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1 ***CFAP70* mutations lead to male infertility due to severe astheno-**
2 **teratozoospermia.**

3
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1 **Abstract**

2 The use of high-throughput sequencing techniques allowed to identify numerous mutations in
3 genes responsible for severe astheno-teratozoospermia due to multiple morphological
4 abnormalities of the sperm flagella (MMAF). However, more than half of the analyzed cases
5 remains unresolved suggesting that many yet uncharacterized gene defects account for this
6 phenotype. Based on whole-exome sequencing data from a large cohort of 167 MMAF-
7 affected subjects, we identified two unrelated affected individuals carrying a homozygous
8 deleterious mutation in *CFAP70*, a gene not previously linked to the MMAF phenotype. One
9 patient had a homozygous splice variant c.1723-1G>T, altering a consensus splice acceptor
10 site of *CFAP70* exon 16 and one had a likely deleterious missense variant in exon 3
11 (p.Phe60Ile). *CFAP70* gene encodes a regulator protein of the outer dynein arms (ODA)
12 strongly expressed in human testis. In the sperm cells from the patient carrying the splice
13 variant, immunofluorescence experiments (IF) confirmed the absence of the protein in the
14 sperm flagellum. Moreover, IF analysis showed the absence of markers for the outer dynein
15 arms and the central-pair complex of the axoneme.. Interestingly, whereas CFAP70 staining
16 was present in sperm cells from patients with mutations in the three other MMAF-related
17 genes *ARMC2*, *FSIP2* and *CFAP43*, we observed an absence of staining in sperm cells from
18 patients mutated in the *WDR66* gene, suggesting a possible interaction between these two
19 axonemal components. In conclusion, this work provides the first evidence that loss of
20 CFAP70 function causes MMAF, and that ODA-related proteins may be crucial for the
21 assembly and/or stability of the flagellum axoneme in addition to its motility.

22 **Key words:** Male infertility, genetics, MMAF, CFAP70, outer dynein arm, gene mutation,
23 flagellum, whole-exome sequencing.

24

1 **Introduction**

2 The past decade has been marked by the emergence of the next generation sequencing
3 (NGS), a fast evolving technology which allowed important advances in human genetics. This
4 technical revolution has proved to be a highly powerful research and diagnostic tool in male
5 infertility and permitted to take a major step forward for the identification of the genetic cause
6 of male infertility (Krausz and Riera-Escamilla, 2018). In particular, NGS has contributed
7 massively to decipher the genetic causes involved in multiple morphological abnormalities of
8 the flagella (MMAF phenotype) which is a subgroup of astheno-teratozoospermia
9 characterized by the presence in the ejaculate of immotile spermatozoa with several flagellar
10 defects including short, coiled, absent and flagella of irregular caliber (Ray *et al.*, 2017). To
11 date, mutations in fourteen genes have been found to be associated with MMAF (*AK7*,
12 *AKAP3*, *AKAP4*, *ARMC2*, *CEP135*, *CFAP43*, *CFAP44*, *CFAP65*, *CFAP69*, *DNAH1*, *FSIP2*,
13 *QRICH2*, *TTC21A* and *WDR66*) (Supplementary Fig. S1) (Baccetti *et al.*, 2005; Ben Khelifa
14 *et al.*, 2014; Sha *et al.*, 2017a; Tang *et al.*, 2017; Auguste *et al.*, 2018; Coutton *et al.*, 2018;
15 Dong *et al.*, 2018; Kherraf *et al.*, 2018; Lorès *et al.*, 2018; Martinez *et al.*, 2018; Coutton *et*
16 *al.*, 2019; Liu *et al.*, 2019; Shen *et al.*, 2019). However, despite these plethoric findings, the
17 etiology of MMAF remains unknown in more than 50% of the cases and the discovery of the
18 remaining genes is a now a major challenge (Coutton *et al.*, 2019).

19 Using different animal models and in particular flagellated protists such as
20 *Chlamydomonas* or *Trypanosoma*, at least five of these known MMAF-related genes
21 (*DNAH1*, *CFAP43*, *CFAP44*, *WDR66* and *CEP135*) have been already reported to encode
22 different components of the axoneme, a central multi-protein complex which is highly
23 conserved throughout evolution and shared by both the sperm flagellum and other motile cilia
24 (Inaba, 2007; Satir and Christensen, 2008). Briefly, the axoneme consists of 9 outer doublet
25 microtubules (DMTs) surrounding 2 central singlets (9+2 structure) known as the central pair

1 complex (CPC). DMTs carry several components that drive and regulate ciliary or flagellar
2 motility including axonemal dynein motors subdivided into inner (IDA) and outer arms
3 (ODA), radial spokes, nexin links and many other (Satir and Christensen, 2008). DNAH1 is
4 located in the inner dynein arm which drives axoneme bending and ciliary or flagellar motility
5 (Ben Khelifa *et al.*, 2014), CFAP43 and CFAP44 seem to be related to the the tether and
6 tether head (T/TH) complex, which connects dynein motor domains to DMTs (Fu *et al.*, 2018;
7 Urbanska *et al.*, 2018), WDR66 is a component of the spoke-associated complex (CSC)
8 which mediates regulatory signals between the radial spokes and dynein arms (Urbanska *et*
9 *al.*, 2015) and CEP135 is located in the centrosomal complex (Ohta *et al.*, 2002). In this
10 work, we analyzed genetic data obtained by whole-exome sequencing (WES) from a large
11 cohort of 167 MMAF patients, and identified two patients carrying a homozygous deleterious
12 mutation in the *CFAP70* gene, which encodes an axonemal protein that is localized at the base
13 of the outer dynein arm and regulates ciliary motility (Shamoto *et al.*, 2018). Therefore, this
14 work shows for the first time the involvement of an ODA-linked protein to the MMAF
15 phenotype and confirmed that the ODA complex, in addition to its motility function, plays an
16 important role in the assembly and/or the stability of the flagellum axoneme.

17 **Case report**

18 WES was performed on a large cohort of 167 MMAF patients as previously described
19 (Coutton *et al.*, 2019). All individuals presented with a typical MMAF phenotype
20 characterized by severe asthenozoospermia (total sperm motility below 10%) with at least
21 three of the following flagellar abnormalities present in >5% of the spermatozoa: short,
22 absent, coiled, bent or irregular flagella (for details see Coutton *et al.*, 2019). Sperm analysis
23 was carried out in the clinical laboratories during routine biological examination of the patient
24 according to World Health Organization (WHO) guidelines (Wang *et al.*, 2014). The

1 morphology of the sperm cells from the *CFAP70* mutated patients was assessed with
2 Papanicolaou staining (Fig. 1A). Detailed semen parameters of the two mutated patients are
3 presented in Table I. All individuals have a normal somatic karyotype (46,XY) with normal
4 bilateral testicular size, normal hormone levels and secondary sexual characteristics. Informed
5 consent was obtained from all the subjects participating in the study according to local
6 protocols and the principles of the Declaration of Helsinki. The study was approved by local
7 ethics committees, and samples were then stored in the CRB Germethèque (certification under
8 ISO-9001 and NF-S 96-900) following a standardized procedure or were part of the
9 Fertithèque collection declared to the French Ministry of health (DC-2015-2580) and the
10 French Data Protection Authority (DR-2016-392).

