Absence of CFAP69 Causes Male Infertility due to Multiple Morphological Abnormalities of the Flagella in Human and Mouse

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The multiple morphological abnormalities of the flagella (MMAF) phenotype is among the most severe forms of sperm defects responsible for male infertility. The phenotype is characterized by the presence in the ejaculate of immotile spermatozoa with severe flagellar abnormalities including flagella being short, coiled, absent, and of irregular caliber. Recent studies have demonstrated that MMAF is genetically heterogeneous, and genes thus far associated with MMAF account for only one-third of cases. Here we report the identification of homozygous truncating mutations (one stop-gain and one splicing variant) in CFAP69 of two unrelated individuals by whole-exome sequencing of a cohort of 78 infertile men with MMAF. CFAP69 encodes an evolutionarily conserved protein found at high levels in the testis. Immunostaining experiments in sperm from fertile control individuals showed that CFAP69 localized to the midpiece of the flagellum, and the absence of CFAP69 was confirmed in both individuals carrying CFAP69 mutations. Additionally, we found that sperm from a Cfp69 knockout mouse model recapitulated the MMAF phenotype. Ultrastructural analysis of testicular sperm from the knockout mice showed severe disruption of flagellum structure, but histological analysis of testes from these mice revealed the presence of all stages of the seminiferous epithelium, indicating that the overall progression of spermatogenesis is preserved and that the sperm defects likely arise during spermiogenesis. Together, our data indicate that CFAP69 is necessary for flagellum assembly/stability and that in both humans and mice, biallelic truncating mutations in CFAP69 cause autosomal-recessive MMAF and primary male infertility.

Introduction

Human male infertility remains a persistent problem, affecting an estimated 15% of couples.1 Male infertility often manifests as decreased sperm count (oligozoospermia), decreased sperm motility (asthenozoospermia), a higher proportion of morphologically defective sperm in the ejaculate (teratozoospermia), or a combination of the above defects. An estimated 30%–50% of male infertility cases have a genetic component.2 Thus, considerable effort has been made to identify and characterize the large number of genes required for sperm development. The advent of high-throughput sequencing (HTS) technologies has greatly facilitated the identification in infertile males of potentially responsible genes, but detailed studies of the functions of these genes in spermatogenesis is generally not possible in humans, requiring animal models instead.3,4 Indeed, many mutant mouse models with defective spermatogenesis have been characterized, and molecular and genetic studies of sperm development in these models have yielded a deeper understanding of spermatogenesis.5

Multiple morphological abnormalities of the flagella (MMAF) phenotype is one of the most severe forms of qualitative sperm defects responsible for male infertility.6 This peculiar phenotype is characterized by the presence in the ejaculate of immotile spermatozoa presenting with several severe abnormalities of the sperm flagellum, including being short, coiled, absent, and of irregular caliber. Whole-exome sequencing (WES) analysis revealed that mutations in DNAH1 (MIM: 603332), CFAP43 (MIM: 617558), and CFAP44 (MIM: 617559) are frequently found in MMAF-affected individuals and account for about one-third of MMAF cases.7–11 These results established a strong...
genetic component for MMAF. They also demonstrated that MMAF is genetically heterogeneous and that other relevant genes still await identification. In a large cohort of 78 infertile male individuals with MMAF, we have now identified two unrelated individuals harboring homozygous truncating mutations in CFAP69 in addition to identifying individuals with mutations in the known MMAF genes, indicating that this gene is likely to be important for sperm flagellum morphogenesis and male fertility. In parallel, we characterized a Cfap69 knockout mouse model, which we found to recapitulate the MMAF phenotype. Overall, our work demonstrates that CFAP69 is required for sperm flagellum assembly/stability and that truncating mutations of CFAP69 induce autosomal-recesive MMAF and primary male infertility.

Subjects and Methods

Human Case and Control Subjects
WES was performed for a large cohort of 78 MMAF-affected individuals as previously described. All individuals presented with a typical MMAF phenotype characterized by severe asthenozoospermia (total sperm motility below 10%) with >5% of sperm having at least three flagellar abnormalities (absent, short, coiled, bent, or irregular flagella).

Individual CFAP69_1 is from Iran and was treated in Tehran at the Royan Institute for primary infertility from 2008 to 2015. Individual CFAP69_2 is from North Africa and consulted for primary infertility at the Cochin University Hospital in Paris (France) from 2015 to the present. Both individuals were born to first-cousin parents, had normal somatic karyotypes (46,XY), and were negative for Y chromosome Azoospermia Factor (AZF) microdeletions.

Sperm analysis was carried out in the source laboratories during routine biological examination of the individuals according to World Health Organization (WHO) guidelines. The morphology of the individuals’ sperm was assessed with Papanicolaou staining (Figures 1A–1C). The detailed semen parameters of the two CFAP69 individuals are presented in Table 1, and the average semen parameters of the studied MMAF cohort according to genotype are described in Table S1.

Informed consent was obtained from all the subjects participating in the study according to local protocols and the principles of the Declaration of Helsinki. Sperm samples were obtained following informed consent from both individuals CFAP69_1 and CFAP69_2. The study was approved by local ethics committees, and the samples were then stored in the CRB Germethque (certification under ISO-9001 and NF-S 96-900) following a standardized procedure. Sperm samples from fertile individuals with normal spermograms were obtained from CRB Germethque. Consent for CRB storage was approved by the CPCR Sud-Ouest of Toulouse (coordinator of the multi-site CRB Germethque).

Whole-Exome Sequencing (WES) and Bioinformatic Analysis
WES and bioinformatic analysis were performed according to our previously described protocol. For details, see Supplemental Material and Methods.

Sanger Sequencing
CFAP69 mutations identified by WES were validated by Sanger sequencing. PCR primers and protocols used for each individual are listed in the Table S2. Sequencing reactions were carried out with BigDye Terminator v3.1 (Applied Biosystems). Sequencing was carried out on ABI 3130XL (Applied Biosystems). Sequences were analyzed using Seqscape software (Applied Biosystems).

