Review

Young Investigator Award

Analysis of the sperm flagellar axoneme using gene-modified mice

Haruhiko MIYATA¹⁾, Akane MOROHOSHI^{1,2)} and Masahito IKAWA^{1–3)}¹⁾Research Institute for Microbial Diseases, Osaka University, 3-1 Yamadaoka, Suita, Osaka 565-0871, Japan²⁾Graduate School of Medicine, Osaka University, 2-2 Yamadaoka, Suita, Osaka 565-0871, Japan³⁾The Institute of Medical Science, The University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108-8639, Japan

Abstract: Infertility is a global health issue that affects 1 in 6 couples, with male factors contributing to 50% of cases. The flagellar axoneme is a motility apparatus of spermatozoa, and disruption of its structure or function could lead to male infertility. The axoneme consists of a “9+2” structure that contains a central pair of two singlet microtubules surrounded by nine doublet microtubules, in addition to several macromolecular complexes such as dynein arms, radial spokes, and nexin-dynein regulatory complexes. Molecular components of the flagellar axoneme are evolutionally conserved from unicellular flagellates to mammals, including mice. Although knockout (KO) mice have been generated to understand their function in the formation and motility regulation of sperm flagella, the majority of KO mice die before sexual maturation due to impaired ciliary motility, which makes it challenging to analyze mature spermatozoa. In this review, we introduce methods that have been used to overcome premature lethality, focusing on KO mouse lines of central pair components.

Key words: central pair, chimeric mice, male fertility

Introduction

Cilia and flagella are evolutionarily conserved organelles that extend from the cell surface and are involved in sensing and locomotion. Defects in these organelles in humans lead to a group of disorders called ciliopathies that affect many tissues, such as lung, kidney, and brain tissues [4, 20, 43]. Primary ciliary dyskinesia (PCD) is one ciliopathy where the formation or motility of motile cilia is impaired, leading to abnormal mucociliary clearance and recurrent respiratory infections. Testes can be affected in PCD as well, and this can result in impairment of the formation and function of sperm flagella, leading to male infertility [22, 28, 55].

Spermatozoa comprise a head region, which contains the paternal genetic information, and flagellum, which is important for migration of spermatozoa in the female

reproductive tract and passage through the zona pellucida, an extracellular matrix that surrounds the oocyte [8, 14, 40]. In contrast to cilia, sperm flagella possess unique accessory components that divide the flagellum into three parts: the midpiece, principal piece, and endpiece (Fig. 1) [13]. The midpiece contains the mitochondrial sheath and outer dense fibers, while the principal piece contains the fibrous sheath and outer dense fibers. No accessory structures are found in the endpiece. The accessory structures of the midpiece and principal piece regulate sperm motility through elastic forces and/or scaffolding molecules that are involved in energy production and/or signaling [13]. The axoneme is a conserved component of cilia and flagella that runs through the entire flagellum and is composed of a “9+2” structure consisting of a central pair (CP) of two singlet microtubules surrounded by nine doublet microtubules (DMTs)

(Received 20 May 2020 / Accepted 28 May 2020 / Published online in J-STAGE 18 June 2020)

Corresponding authors: H. Miyata. e-mail: hmiya003@biken.osaka-u.ac.jp

M. Ikawa. e-mail: ikawa@biken.osaka-u.ac.jp

This is an open-access article distributed under the terms of the Creative Commons Attribution Non-Commercial No Derivatives (by-nc-nd) License <<http://creativecommons.org/licenses/by-nc-nd/4.0/>>.

©2020 Japanese Association for Laboratory Animal Science

Fig. 1. Mouse sperm flagellum. Flagella can be divided into three parts: the midpiece, principal piece, and endpiece. Cross sections of each part are shown. The axoneme runs through the flagellum, which consists of several macromolecular complexes. CP, central pair; DMT, doublet microtubules; IDA, inner dynein arm; N-DRC, nexin-dynein regulatory complex; ODA, outer dynein arm; RS, radial spoke.

(Fig. 1) [19, 64]. In addition to these structures, the axoneme contains several macromolecular complexes, such as outer and inner dynein arms (ODAs and IDAs), radial spokes (RSs), and nexin-dynein regulatory complex (N-DRC).