11 Bioinformatics analysis was performed according to our previously described protocol
12 (Coutton *et al.*, 2019). Data analysis of the whole cohort of 167 MMAF individuals permitted
13 to identify 54 individuals (32.3 %) with harmful mutations in known MMAF-related genes
14 (Coutton *et al.*, 2019). In addition, we identified here two patients (*CFAP70_1* and *CFAP70_2*)
15 with a homozygous deleterious variant in *CFAP70*, a gene not previously associated with any
16 pathology, accounting for 1.2% of our cohort. The *CFAP70* mutations were subsequently
17 validated by Sanger sequencing (Fig. 1B). PCR primers are listed in Supplementary Table S1
18 and a detailed protocol can be found in Coutton *et al.*, (2019). The first variant identified in
19 the patient *CFAP70_1* is a splice variant c.1723-1G>T, altering a consensus splice acceptor
20 site of *CFAP70* exon 16 (Fig. 1C). The second variant identified in individual *CFAP70_2* is a
21 missense variation c.178T>A (p.Phe60Ile) located in exon 3 (Fig. 1C). Both variants were
22 absent from gnomAD, the largest control sequence database regrouping sequence data from
23 over 120 000 individuals. No mRNA analysis or immunostaining could be performed on
24 sperm cells from *CFAP70_2* due to the lack of biological samples. However, using prediction
25 software for non-synonymous SNPs we found that this missense change is predicted to be

1 deleterious by SIFT (score of 0) and probably damaging by PolyPhen (score of 0.996).
2 Moreover, the concerned amino acid (Phe60) was found conserved in *CFAP70* orthologs (Fig.
3 1D). For these two patients, no other candidate variants reported to be associated with cilia,
4 flagella or male fertility were found.

5 *CFAP70* (NM_145170.3, formerly reported as *TTC18*) is located on chromosome 10
6 and contains 27 exons encoding a predicted 1121-amino acid protein (Q5T0N1). *CFAP70* is
7 predominantly expressed in the testis according to data from GTEx (GTEx Consortium, 2015)
8 and described to be associated with cilia and flagella (Shamoto *et al.*, 2018). RT-qPCR
9 experiments performed with a panel of ten human tissues including other ciliated tissues such
10 as trachea confirmed these results showing that *CFAP70* transcripts are most highly expressed
11 in the testis compared to all the other tested tissues (Supplementary Fig. S2). Primers
12 sequences and RT-qPCR conditions are indicated in Supplementary Table S2 and in Coutton
13 *et al.*, (2019). According to the Uniprot server, *CFAP70* contains 8 tetratricopeptide repeats
14 (TPR-repeat), which are known to form scaffolds which mediate protein-protein interactions
15 (The UniProt Consortium, 2017) (Fig. 1C).

16 We performed immunofluorescence (IF) assays on sperm cells from fertile control,
17 patient *CFAP70*₁ carrying the splice variant c.1723-1G>T and other MMAF patients mutated
18 in other genes. For each MMAF patients studied, 200 sperm cells were analyzed by two
19 different experienced operators and the IF staining intensity and pattern were compared to a
20 fertile control. *CFAP70* immunostaining in sperm cells from control was present all along of
21 the flagellum, with a marked signal at the base of the flagellum. In the *CFAP70*₁ patient the
22 *CFAP70* staining was totally absent in all analyzed spermatozoa (Fig. 2) confirming the
23 deleterious effect of this mutation. In order to determine if the loss of *CFAP70* was
24 specifically linked to the *CFAP70* mutation and was not a hallmark of the MMAF phenotype,
25 secondary to structural defects, we performed IF assays on sperm cells from five other

1 MMAF patients including four patients previously identified with a mutation in *ARMC2*,
2 *FSIP2*, *CFAP43*, *WDR66* and one with no genetic cause identified (Fig. 2). Genotype
3 information of the four additional MMAF patients mutated in *ARMC2*, *FSIP2*, *CFAP43* and
4 *WDR66* are indicated in Supplementary Table S3. In all but the *WDR66* mutated patient,
5 CFAP70 immunostaining was comparable with what was observed on fertile controls (Fig. 2),
6 indicating that CFAP70 expression and localization was not altered in the sperm from MMAF
7 patients with mutations in these genes. Interestingly, CFAP70 staining was mostly absent in
8 the *WDR66*-mutated patient irrespective of the sperm morphology (Supplementary Fig. S3)
9 suggesting a possible link, direct or indirect, between these two axonemal components in the
10 sperm flagellum (Fig. 2).

11 To further characterize the molecular defects induced by *CFAP70* mutations in human sperm,
12 we used an indirect approach studying by IF the presence and localization of some proteins
13 belonging to different sub-structures of the axoneme. The presence of the only two following
14 proteins was investigated: SPAG6 as a marker of the CPC and DNAI2 as markers of outer
15 dynein arm (ODA). We observed that in sperm from individual CFAP70₁, staining of
16 SPAG6, an axoneme central pair complex protein (Sapiro *et al.*, 2000), was totally absent
17 from the flagellum (Fig. 3) showing defects in the CPC structure (Fig. 3). This finding is in
18 concordance with previous results showing that the absence of CPC is a frequent
19 characteristic of MMAF spermatozoa (Coutton *et al.*, 2015). Similarly, the staining of DNAI2
20 was totally absent or dramatically reduced in most of the analyzed sperm irrespective of the
21 sperm morphology (Fig. 4). This result is consistent with the function of CFAP70 as a
22 regulatory component of the ODA in motile cilia and flagella (Shamoto *et al.*, 2018). To
23 formally conclude, all the potential ultrastructural defects observed using IF should be further
24 confirmed by Transmission Electron Microscopy (TEM). However, TEM could not be
25 performed in this present work due to a very low amount of sperm cells available. As well,

1 due to limited sample availability, IF analyses could not be repeated on sperm from the other
2 *CFAP70_2* mutated patient.

3

4 **Discussion**

5 In this present work, we identified two infertile patients with typical MMAF
6 phenotype carrying deleterious homozygous mutations in the *CFAP70* gene. The first patient
7 harbors a deleterious splicing mutation leading to the absence of the protein in the sperm
8 flagellum (Fig. 2). The second patient has a missense mutation at position Phe60 predicted to
9 be deleterious using mutation-prediction software. However, bioinformatics predictions are
10 not sufficient to conclude to the pathogenicity of this mutation and additional supporting
11 functional work (e.g. mRNA or protein studies, IF experiments or CRISPR/Cas9-targeted
12 mutagenesis in different animal models) could be useful to reinforce this assumption.
13 Mutations in *CFAP70* appeared as the best candidates to explain the MMAF phenotype
14 observed in these two patients based on robust evidence in the literature supporting its critical
15 function for cilia/flagella formation and function. First, transcriptomic studies showed that
16 *Cfap70* was strongly upregulated in differentiating mouse tracheal epithelial cells (Xu *et al.*,
17 2015). Furthermore, inactivation of *ttc18*, the orthologue of the human *CFAP70* in zebrafish,
18 led to a significant reduced cilia length and number in the Kupffer's vesicle (Dam *et al.*,
19 2017). Moreover, extensive work on *Chlamydomonas FAP70* demonstrated that the protein is
20 a regulator of the ODA (Shamoto *et al.*, 2018). Importantly, the authors demonstrated that
21 FAP70 is not formally a component of the ODA complex but seems to be involved in the
22 coordination of the ODA activity in response to changes in the surrounding mechanical and/or
23 chemical conditions to produce proper ciliary motility (Shamoto *et al.*, 2018). In this work, IF
24 experiments showed ODA defects in sperm cells from *CFAP70_1* patient (Fig. 4). Moreover,
25 previous studies demonstrated that the ODA were not disorganized in sperm flagellum from

