Loss of DRC1 function leads to multiple morphological abnormalities of the sperm flagella and male infertility in human and mouse

Jintao Zhang¹,#, Xiaojin He²,³,#, Huan Wu²,³,#,†, Xin Zhang¹,#, Shenmin Yang⁴,#, Chunyu Liu⁵,⁶,#, Siyu Liu¹, Rong Hua¹, Shushu Zhou¹, Shuqin Zhao⁷, Fan Hu⁸, Junqiang Zhang³,⁹, Wangjie Liu⁵,⁶, Huiru Cheng³,⁹, Yang Gao²,³, Feng Zhang⁵,⁶,*†, Yunxia Cao²,³,⁹,* and Mingxi Liu¹,*†

¹State Key Laboratory of Reproductive Medicine, Department of Histology and Embryology, School of Basic Medical Sciences, Nanjing Medical University, Nanjing 211166, China, ²Reproductive Medicine Center, Department of Obstetrics and Gynecology, the First Affiliated Hospital of Anhui Medical University, Hefei 230022, China, ³NHC Key Laboratory of Study on Abnormal Gametes and Reproductive Tract, Anhui Medical University, Hefei 230032, China, ⁴State Key Laboratory of Reproductive Medicine, Center for Reproduction and Genetics, Suzhou Hospital Affiliated to Nanjing Medical University, Suzhou 215002, China, ⁵Obstetrics and Gynecology Hospital, NHC Key Laboratory of Reproduction Regulation (Shanghai Institute for Biomedical and Pharmaceutical Technologies), State Key Laboratory of Genetic Engineering at School of Life Sciences, Fudan University, Shanghai 200011, China, ⁶Shanghai Key Laboratory of Female Reproductive Endocrine Related Diseases, Shanghai 200011, China, ⁷State Key Laboratory of Reproductive Medicine, Animal Core Facility of Nanjing Medical University, Nanjing 211166, China, ⁸State Key Laboratory of Reproductive Medicine, Nanjing Medical University, Nanjing 211166, China and ⁹Key Laboratory of Population Health Across Life Cycle, Anhui Medical University, Ministry of Education of the People's Republic of China, Hefei 230032, China

*To whom correspondence should be addressed at: Feng Zhang, Obstetrics and Gynecology Hospital, Fudan University, 419 Fangxie Road, Shanghai 200011, China. Tel: +86 2131246783; Email: zhangfeng@fudan.edu.cn; Yunxia Cao, Reproductive Medicine Center, Department of Obstetrics and Gynecology, the First Affiliated Hospital of Anhui Medical University, 218 Jixi Road, Hefei 230022, China. Tel: +86 55165908442; Email: caoyunxia6@126.com; Mingxi Liu, State Key Laboratory of Reproductive Medicine, Nanjing Medical University, 101 Longmian Avenue, Nanjing 211166, China. Tel: +86 2586869387; Email: mingxi.liu@njmu.edu.cn

Abstract

Motile cilia and flagellar defects can result in primary ciliary dyskinesia, which is a multisystemic genetic disorder that affects roughly 1:10 000 individuals. The nexin-dynein regulatory complex (N-DRC) links neighboring doublet microtubules within flagella, serving as a central regulatory hub for motility in Chlamydomonas. Herein, we identified two homozygous DRC1 variants in human patients that were associated with multiple morphological abnormalities of the sperm flagella

1Huan Wu, http://orcid.org/0000-0002-0009-3839
2Feng Zhang, http://orcid.org/0000-0003-4556-8276
3Mingxi Liu, http://orcid.org/0000-0001-6499-7899
4Contributed equally to this work.
Received: May 21, 2021. Revised: June 18, 2021. Accepted: June 21, 2021
© The Author(s) 2021. Published by Oxford University Press.
This is an Open Access article distributed under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/4.0/), which permits unrestricted reuse, distribution, and reproduction in any medium, provided the original work is properly cited.
Introduction

Flagella and motile cilia are evolutionarily ancient structures present in prokaryotic and eukaryotic cells that are involved in sensation and movement (1). These organelles also play essential roles in the development and functionality of key systems including the respiratory, nervous and reproductive systems (2). Genetic defects in the motility of flagella and cilia can cause a multisystem disorder known as primary ciliary dyskinesia (PCD) (3,4), which affects roughly 1:10,000 individuals globally (5,6). The nexin-dynein regulatory complex (N-DRC) functions by linking neighboring doublet microtubules within motile cilia and flagella, stabilizing the axonemal core structure and thereby regulating ciliary motility (7–13). In the flagella of Chlamydomonas cells, at least 11 subunit proteins compose the N-DRC (7,14), and many homologs of these proteins are present in humans and mice. The deletion of DRC genes in mice can result in an array of distinct phenotypes. For example, Drc7 (15) and Drc9 (16) knockout mice exhibit multiple morphological abnormalities of the sperm flagella (MAF) and male infertility, whereas Drc5 deletion (17) causes asthenoospermia and the deletion of Drc6 (15) does not induce significant flagellar abnormalities. A single mutation at amino acid position 89 (Leu89Pro) in DRC3 (LRRC48) can result in classical symptoms of PCD such as mucus accumulation, male sterility and an increased risk of postnatal death (18).

Analyses of Chlamydomonas have identified DRC1, DRC2 and DRC4 as core N-DRC structural components (11). In mice, the loss of DRC4 expression is associated with severe hydrocephaly and lethality within 14–21 days after birth (19). This suggests that this core N-DRC protein is essential for murine survival. No mice harboring knockout mutations for core DRC genes that survive into adulthood have been described to date. However, one case report published in 2013 described the case of a PCD patient exhibiting a homozygous nonsense mutation (c.2056A>T) in DRC1 that was predicted to cause the pre-mature translational arrest of the nascent DRC1 peptide (p.Lys686*) (20). That patient suffered from symptoms including chronic otitis media, sinusitis, recurrent pneumonia and neonatal respiratory distress, all of which are consistent with the diagnosis of PCD. In another recent study, a patient with diffuse panbronchiolitis was found to harbor a large homozygous deletion spanning exons 1–4 of DRC1 (21). In both of these cases, the patients survived into adulthood. No Drc1 knockout mice have yet been developed to explore the functional role of this protein in mammals to date.

Herein, we found that two homozygous DRC1 mutations resulted in MAF and male infertility phenotypes in humans without causing any concomitant respiratory symptoms. While Drc1−/−, Drc1R554X/R554X and Drc1W244X/W244X mice on the C57BL/6 background suffered from pre-pubertal death in all cases, the introduction of the ICR background into these strains enabled some of the mice to survive and to exhibit development and behavior similar to that of wild-type (WT) control mice. The surviving mice recapitulated the MAF phenotypes observed in the context of human DRC1 deficiency. Interestingly, we further found that damage to sperm flagellum was more severe than ciliary damage in Drc1R554X/R554X and Drc1W244X/W244X mice, resulting in damage beyond just the loss of the DRC structure. Altogether, our data suggest that DRC1 mutations can result in dysregulated cilia and flagella formation in humans and mice while also indicating that this protein regulates the structural homeostasis of sperm flagella.

