Bi-allelic Mutations in *ARMC2* Lead to Severe Astheno-Teratozoospermia Due to Sperm Flagellum Malformations in Humans and Mice

Charles Coutton,[1,2,21,*] Guillaume Martinez,[1,2,21] Zine-Eddine Kherraf,[1,3] Amir Amiri-Yekta,[1,3,4] Magalie Boguenet,[1] Antoine Saut,[1] Xiaojin He,[5] Feng Zhang,[6] Marie Cristou-Kent,[1] Jessica Escoffier,[1] Marie Bidart,[7] Véronique Satre,[1,2] Béatrice Conne,[8] Selima Fourati Ben Mustapha,[9] Lazhar Halouani,[9] Ouafi Marrakchi,[9] Mounir Makni,[9] Habib Latrous,[9] Mahmoud Kharouf,[9] Karin Pernet-Gallay,[10] Mélanie Bonhivers,[11] Sylviane Hennebicq,[1,12] Nathalie Rives,[13,14] Emmanuel Dulioust,[15,16] Aminata Touré,[16,17,18] Hamid Gourabi,[4] Yunxia Cao,[5] Raoudha Zouari,[9] Seyedeh Hanieh Hosseini,[19] Serge Nef,[8] Nicolas Thierry-Mieg,[20] Christophe Arnoult,[1,21] and Pierre F. Ray[1,3,21,*]

Male infertility is a major health concern. Among its different causes, multiple morphological abnormalities of the flagella (MMAF) induces asthenozoospermia and is one of the most severe forms of qualitative sperm defects. Sperm of affected men display short, coiled, absent, and/or irregular flagella. To date, six genes (*DNAH1*, *CFAP43*, *CFAP44*, *CFAP69*, *FSIP2*, and *WDR66)* have been found to be recurrently associated with MMAF, but more than half of the cases analyzed remain unresolved, suggesting that many yet-uncharacterized gene defects account for this phenotype. Here, whole-exome sequencing (WES) was performed on 168 infertile men who had a typical MMAF phenotype. Five unrelated affected individuals carried a homozygous deleterious mutation in *ARMC2*, a gene not previously linked to the MMAF phenotype. Using the CRISPR-Cas9 technique, we generated homozygous *Armc2* mutant mice, which also presented an MMAF phenotype, thus confirming the involvement of *ARMC2* in human MMAF. Immunostaining experiments in *AMRC2*-mutated individuals and mutant mice evidenced the absence of the axonemal central pair complex (CPC) proteins SPAG6 and SPEF2, whereas the other tested axonemal and peri-axonemal components were present, suggesting that ARMC2 is involved in CPC assembly and/or stability. Overall, we showed that bi-allelic mutations in *ARMC2* cause male infertility in humans and mice by inducing a typical MMAF phenotype, indicating that this gene is necessary for sperm flagellum structure and assembly.

The characterization of the genetic basis of male infertility represents an important challenge; more than 4,000 genes are thought to be needed for sperm production,[1] and therefore defects in any of these genes can hamper spermatogenesis and induce one of many established sperm phenotypes.[2] Of late, high-throughput sequencing technologies have allowed researchers to identify an increasing number of genes required for sperm production and have thus greatly facilitated efforts to explain the genetic basis of different forms of male infertility.[3] This is especially true for teratozoospermia, qualitative spermatogenesis defects that lead to morphological sperm abnormalities.[4,5] One of the most severe forms of qualitative sperm defects, the MMAF phenotype is responsible for astheno-teratozoospermia,[4] which is characterized by the presence of immotile spermatozoa presenting with a mosaic of sperm flagellum malformations, including short, coiled, and/or absent flagella and/or flagella of irregular caliber.[6] Whole-exome sequencing (WES) analysis revealed that mutations in *DNAH1* (MIM: 603332), *CFAP43* (MIM: 617558), *CFAP44* (MIM: 617559), *CFAP69* (MIM: 617949), *FSIP2* (MIM: 615796), and *WDR66* (MIM: 612573) account for

[1]Team Genetics Epigenetics and Therapies of Infertility, Institute for Advanced Biosciences, Université Grenoble Alpes, Inserm U1209, Centre National de la Recherche Scientifique UMR 5309, Grenoble 38000, France; [2]Unité Médicale (UM) de Génétique Chromosomique, Centre Hospitalier Universitaire Grenoble Alpes, Grenoble 38000, France; [3]Unité Médicale de génétique de l'infertilité et de diagnostic pré-implantatoire (GI-DPI), Centre Hospitalier Universitaire Grenoble Alpes, Grenoble 38000, France; [4]Department of Genetics, Reproductive Biomedicine Research Center, Royan Institute for Reproductive Biomedicine, the Academic Center for Education, Culture, and Research, PO Box 16635-148, Tehran, Iran; [5]Department of Obstetrics and Gynecology, Reproductive Medicine Center, the First Affiliated Hospital of Anhui Medical University, Hefei 230022, China; [6]Institute of Metabolism and Integrative Biology, Obstetrics and Gynecology Hospital, School of Life Sciences, Fudan University, Shanghai 200011, China; [7]Clinatec, Pôle Recherche, Inserm UMR 1205, Centre Hospitalier Universitaire Grenoble, Alpes, Université Grenoble Alpes, Grenoble 38000, France; [8]Department of Genetic Medicine and Development, University of Geneva Medical School, Geneva 1211, Switzerland; [9]Polyclinique les Jasmins, Centre d'Aide Médicale à la Procréation, Centre Urbain Nord, Tunis 1003, Tunisia; [10]Grenoble Neuroscience Institute, Inserm 1216, Grenoble 38000, France; [11]Microbiologie Fondamentale et Pathogénicité, Université de Bordeaux, Centre National de la Recherche Scientifique UMR 5234, Bordeaux 33000, France; [12]Unité fonctionnelle de Biologie de la Procréation, Centre Hospitalier Universitaire de Grenoble, Grenoble 38000, France; [13]EA 4308 Gametogenesis and Gamete Quality, Department of Reproductive Biology-CECOS, Rouen University Hospital, UNIROUEN, Normandie Université, 76000 Rouen, France; [14]Institut de Recherche en Santé, Environnement, et Travail, Inserm U1085, Université de Rennes 1, Rennes, France; [15]Laboratoire d'Histologie Embryologie et de la Biologie de la Reproduction, Groupe Hospitalier Cochin, Broca, et Hôtel Dieu, Assistance Publique-Hôpitaux de Paris, Paris 75014, France; [16]Faculté de Médecine, Université Paris Descartes, Sorbonne Paris Cité, Paris 75014, France; [17]Inserm U1016, Institut Cochin, Paris 75014, France; [18]Centre National de la Recherche Scientifique UMR 8104, Paris 75014, France; [19]Department of Andrology, Reproductive Biomedicine Research Center, Royan Institute for Reproductive Biomedicine, the Academic Center for Education, Culture, and Research, Tehran, Iran; [20]Université Grenoble Alpes, Centre National de la Recherche Scientifique, Téchniques de l'Ingénierie Médicale et de la Complexité et Informatiques, Mathématiques, Applications, Grenoble (TIMC-IMAG), Biologie Computationnelle et Mathématique (BCM), 38000 Grenoble 38000, France
[21]These authors contributed equally to this work
*Correspondence: ccoutton@chu-grenoble.fr (C.C.), pray@chu-grenoble.fr (P.F.R.)
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Table 1. Detailed Semen Parameters for the Five MMAF Individuals Harboring a *ARMC2* Mutation