The components of the axoneme and their functions have been studied extensively in unicellular flagellates such as *Chlamydomonas* [19, 64]. These axonemal proteins are evolutionarily conserved in species that possess cilia and flagella, including mice; however, there are several molecules whose function in mouse spermatozoa remain unknown. One reason for this is the difficulty and cost in generating gene-modified mice using homologous recombination in embryonic stem (ES) cells; however, recent developments in genome-editing technology made it possible to manipulate mouse genomes with ease at low cost [1, 31, 39, 65]. Another reason for this is that knockout (KO) mouse lines often die before sexual maturation because of impaired ciliary motility, such as hydrocephalus, which hampers the analysis of mature spermatozoa. Here, we introduced phenotypes of mouse lines with KO of CP components and methods of overcoming premature lethality caused by impaired ciliary motility.

Structure and Components of the CP

The CP is composed of two singlet microtubules, C1 and C2, that are structurally and biochemically distinguishable [38, 57, 59]. There are several projections on each singlet microtubule and their precise localization

Fig. 2. Structure and components of the CP. There are two singlet microtubules, C1 and C2, that contain several projections (C1a-C1f and C2a-C2e). There is also a bridge that connects C1 and C2 microtubules. *Chlamydomonas* CP components (top) with mouse orthologs (bottom) are indicated.

has been analyzed with cryo-electron tomography (Fig. 2) [6]. The most extended projections on C1 are termed C1a and C1b, and 4 short projections, C1c, C1d, C1e, and C1f, are located between C1a and C1b projections. For C2, there are two long projections, C2a and C2b, and three short projections, C2c, C2d, and C2e. In addition, there is a bridge that connects C1 and C2 microtubules. The molecular components of these projections have been identified and shown to be critical for regulating flagellar motility in *Chlamydomonas* [38, 57, 59]. These components are conserved in mice, and several KO mouse lines have been generated (Table 1); these mouse lines are introduced next.

Analysis of Mature Spermatozoa Using 129 Backgrounds

Chlamydomonas FAP221 (flagellar associated protein 221) is a component of the C1d projection and can bind to calmodulin in a calcium-dependent manner [10, 11]. The mouse ortholog of FAP221 is *Pcdp1* (primary ciliary dyskinesia protein 1), which was identified by analyzing *nm1054* mutant mice that possess an approximately 400-kb deletion on chromosome 1 containing 6 genes [27]. *Nm1054* mutant mice exhibit anemia and cutaneous phenotypes that are caused by *Steap3* (STEAP family member 3) and *Dbi* (diazepam binding inhibitor), respectively, as well as hydrocephalus that is caused by *Pcdp1*. *Nm1054* mutant mice on the C57BL/6 background that possess a *Steap3* transgene to rescue anemia die at around 3 to 5 weeks of age because of hydro-

Table 1. Conservation of CP components and phenotypes of KO mice.

Chlamydomonas	Localization	Mouse	Phenotype of KO mice		Human diseases
			Lethality ^{b)}	Spermatozoa	
<i>CPC1</i>	C1b	<i>Spef2</i>	Premature lethality	Impaired tail formation [54]	PCD and MMAF [29, 30, 52, 63]
<i>FAP42</i>	C1b	<i>Guk1</i>	NA	NA	NA
<i>FAP46</i>	C1d	<i>Cfap46</i>	NA	NA	NA
<i>FAP54</i>	C1d	<i>Cfap54</i>	Premature lethality	Impaired tail formation [33]	NA
<i>FAP69</i>	C1b	<i>Cfap69</i>	Viable	Impaired tail formation [12, 17]	MMAF [12, 17]
<i>FAP74</i>	C1d	<i>Cfap74</i>	NA	NA	NA
<i>FAP101</i>	C1a	<i>Tcte1</i> ^{a)}	Viable	Impaired motility [7]	NA
<i>FAP114</i>	C1a	<i>Ccdc189</i>	NA	NA	NA
<i>FAP119</i>	C1a	<i>Ccdc189</i>	NA	NA	NA
<i>FAP174</i>	C1b	<i>Mychp</i>	NA	NA	NA
<i>FAP221</i>	C1d	<i>Pcdp1</i>	Premature lethality	Impaired tail formation [27]	NA
<i>FAP297</i>	C1d	<i>Wdr93</i>	NA	NA	NA
<i>HYDIN</i>	C2b	<i>Hydin</i>	Premature lethality	Impaired tail formation [47]	PCD [46]
<i>KLP1</i>	C2c/d	<i>Kif9</i>	Viable	Impaired motility [41]	NA
<i>PF6</i>	C1a	<i>Spag17</i>	Neonatal demise	Impaired tail formation [23]	NA
<i>PF16</i>	C1	<i>Spag6</i>	Premature lethality	Impaired tail formation [51]	NA
<i>PF20</i>	Bridge	<i>Spag16l</i>	Viable	Impaired motility [71]	NA

