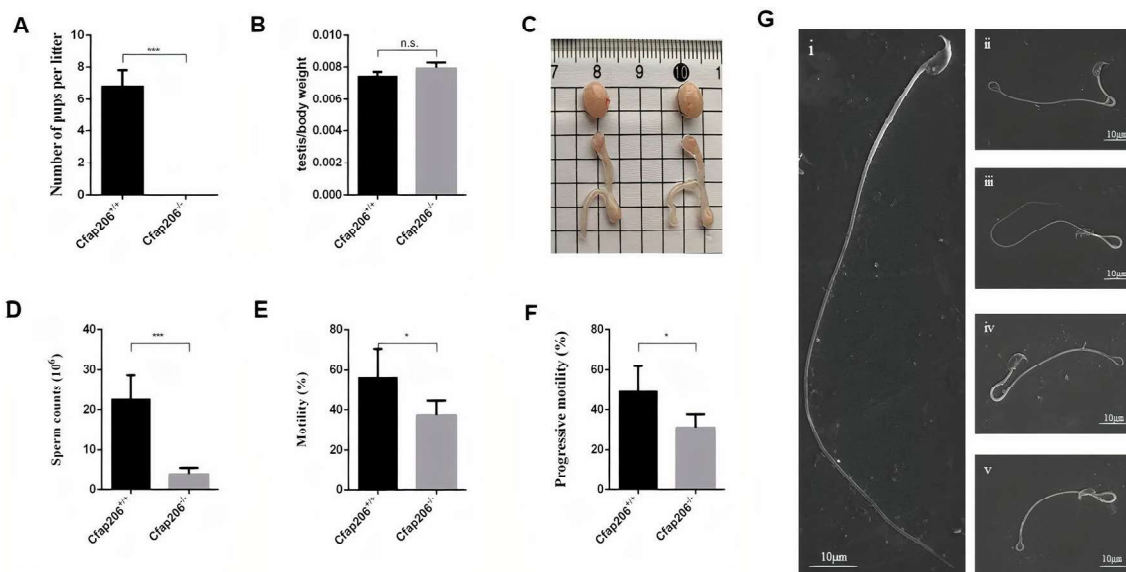
ing is present but displays an abnormal pattern with an irregular signal. Scale bars: 10  $\mu\text{m}$ . **B** Sperm cells from a fertile control individual and the *CFAP206* patient stained with anti-WDR66 (HPA040005, Sigma-Aldrich, rabbit, 1:50, green) and anti-acetylated tubulin (32–2500, ThermoFisher, mouse, 1: 1000, red) antibodies. DNA was counterstained with DAPI II. Contrary to the control, the WDR66 immunostaining is not detectable in the sperm flagellum from the *CFAP206* patient. Scale bars: 10  $\mu\text{m}$

441 along the axoneme, lacked the RS2 and the dynein c, sug-  
 442 gesting that FAP206 is essential for docking the RS2 and  
 443 dynein c to the microtubule. Interestingly, it was also dem-  
 444 onstrated that the assembly of the CSC component FAP91/  
 445 CaM-IP2 into the axoneme was dependent on FAP206  
 446 which was shown to be indirectly associated with the CSC  
 447 through the RSP3 protein (Vasudevan et al. 2015). Consist-  
 448 ently, we previously demonstrated that deleterious variants  
 449 in *MAATS1* encoding *CFAP91*, the human FAP91 ortholog,  
 450 led to a MMAF phenotype with similar axonemal defects  
 451 (lack of WDR66 and RSPH1) to what has been observed  
 452 here in the *CFAP206* affected patient (Martinez et al.  
 453 2020). Altogether, these observations strongly reinforce the

assumption that *CFAP206* is critical for sperm flagellum  
 axoneme assembly and stability through its essential role  
 within the CSC–RS complex and its interaction with other  
 axonemal partners such as *CFAP251/WDR66* or *CFAP91*  
 (Vasudevan et al. 2015; Beckers et al. 2020).