Results

Exome sequencing reveals the presence of homozygous DRC1 variants in MAF patients

Exome sequencing represents a powerful approach to exploring the genetic basis for flagellar defects in sperm, and at least 19 genes associated with MAF phenotypes have been identified to date (22–40). Of our overall 100 MAF patient cohort, we identified 76 patients with harmful variants in known MAF-related genes. After re-analyzing exome data from the remaining patients, we detected two unrelated patients harboring homozygous variants in DRC1. Patient aIV-1 harbored a stop codon gain variant c.C1660T: p.R554X (NM_145038.5) (Fig. 1A; DRC1R554X/R554X) not recorded in the Genome Aggregation Database, while patient bIV-1 harbored a distinct stop-gain variant c.C238T: p.R80X (NM_145038.5) (Fig. 1B; DRC1R80X/R80X) with a minor allele frequency of 3.19e−4 in the Genome Aggregation Database. Morphological analyses confirmed that sperm from both patients exhibited characteristic MAF features (Fig. 1C and D, Table 1). We next generated antisera specific for the homologous N-terminal (11–146 aa) region of human and murine DRC1, which we used to determine that no DRC1 protein was detectable in the sperm of patient aIV-1 (Fig. 2A), consistent with nonsense-mediated mRNA decay (NMD) (41–43). Immunofluorescence analyses similarly confirmed the absence of DRC1 in sperm from this patient, while Ac-tubulin staining revealed that DRC1R554X/R554X sperm presented with short, coiled, absent or irregular flagella (Fig. 2B and C, Supplementary Material, Fig. S1A and B). The sperm of patient bIV-1 exhibited a phenotype similar to that of Patient aIV-1 (Supplementary Material, Fig. S1C). TEM analyses revealed the disordered structure of the flagellar axoneme in DRC1R554X/R554X sperm, with microtubules being scattered in the cytoplasm and with normal centriole implantation and implantation nest formation (Fig. 2D–I). Axoneme structural disorder was also evident in DRC1R80X/R80X sperm (Supplementary Material, Fig. S1C). Following ICSI treatment, Patient aIV-1 was able to obtain fertilized embryos, and one of which was successfully implanted (Table 2), while

(MMAF) and male infertility. Drc1−/−, Drc1R554X/R554X and Drc1W244X/W244X mice on the C57BL/6 background suffered from pre-pubertal mortality. However, when the ICR background was introduced, some of these mice were able to survive and recapitulate the MAF phenotypes detected in human patients. By analyzing these animals, we determined that DRC1 is an essential regulator of N-DRC assembly in cilia and flagella. When DRC1 is absent, this results in the shortening of cilia and consequent impairment of their motility. Damage associated with DRC1 deficiency in sperm flagella was more pronounced than in cilia, as manifested by complete axoneme structural disorder in addition to the loss of the DRC structure. Altogether, these findings suggest that DRC1 is required for the structural stability of flagella but not cilia, emphasizing the key role of this protein in mammalian species.
Figure 1. Biallelic mutations in DRC1 were identified in probands with MMAF. (A and B) Biallelic mutations in DRC1 were identified in the probands from two families. The mutations identified by WES were further verified by Sanger sequencing. Red arrows indicate the positions of point mutations. (C) Light microscopy revealed a spermatozoon with normal morphology from a healthy male. (D and E) Most spermatozoa of DRC1-mutated probands (D: DRC1 R554X/R554X; F: DRC1 R80X/R80X) exhibited flagellar morphological abnormalities.

| Clinical characteristics                        | aIV-1 | bIV-1 |
|------------------------------------------------|-------|-------|
| Semen parameters                               |       |       |
| Ejaculated sperm volume (ml)                    | 2.3   | 2.8   |
| Seminal pH                                      | 7.3   | 7.4   |
| Ejaculated sperm concentration (million/ml)     | 11.3  | 8.84  |
| Progressive motility                            | 0.5%  | 0%    |
| Sperm morphology                               |       |       |
| Abnormal head                                   | 87%   | 100%  |
| Abnormal flagella                               | 99%   | 99%   |
| Serum sex hormone levels                        |       |       |
| FSH (mIU/ml)                                    | 4.01  | ND    |
| LH (mIU/ml)                                     | 4.39  | ND    |
| T (nmol/l)                                      | 8.41  | ND    |
| PRL (ng/ml)                                     | 7.53  | ND    |
| Karyotype                                       | 46XY 46XX | ND    |
| AZF’s deletion                                  | Undetectable | Undetectable |

Patient bIV-1 did not undergo further treatment. The parents of both of these patients were closely related to one another, and the genetic characteristics of the observed DRC1 mutations were consistent with a recessive genetic model, suggesting that a loss of human DRC1 functionality results in MMAF and male infertility.

Drc1 knockout mice on a C57BL/6 background suffer from hydrocephaly and postnatal death

We next evaluated DRC1 sequence conservation among species, revealing DRC1 sequences to be relatively conserved in Chlamydomonas reinhardtii, Drosophila melanogaster, Gallus gallus, Homo sapiens, Macaca fascicularis, Mus musculus, Rattus norvegicus.
Figure 2. The DRC1\textsuperscript{R554X/R554X} mutation causes multiple morphological abnormalities and ultrastructural disorder in sperm flagella. (A) DRC1 is missing in spermatozoa from a DRC1\textsuperscript{R554X/R554X} mutant individual. (B) Spermatozoa from a fertile control individual and from a DRC1\textsuperscript{R554X/R554X} mutant individual were stained with anti-DRC1 and anti-Ac-Tubulin. (C) Spermatozoa from a fertile control individual and from a DRC1\textsuperscript{R554X/R554X} mutant individual were stained with anti-Centrin-1, anti-Ac-Tubulin and PNA. (D–F) The normal axoneme is composed of nine doublets of microtubules circularly arranged around a central-pair complex of microtubules (9+2 organization). (G–I) The DRC1\textsuperscript{R554X/R554X} mutation is associated with severe axonemal disorganization and evidence of unassembled microtubule doublets.

and Xenopus laevis. DRC1 R80 and R554 were both conserved in mice (Supplementary Material, Fig. S2). To understand the functional role of DRC1 in vivo, we then employed a CRISPR/Cas9 approach to generate two Drc1 mutant mouse strains (Supplementary Material, Fig. S3). A stable Drc1 mutant mouse line carrying a 1 bp deletion within exon 13 of this gene (Supplementary Material, Fig. S3A) was established (Drc1\textsuperscript{−/−}). In addition, a mouse model harboring the R554 mutation observed in MMAF patients (Drc1\textsuperscript{R554X/R554X}, Supplementary Material, Fig. S3B) was generated. Unexpectedly, mice harboring both of these mutations experienced pre-puberal death, usually before postnatal day 12 (Table 3). These mice exhibited clear signs of growth retardation and hydrocephaly (Supplementary Material, Fig. S4).

As the Cas9 system has the potential to introduce off-target genetic changes and these two murine lines were constructed
failed to sire any offspring despite copulation with females. The underlying genetic background was altered. We therefore was lethal in inbred strains, whereas survival rates rose when mutant mice onto the C57BL/6 × genes including mutations. We noted that the knockout of other PCD-related background that may influence the penetrance of particular background, inbred C57BL/6 mice present with a simplified of hydrocephaly. While humans exhibit a complex genetic in this study exhibited respiratory symptoms or evidence Neither patient harboring DRC1 stop-gain mutations identified formation in C57BL/6 is necessary for male fertility and sperm flagellum was true when comparing DRC1 protein expression was detected in the testes of Drc1R554X/R554X and Drc1W244X/W244X, mice, with no truncated protein being evident therein (Fig. 3D). Levels of Drc1 mRNA were also significantly lower in these tissue samples, consistent with an NMD phenotype associated with these mutations mRNA (Fig. 3E and F). No differences in gross testis appearance or weight were noted when comparing Drc1+/+ and Drc1R554X/R554X littermates (Fig. 3G and H), and the same was true when comparing Drc1+/+ and Drc1W244X/W244X samples (Fig. 3I and J). Following PAS staining, we found that both Drc1R554X/R554X and Drc1W244X/W244X lacked flagella of normal length within the lumen of seminiferous tubules (Fig. 3K–M). Decreased sperm counts and the presence of short or absent flagella were also observed in cauda epididymis sections from Drc1R554X/R554X and Drc1W244X/W244X mice (Supplementary Material, Fig. S5). Drc1+/+ testis (Supplementary Material, Fig. S5) and epididymis (Supplementary Material, Fig. S5) tissues exhibited phenotypes similar to those of mice harboring the two base mutations detailed above, and as such Drc1R554X/R554X and Drc1W244X/W244X mice were the focus of subsequent experiments.