1 MMAF patients mutated in other axonemal genes suggesting that ODA defects may be
2 specifically linked to *CFAP70* mutations. In *Chlamydomonas*, the lack of FAP70 did not
3 affect the ODA ultrastructure (Shamoto *et al.*, 2018), in contrast to what was observed in the
4 sperm cells from our patient (Fig. 4). However, such discrepancy between human and
5 flagellate flagella was already found for two other genes involved in MMAF (*CFAP43* and
6 *CFAP44*) (Coutton *et al.*, 2018). These IF results should nevertheless be confirmed by TEM
7 analysis in sperm cells from *CFAP70* mutated patients to allow reliable and definitive
8 conclusions. *CFAP70* encodes a TPR protein composed of 8 TPR-repeats located at the C-
9 terminal domain. TPR domains are known to mediate protein–protein interactions and it has
10 been proposed that FAP70 localizes at the base of ODA through the N-terminal end of the
11 protein and the C-terminal region may define the binding partner that regulates ODA function
12 (Shamoto *et al.*, 2018). *CFAP70*'s partners are still unknown but Shamoto *et al.* (2018)
13 suggested that *CFAP70* could interact with various ciliary regulatory proteins such as protein
14 phosphatases (Elam *et al.*, 2011) and calcium-binding proteins (Mizuno *et al.*, 2012).
15 Interestingly, we showed in the present study that the *CFAP70* staining is absent in a patient
16 with mutation in the *WDR66* gene. *WDR66* is a MMAF-related gene previously reported
17 which encodes an axonemal protein containing a calcium-regulating EF-hand domain in its C-
18 terminal end (Auguste *et al.*, 2018; Kherraf *et al.*, 2018). Interestingly, the *WDR66* patient
19 included in this present work harbored the recurrent terminal deletion previously described to
20 remove the C-terminal domain of the protein (Kherraf *et al.*, 2018). Therefore, this
21 observation may suggest an additional role of the C-ter domain of *CFAP251* in the
22 recruitment and assembly of other component of the flagellum as *CFAP70* may be.
23 Moreover, *WDR66* has been described to localize to the calmodulin- and spoke-associated
24 (CSC) complex at the base of radial spoke 3 in *Tetrahymena* and *Chlamydomonas* (Urbanska
25 *et al.*, 2015). Interestingly, the CSC is involved in the modulation of dynein activity and

1 therefore in the regulation of flagellar motility (Heuser *et al.*, 2012). These findings support
2 the fact that WDR66 is a potential partner of CFAP70 and may work in synergy to modulate
3 inner and outer dynein activity. However, further investigations (such as co-
4 immunoprecipitation (co-IP) experiments) are necessary to clarify this hypothesis and will
5 permit to elucidate the mechanisms by which CFAP70 regulates ODA function and flagellum
6 and cilia length in mammals.

7 Last, it has been demonstrated that *Cfap70* inactivation in mouse ependymal cells led
8 to shortened cilia in addition to motility defects (Shamoto *et al.*, 2018). This observation
9 echoes the sperm phenotype observed in our two MMAF patients presenting sperm with short
10 and immotile flagella. Moreover, this supports our findings that CFAP70 may be involved in
11 axoneme biogenesis or stability beyond its function in ODA regulation.

12 As observed for the other MMAF individuals of the cohort (Coutton *et al.*, 2019),
13 analysis of sperm morphology from patients CFAP70₁₋₂ showed a high proportion of
14 spermatozoa with abnormal flagella (Fig. 1, Table I). Interestingly, we also found low sperm
15 concentrations in the ejaculates (oligozoospermia) as was observed for patients mutated in
16 *CFAP69* (Dong *et al.*, 2018). However, the two CFAP70 patients presented a different pattern
17 of morphological defects of the sperm flagellum with only short flagella for the patient 1
18 while a mosaic of defects (short, absent or irregular caliber) was observed in patient 2. In
19 addition, patient CFAP70₁ with the most severe variant presents spermatozoa with 0%
20 motility in contrast with the patient with the missense variant (p.Phe60Ile), who presents a
21 milder phenotype with 13% motility (Table I). This could suggest that the Phe60Ile mutated
22 protein may preserve a residual activity as it was reported for other missense mutations in
23 other MMAF-related genes (Ben Khelifa *et al.*, 2014; Amiri-Yekta *et al.*, 2016). Moreover,
24 this may reinforce the hypothesis of a possible phenotype continuum depending on the
25 severity of mutations in MMAF genes as showed for *DNAH1* (Ben Khelifa *et al.*, 2014).

1 Concerning the prognosis of ICSI using sperm cells from *CFAP70*-mutated subjects, only
2 ICSI outcomes for the patient *CFAP70_1* were available, and one clinical pregnancy with one
3 live birth after two cycles was reported. This positive outcome is consistent with previous
4 studies demonstrating that MMAF affected individuals have a good prognosis following ICSI
5 (*Coutton et al.*, 2015; *Wambergue et al.*, 2016; *Sha et al.*, 2017b).

6 Last, we know that *CFAP70* is expressed in different ciliated tissues such as trachea or
7 ependymal epithelium. However, the two patients with *CFAP70* mutations presented only
8 with primary infertility without any other clinical features, thus excluding a phenotype of
9 Primary Cilia Dyskinesia (PCD) as was observed for all genes associated with a MMAF
10 phenotype. These recurrent observations confirm once again that flagellum biogenesis
11 requires some proteins and pathways that are at least partly different from those necessary for
12 cilia biogenesis. Interestingly, we observed that many of these MMAF-related genes encode
13 proteins which are linked directly or indirectly with the protein complex close to the radial
14 spoke 3 (RS3) (*DNAH1*, *WDR66*, *CFAP43*, *CFAP44* and now *CFAP70*) suggesting that the
15 RS3 biogenesis and function may be one of the major differences between cilia and sperm
16 flagella.

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1 **Supplementary data**

2 Supplementary data are available at Human Reproduction online.

3

4 **Acknowledgments**

5 We thank all patients and control individuals for their participation.

6

7 **Authors' roles**

8 J.B., C.C., G.M., P.F.R., C.A., S.B., and A.T. analyzed the data and wrote the manuscript; Z-
9 E.K., N.T-M., P.F.R. and C.C. performed and analyzed genetic data; J.B., C.C. and G.M.
10 performed IF experiments. G.P., M.B. performed RT-qPCR experiments; A.A-Y., V.S.
11 provided clinical samples and data; C.C., P.F.R., C.A. and G.M. designed the study,
12 supervised all molecular laboratory work, had full access to all of the data in the study and
13 takes responsibility for the integrity of the data and its accuracy. All authors contributed to the
14 report.

15

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20 Rares for the program Séquençage à haut débit 2012.

21

22 **Conflict of interest**

23 The authors have declared that no conflict of interest exists.

24

1 **Figure Legends**

2

3 **Figure 1** Morphology of normal and CFAP70 mutant spermatozoa, and the mutations
4 identified in the *CFAP70* gene. **(A)** Light microscopy analysis of spermatozoa from fertile
5 control and individual CFAP70_1. All spermatozoa from the patient have shorter flagella than
6 controls. Additional features of MMAF spermatozoa such as thick and coiled flagella are also
7 observed. Scale bars 10 μ m. **(B)** Electropherograms of Sanger sequencing showing the two
8 *CFAP70* variants identified in the MMAF cohort. The substituted nucleotides are highlighted
9 in red. **(C)** Location of *CFAP70* mutations in the intron-exon structure and in the protein
10 representation. Blue square stands for tetratricopeptide repeats (TPR) as predicted by InterPro
11 server (<https://www.ebi.ac.uk/interpro/>). Mutations are annotated in accordance to the
12 HGVS's recommendations. **(D)** Amino-acid sequences alignment of CFAP70 in different
13 vertebrate species showing the conservation of the amino-acid (Phe, F) in position 60
14 (highlighted in red) as well as the surrounded amino-acids.