^{a)}*Tcte1* is also the *Chlamydomonas* ortholog of *DRC5*, which is a component of the N-DRC. ^{b)}The most severe phenotypes are shown for cases in which analyses were performed in different genetic backgrounds.

cephalus, which hampers the analysis of mature spermatozoa [27]. Intriguingly, mutants on the 129S6/Sv background develop either mild or no hydrocephalus, suggesting that there are genetic modifiers that characterize these two genetic backgrounds [27]. When the testes of mature *nm1054* mutant mice on the 129S6/Sv background were analyzed, no flagella were observed, indicating that *Pcdp1* is essential for flagellar formation in mice. In contrast to the abnormal flagellar formation, cilia in sinus and tracheal epithelial cells can form with a normal ultrastructure, although the cilia beat slowly, which leads to sinusitis. These results indicate that there are differences in the formation of flagella and cilia.

Milder hydrocephalus phenotypes in 129 backgrounds were observed in other mouse lines with KO of CP components as well. *Chlamydomonas CPC1* (central pair complex 1) is a component of the C1b projection and contains an adenylate kinase domain [37, 69]. The mouse ortholog of *CPC1* is *Spef2* (sperm flagellar protein 2). *Spef2* mutant mice were generated by N-ethyl-N-nitrosourea (ENU) mutagenesis on the C57BL/6 background and named big giant head (*bgh*) because of severe hydrocephalus. Most *bgh* homozygous mice on the C57BL/6 background die at around weaning age because of hydrocephalus, while *bgh* homozygous mice on an F1 mixed background (C57BL/6-129S6/Sv) show no evidence of gross hydrocephalus or no premature mortality [54]. The *bgh* homozygous male mice on the F1 mixed background were infertile because of immotile short flagella. Analyses with transmission electron microscopy (TEM) indicate disorganization or absence of the CP in round spermatids and a complete disorganization

of the axoneme, mitochondrial sheath, and outer dense fibers in early elongating spermatids, indicating that *SPEF2* is essential for the formation of the CP and axoneme in flagella. In contrast, the ultrastructure of tracheal cilia is normal, although their cilia beat slowly [54], supporting the idea that there are differences in flagella and cilia formation.

Mouse CFAP54 (cilia and flagella associated protein 54), an ortholog of *Chlamydomonas* FAP54 that is a component of the C1d projection [5], was also analyzed using a mixed background. *Cfap54* mutant mice on the C57BL/6 background exhibit severe hydrocephalus, while mutant mice on the F1 mixed background (C57BL/6-129S6/Sv) do not develop severe hydrocephalus [33]. *Cfap54* mutant male mice were infertile due to short tails. TEM analysis indicates that mutant flagella are highly disorganized, with axonemal structures mostly absent. Consistent with *nm1054* and *bgh* mutant mice, *Cfap54* mutant mice develop sinusitis, although airway epithelial cilia are present and organized. TEM analysis indicates that the ciliary axoneme is normally organized, but there is an absence of electron-dense material, indicating a loss of the C1d projection in the CP of *Cfap54* KO mice [33].

These studies suggest that the 129 background can be used to overcome premature lethality caused by hydrocephalus. However, genetic modifiers that segregate these strains remain unknown [34], and it is still unclear if the 129 background can rescue all cases. For example, *Spag6* (sperm associated antigen 6), a mouse ortholog of *Chlamydomonas* PF16 that contains armadillo repeats and is associated with the C1 microtubule [56],

was mutated on the mixed background (C57BL/6-129/Sv); however, about 50% of the *Spag6* KO mice died from hydrocephalus before 2 months of age [51]. *Spag6* KO males surviving to sexual maturity were infertile due to impaired flagellar morphology and sperm motility. It is important to understand genetic modifiers that segregate these strains, which may uncover pathways that underlie susceptibility to severe hydrocephalus.