Almost all spermatozoa collected from the epididymal cauda in Drc1R554X/R554X and Drc1W244X/W244X mice exhibited abnormalities including short, bent, curled, thick or absent flagella (Supplementary Material, Fig. S9). Normal sperm acrosome morphology was not detected in Drc1R554X/R554X and Drc1W244X/W244X mice, whereas sperm tails were clearly disordered (Supplementary Material, Fig. S8). These serious tail defects coincided with a complete absence of sperm motility for both murine strains (Supplementary Material, Movies S1–S3). Altogether, these findings indicate that DRC1 is essential for sperm flagellum formation and male fertility.

**Table 2.** Clinical outcomes of the DRC1-mutated patients following ICSI

| Clinical characteristics | | |
|--------------------------|------------------|
| Male age (years)         | 35               |
| Female age (years)       | 28               |
| No. of ICSI cycles       | 1                |
| No. of oocytes injected  | 7                |
| Fertilization rate       | 4/7 (57.14%)     |
| Cleavage rate            | 2/4 (50.0%)      |
| Eight cells embryo development rate | 2/4 (50.0%) |
| Blastocyst development rate | 2/4 (50.0%) |
| No. of frozen-thawed embryos transfer cycles | 1 |
| Number of embryos transferred | 2 |
| Implantation rate (%)    | 1/2 (50%)        |
| Clinical pregnancy Y     | N                |
| Miscarriage              | N                |

**Table 3.** Survival rate after puberty of Drcl mutant mice in different genetic background

| Genotype         | Background | No. of mice with indicated genotype (Hydrocephalus) | Total Alive after puberty |
|------------------|------------|---------------------------------------------------|---------------------------|
| Drcl+/+ × Drcl+/+ | B6         | +/+ 45                                             | 155                       |
| Drcl+/+ × Drcl+/− | B6(ICR)    | +/− 43                                             | 156                       |
| Drcl+/W244X × Drcl+/−W244X | B6       | +/− 22                                             | 63                        |
| Drcl+/R554X × Drcl+/−R554X | B6(ICR) | +/− 76                                             | 353                       |
| Drcl+/R554X × Drcl+/−R554X | B6       | +/− 37                                             | 117                       |
| Drcl+/R554X × Drcl+/−R554X | B6(ICR)  | +/− 92                                             | 352                       |

using the same sgRNA, we additionally attempted to generate an additional murine line simulating human DRC1 mutations using base editing technology. After selecting mutation sites in the gnomAD database capable of causing a loss of DRC1 function and analyzing potential sgRNA designs and conserved residues, we determined that the W244X model would be well-suited to this base editing approach (Supplementary Material, Fig. S3). However, as with these first two mouse models, all DrclW244X/W244X mice died before puberty (Table 3).
Figure 3. The DRC1 mutation results in male infertility in mice of the C57BL/6 × ICR background. (A) Drc1R554X/R554X and Drc1W244X/W244X adult mice did not exhibit any significant hydrocephaly relative to WT controls. (B and C) Average numbers of pups per litter when crossing WT, Drc1R554X/R554X and Drc1W244X/W244X males with WT females, n=3, P<0.01. Data are represented as the mean ± SEM. (D) DRC1 was not detected in Drc1R554X/R554X and Drc1W244X/W244X testis samples by western blotting. (E and F) qRT-PCR analyses of Drc1 levels in WT, Drc1R554X/R554X, and Drc1W244X/W244X testis, n=3, P<0.001. Data are represented as the mean ± SEM. (G, I) Testis from WT, Drc1R554X/R554X and Drc1W244X/W244X adult mice and (H, J) average testis weight/body weight did not differ significantly between groups, n=4. Data are represented as the mean ± SEM. (K-M) Sections of periodic acid Schiff-stained testis, with asterisks being used to denote the lumen of seminiferous tubules, indicating a lack of sperm flagella of normal length.
DRC1 is an essential N-DRC assembly mediator in both flagella and cilia

We next conducted a co-IP experiment confirming the ability of DRC1 to interact with DRC2–5 (Fig. 4A). We then evaluated the expression of other DRC proteins in sperm to understand the impact of DRC1 knockout on N-DRC assembly. While β-Tubulin signals were largely unchanged, DRC1–4 could not be detected in Drcl <sup>R554X/R554X</sup> mature sperm (Fig. 4B). The radial spoke (RS) component RSPH9 was also not detectable in these cells (Fig. 4B). Immunofluorescent staining similarly indicated that CCDC65 (DRC2), GAS8 (DRC4) and RSPH9 signals were largely absent in Drcl <sup>R554X/R554X</sup> spermatozoa (Fig. 4C–K). The same was true for Drcl <sup>W244X/W244X</sup> spermatozoa (Supplementary Material, Fig. S10). Scanning electron microscope (SEM) analyses revealed clear distortion of the flagella of Drcl <sup>R554X/R554X</sup> and Drcl <sup>W244X/W244X</sup> sperm (Fig. 5A–H). Transmission electron microscope (TEM) further indicated that Drcl <sup>R554X/R554X</sup> and Drcl <sup>W244X/W244X</sup> sperm exhibited flagellum appendices such as outer dense fibers separated by microtubules, whereas N-DRC, RSs and dynein arms were not detectable (Fig. S1I).

We additionally assessed respiratory cilia changes in Drcl <sup>R554X/R554X</sup> (Fig. 6) and Drcl <sup>W244X/W244X</sup> mice (Supplementary Material, Fig. S12), revealing them to be significantly shortened (Fig. 6A–E, Supplementary Material, Fig. S12). While these respiratory cilia from Drcl <sup>R554X/R554X</sup> and Drcl <sup>W244X/W244X</sup> mice lacked N-DRC structure formation, they still maintained the 9 + 2 microtubule, RS and dynein arm structural arrangements in contrast to findings in sperm flagella (Fig. 6F–I). In B6 background, respiratory cilia were found no obvious different from mix background by light microscopy (Supplementary Material, Movie S7), while TEM analyses further indicated that the 9 + 2 microtubule was also maintained normally in Drcl <sup>−/−</sup> (Supplementary Material, Fig. S11). Immunofluorescent staining confirmed the absence of DRC1, DRC2 and DRC4 in respiratory tract cilia from Drcl <sup>R554X/R554X</sup> and Drcl <sup>W244X/W244X</sup> mice (Fig. 7 and Supplementary Material, Fig. S13), whereas RSPH9 signal intensity was unchanged in these cilia, suggesting that the RS structure was unaffected by DRC1 deletion in respiratory tract cilia. Cilia lacking the N-DRC structure exhibited abnormal motility (Supplementary Material, Movies S4–S6). These data indicate that DRC1 is necessary for N-DRC complex assembly in both flagella and cilia.