15

16 **Figure 2** CFAP70 immunostaining in human spermatozoa from control and MMAF patients.
17 Sperm cells from a fertile control, the patient CFAP70_1, four other MMAF patients mutated
18 in *ARMC2*, *FSIP2*, *CFAP43*, *WDR66* and one MMAF patient with unknown genetic cause
19 were stained with anti-CFAP70 (HPA037582, Sigma-Aldrich, rabbit, 1:100, green) and anti-
20 acetylated tubulin (32-2500, ThermoFisher, mouse, 1: 1000, red) antibodies. DNA was
21 counterstained with DAPI showing sperm nuclear DNA (blue). The CFAP70 immunostaining
22 (green) uniformly decorates the full-length flagellum from the fertile control, the three
23 patients mutated in *ARMC2*, *FSIP2*, *CFAP43* and the MMAF patient with unknown genetic
24 cause. Conversely, CFAP70 staining is absent in sperm from the CFAP70_1 patient mutated in

1 *CFAP70* and the patient mutated in *WDR66*. Mutations are specified for each patient. Scale
2 bars 10 μm .

3

4 **Figure 3** Outer dynein arms are affected by *CFAP70* mutations.

5 Sperm cells from a fertile control and the patient *CFAP70*₁ were stained with anti-SPAG6
6 (HPA038440, Sigma-Aldrich, rabbit, 1:500, green), a protein located in the CP complex, and
7 anti-acetylated tubulin (32-2500, ThermoFisher, mouse, 1:1000, red) antibodies. DNA was
8 counterstained with DAPI. SPAG6 staining is mostly absent in sperm from the patient
9 mutated in *WDR66* irrespective of the sperm morphology (short, long, coiled or
10 disorganized). The histogram showing the percentage of analyzed spermatozoa with or
11 without the SPAG6 staining according to their morphology are presented (number of sperm
12 cells analyzed = 200). Scale bars 10 μm .

13

14 **Figure 4** The central pair complex are affected by *CFAP70* mutations. Sperm cells from a
15 fertile control and the patient *CFAP70*₁ were stained with anti-DNAI2 (H00064446-M01,
16 Abnova, mouse, 1:400, red), a protein located in the outer dynein arm, and anti-acetylated
17 tubulin (PA5-19489, ThermoFisher, rabbit, 1:500, green) antibodies. DNA was counterstained
18 with DAPI. The DNAI2 immunostaining is mostly absent or dramatically reduced in sperm
19 from the patient *CFAP70*₁ compared to the fertile control and irrespective of the sperm
20 morphology (short, long, coiled or disorganized). The histogram showing the percentage of
21 analyzed spermatozoa with or without the DNAI2 staining according to their morphology are
22 presented (number of sperm cells analyzed = 200). Scale bars 10 μm

23

Tables

Table I Detailed semen parameters for the two MMAF individuals harboring a CFAP70 mutation.

<i>CFAP70</i> mutated patients		Semen parameters																
Patients	<i>CFAP70</i> mutations	Sperm volume (ml)	Sperm conc.(10 ⁶ /ml)	Total motility 1 h	Vitality	Normal spermatozoa	Absent flagella	Short Flagella	Coiled Flagella	Bent Flagella	Flagella of irregular caliber	Tapered head	Thin head	Micro-cephalic	Macro-cephalic	Multiple heads	Abnormal base	Abnormal acrosomal region
CFAP70 ₁	c.1723-1G>T	2.5	2	0	75	0	0	90	0	0	0	10	0	1	0	0	0	0
CFAP70 ₂	c.178T>A	3	6.9	13	72	0	28	24	10	0	58	10	4	0	2	0	54	80
Reference limits^a		1.5 (1.4-1.7)	15 (12-16)	40 (38-42)	58 (55-63)	23 (20-26)	5 (4-6)	1 (0-2)	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
^aReference limits (5th centiles and their 95% confidence intervals) according to the World Health Organization

Supplementary data

Supplementary figures

Figure S1 Structure of mature mammalian sperm cells and localization of proteins whose functional absence leads to MMAF phenotype in human.

Schematic cross sections through a representative segment of the intermediate piece (IP), principal piece (PP) and terminal piece (TP) are shown. The axoneme is enlarged and the offset shows: the 9 outer microtubule doublets of the axoneme with associated inner dynein arms, outer dynein arms, radial spokes, nexin-dynein regulator complex, nexin links and the central pair of microtubule doublets. Proteins encoded by the MMAF-related genes identified by our team (red) and by other teams (green) in human are reported. Proteins with a “?” indicated a possible localization but not yet formally demonstrated. CFAP65 localization is totally unknown and not indicated.

Figure S2 Relative mRNA Expression of human *CFAP70* transcripts.

CFAP70 mRNA levels in a panel of 10 human normal tissues purchased from Life Technologies®. Results are presented as the mean of triplicates (ratio target gene/RPL6) ± Standard Deviation (SD). RT-qPCR data were normalized using the reference gene RPL6 with the $-\Delta\Delta C_t$ method. Heart expression is arbitrary set to 1. *CFAP70* has the strongest expression in testis compared to other organs. Unpaired t-test, *** $P < 0.001$.

Figure S3 CFAP70 immunostaining in human spermatozoa from the *WDR66* mutated patient according to various flagellum defects.

Sperm cells from a fertile control and one patient harboring the homozygous c.3007_3337del mutation in *WDR66* were stained with anti-CFAP70 (HPA037582, Sigma-Aldrich, rabbit, 1:100, green) and anti-acetylated tubulin (32-2500, ThermoFisher, mouse, 1: 1000, red) antibodies. DNA was counterstained with DAPI showing sperm nuclear DNA (blue). CFAP70 staining is mostly absent in sperm from the patient mutated in *WDR66* irrespective of the sperm morphology (short, long, coiled or disorganized). The histogram showing the percentage of analyzed spermatozoa with or without the CFAP70 staining according to their morphology are presented (number of sperm cells analyzed = 200). Scale bars 10µm.

Supplementary tables

Table S1 Primer sequences used for Sanger sequencing verification of *CFAP70* variations and respective melting temperatures (T_m).

Primer names	Primer sequences (5'-3')	T _m
CFAP70-3F	GACTCCAACATAACAGCACTTCA	59°C
CFAP70-3R	GGAAGAAGGCAATCGGGACA	
CFAP70-16F	GCTGATAGTCTACCACCCACC	59°C
CFAP70-16R	TTGTGTTTTTCAGTGCCTAGCA	

Table S2 Primers used for RT-qPCR of *CFAP70* in human.

Primer names	Primer sequences (5'-3')	T _m
CFAP70_qPCR_8F	TGGGACTTGGAAAGTCGCTG	58°C
CFAP70_qPCR_9R	GCAATCGGCAATTCGCTTCT	
RPL6_qPCR_F	TCCATTCGTCAGAGCAAACA	56°C
RPL6_qPCR_R	TACGGAGCAGCGCAAGAT	

Table S3. Genotype information of the four additional MMAF patients mutated in *ARMC2*, *FSIP2*, *CFAP43* and *WDR66* included in this study.