Quantitative Real-Time RT-PCR (RT-qPCR) Analysis
RT-qPCR was performed with cDNAs from various human tissues purchased from Life Technologies. A panel of seven organs was used for experiments: testis, brain, lung, kidney, liver, intestine, and heart. Each sample was assayed in triplicate for each gene on a StepOnePlus (LifeTechnologies) with Power SYBR Green PCR Master Mix (Life Technologies). The PCR cycle was as follows: 10 min at 95°C; 1 cycle for enzyme activation; 15 s at 95°C; 60 s at 60°C with fluorescence acquisition, 40 cycles for the PCR. RT-qPCR data were normalized using the reference housekeeping gene ACTB for human with the $\Delta\Delta C_t$ method. The $2^{-\Delta\Delta C_t}$ value was set at 0 in brain cells, resulting in an arbitrary expression of 1. Primer sequences and RT-qPCR conditions are indicated in Table S3. The efficacy of primers was checked using a standard curve. Melting curve analysis was used to confirm the presence of a single PCR product. Statistics were performed using a two-tailed t test on Prism 4.0 software (GraphPad) to compare the relative expression of CFAP69 transcripts in several organs. Statistical tests with a two-tailed p value ≤ 0.05 were considered significant.

RT-PCR Analysis
Total RNA from whole blood from individual CFAP69_1 was extracted using the mirVana PARIS Kit (Life Technologies) according to the manufacturer’s protocol. Reverse transcription was carried out for individual CFAP69_1 and two healthy control subjects (C1 and C2) with 5 µL of extracted RNA (approximately 500 ng). Hybridization of the oligo-dT was performed by incubation for 5 min at 65°C and quenching on ice in the following mix: 5 µL of RNA, 3 µL of poly T oligo primers (dT)12–18 (10 mM, Pharmaclia), 3 µL of the four dNTPs (0.5 mM, Roche Diagnostics), and 2.2 µL of H2O. Reverse transcription was then carried out for 30 min at 55°C after the addition of 4 µL of 5X buffer, 0.5 µL RNase Inhibitor, and 0.5 µL of Transcriptor Reverse transcriptase (Roche Diagnostics). 2 µL of the obtained cDNA mix was used for subsequent PCR. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a housekeeping gene (internal control). CFAP69 primer sequences and RT-PCR conditions are indicated in the Table S4.

Immunostaining in Human Sperm Cells
Immunofluorescence (IF) experiments were performed using sperm cells from control individuals and from one or both individuals carrying CFAP69 mutations. 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-CFAP69 (ab171156, Abcam, 1:200), mouse monoclonal anti-Hsp60 (ab13532, Abcam, 1:500), rabbit polyclonal...
anti-SPAG6 (HPA038440, Sigma-Aldrich, 1:500), rabbit polyclonal anti-SPEF2 (HPA040343, Sigma-Aldrich, 1:500), rabbit polyclonal anti-DNAH5 (HPA037470, Sigma-Aldrich, 1:100), rabbit polyclonal anti-DNALI1 (HPA028305, Sigma-Aldrich, 1:100), and monoclonal mouse anti-acetylated-α-tubulin (T7451, Sigma-Aldrich, 1:2,000). Washes were performed with 0.1% (v/v) Tween 20-DPBS, followed by 1 hr incubation at room temperature with secondary antibodies. Highly cross-adsorbed secondary antibodies (Dylight 488 and Dylight 549, 1:1,000) were from Jackson Immunoresearch. Appropriate controls were performed, omitting the primary antibodies. Samples were counterstained with 5 mg/mL Hoechst 33342 (Sigma-Aldrich) and mounted with DAKO mounting media (Life Technologies). Fluorescence images were captured with a confocal microscope (Zeiss LSM 710).

Animals
For all experiments involving mice, animals were handled and euthanized in accordance with methods approved by the Animal Care and Use Committees of each applicable institution. All mice used were adult (6 weeks or older) male mice.

Generation of Ckap69
tm1b/tm1b Mice
We obtained Ckap69	m1a mice as previously described. The Ckap69	m1a mouse line carries a Ckap69 knockout first allele, in which a promoterless, gene-trapping cassette including the LacZ and neo genes was inserted in introns 4–5 of Ckap69, resulting in a floxed exon 5. To generate the Ckap69	m1b allele, in which exon 5 and neo are excised, resulting in truncation of Ckap69, but LacZ remains as a reporter, Ckap69	m1a mice were crossed with the E2a-cre mouse line (B6.FVB-Tg(EIIa-cre)C5379Lmgd/J, The Jackson Laboratory), which has early embryonic expression of cre recombinase leading to ubiquitous recombination. Genotyping was performed with two primer pairs: for the Ckap69	m1b allele, CTCCAGTGAAAGCCCACCT (forward) and CGGTCGCTA CCATTACCAGT (reverse) with an expected product size of ~450 bp; for the WT Ckap69 allele, ATGACCTAGGATTCAA...
TAAGCTTGATCT (forward) and CGGCTGCAACTGGAATCAGA (reverse) with an expected product size of ~300 bp.

**X-gal Staining and Imaging**

To collect tissue, mice were deeply anesthetized with Avertin (2, 2, 2-tribromoethanol in 2-methyl-2-butanol) and then perfused transcardially first with PBS (pH 7.4) followed by 4% paraformaldehyde (PFA) in PBS. Testes and epididymides were dissected out, and the tunica albuginea of the testis was punctured several times with a needle at each pole. Tissues were then immersed in 4% PFA at 2 hr at 4°C. For whole-mount X-gal staining, testes and epididymides were washed in PBS and directly immersed in X-gal staining solution (1 mg/mL X-gal, 2 mM MgCl₂, 0.01% sodium deoxycholate, 0.02% NP-40, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide) for 48 hr at 37°C. To prepare tissue cryosections, testes and epididymides were washed in PBS before being cryoprotected in 30% (w/v) sucrose in PBS for 48 hr. The tissues were then embedded in Tissue-Tek O.C.T. (Sakura, 4583) and cut into 12 μm sections, which were adhered to glass slides. Sections were fixed for 5 min in 4% PFA at room temperature, washed in PBS, and immersed in X-gal staining solution for 24 hr at 37°C. Sections were then fixed in 4% PFA at room temperature for 10 min, washed in PBS, and blocked in 5% normal goat serum and 0.1% Triton X-100 in PBS for 1 hr at room temperature. Alexa Fluor 488-conjugated peanut agglutinin (PNA) (Thermo Fisher Scientific, L32458) (1:200) was applied to the sections for 1 hr at room temperature. Alexa Fluor 488-conjugated peanut agglutinin (PNA) (Thermo Fisher Scientific, L32458) (1:200) was diluted in blocking solution and applied to sections overnight at 4°C. Sections were washed in PBS with 0.1% Tween-20, counterstained in DAPI (1 μg/mL in PBS) for 5 min, and mounted in Fluoromount (Sigma-Aldrich, F4680).