The loss of DRC1 disrupts flagellar axoneme assembly and causes nuclear deformation during spermiogenesis

While DRC1-deficient sperm exhibited disordered microtubular arrangement and RS formation, this was not observed in cilia. We therefore hypothesized that DRC1 plays a specific role in

Figure 4. DRC1 is an essential component of N-DRC assembly in sperm flagella. (A) Individual DRC components were coexpressed in HEK293T cells. Interaction model of DRCs according to the structure of N-DRC in Chlamydomonas (11) (left) and immunoprecipitation of HA-DRC1 resulted in the co-precipitation of Flag-DRC2, Flag-DRC3, Flag-DRC4 and Flag-DRC5 (right). (B) Western blotting indicated that DRC1, CCDC65 (DRC2), LRRC48 (DRC3), GAS8 (DRC4) and RSPH9 could not be detected in mature Drcl <sup>R554X/R554X</sup> sperm. (C–E) Immunofluorescence analysis of acetylated-tubulin (green) and CCDC65 (red) in WT and Drcl <sup>R554X/R554X</sup> samples. (F–H) Immunofluorescence analysis of acetylated-tubulin (green) and GAS8 (red) in WT and Drcl <sup>R554X/R554X</sup> samples. (I–K) Immunofluorescence analysis of acetylated-tubulin (red) and RSPH9 (green) in WT and Drcl <sup>R554X/R554X</sup> samples.
Figure 5. Sperm from Drc1 mutant mice exhibit distinct ultrastructural characteristics. (A–C) SEM revealed a mature sperm with a normal flagellum. (D–H) SEM revealed that the flagella of Drc1<sup>W244X/W244X</sup> (D–F) and Drc1<sup>R554X/R554X</sup> (G and H) sperm were distorted, exhibiting reticular, coiled and short phenotypes. (I and J) Transmission electron microscopy (TEM) was used to evaluate flagellar cross-sections for WT (I), Drc1<sup>W244X/W244X</sup> (J) and Drc1<sup>R554X/R554X</sup> (K and L) samples. The typical ‘9 + 2’ microtubule structure was evident for WT samples (I), while in Drc1<sup>W244X/W244X</sup> (J) and Drc1<sup>R554X/R554X</sup> (K and L) samples a disorganized axoneme with outer dense fibers separated by microtubules was evident, with an absence of N-DRC, RIs and dynem arms being evident.

the context of sperm flagellum assembly or stabilization. To test this possibility, we examined flagellum formation during spermiogenesis (Fig. 8A–T), revealing that axoneme assembly occurred normally in early-stage spermatids from Drc1<sup>R554X/R554X</sup> and Drc1<sup>W244X/W244X</sup> mice (Fig. 8G and N), but that with spermatid differentiation, the microtubules within these cells became increasingly disordered such that a normal axoneme structure was no longer present (Fig. 8H–L and O–T). Flagellum lacking DRC1 appeared disordered, whereas the 9 + 2 microtubule structure was still evident in respiratory cilia from these animals, emphasizing that DRC1 plays a key role in regulating the structural stability of sperm flagellar axonemes.

Sperm from patients aIV-1 and bIV-1 exhibited a high frequency of head deformities in addition to the aforementioned tail deformities, and such head deformities were also common in sperm from Drc1<sup>R554X/R554X</sup> and Drc1<sup>W244X/W244X</sup> mice (Supplementary Material, Fig. S8). This may be attributable to the microtubular structural disorder observed in the cytoplasm of spermatids during spermiogenesis. When we further analyzed dynamic changes in the perinuclear manchette microtubule structure during spermiogenesis, we observed a long and narrow manchette distribution around the nucleus in the sperm of Drc1<sup>R554X/R554X</sup> and Drc1<sup>W244X/W244X</sup> mice (Supplementary Material, Fig. S14A–O), resulting in the amorphous head phenotypes of these cells. Overall, these data suggest that sperm flagellar microtubule disorder as a consequence of DRC1 deletion can additionally impact dynamic cytoplasmic microtubule changes in spermatids, thereby causing complex head and tail defects.
Figure 6. DRC1 is an essential component of N-DRC assembly in respiratory cilia. (A and B) Immunofluorescence analysis of acetylated-tubulin (green) in WT (A) and Drc1R554X/R554X (B). (C) Analysis of cilia length in isolated respiratory cilia from WT and Drc1R554X/R554X subjects. Each dot represents the average cilia length of one analyzed specimen (n = 3). Data are represented as the mean ± SEM. (D and E) SEM analyses of respiratory cilia from WT (D) and Drc1R554X/R554X (E) subjects. (F–I) TEM was used to assess respiratory ciliary cross-sections for WT (F and G) and Drc1R554X/R554X (H and I) subjects. The yellow solid line represents the N-DRC structure in WT samples, whereas it was not evident (yellow dotted line) in Drc1R554X/R554X samples.

Discussion

PCD is a multisystem disease that typically arises due to mutations in abnormal genes associated with ciliary movement, with dynein arms, RS, N-DRC and related motility-associated structural defects having been linked to this condition in humans. PCD patients often exhibit a range of genetic defects in genes such as DNAH1 (22), CCDC114 (47) and RSPH4A (48), but phenotypic differences still exist even among individuals that share a given mutation. For example, CEP290 mutations can cause a spectrum of phenotypes that range from retinal degeneration (Leber congenital amaurosis) to embryonic lethality (Meckel–Gruber syndrome) (49–55), and there are often no clear correlations between patient genotype and phenotype (46). Studies of knockout mice have similarly shown that the phenotypes of mice harboring mutations in different PCD-related genes vary depending on the parental background strain (44–46,56). In the present report, we detected no respiratory symptoms harboring...
DRC1 mutations, but we did identify a previously unreported MMAF phenotype in these patients. To confirm the relationship between DRC1 mutations and these phenotypes, we generated three Drc1 mutant mouse strains. The phenotypic manifestations of this mutation were dependent upon murine genetic background such that Drc1<sup>−/−</sup> and Drc1<sup>R554X/R554X</sup> mice on the C57BL/6 background exhibited postnatal death. To determine whether this mutation was mutation-specific, we also assessed an additional DRC1 mutation recorded in gnomAD predicted to cause premature stop codon generation. Prepubertal death was also observed for Drc1<sup>W244X/W244X</sup> mice on the C57BL/6 background and as such we not able to use these mice to model male infertility. When we instead introduced the ICR background into these Drc1<sup>−/−</sup>, Drc1<sup>R554X/R554X</sup> and Drc1<sup>W244X/W244X</sup> strains, we found that some of these mice survived and exhibited weight and behaviors comparable to those of WT control mice. Importantly, these mice recapitulated the MMAF phenotypes detected in humans with a DRC1 deficiency. These results confirmed the ability of DRC1 mutations to directly cause MMAF while also reaffirming the fact that genetic background is a key determinant of PCD pathogenesis. While we did not explore the genomic basis for this finding, it nonetheless underscores the contribution of individual genetic background to PCD patient phenotypes.

Few prior studies have explored spermiogenesis in the context of MMAF pathogenesis. We found that sperm flagellum damage was more severe than ciliary damage in the present study, as evidenced by axoneme structural disorder in addition to DRC structural loss. During the early stages of spermiogenesis, axonemes appeared similar in Drc1<sup>+/-</sup>, Drc1<sup>R554X/R554X</sup> and Drc1<sup>W244X/W244X</sup> round spermatids. However, axoneme structural disorder was evident in Drc1<sup>R554X/R554X</sup> and Drc1<sup>W244X/W244X</sup> elongating spermatids and spermatozoa, and these phenotypic changes cannot be explained by the known functional role of DRC, suggesting that DRC1 to additionally be necessary for flagellar structural stability. We simultaneously detected manchette structural abnormalities in elongating spermatids from mice harboring these DRC1 mutations, further contributing to sperm nuclear transformation and disorder. Similar findings have also been observed in mice exhibiting sperm flagellar dysplasia including Drc7<sup>−/−</sup>, Rsh6a<sup>−/−</sup> and Cnap43<sup>−/−</sup> animals (15,57,58). These data suggest that abnormal microtubule organization in sperm flagella is associated with abnormal microtubule organization in the elongating spermatid manchette, potentially explaining the mixed head and tail malformations observed in the context of teratospermia.

In summary, we herein identified a novel DRC1 mutation that causes MMAF and male infertility, and we found that genetic background profoundly influences the phenotypic manifestation of this mutation. These data offer new insights regarding the genetic basis for phenotypic diversity in PCD patients, emphasizing the fact that differences in genetic background can influence the penetrance of different PCD-related mutations. We also found that DRC1 mutations resulted in decreased flagellum axoneme stability, thereby causing flagellar structural disorder.
while also affecting the manchette of spermatids. These DRC1 mutations also led to distinct phenotypic manifestations in cilia and flagella, providing a foundation for the future study of flagellar and ciliary structural stability (Fig. 9). Overall, our data emphasize the important functions of the N-DRC core component DRC1 in mammals.