***ARMC2*-Mmutated Individuals**		**Semen Parameters**							
Individuals	***ARMC2* Mutation**	**Sperm Volume (ml)**	**Sperm Conc. (10^6/mL)**	**Total Motility 1h**	**Vitality**	**Normal Spermatozoa**	**Absent Flagella**	**Short Flagella**	**Coiled Flagella**
ARMC2$_{_1}$	c.1023+1G>A	4	35	6	NA	0	10	50	16
ARMC2$_{_2}$	c.2279T>A	4.5	4.6	5	64	0	14	30	6
ARMC2$_{_3}$	c.2353_2354delTT	1	58.5	0	16	3	31	37	23
ARMC2$_{_4}$	c.1284_ 1288delACAAA	4.2	2	0	35	2	0	83	0
ARMC2$_{_5}$	c.421C>T	9.2	10.1	1.1	NA	0	20	38	36
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)

Values are percentages unless specified otherwise.
[a]Reference limits (5th centiles and their 95% confidence intervals) according to the World Health Organization.

the main genetic causes of MMAF.[6–13] Rarer recessive mutations in *AK7* (MIM: 615364), *CEP135* (MIM: 611423), and *CFAP65* (MIM: 614270) were also recently identified in different familial cases of MMAF.[10,14,15] Despite these recent findings, more than half of the studied MMAF cases remain without a diagnosis, demonstrating the high genetic heterogeneity of this phenotype and the need for further genetic explorations.[12]

Here, we analyzed by WES a total of 168 MMAF-affected individuals, including 78 who were previously reported[9] and an additional 90 unrelated and unpublished individuals with MMAF. All but one individual were analyzed together with the same analytical pipeline, constituting a 167-strong cohort. In this main cohort, 83 individuals were of North African origin and sought consultation for primary infertility at the Clinique des Jasmins in Tunis, 52 individuals originated from the Middle East (Iran) and were treated in Tehran at the Royan Institute (Reproductive Biomedicine Research Center) for primary infertility, and 32 subjects were recruited in France: 25 at the Cochin Institute in Paris, three in Rouen, two in Grenoble, one in Lille, and one in Caen. All individuals presented with a typical MMAF phenotype, which is characterized by severe asthenozoospermia (total sperm motility below 10%) with at least three of the following flagellar abnormalities present in >5% of the spermatozoa: short, absent, coiled, bent, or irregular flagella (Table 1, Figures 1A–1D). All individuals had a normal somatic karyotype (46, XY) with normal bilateral testicular size, hormone levels, and secondary sexual characteristics. Informed consent was obtained from all the individuals participating in the study according to local protocols and the principles of the Declaration of Helsinki. The study was approved by local ethics committees, and samples were then stored in the CRB Germethèque (certification under ISO-9001 and NF-S 96-900) according to a standardized procedure or were part of the Fertithèque collection declared to the French Ministry of health (DC-2015-2580) and the French Data Protection Authority (DR-2016-392).

WES and bioinformatics analysis were performed according to an improved version of our previously described protocol[9] as described in the Supplemental Material and Methods. Data analysis of the whole cohort of 167 MMAF individuals identified 54 individuals (32.3%) with harmful mutations in known MMAF-related genes (Table S1). In 15 individuals, previously unreported variants were identified in *CFAP43* (n = 2), *CFAP44* (n = 1), *DNAH1* (n = 6), *WDR66* (n = 4), and *FSIP2* (n = 2), thus confirming the importance of these genes in the etiology of the MMAF syndrome (Table S1). In addition, we identified four individuals (ARMC2$_{_1–4}$) with a homozygous variant in *ARMC2*, a gene not previously associated with any pathology; these individuals accounted for 2.4% of our cohort. *ARMC2* (GenBank: NM_032131.5) is located on chromosome 6 and contains 18 exons encoding a predicted 867-amino-acid protein (NCBI: NP8115507.4; UniProtKB: Q8NEN0). Three individuals (ARMC2$_{_1,3,4}$) had a loss-of-function variant, and one (ARMC2$_2$) had a likely deleterious missense variant (Table 1, Table S1). In addition, we identified a fifth individual (ARMC2$_{_5}$) with an *ARMC2* homozygous loss-of-function variant (Table S2). This individual was of Chinese origin and sought consultation for primary infertility at the First Affiliated Hospital of Anhui Medical University (Hefei City, Anhui Province) in 2012. He was born to first-cousin parents and presented a typical MMAF phenotype, and WES analysis for this individual was performed as described in the previous report.[10] Informed consent was obtained from all tested individuals, and the study was approved by the local institutional board.

The five *ARMC2* variants were found in five unrelated individuals, and all were absent from control sequence databases (dbSNP, 1000 Genomes Project, NHLBI Exome Variant Server, gnomAD, and our in-house control database). The variant identified in individual ARMC2$_{_1}$ is a splice variant, c.1023+1G>A, altering a consensus splice donor site of *ARMC2* exon 8 (Figure 1I). To evaluate the deleterious effect of this splicing variant, we performed

Semen Parameters								
Bent Flagella	**Flagella of Irregular Caliber**	**Tapered Head**	**Thin Head**	**Micro-Cephalic**	**Macro-Cephalic**	**Multiple Heads**	**Abnormal Base**	**Abnormal Acrosomal Region**
NA	70	18	28	32	0	2	28	98
16	78	2	2	42	0	4	6	88
NA	39	60	9	0	0	0	31	52
0	0	11	0	2	0	0	0	0
0	0	NA	NA	NA	NA	NA	NA	NA
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)

RT-PCR with RNA extracted from sperm cells from individual ARMC2_1. Amplification of a sequence ranging from cDNA exon 7–10 from a control individual yielded a normal band of 608 bp, whereas a single smaller band was obtained from cDNA of individual ARMC2_1 (Figure S1A). Sequence analysis of the amplified products demonstrated that the c.1023+1G>A variant results in exon 8 skipping (Figure S1B, Figure S1C), causing a shift in the translational reading frame and introducing a premature termination codon (p.Glu283AlafsTer2). Primer sequences and RT-PCR conditions are indicated in Table S3. The variant identified in individual ARMC2_2 is a missense variation, c.2279T>A (p.Ile760Asn), located in exon 16 (Figure 1I). No mRNA analysis or immunostaining could be performed on sperm cells from this individual because of the lack of biological samples. However, using prediction software for non-synonymous SNPs, we found that this missense change is predicted to be deleterious by SIFT (score of 0) and probably damaging by PolyPhen (score of 0.987). Moreover, the concerned amino acid (Ile760) was found to be conserved in *ARMC2* orthologs (Figure S2). The two other variants identified in ARMC2_3 and ARMC2_4 were small frameshift indels, c.2353_2354delTT (p.Leu785MetfsTer5) and c.1284_1288delACAAA (p.Lys428AsnfsTer3), located in exon 17 and exon 10, respectively (Figure 1I), inducing a premature translation termination. The last identified variant (ARMC2_5) is a stop-gain variant, c.421C>T (p.Gln141Ter), located in exon 4 (Figure 1I). Familial study confirmed the presence of the homozygous loss-of-function variant in ARMC2_5 and indicated that his parents were both heterozygous and that his non-affected brother was homozygous wild-type (Figure S3A, B). The last three variants (in individuals ARMC2_3–5) introduce a premature stop codon and are therefore expected to induce nonsense-mediated mRNA decay that is likely to prevent protein synthesis. All *ARMC2* variants are deposited in ClinVar under reference SUB4929442.