Analysis of Mature Spermatozoa Using the Conditional KO Approach

Mouse lines with KO of axonemal components can be lethal due to not only hydrocephalus but also other factors. In such cases, it is difficult to overcome lethality using the 129 background. Mouse SPAG17 is an ortholog of *Chlamydomonas* PF6 that is a component of the C1a projection [15, 49]. *Spag17* KO mice on the C57BL/6-129S4/Sv mixed background exhibit profound respiratory distress associated with lung fluid accumulation and neonatal demise within 12 h of birth, in addition to hydrocephalus [60]. More severe phenotypes of *Spag17* KO mice than other CP component KO mice are probably caused by the proximity of SPAG17 to other macromolecular complexes, such as RSs. To prevent neonatal lethality, the conditional KO approach was utilized with transgenic mouse lines that express CRE recombinase under the *Sox2* promoter [23]. Obtained

mutant mice were infertile due to immotile short flagella. TEM analysis indicates that some flagellar axonemes lack one CP microtubule. These results indicate that SPAG17 is essential for sperm flagellar formation. There are several transgenic mouse lines that express CRE specifically in the testis [32, 44, 62], and the conditional KO approach can be an excellent way to overcome lethality and analyze mature spermatozoa. However, it should be noted that CRE-dependent recombination is not 100% efficient [50, 62], which may make the interpretation of results difficult.

Analysis of Mature Spermatozoa Using Chimeric Mice

Conditional KO approaches can be used to overcome lethality; however, it takes time to generate mouse models and breed them with transgenic mouse lines that express recombinases such as CRE or FLP. To analyze phenotypes of KO spermatozoa faster, chimeric analysis can be utilized, which can analyze mature spermatozoa in one generation (Fig. 3) [45]. In this method, homozygous mutations of target genes are introduced into ES cells using genome-editing technology, and chimeric mice are generated using the KO-ES cells. Because chimeric mice possess wild-type (WT) cells that are derived from host embryos, premature lethality can be rescued. If ES cells express fluorescent proteins, KO spermatozoa

Fig. 3. Chimeric analysis. A gene of interest is knocked out in ES cells that express fluorescent proteins. Chimeric mice are generated with the ES cells, and phenotypes of KO cells that are derived from ES cells are analyzed, such as spermatozoa or blood cells, using the fluorescence as a marker. Because chimeric mice possess wild-type cells derived from host embryos, the mice could overcome premature lethality.

derived from the ES cells can be recognized using the fluorescence as a marker, and their phenotypes can be analyzed. This approach was used to analyze HYDIN, which is a component of the C2b projection [26] and whose mutation leads to premature lethality due to hydrocephalus (FVB background) [9, 25, 35, 48]. *Hydin* was mutated in ES cells that were established by mating 129/Sv mice with RBGS (Red Body Green Sperm) transgenic mice [B6D2-Tg (CAG/Su9-DsRed2, Acr3-EGFP) RBGS002Osb] whose spermatozoa exhibit green fluorescence in the acrosome, a secretory vesicle localized in the head, and red fluorescence in the mitochondria [16]. Chimeric mice that were generated with *Hydin* KO ES cells could reach sexual maturity, and KO spermatozoa with fluorescence signals exhibited short, immotile flagella, indicating that HYDIN is essential for sperm flagellar formation [47]. The next generation (F1) was obtained by injecting KO spermatozoa into B6D2F1 mouse oocytes (intracytoplasmic sperm injection, ICSI). KO mice that were obtained from F1 × F1 mating could not reach sexual maturity, indicating that the KO allele is lethal even in the mixed background.

Although chimeric analysis can be used to analyze the function of axonemal components within a short period, it should be noted that analyses using bulk cell populations are difficult because of the contamination of WT cells derived from host embryos. In this case, chimeric mice that are generated by injecting male ES cells into female embryos can be used because all spermatogenic cells of the chimeric mice are derived from ES cells [21]. Another problem of chimeric analysis is that the next generation cannot be obtained to maintain the line if the gene of interest is essential for male fertility. In that case, assisted reproductive technologies can be utilized to rescue the fertility as ICSI was used for *Hydin* KO spermatozoa [47]. ICSI can often rescue male fertility in mouse lines with KO of axonemal components that exhibit short sperm tails [2, 42, 61, 68].