Materials and Methods

Study participants

A cohort of 100 Han Chinese men with MMAF-related male infertility were enrolled from the First Affiliated Hospital of Anhui Medical University and the Affiliated Suzhou Hospital of Nanjing Medical University in China. All enrolled patients exhibited primary infertility, and patients with PCD were excluded. To be eligible for enrollment, patients had to present with a standard MMAF phenotype characterized by severe asthenozoospermia (total sperm motility < 10%; normal: 40%) with >40% of spermatozoa exhibiting the following flagella abnormalities: short, absent, coiled, bent or irregular flagella. Karyotypic analyses for all enrolled patients were normal (46, XY), as were hormone levels, bilateral testicular size distributions and secondary sex characteristics. The samples of parental DNA were obtained from 84 of the 100 enrolled MMAF patients. An Ex20 kit (AGCU ScienTech Incorporation, Wuxi, China) was used to confirm parental relationships for the enrolled patients. The Institutional review boards of the School of Basic Medical Sciences, Nanjing Medical University, School of Life Sciences at Fudan University, the First Affiliated Hospital of Anhui Medical University and the Affiliated Suzhou Hospital of Nanjing Medical University approved this study. All patients provided informed consent to participate, and the study was performed as per the Declaration of Helsinki.

Whole-exome sequencing

Whole-exome sequencing (WES) was conducted for the 100 enrolled MMAF patients using gDNA isolated from peripheral blood with a DNeasy Blood and Tissue kit (QIAGEN, Duesseldorf, Germany). An Agilent SureSelectXT Human All Exon Kit was utilized to isolate and enrich exonic sequences, after which sequencing was performed on the Illumina HiSeq X-TEN platform. Standard assembly (Burrows-Wheeler Aligner), calling (Genome Analysis Toolkit) and annotation (ANNOVAR) were performed for sequencing analyses as detailed previously (37), and DRC1 mutations identified via this approach were confirmed via Sanger sequencing using primers listed in Supplementary Material, Table S1.

Animals

Mice were housed in a standard animal facility (20–22°C; 50–70% humidity; 12 h light/dark cycle) with free food and water access. The Institutional Animal Care and Use Committees of Nanjing Medical University approved this study (Approval No. IACUC-18100020), and all experiments were performed as per the Guide for the Care and Use of Laboratory Animals and institutional guidelines.

qPCR

Trizol (Thermo Fisher, Waltham, MA, USA) was used to extract RNA samples, after which 1 μg of total RNA was used to prepare
cDNA with a HiScriptIII RT SuperMix (Vazyme, R323, Nanjing, China) as per the manufacturer’s instructions. These cDNA samples were then diluted 1:4 and analyzed via qPCR in a 20 μl volume containing 250 nmol/l of each appropriate primer, 1 μl of cDNA and AceQ qPCR SYBR Green Master Mix (Vazyme, Q131, Nanjing, China). Thermocycler settings were as follows: 50 °C for 2 min; 95 °C for 5 min; 40 cycles of 95 °C for 10 s and 60 °C for 30 s. The 18 s rRNA was utilized as a normalization control, and all primer sequences are compiled in Supplementary Material, Table S2.

Antibodies

Rabbit anti-RSPH9 (23253-1-AP) and anti-CENTRIN1 (12794-1-AP) were obtained from Proteintech (Wuhan, China). Rabbit anti-β-Actin (AC026) and mouse anti-β-Tubulin (AC021) were from Abclonal (Wuhan, China). Rabbit anti-Acetylated Tubulin was purchased from Cell Signaling Technology (MA, USA). Mouse anti-FLAG M2 (F3165), anti-Acetylated Tubulin (T6793) and anti-α-Tubulin (T9026) were from Sigma-Aldrich (St. Louis, MO, USA). Mouse anti-HA-tag (M180–3) and mouse anti-DDDDK-tag (PM020) were from Medical & Biological Laboratories (Nagoya, JP). Antibodies specific for DRC1, CCDC65, LRRC48 and GAS8 were prepared as per previously published protocols (59). Briefly, murine DRC1 (aa 1–146), CCDC65 (aa 1–126), LRRC48 (aa 182–300) and GAS8 (aa 1–478) were expressed as His fusion proteins in *Escherichia coli* using the pET-28a (+) vector, after which the Ni-NTA His Bind Resin was used to affinity purify these proteins. For mice were then immunized with the resultant fusion proteins, yielding the four following antiserum preparations: anti-DRC1, anti-CCDC65, anti-LRRC48 and anti-GAS8.

Drc1 mutant mouse generation

Drc1<sup>−/−</sup> and Drc1<sup>R554X/R554X</sup> mice were prepared via CRISPR/Cas9 genome editing, while Drc1<sup>W244X/ W244X</sup> mice were generated.
using Cytosine base editors and BE3. For Drc1<sup>B554X/B554X</sup> and Dcr1 knock-out mice, appropriate PM sgRNA and donor target sequences were selected to achieve the nonsense mutations and consequent deletion of Drc1 exon 13. For Drc1<sup>W244X/W244X</sup>, the BE3 sgRNA was chosen to introduce a Drc1 nonsense mutation. The PM sgRNA, BE3 sgRNA and Donor sequences used herein were 5'-GACGTTGCGCTGATCGAAGG-3', 5'-CTCCCATTTCTTTATATTGTCG-3' and 5'-CITATACAAGCTGGTAAACTTCTC-3'. Sanger sequencing of PCR products was then performed. A MEGAshortscript Transcription Kit (AM1354, Ambion, Austin, TX, USA) was used for sgRNA preparation, followed by purification based on directions provided with the MEGAClear Kit (AM1908, Ambion, Austin, TX, USA). Cas9 (Addgene No. 44758) and BE3 (Addgene No. 2008) plasmids were linearized using AgeI and PmeI and were purified with the MinElute PCR Purification Kit (28004, Qiagen, Duesseldorf, Germany). BE3 and Cas9 mRNA were generated via in vitro transcription with the mMESSAGE mMACHINE T7 Ultra Kit (AM1345, Ambion, Austin, TX, USA), after which an RNeasy Mini Kit (74104, Qiagen, Duesseldorf, Germany) was used for purification based upon provided directions. One group of murine zygotes were co-injected with Cas9 mRNA (50 ng/μl), PM sgRNA (20 ng/μl) and Donor (50 ng/μl), while another group was co-injected with BE3 mRNA (50 ng/μl) and BE3 sgRNA (20 ng/μl). After injection, these zygotes were transferred into pseudo-pregnant recipients. At 7 days of age, toe-cutting was used to tag newborn mice, and DNA from these excised tissue samples was assessed with the Mouse Direct PCR Kit (B40013, Biotool, Houston, TX, USA). PCR was conducted using model-appropriate primers (Drc1<sup>+/−</sup> and Drc1<sup>B554X/B554X</sup> mice: F 5’-TTGGTGCATGTTCGGTCT-3’, R 5’-GAGTGATGAGGATCAGT-3; Dcr1<sup>W244X/W244X</sup> mice: F 5’-GACAGTAAACGCTCCATTAC-3’, R 5’-GCCCATGTGACAGGAAAT-3’) with the PrimeSTAR HS DNA Polymerase (DR0108, Takara, Tokyo, Japan) and the following thermocycler conditions: 95°C for 5 min; 35 cycles of 95°C for 30 s, 62°C (−0.2°C/Cycle) for 30 s and 72°C for 30 s; 72°C for 5 min. Sanger sequencing of PCR products was then performed.