Overall, a total of 168 exomes from MMAF-affected individuals were analyzed, and five unrelated affected individuals were shown to carry a homozygous deleterious mutation in *ARMC2*. No other candidate variants reported to be associated with cilia, flagella, or male fertility were present in any of the five individuals with *ARMC2* mutatations. We also note that none of the individuals analyzed here carried a homozygous deleterious variant in any two (or more) of the pathological MMAF-associated genes (*DNAH1*, *CFAP43*, *CFAP44*, *CFAP69*, *FSIP2*, *WDR66*, and *ARMC2)*, i.e., the 54 individuals with an established causal mutation did not carry another candidate variant. All mutations identified by WES were validated by Sanger sequencing, as previously described[9,10] and as illustrated in Figure 1H. PCR primers and protocols used for each individual are listed in Table S4.

ARMC2 is preferentially expressed in the testis according to data from ENCODE, FANTOM, and GTEx[16–18] and is described as being associated with cilia and flagella.[19] We confirmed these data by RT-qPCR experiments in a panel of human tissues; these experiments indicated that expression of *ARMC2* mRNA in testis is significantly higher than in other tested tissues (Figure S4). Primer sequences and RT-qPCR conditions are indicated in Table S5. According to the Uniprot server, ARMC2 is an armadillo protein composed of 12 armadillo repeats (ARM-repeat) flanked by unique C-terminal and N-terminal domains[20] (Figure 1I).

Sperm analysis was carried out in the source laboratories during routine biological examination of the individuals according to World Health Organization (WHO) guidelines.[21] The morphology of the sperm cells was assessed with Papanicolaou staining (Figures 1A–1D). Detailed semen parameters of the five individuals with *ARMC2* mutations are presented in Table 1, and the average semen parameters of the studied MMAF cohort, separated by genotypes, are described in the Table S6. Among the different parameters studied, only viability, total motility, and "lack of flagellum" presented a statistical difference

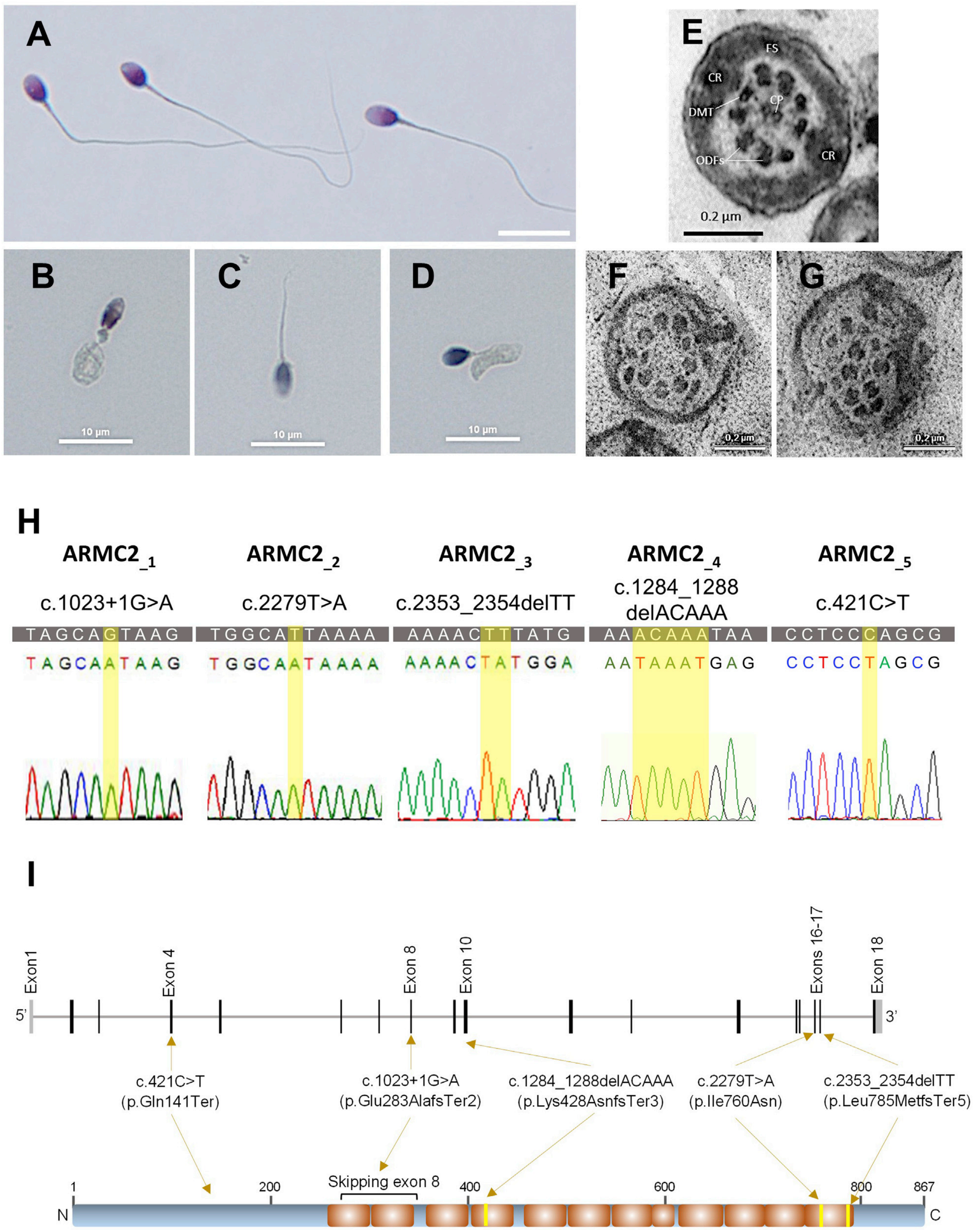

Figure 1. Morphology of Normal and *ARMC2*-Mutant Spermatozoa, and the Mutations Identified in Individuals with *ARMC2* Mutations
Light-microscopy analysis of spermatozoa from a fertile control individual (A) and individual ARMC_1 (B–D). Most spermatozoa from individuals with *ARMC2* mutations have flagella that are coiled (B), short (C), and/or of irregular caliber (D).
Transmission-electron-microscopy analyses of sperm cells from a control individual (E) and individual ARMC2_1 (F–G).
(E) Cross-sections of the principal piece from a fertile control individual. The axoneme is composed of nine doublets of microtubules (DMTs) circularly arranged around a central-pair complex (CPC) of microtubules (9 + 2 organization). The axoneme is surrounded by