KO Mouse Lines that Do Not Exhibit Premature Lethality

Although KO mouse lines that show premature lethality have been introduced so far, there are some KO mouse lines that have been reported to be viable. *Chlamydomonas* PF20 possesses five WD repeats that are important for protein interactions and is a component of a bridge that connects C1 and C2 microtubules [58]. The mouse ortholog of *PF20* is *Spag16*, which encodes two major transcripts, *Spag16l* (2.5 kb) and *Spag16s* (1.4 kb) [72]. SPAG16L is localized in the central pair of sperm flagella, while SPAG16S is localized abundantly in the

nuclei of round and condensing spermatids [70, 72]. Deletion of *Spag16* exon 11, which disrupts both *Spag16l* and *Spag16s*, causes impaired spermatogenesis in chimeric mice due to haploinsufficiency [70]. In contrast, deletion of exons 2 and 3, which disrupts only *Spag16l*, leads to male infertility due to impaired sperm motility with a normal axoneme ultrastructure (mixed background C57BL/6-129/Sv) [71]. *Spag16l* KO mice do not show hydrocephalus or sinusitis [71]. This mild phenotype might be due to a mixed background, or it is possible that SPAG16L does not play critical roles in regulating ciliary motility.

Milder phenotypes were also observed in *Kif9* (kinesin family member 9) KO mice (B6D2 background). KIF9 contains a motor domain like other kinesin superfamily proteins [18] and is an ortholog of *Chlamydomonas* KLP1 (kinesin like protein 1), which is a component of the C2c/d projections [3, 67]. *Kif9* KO male mice were subfertile due to impaired sperm motility, while no overt hydrocephalus was observed [41]. This mild phenotype may be because KIF9 does not play critical roles in axonemal formation and/or motility. It is also possible that KIF6, a paralog of KIF9, plays more important roles in regulating ciliary motility [24]. Two paralogs playing separate roles in cilia and flagella have been reported in other axonemal components. For example, *Rsph4a* (radial spoke head 4 homolog A) is essential for regulating ciliary motility, disruption of which leads to hydrocephalus [53, 66], while its paralog, *Rsph6a*, is essential for sperm flagellar formation [2].

Mice with KO of *Cfap69*, an ortholog of *Chlamydomonas* FAP69, which is a component of the C1b projection [36], could survive to sexual maturity as well and were infertile due to abnormal flagellar formation (mixed background C57BL/6-FVB) [12]. Variations in *CFAP69* were also found in infertile men who exhibited severe flagellar abnormalities, including flagella being short, coiled, absent, and of irregular caliber, which is referred to as multiple morphological abnormalities of the flagella (MMAF) [12, 17]. Variations in another CP component, *SPEF2*, were found in MMAF patients as well [29, 30, 52, 63], suggesting that CP components are important for sperm flagellar formation not only in mice but also humans.

Conclusion

Generation and analysis of KO mice with various methods indicate that most CP components were essential for sperm flagellar formation. These phenotypes in flagella are different from those in cilia that can form but exhibit impaired motility. The difference could be

because sperm tail elongation is associated with the formation of accessory structures such as mitochondrial sheaths, fibrous sheaths, and outer dense fibers that are not found in cilia. Supporting this idea, mice with KO of CP components exhibit not only a disrupted axoneme but also the absence or disorganized accessory structures. Further, *Spef2* KO mice exhibit more disorganized axonemes in round spermatids when accessory structures are being built [54]. Recently, more candidate components of the CP were identified in *Chlamydomonas* with proteomics analysis [73]. Understanding the function of these CP components may lead us to understand how CP components are involved in flagellar formation and to uncover the causes of male infertility, such as MMAF.

In this review, we introduced three methods that could overcome premature lethality: 129 backgrounds, the conditional KO approach, and chimeric analysis. These methods can be applied to understand the functions of not only CP but also other axonemal components. For example, chimeric analysis has been utilized to analyze DNAJB13, a component of RS [45]. Chimeric analysis can even be applied to examine other cell types, such as blood cells or immune cells, if analysis in mature KO mice is difficult to perform due to lethality (Fig. 3). Using the methods introduced here and genome-editing technology, the regulatory mechanism of flagellar formation and motility in mammals will be unveiled further.