Histological analysis

Mouse testes, epididymal and tracheal tissues were collected from a minimum of three mice per genotype. Modified Davidson’s fluid was used to fix testis and epididymis samples for up to 24 h, whereas 4% PFA was used to fix tracheal samples overnight followed by storage in 70% ethanol. Samples were then dehydrated via ethanol gradient, paraffin-embedded and 5 μm thick tissue sections were mounted on glass slides. H&E staining was conducted as per standard protocols while Periodic Acid-Schiff (PAS) staining was conducted with the PAS staining kit (395B, Sigma-Aldrich, St. Louis, MO, USA).

Murine tracheal epithelial cell isolation

A tracheal brushing approach was used to isolate murine tracheal epithelial cell (mTECs) as in prior studies (60). Briefly, tracheal brushing was conducted to isolate multiciliated airway cells, which were fixed for 30 min with 4% PFA, spread on glass slides and allowed to air-dry.

Western blotting

A lysis buffer (50 mM Tris–HCl pH 8.2, 75 Mm NaCl, 8 M urea) containing a 1× Complete EDTA-free Protease Inhibitor Cocktail (Roche, Basel, Switzerland) was used to extract proteins, which were then separated via SDS-PAGE and transferred onto PVDF membranes that were blocked for 2 h with 5% non-fat milk in TBS at room temperature, followed by overnight incubation with appropriate primary antibodies at 4°C. Blots were then washed thrice with TBST, probed for 2 h at room temperature with appropriate secondary antibodies and protein bands were then detected with the high-sig ECL western blotting substrate (Tanon, Shanghai, China).

Cell culture

HEK293T cells were grown in high-glucose DMEM containing 10% FBS (Gibco, Grand Island, NY, USA) and penicillin/streptomycin (100 U/ml, Thermo Fisher, Waltham, MA, USA). Lipofectamine 2000 (11668019, Thermo Fisher) was used for cellular transfection based upon provided directions.

Immunoprecipitation

Lipofectamine 2000 was used to transfect HEK293T cells with DRC expression plasmids. At 2 days post-transfection, cells were lysed for 40 min using RIPA buffer (P0013C, Beyotime, Shanghai, China) containing 1× Complete EDTA-free Protease Inhibitor Cocktail (Roche, Basel, Switzerland) at 4°C, after which samples were spun down for 20 min at 12000×g. Supernatants from these lysates were then preclared for 1 h with 10 μl Protein G magnetic beads (10004D, Thermo Fisher, Waltham, MA, USA) at 4°C, after which they were combined with anti-HA-tag antibodies overnight at 4°C. They were then mixed with 50 μl of Protein G magnetic beads for 3 h at 4°C. Beads were washed thrice with RIPA buffer, boiled for 10 min in 1× SDS loading buffer and proteins were then subjected to SDS-PAGE analysis.

Immunofluorescence

Immunofluorescent staining of testis and tracheal tissue sections was conducted as detailed previously (61). Spermatocytes and mTECs were obtained as detailed above. Samples were washed thrice with PBS (10 min/wash), and antigen retrieval was conducted for 10 min in 10 mM citrate buffer (pH 6.0) in
a microwave oven. Following three additional PBS washes, 5% BSA was used to block slides for 2 h, after which they were stained overnight with appropriate primary antibodies at 4°C. Following secondary antibody staining for 2 h at room temperature, Hoechst 33342 counterstaining was performed for 5 min. Slides were then rinsed with PBS and mounted using glycerol prior to imaging with an LSM800 confocal microscope (Carl Zeiss AG, Jena, Germany) or a TCS SP8X confocal microscope (Leica Microsystems, Wetzlar, Germany).

**Analysis of tracheal ciliary length**

Tracheas were excised from three 12-day-old Drc1+/+, Drc1+/Drc1+Drc1X mice, and isolated mTECs were stained with anti-acetylated α-tubulin as above. Cells were then imaged via confocal microscope (Leica TCS SP8X), and LAS X was used to measure cilia length by assessing the ciliary tuft length for each cell, with 20 cells being analyzed per animal.

**Assessment of tracheal ciliary motility**

Murine tracheal tissues were dissected, added to high-glucose DMEM containing 10% FBS (Gibco, Grand Island, NY, USA), opened on the dorsal side and minced under stereoscopic magnification to yield ~5 mm tissue fragments. These tissues were then transferred to a confocal dish (BDD012035, BIOFIL, Guangzhou, China) and a scotch tape spacer was used to facilitate their imaging under a 40× objective (CFI S Plan Flour ELWD NAMC) with an inverted microscope (Eclipse Ti2-U, Nikon, Tokyo, Japan).

**Transmission electron microscopy**

For ultrastructural analyses, 2.5% glutaraldehyde was used to fix tracheal and epididymal tissue samples overnight followed by 2% OsO4 post-fixing and embedding in Araldite. Ultrathin 80 nm sections were then stained using uranyl acetate and lead citrate, followed by analysis with an electron microscope (JEM.1010, JEOL, Tokyo, Japan).

**Scanning electron microscopy**

Spermatozoa and tracheal samples were fixed for 2 h with 2.5% phosphate-buffered glutaraldehyde at 4°C. Spermatozoa were then allowed to attach to coverslips coated with poly-L-lysine. Both sample types were then washed with PBS, dehydrated with a chilled ethanol gradient (30, 50, 70, 80, 90 and 100%) and subjected to critical point drying with a Lecia EM CPD300 Critical Point Dryer (Wetzlar, Germany). Samples were then attached to a Helios G4 CX scanning electron microscopy (SEM) (Thermo Scientific, Waltham, MA, USA) and observed with a high-resolution scanning electron microscope (LEO 982, Carl Zeiss, Jena, Germany).

**Statistical analysis**

Experiments were conducted in triplicate. Data are given as means ± standard error and were compared by one-way ANOVAs and unpaired two-tailed t-tests. P < 0.05 was the significance threshold. Microsoft Excel and GraphPad Prism 6.0 were utilized for all statistical testing.

**Supplementary Material**

Supplementary Material is available at HMG online.
5. Rubbo, B. and Lucas, J.S. (2017) Clinical care for primary ciliary dyskinesia: current challenges and future directions. Eur. Respir. Rev., 26, 170023.

6. Lucas, J.S., Burgess, A., Mitchison, H.M., Moya, E., Williamson, M., Hogg, C. and National Pcd Service, U.K. (2014) Diagnosis and management of primary ciliary dyskinesia. Arch. Dis. Child., 99, 850–856.

7. Bower, R., Tritschler, D., Vanderwaal, K., Perrone, C.A., Mueller, J., Fox, L., Sale, W.S. and Porter, M.E. (2013) The N-DRC forms a conserved biochemical complex that maintains outer doublet alignment and limits microtubule sliding in motile axonemes. Mol. Biol. Cell, 24, 1134–1152.

8. Satir, P. (1968) Studies on the eel sperm flagellum. I. The structure of the inner dynein arm complex. J. Cell Sci., 110, 85–94.

9. Summers, K.E. and Gibbons, I.R. (1971) Adenosine triphosphate-induced sliding of tubules in trypsin-treated flagella of sea-urchin sperm. Proc. Natl. Acad. Sci. U. S. A., 68, 3092–3096.

10. Woolley, D.M. (1997) Studies on the eel sperm flagellum. I. The structure of the inner dynein arm complex. J. Cell Sci., 110, 85–94.

11. Gui, L., Song, K., Tritschler, D., Bower, R., Yan, S., Dai, A., Augspurger, K., Sakizadeh, J., Grzemska, M., Ni, T., Porter, M.E. and Nicastro, D. (2019) Scaffolding subunits support associated subunit assembly in the Chlamydomonas ciliary nexin-dynein regulatory complex. Proc. Natl. Acad. Sci. 116, 23152-23162.