Fluorescent X-gal imaging using confocal microscopy was performed as previously described. Imaging was performed on a Zeiss LSM 700 confocal microscope. X-gal inclusions were excited with a 637 nm laser, and emitted fluorescent signal between 650 and 770 nm was recorded. Transmitted light signal was also recorded. PNA and DAPI were simultaneously imaged using standard methods. To produce the final fluorescent image of X-gal staining, the transmitted light image was subtracted from the emitted light image using the “Image Calculator” function in ImageJ.

**Western Blotting**

Whole testes were homogenized in 2× Laemmli buffer (65.8 mM Tris-HCl, 26.3% glycerol, 2.1% SDS, 0.01% bromophenol blue, 10% 2-mercaptoethanol [pH 6.8]) using a dounce homogenizer and then heated at 95°C for 10 min. Lysates were fractionated by SDS-PAGE on 8% polyacrylamide gels and transferred to polyvinylidene fluoride membranes by wet transfer in Towbin Buffer without methanol (2,2,2-tribromoethanol in 2-methyl-2-butanol) and then washed in PBS. The tissue was incubated at 37°C for 30 min before the sperm-containing supernatant was collected.

**Mouse Epididymal Sperm Collection**

Male mice were deeply anesthetized with Avertin. To collect sperm, the cauda epididymides were dissected out and minced in PBS. The tissue was incubated at 37°C for 30 min before the sperm-containing supernatant was collected.

**Scanning Electron Microscopy**

Sperm were deposited on poly-L-lysine coated coverslips, fixed in 2.5% glutaraldehyde, and post-fixed in osmium tetroxide. Coverslips were washed in distilled water and dehydrated through cold 50%, 70%, 95%, and 100% ethanol. Coverslips were then dried at critical point in a Tousimis Autosamdri-810 Critical Point Dryer, mounted onto specimen stubs, and sputter-coated with palladium before being viewed with a FEI Quanta ESEM 200 scanning electron microscope.

**Transmission Electron Microscopy**

Tissue was prepared for transmission electron microscopy (TEM) according to standard protocols. Briefly, mice were deeply anesthetized with Avertin and perfused transcardially with PBS and then 5% glutaraldehyde (in 0.1 M phosphate buffer [pH 7.4]). Testes were dissected out and cut into pieces no more than 2 mm thick in any dimension before being immersed in 5% glutaraldehyde overnight at 4°C. Samples were then washed in phosphate buffer, post-fixed in osmium tetroxide for 1 hr at 4°C, washed again, and dehydrated through 1 change each of cold 50%, 70%, 95% ethanol, and 3 changes of 100% ethanol for 15 min each. Tissue fragments were then immersed in 2 changes of propylene oxide, 15 min each, before being placed in a 1:1 propylene oxide: EMbed812 (Electron Microscopy Sciences, 14120) mixture overnight under vacuum. Samples were further infiltrated with EMbed812 for 6 hr under vacuum before being placed in fresh EMbed812. Blocks were polymerized at 60°C for 48 hr. Pale gold thin sections were cut, collected on copper grids, and stained with uranyl acetate and lead citrate. Images were obtained using a Phillips EM 420 transmission electron microscope.

**Histology**

Tissue was prepared the same way as for TEM analysis. Semi-thin sections 1–2 μm in thickness were cut from EMbed812-embedded testis blocks with glass knives and placed on drops of water on glass slides. The slides were then placed on a hot plate at low heat for about 30 s to dry the water and adhere the sections to the slides. The sections were then covered in a 1% solution of toluidine blue in 2% sodium tetraborate for an additional 30 s. The slides were then removed from the hot plate, rinsed in distilled water, and mounted in EMbed812.

**Results**

**Whole-Exome Sequencing (WES) Identifies Homozygous Truncating Mutations in **CFAP69** in MMAF Individuals**

In the cohort of 78 MMAF-affected individuals we analyzed, 22 individuals were identified with harmful mutations in the known MMAF-related genes DNAH1, CFAP43, or CFAP44. After applying stringent filters and reanalysis of the remaining exomes, we identified two individuals with truncating mutations in CFAP69, which
accounts for 2.5% of our cohort (Figure 1D, Table S5). *Cfap69* was reported in public databases to be strongly expressed in the testis and connected with cilia or the flagellum. We confirmed these data by RT-qPCR experiments in human tissue panels, which indicate that expression of *Cfap69* mRNA in testis is predominant and is significantly higher than in other tested tissues (Figure S1). For both individuals, no variants with low frequency in control databases were identified in other genes reported to be associated with cilia, flagella, or male fertility. We therefore focused on *Cfap69*, which appeared to be an excellent MMAF candidate.

*Cfap69* (formerly known as *c7orf63*; GenBank: NM_001039706) is located on chromosome 7 and contains 23 exons encoding a predicted 941-amino acid protein (ASD8W1). The two *Cfap69* variants were found in two unrelated individuals. These two variants were absent from control sequence databases (dbSNP, 1000 Genomes Project, NHLBI Exome Variant Server, gnomAD, and inhouse database). The variant identified in individual *Cfap69*_1 is a splicing variant c.860+1G>A, altering a consensus splice donor site of *Cfap69* exon 8. The variant identified in individual *Cfap69*_2 was a stop-gain mutation c.763C>T (p.Gln255Ter) located in exon 8 (Figure 1D, Table S5). The presence of the two variants was confirmed by Sanger sequencing of *Cfap69* exon 8 in both individuals (Figure S2). According to the Human Splicing Finder, the c.860+1G>A mutation abrogates the consensus donor site, leading to altered splicing and subsequent frameshift. No *Cfap69* mRNA could be detected by RT-PCR in individual *Cfap69*_1 (Figure S3), possibly indicating a frameshift and premature translation termination leading to nonsense-mediated decay.