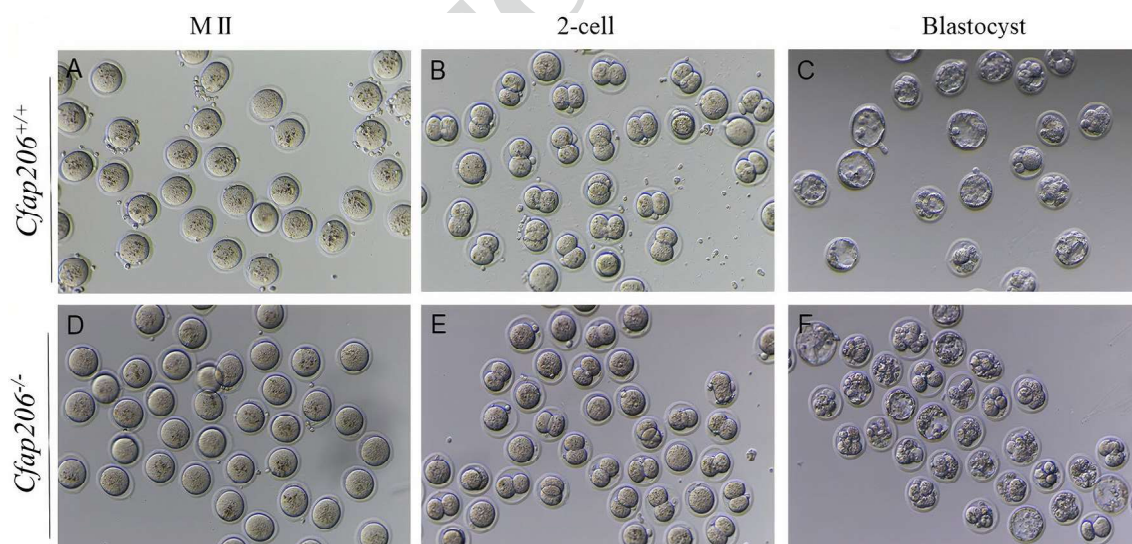
These data further reinforce the hypothesis that the  
 RS3–CSC complex is critical for the assembly and stability  
 of the sperm flagellum axoneme and its alteration constantly  
 lead to the MMAF phenotype. In our cohort, variants in  
 CSC-related genes (*CFAP251/WDR66*, *MAATS1* and now  
*CFAP206*) are found in about 11% of the tested subjects  
 suggesting that the CSC is the main structure affected in  
 MMAF patients (Kherraf et al. 2018; Martinez et al. 2020).





**Fig. 3** *Cfaf206* deficiency induces sperm flagellar abnormalities and infertility in male mice. **A** The mean number of the pups per litter was  $6.83 \pm 0.40$  in *Cfaf206*<sup>+/+</sup> male mice, whereas all the four *Cfaf206*<sup>-/-</sup> male mice were completely infertile. \*\*\* $P < 0.001$ . **B–C** The ratio of testes to body weight was comparable between *Cfaf206*<sup>+/+</sup> and *Cfaf206*<sup>-/-</sup> male mice. **D** Sperm counts ( $\times 10^6$ ) of *Cfaf206*<sup>-/-</sup> male mice was significantly lower compared to *Cfaf206*<sup>+/+</sup> male mice ( $3.92 \pm 0.64$  versus  $22.63 \pm 2.47$ , respectively; \*\*\* $P < 0.001$ ). **E** Sperm motility rate in *Cfaf206*<sup>+/+</sup> male mice was