Acknowledgments

The authors would like to thank Dr. Julio M. Castaneda for critical reading of the manuscript. This research was supported by Ministry of Education, Culture, Sports, Science and Technology (MEXT)/Japan Society for the Promotion of Science (JSPS) KAKENHI grants (JP17H04987 to H.M., JP17H01394 to M.I.).

References

- Abbasi, F., Miyata, H. and Ikawa, M. 2017. Revolutionizing male fertility factor research in mice by using the genome editing tool CRISPR/Cas9. *Reprod. Med. Biol.* 17: 3–10. [Medline] [CrossRef]
- Abbasi, F., Miyata, H., Shimada, K., Morohoshi, A., Nozawa, K., Matsumura, T., Xu, Z., Pratiwi, P. and Ikawa, M. 2018. RSPH6A is required for sperm flagellum formation and male fertility in mice. *J. Cell Sci.* 131: jcs221648. [Medline] [CrossRef]
- Bernstein, M., Beech, P.L., Katz, S.G. and Rosenbaum, J.L. 1994. A new kinesin-like protein (Klp1) localized to a single microtubule of the *Chlamydomonas* flagellum. *J. Cell Biol.* 125: 1313–1326. [Medline] [CrossRef]
- Bisgrove, B.W. and Yost, H.J. 2006. The roles of cilia in developmental disorders and disease. *Development* 133: 4131–4143. [Medline] [CrossRef]
- Brown, J.M., DiPetrillo, C.G., Smith, E.F. and Witman, G.B. 2012. A FAP46 mutant provides new insights into the function and assembly of the C1d complex of the ciliary central apparatus. *J. Cell Sci.* 125: 3904–3913. [Medline] [CrossRef]
- Carbajal-González, B.I., Heuser, T., Fu, X., Lin, J., Smith, B.W., Mitchell, D.R. and Nicastro, D. 2013. Conserved structural motifs in the central pair complex of eukaryotic flagella. *Cytoskeleton (Hoboken)* 70: 101–120. [Medline] [CrossRef]
- Castaneda, J.M., Hua, R., Miyata, H., Oji, A., Guo, Y., Cheng, Y., Zhou, T., Guo, X., Cui, Y., Shen, B., Wang, Z., Hu, Z., Zhou, Z., Sha, J., Prunskaitė-Hyyryläinen, R., Yu, Z., Ramirez-Solis, R., Ikawa, M., Matzuk, M.M. and Liu, M. 2017. TCTE1 is a conserved component of the dynein regulatory complex and is required for motility and metabolism in mouse spermatozoa. *Proc. Natl. Acad. Sci. USA* 114: E5370–E5378. [Medline] [CrossRef]
- Chung, J.J., Shim, S.H., Everley, R.A., Gygi, S.P., Zhuang, X. and Clapham, D.E. 2014. Structurally distinct Ca(2+) signaling domains of sperm flagella orchestrate tyrosine phosphorylation and motility. *Cell* 157: 808–822. [Medline] [CrossRef]
- Davy, B.E. and Robinson, M.L. 2003. Congenital hydrocephalus in hy3 mice is caused by a frameshift mutation in Hydin, a large novel gene. *Hum. Mol. Genet.* 12: 1163–1170. [Medline] [CrossRef]
- DiPetrillo, C.G. and Smith, E.F. 2010. Pcdp1 is a central apparatus protein that binds Ca(2+)-calmodulin and regulates ciliary motility. *J. Cell Biol.* 189: 601–612. [Medline] [CrossRef]
- DiPetrillo, C.G. and Smith, E.F. 2011. The Pcdp1 complex coordinates the activity of dynein isoforms to produce wild-type ciliary motility. *Mol. Biol. Cell* 22: 4527–4538. [Medline] [CrossRef]
- Dong, F.N., Amiri-Yekta, A., Martinez, G., Saut, A., Tek, J., Stouvenel, L., Lorès, P., Karaouzen, T., Thierry-Mieg, N., Satre, V., Brouillet, S., Daneshpour, A., Hosseini, S.H., Bonhivers, M., Gourabi, H., Dulioust, E., Arnoult, C., Touré, A., Ray, P.F., Zhao, H. and Coutton, C. 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. [Medline] [CrossRef]
- Eddy, E.M., Toshimori, K. and O'Brien, D.A. 2003. Fibrous sheath of mammalian spermatozoa. *Microsc. Res. Tech.* 61: 103–115. [Medline] [CrossRef]
- Fujihara, Y., Miyata, H. and Ikawa, M. 2018. Factors controlling sperm migration through the oviduct revealed by genome-modified mouse models. *Exp. Anim.* 67: 91–104. [Medline] [CrossRef]
- Goduti, D.J. and Smith, E.F. 2012. Analyses of functional domains within the PF6 protein of the central apparatus reveal a role for PF6 sub-complex members in regulating flagellar beat frequency. *Cytoskeleton (Hoboken)* 69: 179–194. [Medline] [CrossRef]
- Hasuwa, H., Muro, Y., Ikawa, M., Kato, N., Tsujimoto, Y. and Okabe, M. 2010. Transgenic mouse sperm that have green acrosome and red mitochondria allow visualization of sperm and their acrosome reaction in vivo. *Exp. Anim.* 59: 105–107. [Medline] [CrossRef]
- He, X., Li, W., Wu, H., Lv, M., Liu, W., Liu, C., Zhu, F., Li, C., Fang, Y., Yang, C., Cheng, H., Zhang, J., Tan, J., Chen, T., Tang, D., Song, B., Wang, X., Zha, X., Wang, H., Wei, Z., Yang, S., Saiyin, H., Zhou, P., Jin, L., Wang, J., Zhang, Z., Zhang, F. and Cao, Y. 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. [Medline] [CrossRef]
- Hirokawa, N., Noda, Y., Tanaka, Y. and Niwa, S. 2009. Kinesin superfamily motor proteins and intracellular transport. *Nat. Rev. Mol. Cell Biol.* 10: 682–696. [Medline] [CrossRef]
- Inaba, K. 2011. Sperm flagella: comparative and phylogenetic perspectives of protein components. *Mol. Hum. Reprod.* 17: 524–538. [Medline] [CrossRef]
- Inaba, K. and Mizuno, K. 2015. Sperm dysfunction and cili-