We compared semen parameters of individuals carrying *Cfap69* mutations with those of individuals carrying mutations in previously described MMAF-related genes in order to test for potential phenotype-genotype correlation (Table S5). Although there were no significant statistical differences between the semen parameters of the *Cfap69* individuals and MMAF-affected individuals with mutations in DNAH1, *Cfap43*, or *Cfap44*, we observe very low sperm concentrations and total sperm counts in the ejaculates from both *Cfap69*-mutant individuals. Compared to sperm from control samples (Figure 1A), sperm from both individuals showed severe defects characteristic of MMAF (Figures 1B and 1C). A high rate of head malformations, in particular thin heads and an abnormal acrosomal region, were also observed (Figure 1C, Tables 1 and S1). Importantly, when selecting sperm for intracytoplasmic sperm injection by density gradient centrifugation from individual *Cfap69* _2, numerous isolated sperm heads were recovered, suggesting a fragility of the head-flagellum connection.

### CFAP69 Is Located in the Midpiece of the Human Sperm Flagellum, and Its Absence Is Associated with Axonemal Defects

To further investigate the pathogenicity of the *Cfap69* variants identified, we examined the distribution of *Cfap69* in sperm cells from control and *Cfap69*-mutant individuals by immunofluorescence staining. In sperm from control individuals, we observed *Cfap69* concentrated in the midpiece of the sperm flagellum (Figure 2A). This localization was confirmed by co-staining of the sperm mitochondrial protein HSP60 (Figure 2B), which visualizes the mitochondrial sheath in the midpiece. Importantly, *Cfap69* was absent from all sperm cells from both individuals *Cfap69*_1 and *Cfap69*_2 (Figure 2A), consistent with the absence of *Cfap69* transcript observed in individual *Cfap69*_1 (Figure S3). We next investigated the flagellar and axonemal defects of *Cfap69*-mutant individuals. Due to sample availability, these analyses were carried out only for individual *Cfap69*_2. We observed that in sperm from individual *Cfap69*_2, staining of SPAG6, an axoneme central pair complex (CPC) protein, was absent from the flagellum or displayed abnormal localization in the midpiece and the acrosomal region of the spermatozoon (Figure 3A). In contrast, immunostaining of DNALI1 and DNAH5 in sperm from individual *Cfap69*_2 was comparable with that observed in control cells, suggesting that outer dynein arms (ODAs) and inner dynein arms (IDAs), respectively, were not directly affected by mutations in *Cfap69* (Figures S4A and S4B). We also examined the localization of SPEF2, a protein required for sperm flagellum assembly in the mouse and described as a putative partner to *Cfap69*.  

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**Table 1. Detailed Semen Parameters in the Two MMAF-Affected Individuals Harboring a CFAP69 Mutation**

<table>
<thead>
<tr>
<th>CFAP69 Mutated Individuals</th>
<th>Semen Parameters</th>
<th>Reference Limits a</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Individuals</strong></td>
<td></td>
<td>Sperm Volume (mL)</td>
</tr>
<tr>
<td>Age (years)</td>
<td></td>
<td>Sperm Concentration (10⁶/mL)</td>
</tr>
<tr>
<td>CFAP69_1</td>
<td></td>
<td>Total Motility 1 hr</td>
</tr>
<tr>
<td>c.860+1G&gt;A</td>
<td></td>
<td>Vitality</td>
</tr>
<tr>
<td>42</td>
<td></td>
<td>Normal Spermatozoa</td>
</tr>
<tr>
<td>CFAP69_2</td>
<td></td>
<td>Absent Flagella</td>
</tr>
<tr>
<td>c.763C&gt;T</td>
<td></td>
<td>Short Flagella</td>
</tr>
<tr>
<td>51</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values are percentages unless specified otherwise.

"Reference limits (5th centiles and their 95% confidence intervals) according to the World Health Organization (WHO) standards" and the distribution range of morphologically normal spermatozoa observed in 926 fertile individuals.

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In control sperm, SPEF2 immunostaining appeared very strongly at the base of the flagellum, likely corresponding to the basal body, and lightly throughout the whole sperm flagellum, but in individual CFAP69-/-, SPEF2 staining was weak or completely absent whereas tubulin staining remained detectable in the axoneme (Figure 3B).

**CFAP69 Is Found in the Mouse Testis and Is Required for Male Fertility**

Various expression\textsuperscript{24} and RNA-seq\textsuperscript{25,26} datasets indicate that a *Cfap69* transcript is highly expressed in the mouse testis, and the availability of a *Cfap69* knockout mouse\textsuperscript{15} allowed us to investigate the function of CFAP69 in male mouse reproduction. We investigated *Cfap69* expression in the male mouse reproductive system by X-gal staining in mice heterozygous for a *Cfap69* knockout-reporter allele (*Cfap69*\textsuperscript{tm1b/+} mice; see Subjects and Methods). In whole-mount preparations from male *Cfap69*\textsuperscript{tm1b/+}, but not wild-type, mice, strong X-gal staining was observed in the testes (Figure 4A). The epididymis is known to express endogenous β-galactosidase,\textsuperscript{27,28} and accordingly, we found X-gal staining in whole-mount epididymis (Figure 4A) and in the epithelial cells of cauda epididymis from both wild-type and *Cfap69*\textsuperscript{tm1b/+} mice (Figure 4B). This suggests that the X-gal staining observed in the *Cfap69*\textsuperscript{tm1b/+} epididymal cells is most likely due to endogenous β-galactosidase expression and not *Cfap69* expression. However, sperm cells within *Cfap69*\textsuperscript{tm1b/+}, but not in wild-type, epididymides were positive for X-gal staining (Figure 4B), suggesting that sperm cells or their precursors express the reporter gene. In testis cryosections, X-gal staining was observed in all seminiferous tubules of *Cfap69*\textsuperscript{tm1b/+} testes, but not in wild-type testes (Figure 4C). The staining first appeared in pachytene spermatocytes and increased in intensity in early and late spermatids (Figure 4D).

Western blotting analysis of wild-type adult mouse testis lysates using a custom antibody against CFAP69\textsuperscript{15} detects a band around 100 kD (Figure 4E), consistent with the predicted molecular weight of the protein encoded by the testis *Cfap69* transcript. The corresponding band was absent from testes of *Cfap69* knockout mice (*Cfap69*\textsuperscript{tm1b/tm1b}; see Subjects and Methods) (Figure 4E).

(Note: for unknown reasons, this antibody gives no staining when used in immunostaining in sections of reproductive tissues.)