$56.33 \pm 5.67\%$ , whereas the motility decreased to  $37.33 \pm 2.94\%$  in *Cfaf206*<sup>-/-</sup> male mice. \* $P < 0.05$ . **F** The progressive motility was significantly reduced in *Cfaf206*<sup>-/-</sup> male mice compared to the wild-type ( $31.00 \pm 2.75\%$  and  $49.17 \pm 5.17\%$  respectively, \* $P < 0.05$ ). **G** Sperm morphology using scanning electron microscopy of the *Cfaf206*<sup>+/+</sup> (**i**) and *Cfaf206*<sup>-/-</sup> male mice (**ii–v**). Most spermatozoa of *Cfaf206*<sup>-/-</sup> male mice presented with coiled and bent flagellum. Scale bars: 10  $\mu\text{m}$ . n.s. not significant



**Fig. 4** Representative two-cell embryos and blastocysts obtained following intracytoplasmic sperm injection (ICSI) carried out with spermatozoa from *Cfaf206*<sup>+/+</sup> and *Cfaf206*<sup>-/-</sup> male mice

467 Interestingly, although the axoneme is a common structure  
468 shared between cilia and flagellum, we observed that MMAF  
469 patients only present with an isolated infertility without  
470 any other clinical features or ciliopathies suggesting that,

in other ciliated cells, these axonemal MMAF-related pro- 471  
teins are dispensable for the structure and function of cilia 472  
and that axonemal biogenesis/structure of sperm flagella 473  
and cilia may require different proteins and mechanisms 474

475 in particular regarding the RS3–CSC complex (Touré  
476 et al. 2021). Interestingly, another team recently published  
477 another mouse model inactivated for the *Cfap206* gene and  
478 evidenced a similar sperm phenotype. Electron tomogra-  
479 phy on cryo-conserved sperm flagella revealed defects in  
480 the repetitive pattern of radial spokes with only one RS per  
481 96 nm repeat confirming that CFAP206 is needed for the  
482 establishment of radial spokes in mammalian sperm flagella.  
483 In addition, the authors reported that about 80% of KO mice  
484 developed externally visible enlarged cranial vaults, suggest-  
485 ing ventricular dilatation and hydrocephalus. In addition,  
486 these mutant mice presented mucus accumulation in nasal  
487 cavities and a significant increase in ciliary beat frequency  
488 compared to wild-type (Beckers et al. 2020). These extra-  
489 reproductive observations are in opposition with the clinical  
490 features observed in our mouse model and in our patient carry-  
491 ing the bi-allelic truncating variations in *CFAP206* who  
492 presented only isolated infertility without any other clinical  
493 features. We do not believe that this phenotypic discrep-  
494 ancy can be caused by a residual CFAP206 activity in our  
495 patient nor in our KO mice, as all carried a clear cut loss of  
496 function variant similar to that induced in Beckers et al.'s  
497 mice. Such phenotypic discrepancies between patients with  
498 MMAF phenotype or two different mouse models inactiv-  
499 ated for the same gene have been described previously  
500 (Ben Khelifa et al. 2014; Coutton et al. 2018; Morimoto  
501 et al. 2019; Rachev et al. 2020; Touré et al. 2021). These  
502 observations might be explained by different mouse genetic  
503 background, various genome-editing method or unknown  
504 off-target effect. Alternatively, we cannot exclude a slight  
505 effect on ciliary beating in MMAF patients without patho-  
506 logical consequences, or at least none that have been noticed  
507 by the affected men themselves. Nasal brushings or curette  
508 biopsies from affected MMAF individuals could be useful  
509 to formally explore this possibility.

510 It was generally accepted that MMAF affected individu-  
511 als have a good prognosis following ICSI, in particular for  
512 patients carrying mutations in genes encoding axonemal pro-  
513 teins (Wambergue et al. 2016; Touré et al. 2021). In contrast  
514 to what was observed for most other MMAF patients, the  
515 patient carrying the *CFAP206* mutation did not achieve any  
516 pregnancy when his sperm was used for ICSI. Furthermore,  
517 ICSI experiments performed in *Cfap206*<sup>-/-</sup> mouse showed a  
518 dramatic decrease in early division and blastocyst rate com-  
519 pared to WT, suggesting that CFAP206 deficiency may also  
520 affect early embryonic development (Fig. 4; Table S6). This  
521 effect on early embryonic development following IVF was  
522 also observed in the previously published *Cfap206*<sup>-/-</sup> mouse  
523 line (Beckers et al. 2020). This very low rate of embryo  
524 development is a clear additional factor compromising the  
525 fertility of *CFAP206*-mutated patients as it was observed  
526 in the *Cfap206*<sup>-/-</sup> mouse. Beckers et al. (2020) previously  
527 demonstrated that CFAP206 also localized to the basal body/