Gene	Variant coordinates	Transcript	cDNA Variation	Amino acid variation	Allelic status	References
<i>CFAP43</i>	chr10:105912486	ENST00000357060	c.3541-2A>C	p.Ser1181Lysfs*4	Homozygous	Coutton <i>et al.</i> , 2018
<i>WDR66</i>	chr12:122355768_122441833	ENST00000288912.8	c.[3007_3337del]	p.Ile1003Lysfs26	Homozygous	Kherraf <i>et al.</i> , 2018
<i>FSIP2</i>	chr2:185805693	ENST00000424728	c.[16389_16392delAATA]	p.Glu5463GlufsTer7	Homozygous	Martinez <i>et al.</i> , 2018
<i>ARMC2</i>	chr6:108904406	ENST00000392644.8	c.1023+1G>A	splice_donor_variant	Homozygous	Coutton <i>et al.</i> , 2019

REFERENCES

- Amiri-Yekta A, Coutton C, Kherraf Z-E, Karaouzène T, Le Tanno P, Sanati MH, Sabbaghian M, Almadani N, Sadighi Gilani MA, Hosseini SH, *et al.* Whole-exome sequencing of familial cases of multiple morphological abnormalities of the sperm flagella (MMAF) reveals new DNAH1 mutations. *Hum Reprod Oxf Engl* 2016;**31**:2872–2880.
- Auguste Y, Delague V, Desvignes J-P, Longepied G, Gnisci A, Besnier P, Levy N, Beroud C, Megarbane A, Metzler-Guillemain C, *et al.* Loss of Calmodulin- and Radial-Spoke-Associated Complex Protein CFAP251 Leads to Immotile Spermatozoa Lacking Mitochondria and Infertility in Men. *Am J Hum Genet* 2018;**103**:413–420.
- Baccetti B, Collodel G, Estenoz M, Manca D, Moretti E, Piomboni P. Gene deletions in an infertile man with sperm fibrous sheath dysplasia. *Hum Reprod Oxf Engl* 2005;**20**:2790–2794.
- Ben Khelifa M, Coutton C, Zouari R, Karaouzène T, Rendu J, Bidart M, Yassine S, Pierre V, Delaroche J, Hennebicq S, *et al.* Mutations in DNAH1, which encodes an inner arm heavy chain dynein, lead to male infertility from multiple morphological abnormalities of the sperm flagella. *Am J Hum Genet* 2014;**94**:95–104.
- Coutton C, Escoffier J, Martinez G, Arnoult C, Ray PF. Teratozoospermia: spotlight on the main genetic actors in the human. *Hum Reprod Update* 2015;**21**:455–485.
- Coutton C, Martinez G, Kherraf Z-E, Amiri-Yekta A, Boguenet M, Saut A, He X, Zhang F, Cristou-Kent M, Escoffier J, *et al.* Bi-allelic Mutations in ARMC2 Lead to Severe Astheno-Teratozoospermia Due to Sperm Flagellum Malformations in Humans and Mice. *Am J Hum Genet* 2019;**104**:331–340.
- Coutton C, Vargas AS, Amiri-Yekta A, Kherraf Z-E, Ben Mustapha SF, Le Tanno P, Wambergue-Legrand C, Karaouzène T, Martinez G, Crouzy S, *et al.* Mutations in

- CFAP43 and CFAP44 cause male infertility and flagellum defects in *Trypanosoma* and human. *Nat Commun* 2018;**9**:686.
- Dam TJP van, Kennedy J, Lee R van der, Vrieze E de, Wunderlich KA, Rix S, Dougherty GW, Lambacher NJ, Li C, Jensen VL, *et al.* CiliaCarta: An Integrated And Validated Compendium Of Ciliary Genes. *bioRxiv* 2017;123455.
- Dong FN, Amiri-Yekta A, Martinez G, Saut A, Tek J, Stouvenel L, Lorès P, Karaouzène T, Thierry-Mieg N, Satre V, *et al.* Absence of CFAP69 Causes Male Infertility due to Multiple Morphological Abnormalities of the Flagella in Human and Mouse. *Am J Hum Genet* 2018;**102**:636–648.
- Elam CA, Wirschell M, Yamamoto R, Fox LA, York K, Kamiya R, Dutcher SK, Sale WS. An axonemal PP2A B-subunit is required for PP2A localization and flagellar motility. *Cytoskelet Hoboken NJ* 2011;**68**:363–372.
- Fu G, Wang Q, Phan N, Urbanska P, Joachimiak E, Lin J, Wloga D, Nicastro D. The II dynein-associated tether and tether head complex is a conserved regulator of ciliary motility. *Mol Biol Cell* 2018;**29**:1048–1059.
- GTEX Consortium. Human genomics. The Genotype-Tissue Expression (GTEx) pilot analysis: multitissue gene regulation in humans. *Science* 2015;**348**:648–660.
- Heuser T, Dymek EE, Lin J, Smith EF, Nicastro D. The CSC connects three major axonemal complexes involved in dynein regulation. *Mol Biol Cell* 2012;**23**:3143–3155.
- Inaba K. Molecular basis of sperm flagellar axonemes: structural and evolutionary aspects. *Ann N Y Acad Sci* 2007;**1101**:506–526.
- Kherraf Z-E, Amiri-Yekta A, Dacheux D, Karaouzène T, Coutton C, Christou-Kent M, Martinez G, Landrein N, Le Tanno P, Fourati Ben Mustapha S, *et al.* A Homozygous Ancestral SVA-Insertion-Mediated Deletion in WDR66 Induces Multiple

- Morphological Abnormalities of the Sperm Flagellum and Male Infertility. *Am J Hum Genet* 2018;**103**:400-412
- Krausz C, Riera-Escamilla A. Genetics of male infertility. *Nat Rev Urol* 2018;**15**:369–384.
- Liu W, He X, Yang S, Zouari R, Wang J, Wu H, Kherraf Z-E, Liu C, Coutton C, Zhao R, *et al.* Bi-allelic Mutations in TTC21A Induce Asthenoteratospermia in Humans and Mice. *Am J Hum Genet* 2019;**104**:738–748.
- Lorès P, Coutton C, El Khouri E, Stouvenel L, Givelet M, Thomas L, Rode B, Schmitt A, Louis B, Sakheli Z, *et al.* Homozygous missense mutation L673P in adenylate kinase 7 (AK7) leads to primary male infertility and multiple morphological anomalies of the flagella but not to primary ciliary dyskinesia. *Hum Mol Genet* 2018;**27**:1196–1211.
- Martinez G, Kherraf Z-E, Zouari R, Fourati Ben Mustapha S, Saut A, Pernet-Gallay K, Bertrand A, Bidart M, Hograindleur JP, Amiri-Yekta A, *et al.* Whole-exome sequencing identifies mutations in FSIP2 as a recurrent cause of multiple morphological abnormalities of the sperm flagella. *Hum Reprod Oxf Engl* 2018;**33**:1973-1984
- Mizuno K, Shiba K, Okai M, Takahashi Y, Shitaka Y, Oiwa K, Tanokura M, Inaba K. Calaxin drives sperm chemotaxis by Ca²⁺-mediated direct modulation of a dynein motor. *Proc Natl Acad Sci U S A* 2012;**109**:20497–20502.
- Ohta T, Essner R, Ryu J-H, Palazzo RE, Uetake Y, Kuriyama R. Characterization of Cep135, a novel coiled-coil centrosomal protein involved in microtubule organization in mammalian cells. *J Cell Biol* 2002;**156**:87–99.
- Ray PF, Toure A, Metzler-Guillemain C, Mitchell MJ, Arnoult C, Coutton C. Genetic abnormalities leading to qualitative defects of sperm morphology or function. *Clin Genet* 2017;**91**:217–232.