12. Gui, M., Ma, M., Sze-Tu, E., Wang, X., Koh, F., Zhong, E.D., Berger, B., Davis, J.H., Dutcher, S.K., Zhang, R. et al. (2020) Structures of radial spokes and associated complexes important for ciliary motility. Nat. Struct. Mol. Biol., 28, 29–37.

13. Heuser, T., Raytchev, M., Krell, J., Porter, M.E. and Nicastro, D. (2009) The dynein regulatory complex is the nexin link and a major regulatory node in cilia and flagella. J. Cell Biol., 187, 921–933.

14. Lin, J., Tritschler, D., Song, K., Barber, C.F., Cobb, J.S., Porter, M.E. and Nicastro, D. (2011) Building blocks of the nexin-dynein regulatory complex in Chlamydomonas flagella. J. Biol. Chem., 286, 29175–29191.

15. Dutcher, S.K., Morohoshi, A., Miyata, H., Shimada, K., Nozawa, K., Matsumura, T., Yanase, R., Shibata, K., Inaba, K. and Ikawa, M. (2020) Nixin-dynein regulatory complex component DRC7 but not FBXL13 is required for sperm flagellum formation and male fertility in mice. PLoS Genet., 16, e1008585.

16. Wood, C.D., Li, R.-K., Tan, J.-L., Chen, L.-T., Feng, J.-S., Liang, W.-X., Guo, X.-J., Liu, P., Chen, Z., Sha, J.-H. et al. (2014) IQcg is essential for sperm flagellum formation in mice. PLoS One, 9, e86053.

17. Castaneda, J.M., Hua, R., Miyata, H., Oji, A., Guo, Y., Cheng, Y., Zhou, T., Guo, X., Cui, Y., Shen, B. et al. (2017) TTC1E is a conserved component of the dynein regulatory complex and is required for motility and metabolism in mouse spermatozoa. Proc. Natl. Acad. Sci. U. S. A., 114, ES370–ES378.

18. Ha, S., Lindsay, A.M., Timms, A.E. and Beier, D.R. (2016) Mutations in Dnaaf1 and Lrcc48 cause hydrocephalus, laterality defects, and sinusitis in mice. G3. 6, 2479–2487.

19. Lewis, W.R., Malarkey, E.B., Tritschler, D., Bower, R., Pasek, R.C., Porath, J.D., Birket, S.E., Saunier, S., Artignac, C., Knowles, M.R. et al. (2016) Mutation of growth arrest specific 8 reveals a role in motile cilia function and human disease. PLoS Genet., 12, e1006220.

20. Wirschell, M., Olbrich, H., Werner, C., Tritschler, D., Bower, R., Sale, W.S., Loges, N.T., Pennekamp, P., Lindberg, S., Stenram, U. et al. (2013) The nixin-dynein regulatory complex subunit DRC1 is essential for motile cilia function in algae and humans. Nat. Genet., 45, 262–268.

21. Morimoto, K., Hikijaka, M., Zariwala, M.A., Nykamp, K., Inaba, A., Guo, T.C., Yamada, H., Truty, R., Sasaki, Y. and Ohta, K. (2019) Recurring large deletion in DRC1 (CCDC616) identified as causing primary ciliary dyskinesia in two Asian patients. Mol. Genet. Genomic Med., 7, e838.

22. Ben Khelifa, M., Coutton, C., Zouari, R., Karouzouzene, T., Rendu, J., Bidart, M., Yassine, S., Pierre, V., Delaroche, J., Hennebicq, S. et al. (2014) Mutations in DNH1, 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.

23. Beurois, J., Martinez, G., Cazin, C., Kherraf, Z.E., Amiri-Yekta, A., Thierry-Mieg, N., Bidart, M., Petre, G., Satre, V., Brouillet, S. et al. (2019) CAF70 mutations lead to male infertility due to severe asthene-teratozoospermia. A case report. Hum. Reprod., 34, 2071–2079.

24. Coutton, C., Martinez, G., Kherraf, Z.E., Amiri-Yekta, A., Bougenet, M., Saunier, S., Antignac, C., Escoffier, J. et al. (2019) Bi-allelic mutations in ARM2C lead to severe astheno-teratozoospermia due to sperm flagellum malformations in humans and mice. Am. J. Hum. Genet., 104, 331–340.

25. He, X., Li, W., Wu, H., Lv, M., Liu, W., Liu, C., Zhu, F., Li, C., Fang, Y., Yang, C. et al. (2019) Novel homozygous CFAP69 mutations in humans and mice cause severe asthenoteratozoospermia with multiple morphological abnormalities of the sperm flagella. J. Med. Genet., 56, 96–103.

26. He, X., Liu, C., Yang, X., Lv, M., Ni, X., Li, Q., Cheng, H., Liu, W., Tian, S., Wu, H. et al. (2020) Bi-allelic loss-of-function variants in CFAP58 cause flagellar axoneme and mitochondrial sheath defects and asthenoterozoospermia in humans and mice. Am. J. Hum. Genet., 107, 514–526.

27. Li, W., Wu, H., Li, F., Tian, S., Kherraf, Z.E., Zhang, J., Ni, X., Lv, M., Liu, C., Tan, Q. et al. (2020) Biallelic mutations in CFAP65 cause male infertility with multiple morphological abnormalities of the sperm flagella in humans and mice. J. Med. Genet., 57, 89–95.

28. Liu, C., He, X., Liu, W., Yang, S., Wang, L., Li, W., Wu, H., Tang, S., Ni, X., Wang, J. et al. (2019) Bi-allelic mutations in TCTC2 cause male subfertility with asthenoteratozoospermia in humans and mice. Am. J. Hum. Genet., 105, 1168–1181.

29. Liu, C., Miyata, H., Gao, Y., Sha, Y., Tang, S., Xu, Z., Whitfield, M., Patrat, C., Wu, H., Duliouest, E. et al. (2020) Bi-allelic DNAH8 variants lead to multiple morphological abnormalities of the sperm flagella and primary male infertility. Am. J. Hum. Genet., 107, 330–341.

30. Liu, W., He, X., Yang, S., Zouari, R., Wang, J., Wu, H., Kherraf, Z.E., Liu, C., Coutton, C., Zhao, R. et al. (2019) Bi-allelic mutations in TCTC21A induce asthenoteratozoospermia in humans and mice. Am. J. Hum. Genet., 104, 738–748.

31. Liu, W., Sha, Y., Li, Y., Mei, L., Lin, S., Huang, X., Lu, J., Ding, L., Kong, S. and Lu, Z. (2019) Loss-of-function mutations in SFE2 cause multiple morphological abnormalities of the sperm flagella (MMAF). J. Med. Genet., 56, 678–684.

32. Lorès, P., Coutton, C., El Khouri, E., Stouvenel, L., Givelet, M., Thomas, L., Roe, 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.

33. Lv, M., Liu, W., Chi, W., Ni, X., Wang, J., Cheng, H., Li, W., Yang, C., Wu, H., Zhang, J. et al. (2020) Homozygous...
mutations in DZIP1 can induce asthenotatospermia with severe MMAF. J. Med. Genet., 57, 455–453.

34. Martinez, G., Kherraf, Z.E., Zouari, R., Fourati Ben Mustapha, S., Saut, A., Pernet-Gallay, K., Bertrand, A., Bidart, M., Hograindeur, 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.

35. Sha, Y.W., Xu, X., Mei, L.B., Li, P., Su, Z.Y., He, X.O. and Li, L. (2017) A homozygous CEP135 mutation is associated with multiple morphological abnormalities of the sperm flagella (MMAF). Gene, 633, 48–53.

36. Shen, Y., Zhang, F., Li, F., Jiang, X., Yang, Y., Li, X., Li, W., Wang, X., Cheng, J., Liu, M. et al. (2019) Loss-of-function mutations in QRICH2 cause male infertility with multiple morphological abnormalities of the sperm flagella. Nat. Commun., 10, 433.