(legend continued on next page)

between the different groups, according to their genotype (one-way ANOVA test). For parameters with a positive ANOVA test, a pairwise statistical Fisher's LSD test was employed so that significant differences between individuals with different genotypes could be identified (Figure S5). Concerning vitality, sperm from individuals with *WDR66* mutations and non-characterized individuals (unknown) presented a significant increase in comparison with individuals harboring *CFAP69, CFAP44, FSIP2,* and *ARMC2* mutations. With regard to motility, *CFAP43* and *CFAP44* showed the most pronounced alteration, as a result of a significant increase in sperm without a flagellum, and individuals harboring *CFAP44* mutations displayed a significant increase in "no tail" sperm (Figure S5).

Sperm samples for additional phenotypic characterization could only be obtained from individual ARMC2_1. We studied the ultrastructure of sperm cells from individual ARMC2_1 by transmission electron microscopy (TEM) (Figures 1E–1G) according to the protocol previously described.[9] For details, see Supplemental Material and Methods. Because of the low number of sperm cells available, only a few cross-sections (<10) were of sufficient quality for analysis. Among these sections, all were abnormal, and the main defect observed was the absence of the CPC (9 + 0 conformation) (Figure 1F). In some sections we observed a dramatic axonemal disorganization associated with peri-axonemal structural defects such as unassembled outer dense fibers (ODFs) (Figure 1G), a defect already observed in sperm from MMAF-affected individuals carrying mutations in other genes. Observation of rare longitudinal sections showed severe abnormalities such as truncated flagella or the presence of cytoplasmic structures encompassing unassembled axonemal components (not shown).

To assess the impact the absence of ARMC2 has on mouse spermatogenesis, we used the CRISPR-Cas9 technology (as previously described) to generate *Armc2* mutant animals.[9,22] For all experiments involving mice, animals were handled and euthanized in accordance with methods approved by the animal ethics committees of Grenoble and Geneva. All mice were adult (6 weeks or older) mice. We generated a strain with a one-nucleotide duplication in exon 4 (DupT), inducing a translational frameshift expected to lead to the complete absence of the protein or the production of a truncated protein. mRNA was extracted from *Armc2* homozygous-mutant mice (*Armc2*^{mutant}) and amplified by RT-PCR. The level of *Armc2*^{mutant} mRNA amplification was much lower in mutant animals than in controls (Figure S6). Sanger sequencing of mRNA from *Armc2* homozygous-mutant mice confirmed the production of abnormal transcripts with a premature stop codon 12 nucleotides after the first modified codon at position 135 (GenBank: NM_001034858.3 [c.403dupT]; NCBI: NP_001030030.2 [p.Tyr135LeufsTer12]) (Figure S6). The guide RNA sequence for CRISPR-Cas9 mice generation and the list of primers used for mice genotyping and RT-PCR are indicated in Table S7 and Table S8. We studied sperm morphology and observed that in contrast to what is observed in WT animals (Figure 2A), epididymal sperm from *Armc2*^{mutant} males displayed a phenotype identical to the typical human MMAF phenotype: 100% of spermatozoa had short, thick, and/or coiled flagella, whereas sperm heads conserved an overall typical hooked shape (Figures 2B–2D). As could be expected, homozygous *Armc2*^{mutant} males exhibited complete infertility when mated with WT females (Figure 2E), whereas homozygous *Armc2*^{mutant} females were fully fertile and gave litters of normal size compared to those of WT females (8.33 ± 1.11 versus 9.67 ± 1.01 pups/litter [mean ± SE, n = 6 versus n = 3]). There was no obvious testicular anomaly; there was no difference in weight between *Armc2*^{mutant} and WT testes (94 ± 20 and 92 ± 10.67 mg per testis; mean ± SE, n = 6 and n = 3, respectively) (Figure 2F). Structural defects were observed in close to 100% of spermatozoa from *Armc2*^{mutant} males (Figure 2G) and were associated with a complete motility deficiency (Figure 2H). Sperm production was also affected, as shown by *Armc2*^{mutant} sperm concentrations of 4.47 ± 1.29 × 10^6 sperm/mL versus concentrations of 20.75 ± 8.33 × 10^6 sperm/mL (mean ± SE, n = 6 and n = 3) in WT littermates (Figure 2I).

To define the ultrastructural defects evidenced by TEM and to characterize the molecular defects induced by *ARMC2* mutation in human sperm, we studied by immunofluorescence the presence and localization of several proteins belonging to different sub-structures of the axoneme. We investigated the presence of the following proteins: SPAG6 as a marker of the CPC; DNAI2 and DNALI1 as markers of outer and inner dynein arms (ODAs and IDAs), respectively; RSPH1 as a marker of the radial spokes (RS), GAS8 as a marker of the nexin-dynein regulatory complex (N-DRC), and AKAP4 as marker of the fibrous sheath (FS). We observed that in sperm from individual ARMC2_1, staining of SPAG6, an axoneme central-pair complex protein,[23] was totally absent from the flagellum (Figure 3A). In contrast, immunostaining for AKAP4, DNALI1, DNAH5, RSPH1, and GAS8 was similar to that of controls, suggesting that FS, IDAs, ODAs, RS,

seven outer dense fibers (ODFs) and by the fibrous sheath (FS) composed of two longitudinal columns (LCs) connected by circumferential ribs (CRs).
(F) A cross-section of a sperm flagellum from individual ARMC2_1 shows a 9 + 0 axoneme lacking the CPC.
(G) A cross-section of a sperm flagellum from individual ARMC2_1 shows a severe axonemal disorganization with unassembled ODFs and DMTs. Scale bars: 10 μm (A–D) and 200 nm (E–G).
(H) Electrophoregrams of Sanger sequencing for the five ARMC2-mutated individuals are compared to the reference sequence.
(I) Location and nature of *ARMC2* mutations in *ARMC2* and of alterations in the protein. Colored squares stand for armadillo repeats as predicted by the Uniprot server. Mutations are annotated in accordance to the HGVS's recommendations.