- Sapiro R, Tarantino LM, Velazquez F, Kiriakidou M, Hecht NB, Bucan M, Strauss JF. Sperm antigen 6 is the murine homologue of the *Chlamydomonas reinhardtii* central apparatus protein encoded by the PF16 locus. *Biol Reprod* 2000;**62**:511–518.
- Satir P, Christensen ST. Structure and function of mammalian cilia. *Histochem Cell Biol* 2008;**129**:687–693.
- Sha Y-W, Xu X, Mei L-B, Li P, Su Z-Y, He X-Q, Li L. A homozygous CEP135 mutation is associated with multiple morphological abnormalities of the sperm flagella (MMAF). *Gene* 2017a;**633**:48–53.
- Sha Y-W, Xu X, Mei L-B, Li P, Su Z-Y, He X-Q, Li L. A homozygous CEP135 mutation is associated with multiple morphological abnormalities of the sperm flagella (MMAF). *Gene* 2017b;**633**:48–53.
- Shamoto N, Narita K, Kubo T, Oda T, Takeda S. CFAP70 Is a Novel Axoneme-Binding Protein That Localizes at the Base of the Outer Dynein Arm and Regulates Ciliary Motility. *Cells* 2018;**7**(9).
- Shen Y, Zhang F, Li F, Jiang X, Yang Y, Li X, Li W, Wang X, Cheng J, Liu M, *et al.* Loss-of-function mutations in QRICH2 cause male infertility with multiple morphological abnormalities of the sperm flagella. *Nat Commun* 2019;**10**:433.
- Tang S, Wang X, Li W, Yang X, Li Z, Liu W, Li C, Zhu Z, Wang L, Wang J, *et al.* Biallelic Mutations in CFAP43 and CFAP44 Cause Male Infertility with Multiple Morphological Abnormalities of the Sperm Flagella. *Am J Hum Genet* 2017;**100**:854–864.
- The UniProt Consortium. UniProt: the universal protein knowledgebase. *Nucleic Acids Res* 2017;**45**:D158–D169.

Urbanska P, Joachimiak E, Bazan R, Fu G, Poprzeczko M, Fabczak H, Nicastro D, Wloga D.

Ciliary proteins Fap43 and Fap44 interact with each other and are essential for proper cilia and flagella beating. *Cell Mol Life Sci CMLS* 2018;**75**:4479–4493.

Urbanska P, Song K, Joachimiak E, Krzemien-Ojak L, Koprowski P, Hennessey T, Jerka-

Dziadosz M, Fabczak H, Gaertig J, Nicastro D, *et al.* The CSC proteins FAP61 and FAP251 build the basal substructures of radial spoke 3 in cilia. *Mol Biol Cell* 2015;**26**:1463–1475.

Wambergue C, Zouari R, Fourati Ben Mustapha S, Martinez G, Devillard F, Hennebicq S,

Satre V, Brouillet S, Halouani L, Marrakchi O, *et al.* Patients with multiple morphological abnormalities of the sperm flagella due to DNAH1 mutations have a good prognosis following intracytoplasmic sperm injection. *Hum Reprod Oxf Engl* 2016;**31**:1164–1172.

Wang Y, Yang J, Jia Y, Xiong C, Meng T, Guan H, Xia W, Ding M, Yuchi M. Variability in the morphologic assessment of human sperm: use of the strict criteria recommended by the World Health Organization in 2010. *Fertil Steril* 2014;**101**:945-949

Xu Y, Cao J, Huang S, Feng D, Zhang W, Zhu X, Yan X. Characterization of tetratricopeptide repeat-containing proteins critical for cilia formation and function. *PloS One* 2015;**10**:e0124378.