- opathy. *Reprod. Med. Biol.* 15: 77–94. [Medline] [CrossRef]
21. Isotani, A., Nakanishi, T., Kobayashi, S., Lee, J., Chuma, S., Nakatsuji, N., Ishino, F. and Okabe, M. 2005. Genomic imprinting of XX spermatogonia and XX oocytes recovered from XX $\leftrightarrow$ XY chimeric testes. *Proc. Natl. Acad. Sci. USA* 102: 4039–4044. [Medline] [CrossRef]
 22. Ji, Z.Y., Sha, Y.W., Ding, L. and Li, P. 2017. Genetic factors contributing to human primary ciliary dyskinesia and male infertility. *Asian J. Androl.* 19: 515–520. [Medline] [CrossRef]
 23. Kazarian, E., Son, H., Sapao, P., Li, W., Zhang, Z., Strauss, J.F. and Teves, M.E. 2018. SPAG17 Is Required for Male Germ Cell Differentiation and Fertility. *Int. J. Mol. Sci.* 19: 1252. [Medline] [CrossRef]
 24. Konjikusic, M.J., Yeetong, P., Boswell, C.W., Lee, C., Robertson, E.C., Itiwut, R., Suphapeetiporn, K., Ciruna, B., Gurnett, C.A., Wallingford, J.B., Shotelersuk, V. and Gray, R.S. 2018. Mutations in Kinesin family member 6 reveal specific role in ependymal cell ciliogenesis and human neurological development. *PLoS Genet.* 14: e1007817. [Medline] [CrossRef]
 25. Lechtreck, K.F., Delmotte, P., Robinson, M.L., Sanderson, M.J. and Witman, G.B. 2008. Mutations in Hydin impair ciliary motility in mice. *J. Cell Biol.* 180: 633–643. [Medline] [CrossRef]
 26. Lechtreck, K.F. and Witman, G.B. 2007. Chlamydomonas reinhardtii hydin is a central pair protein required for flagellar motility. *J. Cell Biol.* 176: 473–482. [Medline] [CrossRef]
 27. 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. [Medline] [CrossRef]
 28. Linck, R.W., Chemes, H. and Albertini, D.F. 2016. The axoneme: the propulsive engine of spermatozoa and cilia and associated ciliopathies leading to infertility. *J. Assist. Reprod. Genet.* 33: 141–156. [Medline] [CrossRef]
 29. Liu, C., Lv, M., He, X., Zhu, Y., Amiri-Yekta, A., Li, W., Wu, H., Kherraf, Z.E., Liu, W., Zhang, J., Tan, Q., Tang, S., Zhu, Y.J., Zhong, Y., Li, C., Tian, S., Zhang, Z., Jin, L., Ray, P., Zhang, F. and Cao, Y. 2020. Homozygous mutations in *SPEF2* induce multiple morphological abnormalities of the sperm flagella and male infertility. *J. Med. Genet.* 57: 31–37. [Medline] [CrossRef]
 30. 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 *SPEF2* cause multiple morphological abnormalities of the sperm flagella (MMAF). *J. Med. Genet.* 56: 678–684. [Medline] [CrossRef]
 31. Mashiko, D., Fujihara, Y., Satouh, Y., Miyata, H., Isotani, A. and Ikawa, M. 2013. Generation of mutant mice by pronuclear injection of circular plasmid expressing Cas9 and single guided RNA. *Sci. Rep.* 3: 3355. [Medline] [CrossRef]