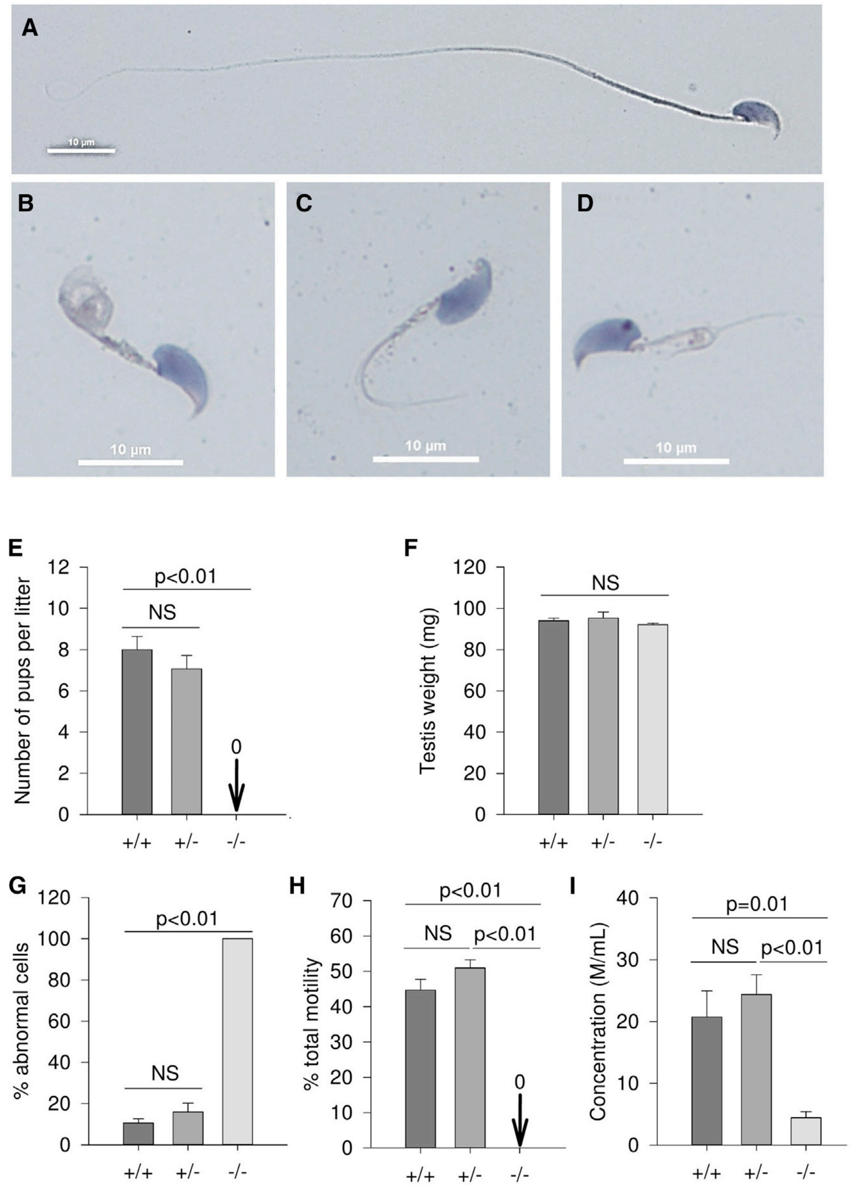

Figure 2. Reproductive Phenotype of Heterozygous and Homozygous *Armc2* Male Mice

(A) A spermatozoon with normal morphology from a wild-type male mouse.
(B–D) Sperm from *Armc2*-deficient mice show severe morphological defects. All spermatozoa had flagellar abnormalities: short, coiled, absent, and/or of irregular caliber consistent with the MMAF phenotype reported in the *ARMC2*-mutated individuals. Scale bars: 10 μm.
(E) Fertility of wild-type (WT), *Armc2* heterozygous-mutant ($Armc2^{+/-}$), and *Armc* homozygous-mutant ($Armc2^{-/-}$) males.
(F) Testis weight from WT, $Armc2^{+/-}$, and $Armc2^{-/-}$ males.
(G) Total motility of sperm extracted from the cauda epididymis of WT, $Armc2^{+/-}$, and $Armc2^{-/-}$ males.
(H) Concentrations of sperm from the cauda epididymis from WT, heterozygous, and homozygous *Armc2* males.
(I) Concentration of morphologically abnormal sperm in WT, heterozygous, and homozygous *Armc2* males, evaluated after Harris-Schorr coloration (expressed in million/mL [M/mL]). Data represent means ± SE; statistical differences were assessed with a t test; a probability value of less than 0.05 was considered to be statistically significant.

and the N-DRC, respectively, were not directly affected by mutations in *ARMC2* (Figure S7). Because of limited sample availability, these analyses could not be repeated on sperm from other individuals with *ARMC2* mutations. Also, we regret that we could not obtain any specific ARMC2 antibodies allowing the localization of the protein in human and mouse sperm. In addition, IF experiments performed in $Armc2^{\text{mutant}}$ animals showed that SPEF2 staining, another marker of the CPC,[24] was totally absent, supporting the CPC defects observed in sperm samples from the MMAF-affected individual $\text{ARMC2}_{\_1}$ (Figure 3B).

We showed that the presence of bi-allelic *ARMC2* mutations induces a typical MMAF phenotype in both humans and mice, indicating that this gene is necessary for spermatogenesis and, in particular, for sperm flagellum structure and motility. Bioinformatic analysis suggests that *ARMC2* encodes a protein belonging to the ARM-repeat-containing protein family[20] (Figure 1I). ARM repeats are typically characterized by a 42-amino-acid motif composed of three α helices.[25] Tandem ARM-repeat units fold together as "superhelixes" serving as platforms for protein-protein interactions.[26] ARM-repeat proteins are involved in a wide range of cellular functions, including intracellular signaling, cytoskeletal regulation, and protein degradation or folding.[27] ARM-repeat proteins are also involved in different functions in cilia and flagella; such functions include intraflagellar transport and assembly and/or stability of different axonemal components.[26,27] Moreover, several ARM-repeat proteins have been described to be involved in ciliopathies or male infertility.[4,23,28–30] In particular, in cattle and mice, mutations in *ARMC3* and *SPAG6* (a protein with eight contiguous armadillo repeats) are involved in male infertility due to sperm flagellum malformations.[23,31,32] More recently, we also demonstrated that the absence of *CFAP69*, another ARM-repeat flagellum-associated protein, leads to an MMAF phenotype in humans and mice.[11] These data clearly demonstrate that armadillo-domain repeat proteins are critical for correct spermatogenesis and flagellum formation, but the precise function of ARMC2 and of the other ARM-repeat flagellum-associated protein remains to be elucidated.