centrosome of motile cilia. Interestingly, unsuccessful ICSI 528  
were already reported in MMAF patients with mutations 529  
impacting centrosomal proteins (Touré et al. 2021). These 530  
data provide further support to the assumption that a poor 531  
ICSI prognosis is expected for MMAF patients with muta- 532  
tion in genes encoding centrosomal proteins which persist 533  
after fertilization and are required for embryo development 534  
conversely to axonemal proteins. However, no basal body 535  
defects could be evidenced in *Cfap206*<sup>-/-</sup> sperm cells using 536  
IF and TEM (data not shown). These results should be con- 537  
firmed with further cases and experimentation but suggest 538  
that *CFAP206* mutations may constitute an adverse factor to 539  
obtain pregnancies with ICSI and may modify the clinical 540  
management of *CFAP206*-mutated MMAF patient. 541

542 Overall, these data demonstrate that CFAP206 is essential  
543 for normal sperm flagellum structure and function in human  
544 and mouse, and that variants in *CFAP206* lead to severe fla-  
545 gellum malformations and may also cause poor early embryo  
546 development resulting in primary male infertility.

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551 **Author contribution** All authors contributed to the study conception  
552 and design. CCo, YXC, PFR, FZ, XJH and XA designed and super-  
553 vised the study. AA-Y, VS recruited the patients, performed clinical  
554 analysis and characterization. NTM, ZEK, PFR, TC and CCo per-  
555 formed exome and bioinformatics analysis. GM, JB, CCA, MB and  
556 TC performed patient's experimental work. HBL and QSS provided  
557 *Cfap206* knockout mice. BS, MRL and QSS performed animal's experi-  
558 mental work. QSS and DL performed ICSI of mice. GM, CCA, KKL,  
559 FBT and XJH performed data illustrations. XA, FZ, PFR, AT, MW,  
560 CA, HW and CCo analyzed the data. XA, GM, QSS and CCo wrote  
561 the manuscript. The first draft of the manuscript was written by XA,  
562 GM and CCo, and all authors commented on previous versions of the  
563 manuscript. All authors read and approved the final manuscript.

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572 **Data availability** The datasets generated during and/or analyzed dur-  
573 ing the current study are available from the corresponding author on  
574 reasonable request. Accession numbers: The *CFAP206* variant reported  
575 in this manuscript is accessible in ClinVar with the submission number  
576 SUB9294549.


577 **Declarations**

578 **Conflict of interest** On behalf of all authors, the corresponding author  
579 states that there is no conflict of interest.

- 580 **Ethical approval** The study was approved by local ethics committees,  
581 and samples were then stored in the CRB Germethèque (certification  
582 under ISO-9001 and NF-S 96-900) following a standardized procedure  
583 or were part of the Fertithèque collection declared to the French  
584 Ministry of health (DC-2015-2580) and the French Data Protection  
585 Authority (DR-2016-392).
- 586 **Animal research** All animal procedures were run according to the Chi-  
587 nese guidelines on the use of animals in scientific investigations with  
588 the approval of the animal Ethics Committee at the First Affiliated  
589 Hospital of Anhui Medical University (P2020-12-36).
- 590 **Consent to participate** Informed consent was obtained from all indi-  
591 viduals included in the study.
- 592 **Consent to publish** Agreement for data publication was obtained from  
593 all individuals included in the study.
- 594 **Web resources** ClinVar, <https://www.ncbi.nlm.nih.gov/clinvar/>  
595 gnomAD Browser, <http://gnomad.broadinstitute.org>  
596 GTEx, <https://gtexportal.org>  
597 Uniprot, <https://www.uniprot.org/>
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## Authors and Affiliations