37. 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.

38. Auguste, Y., Delague, V., Desvignes, J.-P., Longepied, G., Gniisci, A., Bessier, P., Levy, N., Beroud, C., Megarbane, A., Metzler-Guillenain, C. et al. (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.

39. Li, W., He, X., Yang, S., Liu, C., Wu, H., Liu, W., Lv, M., Tang, D., Tan, J., Tang, S. et al. (2019) Biallelic mutations of CFAP251 cause sperm flagellar defects and human male infertility. J. Hum. Genet., 64, 54–69.

40. Martinez, G., Beurrous, J., Dacheux, D., Cazin, C., Bidart, M., Kherraf, Z.-E., Robinson, D.R., Satre, V., Le Gac, G. and Ka, M.-A. (2020) Biallelic variants in MAATS1 encoding CFAP91, a calmodulin-associated and spoke-associated complex protein, cause severe asthenoteratospermia and male infertility. J. Med. Genet., 57, 708–716.

41. Chang, Y.F., Imam, J.S. and Wilkinson, M.F. (2007) The nonsense-mediated decay RNA surveillance pathway. Annu. Rev. Biochem., 76, 51–74.

42. Fanourgakis, G., Lesche, M., Akpinar, M., Dahl, A. and Jessorberger, R. (2016) Chromatoid body protein TDRD6 supports long 3' UTR triggered nonsense mediated mRNA decay. PLoS Genet., 12, e1005857.

43. Mühlemann, O. (2016) Spermatogenesis studies reveal a distinct nonsense-mediated mRNA decay (NMD) mechanism for mRNAs with long 3'UTRs. PLoS Genet., 12, e1005979.

44. Lee, L., Campagna, D.R., Pinkus, J.L., Mulhern, H., Wyatt, T.A., Sisson, J.H., Pavlik, J.A., Pinkus, G.S. and Fleming, M.D. (2008) Primary ciliary dyskinesia in mice lacking the novel ciliary protein Pcdp1. Mol. Cell. Biol., 28, 949–957.

45. Robinson, A.M., Takahashi, S., Brotslaw, E.J., Ahmad, A., Ferre, E., Procius, D., Richter, C.-P., Cheatham, M.A., Mitchell, B.J. and Zheng, J. (2020) CAMSAP3 facilitates basal body polarity and the formation of the central pair of microtubules in motile cilia. Proc. Natl. Acad. Sci., 117, 13571–13579.

46. Rachel, R.A., Yamamoto, E.A., Dewanjee, M.K., May-Simera, H.L., Sergeev, Y.V., Hackett, A.N., Fohida, K., Munasinghe, J., Gotoh, N., Wickstead, B. et al. (2015) CEP290 alleles in mice disrupt tissue-specific cilia biogenesis and recapitulate features of syndromic ciliopathies. Hum. Mol. Genet., 24, 3775–3791.

47. Onouchiadis, A., Pasf, T., Antony, D., Shoemark, A., Micha, D., Kuyt, B., Schmidt, M., Petridi, S., Dankert-Roelse, J.E., Haarman, E.G. et al. (2013) Splice-site mutations in the axonemal outer dynein arm docking complex gene CCDC61 cause primary ciliary dyskinesia. Am. J. Hum. Genet., 92, 88–98.

48. Moryan, A., Guay, A.T., Kurtz, S. and Nowak, P.J. (1985) Familial ciliary dyskinesia: a cause of infertility without respiratory disease. Fertil. Steril., 44, 539–542.

49. Baal, L. Audollent, S., Martinovic, J., Ozlou, C., Babron, M.C., Sivanandamoorthy, S., Saunier, S., Salomon, R., Gonzales, M., Rattenberry, E. et al. (2007) Pleiotropic effects of CEP290 (NPHP6) mutations extend to Meckel syndrome. Am. J. Hum. Genet., 81, 170–179.

50. Coppieters, F., Lefever, S., Leroy, B.P. and De Baere, E. (2010) CEP290, a gene with many faces: mutation overview and presentation of CEP290base. Hum. Mutat., 31, 1097–1108.

51. den Hollander, A.I., Koenekoop, R.K., Yzer, S., Lopez, I., Arends, M.L., Voesenek, K.E., Zonneveld, M.N., Strom, T.M., Mettler, T., Brunner, H.G. et al. (2006) Mutations in the CEP290 (NPHP6) gene are a frequent cause of Leber congenital amaurosis. Am. J. Hum. Genet., 79, 556–561.

52. Drivas, T.G., Wojno, A.P., Tucker, B.A., Stone, E.M. and Bennett, J. (2015) Basal exon skipping and genetic pleiotropy: a predictive model of disease pathogenesis. Sci. Transl. Med., 7, 291ra297.

53. Leitch, C.C., Zaghoul, N.A., Davis, E.E., Stoezel, C., Diaz-Font, A., Rix, S., Alfadhel, M., Lewis, R.A., Eysid, W., Banin, E. et al. (2008) Hypomorphic mutations in syndromic encaphalocele genes are associated with Bardet-Biedl syndrome. Nat. Genet., 40, 443–448.

54. Sayer, J.A., Otto, E.A., O'Toole, J.F., Nurnberg, G., Kennedy, M.A., Becker, C., Hennies, H.C., Helou, J., Attanasio, M., Fausett, B.V. et al. (2006) The centrosomal protein nephrocystin-6 is mutated in Joubert syndrome and activates transcription factor ATF4. Nat. Genet., 38, 674–681.

55. Valente, E.M., Silhavy, J.L., Brancati, F., Barrano, G., Krishnaswami, S.R., Castori, M., Lancaster, M.A., Bolthausen, E., Boccone, L, Al-Gazali, L. et al. (2006) Mutations in CEP290, which encodes a centrosomal protein, cause pleiotropic forms of Joubert syndrome. Nat. Genet., 38, 623–625.

56. Ramsbottom, S.A., Thelwall, P.E., Wood, K.M., Clowry, G.J., Devlin, L.A., Silbermann, F., Spiewak, H.L., Shril, S., Molinari, E., Hildebrandt, F. et al. (2020) Mouse genetics reveals Barttin as a genetic modifier of Joubert syndrome. Proc. Natl. Acad. Sci., 117, 1113–1118.

57. Abbasi, F., Miyata, H., Shimada, K., Morohoshi, A., Nozawa, K., Matsumura, T., Xu, Z., Pratwi, P. and Ikawa, M. (2018) RSPH6A is required for sperm flagellum formation and male fertility in mice. J. Cell Sci., 131, jcs221648.

58. Yu, Y., Wang, J., Zhou, L., Li, H., Zheng, B. and Yang, S. (2020) CEP43-mediated intra-manchette transport is required for sperm head shaping and flagella formation. Zygote, 29, 75–81.

59. Liu, M., Shi, X., Bi, Y., Qi, L., Guo, X., Wang, L., Zhou, Z. and Sha, J. (2014) SHCBP1L, a conserved protein in mammals, is required for sperm flagellum development and male fertility in mice. J. Cell Sci., 131, jcs221648.

60. Rachev, E., Schuster-Gossler, K., Puhl, F., Ott, T., Terwiakhina, L., Beckers, A., Hegermann, J., Boldt, K., Mai, M., Kremmer, E. et al. (2020) CEP43 modulates ciliary beating in mouse and Xenopus. Dev. Biol., 459, 109–125.

61. Castañeda, J., Genzor, P., van der Heijden, G.W., Sarkeshik, A., Yates, J.R., III, Ingolia, N.T. and Bortvin, A. (2014) Reduced pachytene piRNAs and translation underlie spermigenic arrest in Maelstrom mutant mice. EMBO J., 33, 1999–2019.