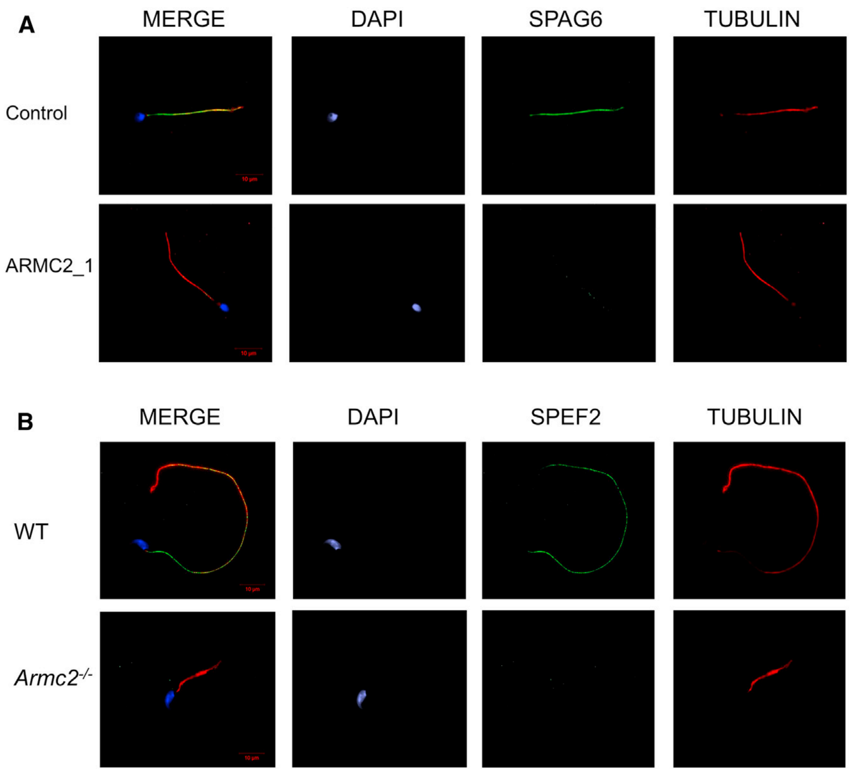

Figure 3. Immunostaining of SPAG6 and SPEF2 Revealed that the Central-Pair Complex Is Affected by Mutations in *ARMC2* in Humans and Mice

(A) Sperm cells from a fertile control individual and from individual ARMC2$_{_1}$ were stained with anti-SPAG6 (rabbit polyclonal, HPA38440, Sigma-Aldrich, 1:500, green), which detects a protein located in the C1 microtubule, and anti-acetylated tubulin (monoclonal mouse T7451, Sigma-Aldrich, 1:2000, red) antibodies. SPAG6 staining uniformly decorates the full-length flagellum in the fertile control individual, whereas it is absent from the flagellum of sperm from individual ARMC2$_{_1}$.

(B) Mouse sperm cells from a WT male and *Armc2*$^{-/-}$ males stained with anti-SPEF2 (rabbit polyclonal, HPA040343, Sigma-Aldrich, 1:1000, green), a marker of the projection 1b of singlet C1, and anti-acetylated tubulin (monoclonal mouse, T7451, Sigma-Aldrich, 1:500, red) antibodies. DNA was counterstained with DAPI. Contrary to the WT, the SPEF2 immunostaining is not detectable in the sperm flagellum from the *Armc2*$^{-/-}$ male. Scale bars: 10 μm.

Immunostaining experiments in sperm cells from individual ARMC2$_{_1,}$ who harbors a splicing mutation, evidenced an absence of the SPAG6 protein, which normally locates to the C1 singlet,[23,28] strongly suggesting defects in the CPC structure (Figure 3A). These findings are in concordance with TEM analysis, which revealed the absence of the central pair in the few cross-sections observed in sperm from individual ARMC2$_{_1}$ (Figure 1F). Similar observations were described in the *Armc2*$^{\text{mutant}}$ mouse, which showed abnormal staining of the SPEF2 protein (Figure 3B). IF experiments showed that other axonemal or peri-axonemal structures did not appear to be affected by the *ARMC2* mutation (Figure S7), suggesting that in the absence of ARMC2, these proteins were correctly addressed and were able to maintain their normal localization within the axoneme. These observations suggest that ARMC2 might be specifically involved in the CPC assembly or stability. Interestingly, the two other armadillo-repeat proteins, SPAG6 and CFAP69, associated with MMAF phenotype are also linked to the CPC.[11,24] The CPC complex consists of two singlets of microtubules, named C1 and C2, which are structurally and biochemically distinct and are surrounded by complex protein structures, known as projections, that are unique for each microtubule.[24] It is worth noting that cilia without the CPC exist in different tissues (9 + 0 organization), indicating that the CPC is not necessary for axoneme growth and integrity; interestingly those 9 + 0 cilia are most often non-motile cilia. In contrast to cilia, the CPC seems to play a key role in maintaining the global organization of the sperm flagellum throughout spermiogenesis. Moreover, inactivation of different genes encoding proteins directly or indirectly associated with the CPC was described to lead to the MMAF phenotype in humans and in several animal models,[4] confirming the importance of the CPC in sperm flagellum assembly and/or structure. Further experiments, including the development of functional antibodies, should now be performed so that the axonemal localization can be precisely determined and the putative role of ARMC2 in the CPC assembly or stability can be confirmed.

We present here a cohort of 167 individuals. Overall, we identified 50 individuals (29.9%) with a mutation in known MMAF-related genes; these individuals included the 35 who were previously reported (Table S1). We identified 10 different mutations in *DNAH1* in 10 individuals (6%), 12 mutations in *CFAP43* in 12 individuals (7.2%), two mutations in *WDR66* in 11 individuals (6.6%), six mutations in *CFAP44* in eight individuals (4.8%), six mutations in *FSIP2* in seven individuals (4.2%), and two mutations in *CFAP69* in two individuals (1.2%) (Table S1). When adding the four *ARMC2*-mutated individuals of the cohort, we obtained a diagnostic efficiency of 32.3% (54/167). We note that, for previously published genes, the newly described variants only include the homozygous loss-of-function variants, and we did not report the unpublished variants of questionable significance (missense variants, in-frame deletions, or variants located in splice regions other than the consensus splice sites) because we feel these need additional confirmatory work. From these results, *CFAP43, WDR66,* and *DNAH1* appear to be the most frequently mutated genes in MMAF-affected individuals. Ten of the 11 *WDR66* subjects, however, harbored the same founder deletion,[13] found only in Tunisian subjects (Table S1). The contribution of this gene in other populations could therefore be limited, although *WDR66* has been reported by others to induce MMAF.[33] WES sequencing in MMAF continues to permit the identification of new genes and pathological variants, demonstrating the efficiency of WES for investigating the genetic causes of MMAF syndrome. However, we observe here that despite regular new gene identification, the genetic causes of MMAF remain unknown for about two thirds of affected individuals. This highlights the high genetic heterogeneity of the phenotype and is consistent with the large number of genes involved in spermatogenesis.[34] As discussed above, some variants of unknown significance in identified MMAF-associated genes could also be responsible for the infertility of some of the investigated individuals. Techniques that reliably validate the pathogenicity of these variations are currently cruelly lacking. These results also suggest that the WES approach cannot be expected to provide 100% positive diagnoses. This could also be explained in part by the fact that some variants are not detected by the technique used (e.g., such variants might include non-sequenced deep intronic variants or some exonic variants because only approximately 90% of coding nucleotides were covered) or by the current bio-informatic pipeline used for the analysis (e.g., such variants might include small duplications, structural variations, and rearrangements). To improve this diagnosis rate and to detect new variants in as-yet-uncharacterized genes, one can now envisage more powerful techniques, such as whole-genome sequencing (WGS), for MMAF-affected individuals for whom no variants were identified.[35] Moreover, although the use of new sequencing technologies now permits easy identification of new candidate genes, the resulting high flow of data can easily lead to erroneous diagnoses. Therefore, human genetic studies pertaining to new candidate genes and missense variants should be confirmed by functional work and/or phenotypic characterization using animal models. Such studies are also essential for improving our understanding of the function of the studied genes and their role in spermatogenesis.[22]