Qunshan Shen<sup>1,2,3</sup> · Guillaume Martinez<sup>4,5</sup> · Hongbin Liu<sup>6</sup> · Julie Beurois<sup>4</sup> · Huan Wu<sup>1,2,3</sup> · Amir Amiri-Yekta<sup>7</sup> · Dan Liang<sup>1,2,3</sup> · Zine-Eddine Kherraf<sup>4,8</sup> · Marie Bidart<sup>4,9</sup> · Caroline Cazin<sup>4</sup> · Tristan Celse<sup>4,5</sup> · Véronique Satre<sup>4,5</sup> · Nicolas Thierry-Mieg<sup>10</sup> · Marjorie Whitfield<sup>4</sup> · Aminata Touré<sup>4</sup> · Bing Song<sup>1,2,3</sup> · Mingrong Lv<sup>1,2,3</sup> · Kuokuo Li<sup>1,2,3</sup> · Chunyu Liu<sup>11,12</sup> · Fangbiao Tao<sup>2,3</sup> · Xiaojin He<sup>1,2,3</sup> · Feng Zhang<sup>11,12</sup> · Christophe Arnoult<sup>4</sup> · Pierre F. Ray<sup>4,8</sup> · Yunxia Cao<sup>1,2,3</sup> · Charles Coutton<sup>4,5,13</sup> 

<sup>1</sup> Reproductive Medicine Center, Human Sperm Bank, Department of Obstetrics and Gynecology, the First Affiliated Hospital of Anhui Medical University, Hefei 230022, China

<sup>2</sup> NHC Key Laboratory of Study on Abnormal Gametes and Reproductive Tract (Anhui Medical University), Hefei 230032, China

<sup>3</sup> Key Laboratory of Population Health Across Life Cycle (Anhui Medical University), Ministry of Education of the People's Republic of China, Hefei 230032, China

<sup>4</sup> Université Grenoble Alpes, INSERM U1209, CNRS UMR 5309, Institute for Advanced Biosciences, Team Genetics Epigenetics and Therapies of Infertility, 38000 Grenoble, France

<sup>5</sup> CHU Grenoble Alpes, UM de Génétique Chromosomique, 38000 Grenoble, France

<sup>6</sup> Center for Reproductive Medicine, Cheeloo College of Medicine, Shandong University, Jinan 250012, China

<sup>7</sup> Department of Genetics, Reproductive Biomedicine Research Center, Royan Institute for Reproductive Biomedicine, ACECR, Tehran, Iran

<sup>8</sup> CHU Grenoble Alpes, UM GI-DPI, 38000 Grenoble, France

<sup>9</sup> Unité Médicale de Génétique Moléculaire: Maladies Héritaires et Oncologie, Pôle Biologie, Institut de Biologie et de Pathologie, CHU Grenoble Alpes, 38000 Grenoble, France

<sup>10</sup> Université Grenoble Alpes, CNRS UMR 5525, TIMC-IMAG/BCM, 38000 Grenoble, France

<sup>11</sup> Obstetrics and Gynecology Hospital, NHC Key Laboratory of Reproduction Regulation (Shanghai Institute of Planned Parenthood Research), State Key Laboratory of Genetic Engineering at School of Life Sciences, Fudan University, Shanghai 200011, China

<sup>12</sup> Shanghai Key Laboratory of Female Reproductive Endocrine Related Diseases, Shanghai 200011, China

<sup>13</sup> Laboratoire de Génétique Chromosomique, Hôpital Couple-Enfant, CHU de Grenoble, 38043 Grenoble, France