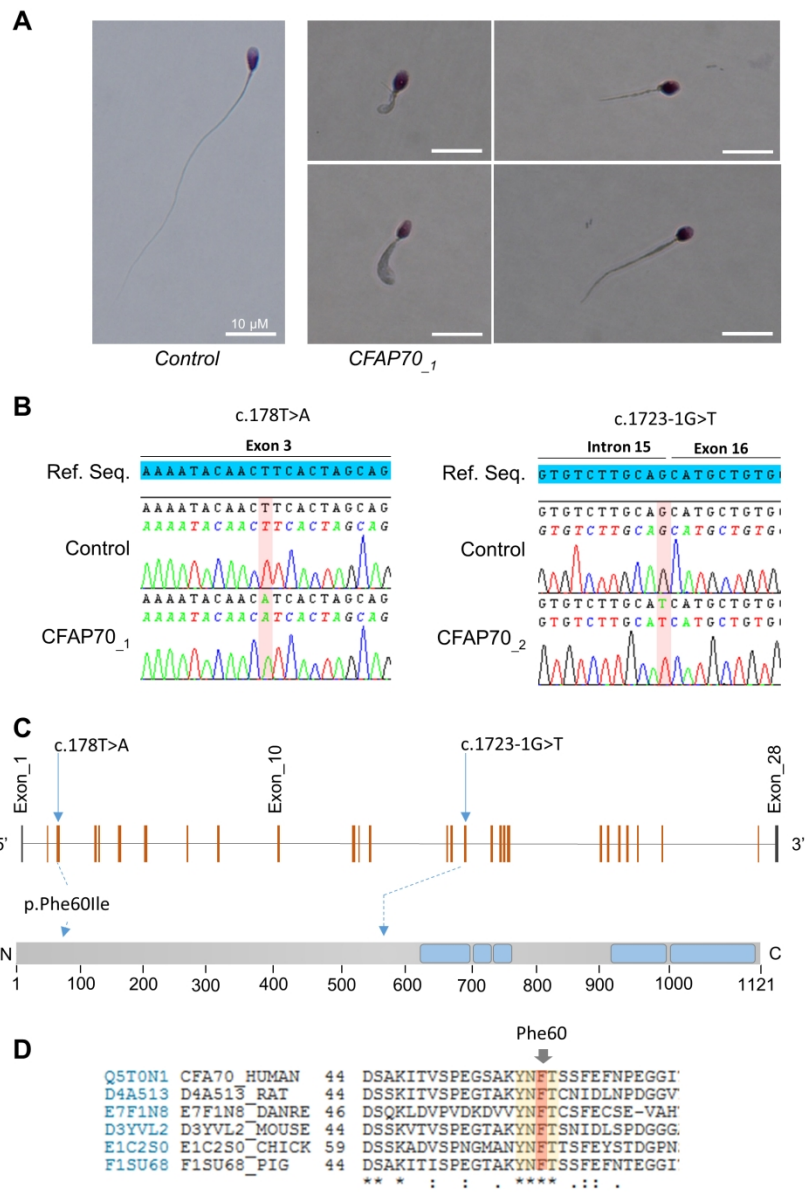


Figure 1

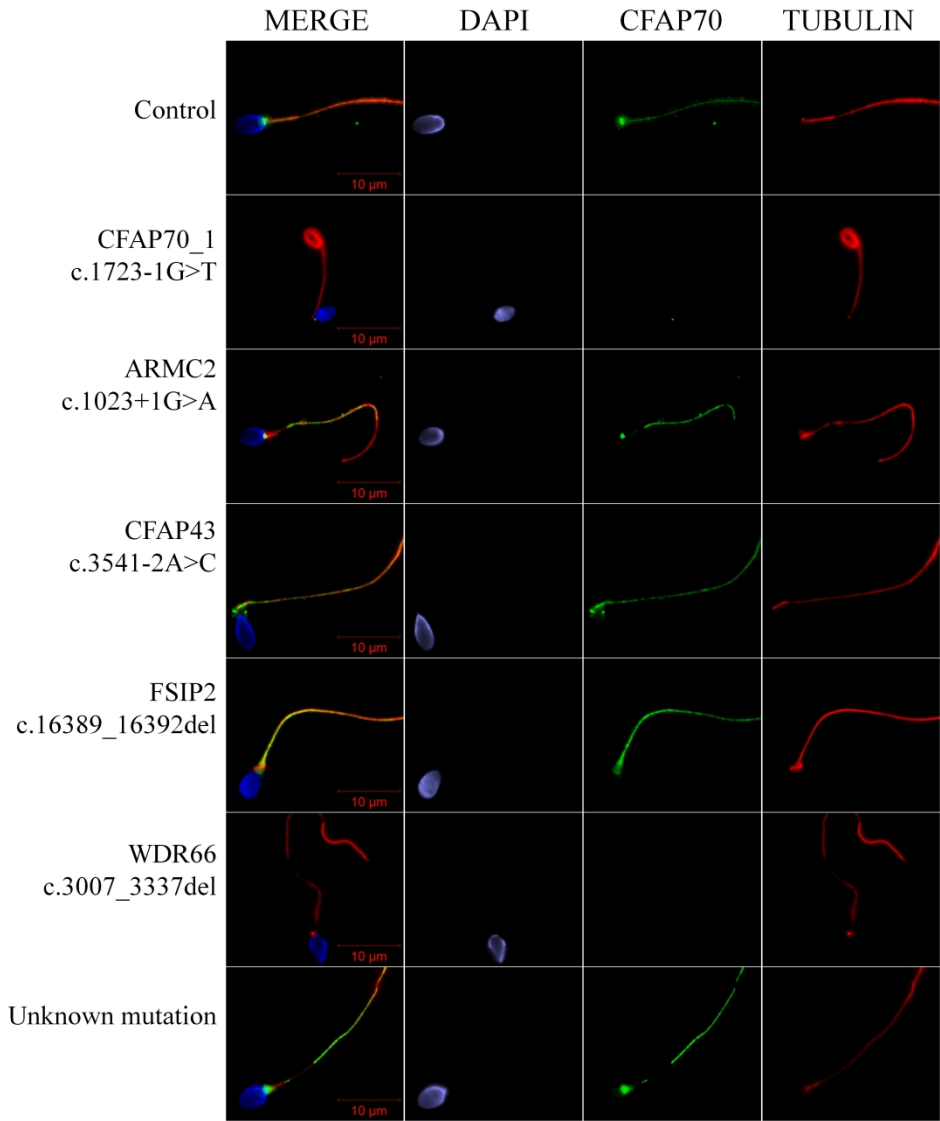


figure 2

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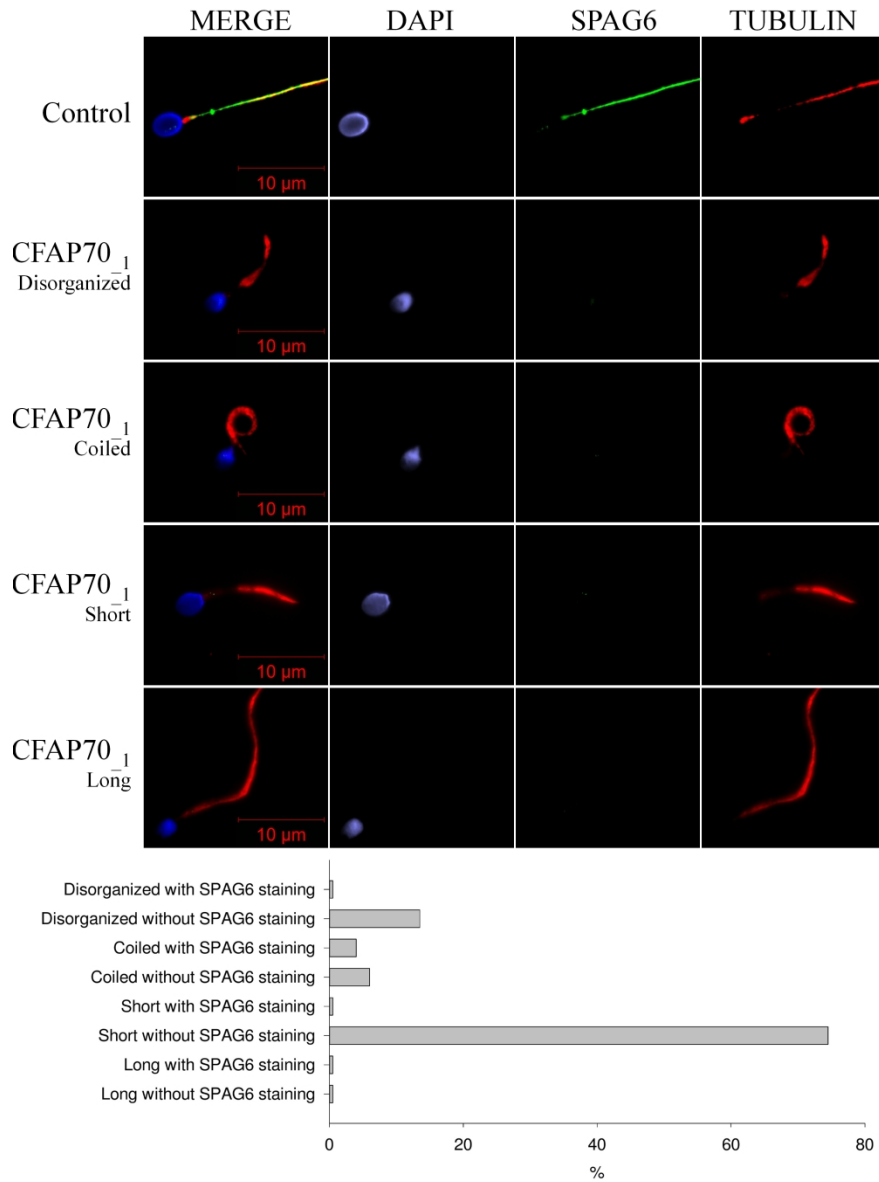


Figure 3

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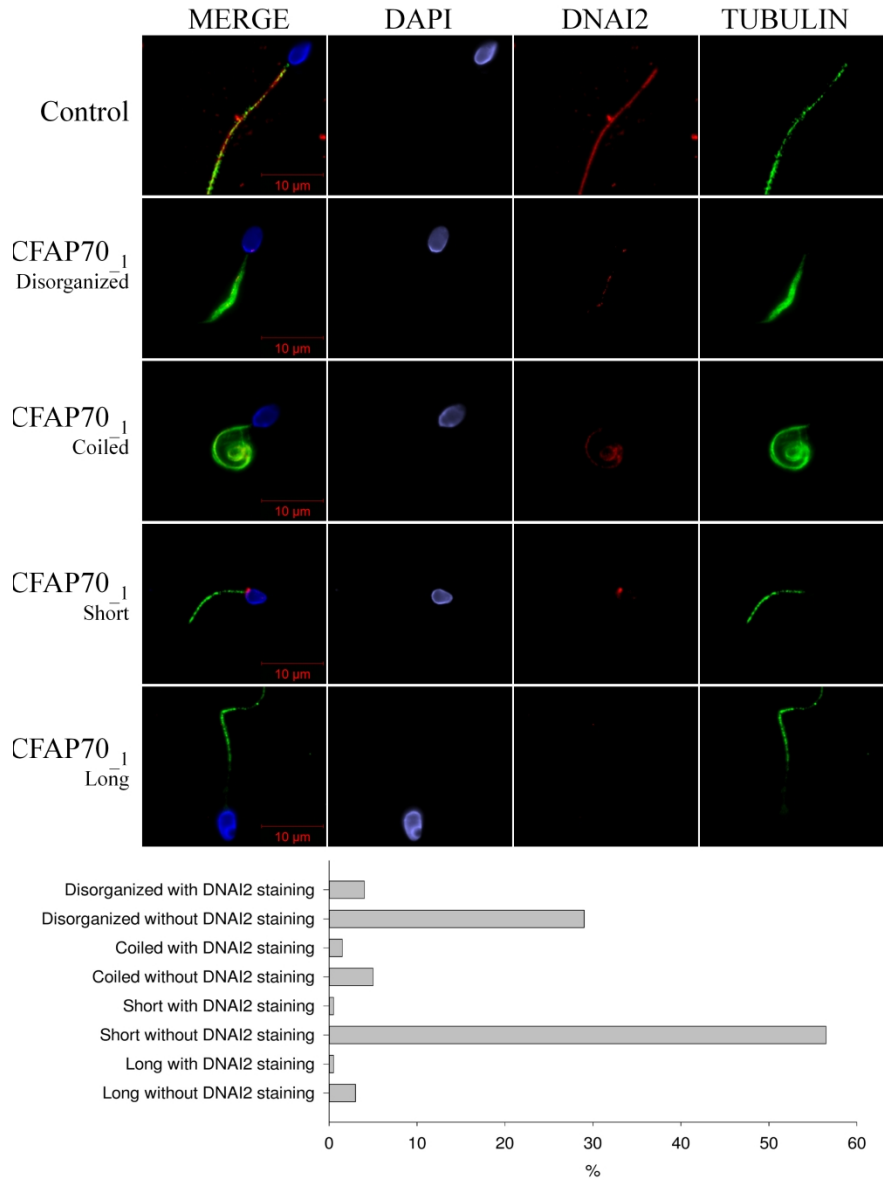