Like all the other MMAF-affected individuals included in this study, the five individuals with *ARMC2* mutations presented only with primary infertility and no other clinical features, thus excluding a phenotype of primary cilia dyskinesia (PCD). This observation clearly supports the idea that sperm flagellum biogenesis requires a pool of proteins that are different from the pool necessary for cilia biogenesis. In addition, this also suggests that some assembly mechanisms might be specific to the sperm flagellum. We did not observe any significant differences between the semen parameters of the five individuals carrying mutations in *ARMC2* and the parameters of individuals with mutations in other MMAF-related genes. This analysis, however, revealed that sperm from *CFAP43*-mutated and *CFAP44*-mutated individuals present the highest level of defects, whereas sperm from the unknown group seems the least altered (Figure S5). Finally, some questions remain about the prognosis of using intracytoplasmic sperm injection (ICSI) with sperm cells from *ARMC2*-mutated individuals. Several studies previously demonstrated that MMAF-affected individuals had a good prognosis after ICSI.[4,15,36] However, it would be interesting to take all MMAF genotypes into account and compare the success rates after ICSI. Altogether, these data demonstrate that ARMC2 is essential for sperm-tail biogenesis in humans and mice and that mutations in this gene lead to drastic flagellum malformations that result in severe asthenoteratozoospermia and primary male infertility.

Accession Numbers

All *ARMC2* variants reported in this manuscript are accessible in ClinVar under the accession number SUB4929442.

Supplemental Data

Supplemental Data include Supplemental Material and Methods, seven figures, and eight tables and can be found with this article online at https://doi.org/10.1016/j.ajhg.2018.12.013.

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Declaration of Interests

The authors declare no competing interests.

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Web Resources

1000 Genomes, http://www.internationalgenome.org
ClinVar, https://www.ncbi.nlm.nih.gov/clinvar/
dbSNP, https://www.ncbi.nlm.nih.gov/projects/SNP
ENCODE, https://www.encodeproject.org
ExAC Browser, http://exac.broadinstitute.org/
FANTOM, http://fantom.gsc.riken.jp
gnomAD Browser, http://gnomad.broadinstitute.org
GTEx, http://www.gtexportal.org
NHLBI Exome Sequencing Project (ESP) Exome Variant Server, http://evs.gs.washington.edu/EVS/
Online Mendelian Inheritance in Man, https://www.omim.org
PolyPhen-2, http://genetics.bwh.harvard.edu/pph2/bgi.shtml
SIFT, http://sift.jcvi.org