Altogether, these results indicate that CFAP69 is found in germ cells of adult mouse testis, possibly beginning in pachytene spermatocytes and at higher levels after meiosis.

We then assessed fertility and reproductive behavior of *Cfap69* knockout mice. When male *Cfap69* knockout mice 6 weeks of age or older were mated to similarly aged wild-type females, they show normal mounting and produce copulatory plugs after mating, indicating that mating behavior is unimpaired in the absence of CFAP69. However, *Cfap69* knockout male mice failed to produce any offspring over 3 months of breeding (8 *Cfap69* mutant males mated with 2 wild-type females each, no offspring produced over 3 months), whereas wild-type males routinely produced offspring (8 wild-type males mated with 2 wild-type females each, 3–4 litters produced per female over 3 months, 6.93 ± 0.13 pups per litter). *Cfap69* knockout female mice produced offspring at a rate similar to that of wild-type female mice when mated with wild-type males (data not shown). These results indicate that CFAP69 is required for male fertility in the mouse, consistent with phenotype analysis by The International Mouse Phenotyping Consortium.

**Sperm of *Cfap69* Knockout Mice Have Profound Flagellum Morphology Defects**

We next examined *Cfap69* knockout sperm by collecting sperm from the cauda epididymides of both wild-type and *Cfap69* knockout mice. Samples from *Cfap69* knockout mice showed a complete lack of normal-looking sperm, and whereas we could observe sperm swimming in samples collected from wild-type mice, no sperm were observed to be motile in *Cfap69* mutant samples. When observed by scanning electron microscopy (SEM), wild-type mouse sperm showed a hook-shaped head and a long flagellum with a clearly defined midpiece, principal piece, and end piece (Figure 5A), but all *Cfap69* mutant sperm had severe morphology defects of the flagellum (Figure 5B). These defects are highly varied and affect all parts of the flagellum. In general, *Cfap69* mutant sperm display shortening of...
both midpiece and principal piece, leading to overall shorter flagella. The flagella frequently show splaying or perforation or are missing. Additionally, head morphology defects ranging from mild to severe are observed (Figure 5B, III and V), though only a subset of sperm is affected. These defects likely account for the immotility of \textit{Cfap69} mutant sperm and the infertility of \textit{Cfap69} mutant mice and recapitulate the MMAF phenotype.

**Sperm Flagellum Development during Spermiogenesis Requires CFAP69**

Given the presence of CFAP69 in sperm precursor cells (Figure 4), we sought to gain an understanding of when and how CFAP69 functions during sperm development by examining the histology of \textit{Cfap69} knockout testes. Toluidine blue staining of semi-thin testis sections indicates that in knockout mice, the cell composition of the seminiferous epithelium is unaltered as compared to wild-type: all types of germ cells could be observed, including spermatogonia, spermatocytes, spermatids, and spermatozoa (Figure 6A). The somatic Sertoli cells are also present. Staging of seminiferous epithelia by acrosome and nuclear morphology as well as by cell composition reveals that all 12 stages of the mouse seminiferous epithelium are present in \textit{Cfap69} knockout testes (Figure 6B). These findings indicate that the overall progression of spermatogenesis is

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**Figure 2. CFAP69 Immunostaining in Human Spermatozoa from Control Subjects and CFAP69 Mutant Individuals**

(A) Sperm cells from a fertile control individual and the two CFAP69-mutant individuals stained with anti-CFAP69 (green) and anti-acetylated tubulin (red) antibodies. DNA was counterstained with Hoechst 33342. In the fertile control, the CFAP69 immunostaining (green) is concentrated in the midpiece of the spermatozoa (white arrows) and is not detectable in the principle piece. CFAP69 staining is absent in sperm flagellum from individual CFAP69_1 and individual CFAP69_2. Scale bars: 10 μm.

(B) Sperm cell from a fertile control individual stained with anti-HSP60 (red), which detects a mitochondrial protein located in the midpiece, and anti-CFAP69 (green) antibodies. The merged image shows that in control sperm, CFAP69 and HSP60 staining superimpose. Scale bars: 5 μm.
preserved and that many spermatogenesis and spermiogenesis processes are nominally intact, including meiosis (Figure 6B, xii), acrosome development, and nuclear condensation and elongation (Figures 6B and S5). However, the absence of long flagella in the lumens of Cfap69 knockou...
knockout sperm reveal flagellum components in a dramatic state of disarray within bodies of cytoplasm (Figures 6G–6K). Although some mitochondria can be observed to localize to regions of mutant sperm flagella that would ordinarily be the midpiece, many appear throughout the cytoplasm (Figure 6G), in contrast to what was observed in wild-type sperm. In addition, in Cfap69 knockout sperm, ODFs are irregular in number and organization (Figures 6G–6K). Notably, in longitudinal sections, the ODFs show coiled and tangled arrangements resembling the unusual flagellum morphology observed in whole Cfap69 knockout sperm (Figure 6G). Additionally, the fibrous sheath is disorganized and largely absent (Figure 6G). Finally, axoneme organization is severely disrupted. Doublet and singlet microtubules are still frequently found to associate with ODFs, but their numbers and their arrangement vary (Figures 6H–6K). Microtubule doublets that have been split are also observed (Figure 6I, asterisk). Large numbers of singlet microtubules, likely from the manchette, were also frequently observed (Figure 6K), indicating an abnormal confluence of the manchette and flagellum components. The abnormal organization of all sperm flagellum components indicates a failure of sperm flagellum development during spermiogenesis in Cfap69 knockout mice and likely accounts for the observed sperm morphology defects.

Discussion

We showed that, in humans and mice, the presence of biallelic truncating mutations in CFAP69 induces male infertility owing to MMAF, thus establishing CFAP69 as a MMAF-related gene along with DNAH1, CFAP43, and CFAP44. The two individuals in our cohort with CFAP69 mutations presented only with primary infertility without other clinical features, similar to the individuals with DNAH1, CFAP43, and CFAP44 mutations.7–11 Although CFAP69 regulates olfactory response kinetics, the individuals did not believe they had a deficient sense of smell and did not wish to participate in any olfaction-related studies.
In both humans and mice, dramatic sperm flagellum defects were observed (Figures 1 and 5). In the adult mouse testis, CFAP69 expression begins during meiosis and strongly increases in subsequent stages (Figure 4). Major processes of the spermatogenic cycle and some spermiogenesis processes appeared normal. TEM analysis of the testis showed dramatic disorganization of all sperm flagellum components (Figure 5), suggesting an essential role for CFAP69 in normal flagellum formation during spermiogenesis.