Figure 4

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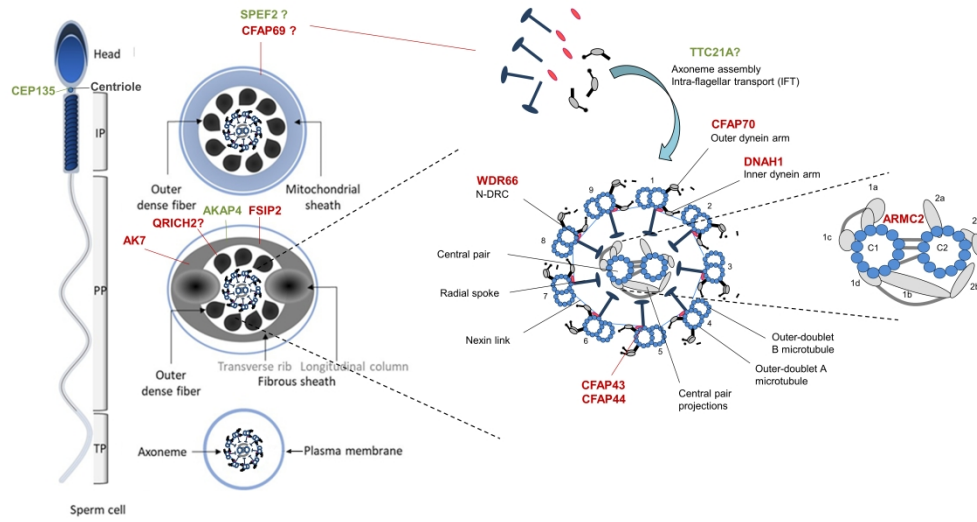
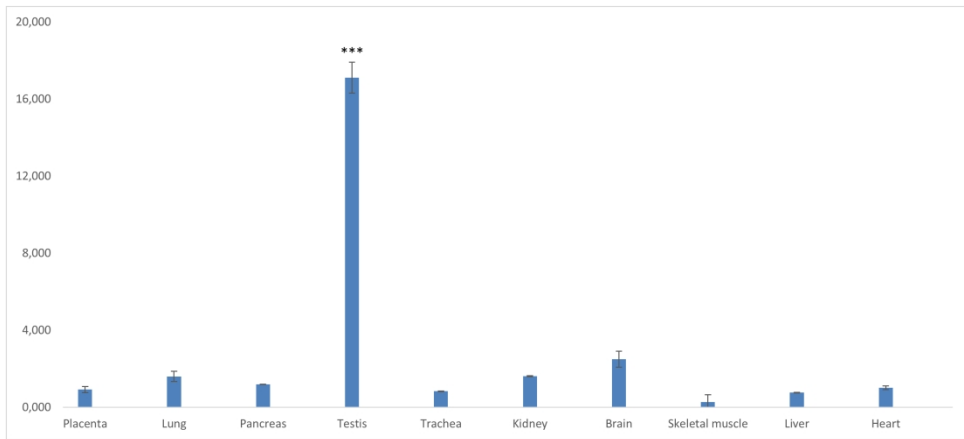
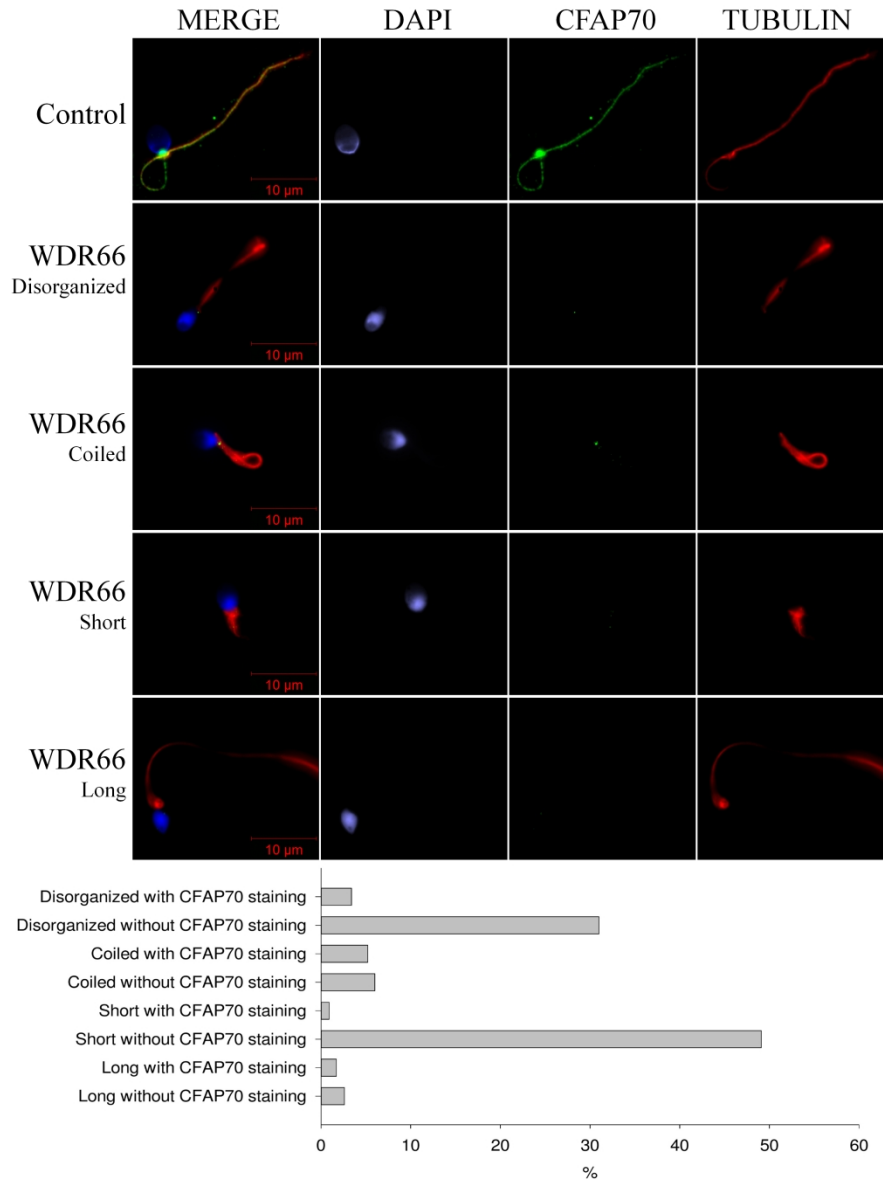


Figure S1





1098x1405mm (96 x 96 DPI)