References

1. Jan, S.Z., Vormer, T.L., Jongejan, A., Röling, M.D., Silber, S.J., de Rooij, D.G., Hamer, G., Repping, S., and van Pelt, A.M.M. (2017). Unraveling transcriptome dynamics in human spermatogenesis. Development *144*, 3659–3673.
2. Tüttelmann, F., Ruckert, C., and Röpke, A. (2018). Disorders of spermatogenesis: Perspectives for novel genetic diagnostics after 20 years of unchanged routine. Med. Genetik *30*, 12–20.
3. Krausz, C., and Riera-Escamilla, A. (2018). Genetics of male infertility. Nat. Rev. Urol. *15*, 369–384.
4. Coutton, C., Escoffier, J., Martinez, G., Arnoult, C., and Ray, P.F. (2015). Teratozoospermia: spotlight on the main genetic actors in the human. Hum. Reprod. Update *21*, 455–485.
5. Ray, P.F., Toure, A., Metzler-Guillemain, C., Mitchell, M.J., Arnoult, C., and Coutton, C. (2017). Genetic abnormalities leading to qualitative defects of sperm morphology or function. Clin. Genet. *91*, 217–232.
6. Ben Khelifa, M., Coutton, C., Zouari, R., Karaouzène, T., Rendu, J., Bidart, M., Yassine, S., Pierre, V., Delaroche, J., Hennebicq, S., et al. (2014). 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. *94*, 95–104.
7. Amiri-Yekta, A., Coutton, C., Kherraf, Z.-E., Karaouzène, T., Le Tanno, P., Sanati, M.H., Sabbaghian, M., Almadani, N., Sadighi Gilani, M.A., Hosseini, S.H., et al. (2016). Whole-exome sequencing of familial cases of multiple morphological abnormalities of the sperm flagella (MMAF) reveals new DNAH1 mutations. Hum. Reprod. *31*, 2872–2880.
8. Wang, X., Jin, H., Han, F., Cui, Y., Chen, J., Yang, C., Zhu, P., Wang, W., Jiao, G., Wang, W., et al. (2017). Homozygous DNAH1 frameshift mutation causes multiple morphological anomalies of the sperm flagella in Chinese. Clin. Genet. *91*, 313–321. Published online November 24, 2016.
9. Coutton, C., Vargas, A.S., Amiri-Yekta, A., Kherraf, Z.-E., Ben Mustapha, S.F., Le Tanno, P., Wambergue-Legrand, C., Karaouzène, T., Martinez, G., Crouzy, S., et al. (2018). Mutations in CFAP43 and CFAP44 cause male infertility and flagellum defects in Trypanosoma and human. Nat. Commun. *9*, 686.
10. Tang, S., Wang, X., Li, W., Yang, X., Li, Z., Liu, W., Li, C., Zhu, Z., Wang, L., Wang, J., et al. (2017). Biallelic mutations in CFAP43 and CFAP44 cause male infertility with multiple morphological abnormalities of the sperm flagella. Am. J. Hum. Genet. *100*, 854–864.
11. Dong, F.N., Amiri-Yekta, A., Martinez, G., Saut, A., Tek, J., Stouvenel, L., Lorès, P., Karaouzène, T., Thierry-Mieg, N., Satre, V., et al. (2018). Absence of CFAP69 causes male infertility due to multiple morphological abnormalities of the flagella in human and mouse. Am. J. Hum. Genet. *102*, 636–648.
12. Martinez, G., Kherraf, Z.-E., Zouari, R., Fourati Ben Mustapha, S., Saut, A., Pernet-Gallay, K., Bertrand, A., Bidart, M., Hograindleur, J.P., Amiri-Yekta, A., et al. (2018). Whole-exome sequencing identifies mutations in FSIP2 as a recurrent cause of multiple morphological abnormalities of the sperm flagella. Hum. Reprod. *33*, 1973–1984.
13. 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. (2018). A homozygous ancestral SVA-insertion-mediated deletion in WDR66 induces multiple morphological abnormalities of the sperm flagellum and male infertility. Am. J. Hum. Genet. *103*, 400–412.
14. Lorès, P., Coutton, C., El Khouri, E., Stouvenel, L., Givelet, M., Thomas, L., Rode, B., Schmitt, A., Louis, B., Sakheli, Z., et al. (2018). 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. *27*, 1196–1211.
15. Sha, Y.-W., Xu, X., Mei, L.-B., Li, P., Su, Z.-Y., He, X.-Q., and Li, L. (2017). A homozygous CEP135 mutation is associated with multiple morphological abnormalities of the sperm flagella (MMAF). Gene *633*, 48–53.
16. Gerstein, M.B., Kundaje, A., Hariharan, M., Landt, S.G., Yan, K.-K., Cheng, C., Mu, X.J., Khurana, E., Rozowsky, J., Alexander, R., et al. (2012). Architecture of the human regulatory network derived from ENCODE data. Nature *489*, 91–100.
17. Lizio, M., Harshbarger, J., Abugessaisa, I., Noguchi, S., Kondo, A., Severin, J., Mungall, C., Arenillas, D., Mathelier, A., Medvedeva, Y.A., et al. (2017). Update of the FANTOM web resource: high resolution transcriptome of diverse cell types in mammals. Nucleic Acids Res. *45* (D1), D737–D743.
18. GTEx Consortium (2015). Human genomics. The Genotype-Tissue Expression (GTEx) pilot analysis: multitissue gene regulation in humans. Science *348*, 648–660.
19. Ivliev, A.E., 't Hoen, P.A.C., van Roon-Mom, W.M.C., Peters, D.J.M., and Sergeeva, M.G. (2012). Exploring the transcriptome of ciliated cells using in silico dissection of human tissues. PLoS ONE *7*, e35618.
20. The UniProt Consortium (2017). UniProt: the universal protein knowledgebase. Nucleic Acids Res. *45* (D1), D158–D169.
21. Wang, Y., Yang, J., Jia, Y., Xiong, C., Meng, T., Guan, H., Xia, W., Ding, M., and Yuchi, M. (2014). Variability in the morphologic assessment of human sperm: use of the strict criteria recommended by the World Health Organization in 2010. Fertil. Steril. *101*, 945–949.
22. Kherraf, Z.-E., Conne, B., Amiri-Yekta, A., Kent, M.C., Coutton, C., Escoffier, J., Nef, S., Arnoult, C., and Ray, P.F. (2018). Creation of knock out and knock in mice by CRISPR/Cas9 to validate candidate genes for human male infertility, interest, difficulties and feasibility. Mol. Cell. Endocrinol. *468*, 70–80.
23. Sapiro, R., Tarantino, L.M., Velazquez, F., Kiriakidou, M., Hecht, N.B., Bucan, M., and Strauss, J.F., 3rd. (2000). Sperm antigen 6 is the murine homologue of the Chlamydomonas reinhardtii central apparatus protein encoded by the PF16 locus. Biol. Reprod. *62*, 511–518.
24. Teves, M.E., Nagarkatti-Gude, D.R., Zhang, Z., and Strauss, J.F., 3rd. (2016). Mammalian axoneme central pair complex proteins: Broader roles revealed by gene knockout phenotypes. Cytoskeleton (Hoboken) *73*, 3–22.
25. Peifer, M., Berg, S., and Reynolds, A.B. (1994). A repeating amino acid motif shared by proteins with diverse cellular roles. Cell *76*, 789–791.
26. Coates, J.C. (2003). Armadillo repeat proteins: beyond the animal kingdom. Trends Cell Biol. *13*, 463–471.
27. Tewari, R., Bailes, E., Bunting, K.A., and Coates, J.C. (2010). Armadillo-repeat protein functions: questions for little creatures. Trends Cell Biol. *20*, 470–481.
28. Sapiro, R., Kostetskii, I., Olds-Clarke, P., Gerton, G.L., Radice, G.L., and Strauss, J.F., III. (2002). Male infertility, impaired sperm motility, and hydrocephalus in mice deficient in sperm-associated antigen 6. Mol. Cell. Biol. *22*, 6298–6305.
29. Onoufriadis, A., Shoemark, A., Munye, M.M., James, C.T., Schmidts, M., Patel, M., Rosser, E.M., Bacchelli, C., Beales, P.L., Scambler, P.J., et al.; UK10K (2014). Combined exome

and whole-genome sequencing identifies mutations in ARMC4 as a cause of primary ciliary dyskinesia with defects in the outer dynein arm. J. Med. Genet. *51*, 61–67.

30. Hjeij, R., Lindstrand, A., Francis, R., Zariwala, M.A., Liu, X., Li, Y., Damerla, R., Dougherty, G.W., Abouhamed, M., Olbrich, H., et al. (2013). ARMC4 mutations cause primary ciliary dyskinesia with randomization of left/right body asymmetry. Am. J. Hum. Genet. *93*, 357–367.
31. Pausch, H., Venhoranta, H., Wurmser, C., Hakala, K., Iso-Touru, T., Sironen, A., Vingborg, R.K., Lohi, H., Söderquist, L., Fries, R., and Andersson, M. (2016). A frameshift mutation in ARMC3 is associated with a tail stump sperm defect in Swedish Red (Bos taurus) cattle. BMC Genet. *17*, 49.
32. Zhang, Z., Sapiro, R., Kapfhamer, D., Bucan, M., Bray, J., Chennathukuzhi, V., McNamara, P., Curtis, A., Zhang, M., Blanchette-Mackie, E.J., and Strauss, J.F., 3rd. (2002). A sperm-associated WD repeat protein orthologous to Chlamydomonas PF20 associates with Spag6, the mammalian orthologue of Chlamydomonas PF16. Mol. Cell. Biol. *22*, 7993–8004.
33. Auguste, Y., Delague, V., Desvignes, J.-P., Longepied, G., Gnisci, A., Besnier, P., Levy, N., Beroud, C., Megarbane, A., Metzler-Guillemain, C., and Mitchell, M.J. (2018). Loss of Calmodulin- and Radial-Spoke-Associated Complex Protein CFAP251 Leads to Immotile Spermatozoa Lacking Mitochondria and Infertility in Men. Am. J. Hum. Genet. *103*, 413–420.
34. Matzuk, M.M., and Lamb, D.J. (2008). The biology of infertility: research advances and clinical challenges. Nat. Med. *14*, 1197–1213.
35. Meienberg, J., Bruggmann, R., Oexle, K., and Matyas, G. (2016). Clinical sequencing: is WGS the better WES? Hum. Genet. *135*, 359–362.
36. Wambergue, C., Zouari, R., Fourati Ben Mustapha, S., Martinez, G., Devillard, F., Hennebicq, S., Satre, V., Brouillet, S., Halouani, L., Marrakchi, O., et al. (2016). Patients with multiple morphological abnormalities of the sperm flagella due to DNAH1 mutations have a good prognosis following intracytoplasmic sperm injection. Hum. Reprod. *31*, 1164–1172.