We observed no significant differences between the semen parameters of the two individuals carrying mutations in CFAP69 compared to the parameters of individuals with mutations in other MMAF genes (DNAH1, CFAP43, and CFAP44). However, we found low sperm concentrations in the ejaculates (oligozoospermia) associated with a high rate of head malformations in both CFAP69-mutant individuals (Figure 1, Tables 1 and S1), which were also observed in the Cfa69 knockout mouse model (Figure 5). These observations suggest that mutations in CFAP69 lead to an atypical and more severe MMAF phenotype, and that CFAP69 could also be important for sperm head shaping during spermiogenesis. Whether CFAP69 is required for a process common to head and flagellum development or in distinct processes is not known.

Interestingly, immunostaining experiments with sperm from fertile control individuals showed that CFAP69 is located in the midpiece of the sperm flagellum (Figure 2). The midpiece-specific staining differs from the whole-flagellum staining observed for other axonemal components involved in the MMAF phenotype such as DNAH1, CFAP43, or CFAP44 (Figure S6). This localization suggests that CFAP69 is unlikely to belong to the core axoneme. The midpiece of the mammalian sperm flagellum is composed of the mitochondrial sheath surrounding nine outer dense fibers and the axoneme. However, CFAP69 is not necessarily, and may most likely not be, a mitochondrial or an ODF protein, as CFAP69 is found in other cilia in the mouse and in other organisms that contain neither outer dense fibers nor mitochondria. For example, we recently showed that CFAP69 is present in the cilia of mouse olfactory sensory neurons, which do not contain mitochondria, and that it plays a role in regulating the...
odor-response kinetics of olfactory sensory neurons. Interestingly, in contrast to sperm flagella, which have severe morphology defects, the olfactory cilia lacking CFAP69 in mouse appeared morphologically normal, illustrating that the sperm flagellum is assembled and organized differently from other cilia to fulfill its unique functions. Additionally, proteomic analyses in Chlamydomonas rheinhardtii found CFAP69 enriched in the flagellum. The role of CFAP69 in the function and assembly of these other cilia and its relationship to CFAP69 function in sperm flagellum assembly and function are under investigation.

The human and mouse sperm head and flagellum defects (Figures 1 and 5), as well as abnormally localized manchette microtubules (Figure 6), are reminiscent of defects arising from disruption of intramanchette transport (IMT) or intraflagellar (IFT) transport. Interestingly, IF staining of SPEF2, which is involved in IMT, sperm head shaping, and CPC assembly during spermiogenesis, is strongly reduced or totally absent from CFAP69-mutant individuals’ sperm cells (Figure 3B). Truncating mutations in Spef2 are also known to cause male infertility in the pig and mouse due to a short sperm tail phenotype associated with severe ultrastructural axonemal and peri-axonemal defects which closely resemble the MMAF phenotype observed in the Cfap69 knockout mouse model. Additionally, reduced and atypical staining of the CPC protein SPAG6 in spermatozoa from our CFAP69-mutant individuals suggests that CFAP69 may be also involved in CPC organization (Figure 3A). We can therefore speculate that CFAP69 might partake in sperm tail biogenesis and CPC assembly through a role in flagellar protein transport (Figure S6). Although mass spectrometry analysis recently suggested that SPEF2 might interact with CFAP69 (Q8BH53), no protein interaction partners for CFAP69 have been confirmed. Bioinformatic analysis does suggest that CFAP69 contains an ARM-repeat domain and an AH/BAR domain (Figure 1D), indicating that CFAP69 function may indeed involve interaction with other proteins. The identity of these proteins and the processes they affect remain to be determined.
Supplemental Data include Supplemental Material and Methods, six figures, and five tables and can be found with this article online at https://doi.org/10.1016/j.ajhg.2018.03.007.

Acknowledgments

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Web Resources

1000 Genomes, http://www.internationalgenome.org/
ExAC Browser, http://exac.broadinstitute.org/
ExSQLibur, https://github.com/tkaraouzene/ExSQLibur
ExAC Browser, http://exac.broadinstitute.org/
International Mouse Phenotyping Consortium, http://www.mousephenotype.org/data/genes/
InterPro, https://www.ebi.ac.uk/interpro/
PolyPhen-2, http://genetics.bwh.harvard.edu/pph2/
UCSC Genome Browser, http://genome.ucsc.edu
UniProt, http://www.uniprot.org/

References


Supplemental Data

Absence of CFAP69 Causes Male Infertility
due to Multiple Morphological Abnormalities
of the Flagella in Human and Mouse

Supplemental material and methods

Whole-Exome Sequencing (WES) and bioinformatic analysis

Genomic DNA was isolated from saliva using the Oragen DNA extraction kit (DNAgenotech®, Ottawa, Canada). Coding regions and intron/exon boundaries were enriched using the “all Exon V5 kit“ (Agilent Technologies, Wokingham, UK). DNA sequencing was undertaken at the Genoscope, Evry, France, on the HiSeq 2000 from Illumina®. Sequence reads were aligned to the reference genome (hg19) using MAGIC\(^1\). MAGIC produces quality-adjusted variant and reference read counts on each strand at every covered position in the genome. Duplicate reads and reads that mapped to multiple locations in the genome were excluded from further analysis. Positions whose sequence coverage was below 10 on either the forward or reverse strand were marked as low confidence, and positions whose coverage was below 10 on both strands were excluded. Single nucleotide variations (SNV) and small insertions/deletions (indels) were identified and quality-filtered using in-house scripts. Briefly, for each variant, independent calls were made on each strand, and only positions where both calls agreed were retained. The most promising candidate variants were identified using an in-house bioinformatics pipeline, as follows. Variants with a minor allele frequency greater than 5% in the NHLBI ESP6500 [Exome Variant Server, NHLBI GO Exome Sequencing Project (ESP), Seattle, WA] or in 1000 Genomes Project phase 1 datasets\(^2\), or greater than 1% in ExAC\(^3\) or gnomAD (http://gnomad.broadinstitute.org/) were discarded. We also compared these variants to an in-house database of 94 control exomes obtained from subjects from North Africa (n=60) and the Middle East (n=34), corresponding to the geographical origin of most individuals in this study and which is under-represented in public SNP databases. All these control subjects presented a sperm phenotype clearly different (azoospermia) from those of the MMAF patients. All variants present in homozygous state in this database were excluded. We used Variant Effect Predictor (VEP version 81\(^4\)) to predict the impact of the selected variants. We only retained variants impacting splice donor or acceptor sites or causing frame shifts, in-frame insertions or deletions, stop gain, stop loss or missense variants except those scored as "tolerated" by SIFT\(^5\) (sift.jcvi.org) and as "benign" by Polyphen-2\(^6\) (genetics.bwh.harvard.edu/pph2). To predict the impact of mutations within the 5′ and 3′ splicing consensus, we used the Human Splicing Finder server v 3.0. All steps from sequence mapping to variant selection were performed using the ExSQLibur pipeline.
Figure S1. Relative mRNA Expression of human CFAP69 transcripts. CFAP69 mRNA levels in a panel of human normal tissues. Results are presented as the mean of triplicates (ratio target gene/ACTB) ± Standard Deviation (SD). RT-qPCR data were normalized using the reference gene ACTB with the -ΔΔCt method. Brain expression is arbitrary set to 1. In human, CFAP69 has the strongest expression in testis compared to other organs. Unpaired t-test, ***P< 0.001.
Figure S2. Electropherograms of Sanger sequencing for the two CFAP69-mutated individuals compared to reference sequence.
Figure S3. RT-PCR analyses on peripheral whole blood cells from CFAP69_1 patient showing mRNA decay. RT–PCR analysis of CFAP69_1 patients with the c.860+1G>A and control subjects from the general population (C1 and C2). (A) Electrophoresis showing the RT–PCR amplification of CFAP69 exons 12-13. C1 and C2 controls yield a normal fragment of 282 bp, whereas patient CFAP69_1 shows no amplification. There is no amplification from the RT-negative control (Blank). (B) Electropherogram showing the amplification of the same cDNAs amplified with GAPDH primers. Bands of equivalent intensity are obtained from all samples except the RT-negative control (Blank).
Figure S4. Axonemal inner and outer dynein arms are not affected by the absence of CFAP69. (A) Sperm cells from fertile controls and CFAP69\_2 stained with anti-DNALI1 (Green), which detects a protein located in the inner dynein arm, and anti-acetylated tubulin (red) antibodies. DNA was counterstained with Hoechst 33342. (B) Sperm cells from a fertile control and CFAP69\_2 stained with anti-DNAH5 (Green), which detects a protein located in the outer dynein arm, and anti-acetylated tubulin (red) antibodies. DNA was counterstained with Hoechst 33342. Immunostaining for DNALI1, DNAH5 were comparable with controls, suggesting that outer dynein arms (ODAs) and inner dynein arms (IDAs) respectively were not directly affected by mutations in CFAP69. Scale bars 10µm.
Figure S5. Normal acrosome development and nuclear elongation can be observed in testes of Cfap69 KO mice. Transmission electron micrographs of spermatids during spermiogenesis. Yellow arrows indicate the acrosome, and yellow “N” indicates the nucleus. Scale bars: C and D, 1 µm.
Figure S6. Schematic cross-section of human sperm flagellar axoneme and localization of MMAF-related proteins. The outer and inner dynein arms, nexin-dynein regulator complex (N-DRC) and radial spokes are attached to the nine outer doublet microtubules. The 5-6 bridge likely links the outer doublet microtubules 5-6 and potentially with other extra-axonemal structures. Genes formally identified for the MMAF phenotype in human are reported in red.
Supplementary Tables

**Table S1.** Average semen parameters in different genotype groups for the 78 included MMAF subjects in the present study.

<table>
<thead>
<tr>
<th>Semen parameters</th>
<th>MMAF with CFAP69 mutation</th>
<th>MMAF with other mutations&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Overall MMAF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean age (years)</td>
<td>n=2</td>
<td>n=22</td>
<td>n=78</td>
</tr>
<tr>
<td>Mean age (years)</td>
<td>46.5 ± 6.3</td>
<td>39.8 ± 7</td>
<td>41.6 ± 7.7</td>
</tr>
<tr>
<td>Sperm volume (ml)</td>
<td>n=2</td>
<td>n=20</td>
<td>n=75</td>
</tr>
<tr>
<td>Sperm volume (ml)</td>
<td>4.5 ± 0.7</td>
<td>3.4 ± 1.2</td>
<td>3.5 ± 1.4</td>
</tr>
<tr>
<td>Sperm concentration (10&lt;sup&gt;6&lt;/sup&gt;/ml)</td>
<td>n=2</td>
<td>n=20</td>
<td>n=75</td>
</tr>
<tr>
<td>Sperm concentration (10&lt;sup&gt;6&lt;/sup&gt;/ml)</td>
<td>5 ± 1.4</td>
<td>20.1 ± 18.8</td>
<td>25.6 ± 32.1</td>
</tr>
<tr>
<td>Total motility 1 h</td>
<td>n=2</td>
<td>n=21</td>
<td>n=76</td>
</tr>
<tr>
<td>Total motility 1 h</td>
<td>5.5 ± 6.3</td>
<td>0.7 ± 2.4</td>
<td>3.9 ± 5.6</td>
</tr>
<tr>
<td>Vitality</td>
<td>n=2</td>
<td>n=19</td>
<td>n=72</td>
</tr>
<tr>
<td>Vitality</td>
<td>37.5 ± 34.6</td>
<td>50.5 ± 22.7</td>
<td>52.7 ± 20</td>
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<tr>
<td>Normal spermatozoa</td>
<td>n=2</td>
<td>n=20</td>
<td>n=61</td>
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<tr>
<td>Normal spermatozoa</td>
<td>6 ± 8.5</td>
<td>0.5 ± 2.3</td>
<td>1.6 ± 2.7</td>
</tr>
<tr>
<td>Absent flagella</td>
<td>n=2</td>
<td>n=15</td>
<td>n=66</td>
</tr>
<tr>
<td>Absent flagella</td>
<td>6.5 ± 7.8</td>
<td>28.1 ± 14.4</td>
<td>20.7 ± 15.7</td>
</tr>
<tr>
<td>Short Flagella</td>
<td>n=2</td>
<td>n=19</td>
<td>n=72</td>
</tr>
<tr>
<td>Short Flagella</td>
<td>46 ± 46.7</td>
<td>57.1 ± 27.9</td>
<td>43.7 ± 27.3</td>
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<tr>
<td>Coiled Flagella</td>
<td>n=2</td>
<td>n=16</td>
<td>n=69</td>
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<tr>
<td>Coiled Flagella</td>
<td>4 ± 4.2</td>
<td>10.4 ± 6.6</td>
<td>12.8 ± 9.4</td>
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<tr>
<td>Bent Flagella</td>
<td>n=2</td>
<td>n=6</td>
<td>n=26</td>
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<tr>
<td>Bent Flagella</td>
<td>2.5 ± 3.5</td>
<td>8.7 ± 5.8</td>
<td>4.2 ± 8.4</td>
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<tr>
<td>Flagella of irregular caliber</td>
<td>n=2</td>
<td>n=15</td>
<td>n=67</td>
</tr>
<tr>
<td>Flagella of irregular caliber</td>
<td>4 ± 4.2</td>
<td>27.9 ± 19.4</td>
<td>31.7 ± 25.1</td>
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<tr>
<td>Tapered head</td>
<td>n=2</td>
<td>n=13</td>
<td>n=68</td>
</tr>
<tr>
<td>Tapered head</td>
<td>10.5 ± 13.4</td>
<td>22.5 ± 29</td>
<td>16.5 ± 20.2</td>
</tr>
<tr>
<td>Condition</td>
<td>Mean ± SD (n') =</td>
<td>Mean ± SD (n') =</td>
<td>Mean ± SD (n') =</td>
</tr>
<tr>
<td>-------------------------------</td>
<td>-----------------</td>
<td>-----------------</td>
<td>-----------------</td>
</tr>
<tr>
<td>Thin head</td>
<td>25.5 ± 16.2</td>
<td>10.8 ± 13.7</td>
<td>11.1 ± 13.4</td>
</tr>
<tr>
<td></td>
<td>(n' = 2)</td>
<td>(n' = 13)</td>
<td>(n' = 65)</td>
</tr>
<tr>
<td>Microcephalic</td>
<td>0.5 ± 0.7</td>
<td>3.8 ± 2.4</td>
<td>4.3 ± 4.8</td>
</tr>
<tr>
<td></td>
<td>(n' = 2)</td>
<td>(n' = 14)</td>
<td>(n' = 67)</td>
</tr>
<tr>
<td>Macrocephalic</td>
<td>0</td>
<td>0.15 ± 0.5</td>
<td>0.6 ± 1.8</td>
</tr>
<tr>
<td></td>
<td>(n' = 2)</td>
<td>(n' = 13)</td>
<td>(n' = 66)</td>
</tr>
<tr>
<td>Multiple heads</td>
<td>0</td>
<td>1.9 ± 4</td>
<td>1.9 ± 3.7</td>
</tr>
<tr>
<td></td>
<td>(n' = 2)</td>
<td>(n' = 15)</td>
<td>(n' = 67)</td>
</tr>
<tr>
<td>Abnormal base</td>
<td>21 ± 8.5</td>
<td>36.3 ± 26.4</td>
<td>31.2 ± 20.8</td>
</tr>
<tr>
<td></td>
<td>(n' = 2)</td>
<td>(n' = 13)</td>
<td>(n' = 64)</td>
</tr>
<tr>
<td>Abnormal acrosomal region</td>
<td>67 ± 4.2</td>
<td>58.3 ± 31.5</td>
<td>61.9 ± 26.7</td>
</tr>
<tr>
<td></td>
<td>(n' = 2)</td>
<td>(n' = 15)</td>
<td>(n' = 68)</td>
</tr>
</tbody>
</table>

* Other mutations correspond to individuals mutated in CFAP43, CFAP44 and DNAH1. Values are percentages unless specified otherwise. Values are mean +/- SD; n= total number of individuals in each group; n’= number of individuals used to calculate the average based on available data.
Table S2. Primer sequences used for Sanger sequencing verification of CFAP69 variations and respective melting temperatures (Tm).

<table>
<thead>
<tr>
<th>Primer names</th>
<th>Primer sequences (5’-3’)</th>
<th>Tm</th>
</tr>
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<tbody>
<tr>
<td>CFAP69-Ex8F</td>
<td>AAAAATGTCAATATTGTAAGCACA</td>
<td>58°C</td>
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<tr>
<td>CFAP69-Int8R</td>
<td>TGTGGCTTTATTTGTCGAG</td>
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Table S3. Primers used for RT-qPCR of CFAP69 in human.

<table>
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<th>Primer names</th>
<th>Primer sequences (5’-3’)</th>
<th>Tm</th>
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</thead>
<tbody>
<tr>
<td>CFAP69-RTqPCR-Ex12F</td>
<td>ATTGACTGGTCTGCAGCACAC</td>
<td>60°C</td>
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<tr>
<td>CFAP69-RTqPCR-Ex13R</td>
<td>ACTGTAACGCATCTGGGCAA</td>
<td></td>
</tr>
<tr>
<td>GAPDH-RTqPCR-F</td>
<td>AGCCACATCGCTAGACAC</td>
<td>60°C</td>
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<tr>
<td>GAPDH-RTqPCR-R</td>
<td>GCCCAATACGACCAATCC</td>
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Table S4. Primer sequences used in human CFAP69 RT-PCR and respective melting temperatures (Tm).

<table>
<thead>
<tr>
<th>Primer names</th>
<th>Primer sequences (5’-3’)</th>
<th>Tm</th>
</tr>
</thead>
<tbody>
<tr>
<td>CFAP69-RT-Ex7F</td>
<td>TTCTGCAGCATCTCTCAACTTC</td>
<td>57°C</td>
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<td>CFAP69-RT-Ex10R</td>
<td>CAAATCCTTGTAAGGCCACA</td>
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Table S5. All CFAP69 (C7orf63) variations identified by WES.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Patients</th>
<th>Variant coordinates</th>
<th>Canonical Transcripts</th>
<th>cDNA Variations</th>
<th>Amino acid variations</th>
<th>Exon</th>
<th>Nationality</th>
<th>Allelic status</th>
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Supplemental References