 32. Matsumura, H., Hasuwa, H., Inoue, N., Ikawa, M. and Okabe, M. 2004. Lineage-specific cell disruption in living mice by Cre-mediated expression of diphtheria toxin A chain. *Biochem. Biophys. Res. Commun.* 321: 275–279. [Medline] [CrossRef]
 33. McKenzie, C.W., Craige, B., Kroeger, T.V., Finn, R., Wyatt, T.A., Sisson, J.H., Pavlik, J.A., Strittmatter, L., Hendricks, G.M., Witman, G.B. and Lee, L. 2015. CFAP54 is required for proper ciliary motility and assembly of the central pair apparatus in mice. *Mol. Biol. Cell* 26: 3140–3149. [Medline] [CrossRef]
 34. McKenzie, C.W., Preston, C.C., Finn, R., Eyster, K.M., Faustino, R.S. and Lee, L. 2018. Strain-specific differences in brain gene expression in a hydrocephalic mouse model with motile cilia dysfunction. *Sci. Rep.* 8: 13370. [Medline] [CrossRef]
 35. McLone, D.G., Bondareff, W. and Raimondi, A.J. 1971. Brain edema in the hydrocephalic hy-3 mouse: submicroscopic morphology. *J. Neuropathol. Exp. Neurol.* 30: 627–637. [Medline] [CrossRef]
 36. Mitchell, B.F., Pedersen, L.B., Feely, M., Rosenbaum, J.L. and Mitchell, D.R. 2005. ATP production in Chlamydomonas reinhardtii flagella by glycolytic enzymes. *Mol. Biol. Cell* 16: 4509–4518. [Medline] [CrossRef]
 37. Mitchell, D.R. and Sale, W.S. 1999. Characterization of a Chlamydomonas insertional mutant that disrupts flagellar central pair microtubule-associated structures. *J. Cell Biol.* 144: 293–304. [Medline] [CrossRef]
 38. Mitchell, D.R. and Smith, B. 2009. Analysis of the central pair microtubule complex in Chlamydomonas reinhardtii. *Methods Cell Biol.* 92: 197–213. [Medline] [CrossRef]
 39. Miyata, H., Castaneda, J.M., Fujihara, Y., Yu, Z., Archambeault, D.R., Isotani, A., Kiyozumi, D., Kriseman, M.L., Mashiko, D., Matsumura, T., Matzuk, R.M., Mori, M., Noda, T., Oji, A., Okabe, M., Prunskaitė-Hyyryläinen, R., Ramirez-Solis, R., Satouh, Y., Zhang, Q., Ikawa, M. and Matzuk, M.M. 2016. Genome engineering uncovers 54 evolutionarily conserved and testis-enriched genes that are not required for male fertility in mice. *Proc. Natl. Acad. Sci. USA* 113: 7704–7710. [Medline] [CrossRef]
 40. Miyata, H., Satouh, Y., Mashiko, D., Muto, M., Nozawa, K., Shiba, K., Fujihara, Y., Isotani, A., Inaba, K. and Ikawa, M. 2015. Sperm calcineurin inhibition prevents mouse fertility with implications for male contraceptive. *Science* 350: 442–445. [Medline] [CrossRef]
 41. Miyata, H., Shimada, K., Morohoshi, A., Oura, S., Matsumura, T., Xu, Z., Oyama, Y. and Ikawa, M. 2020. Testis-enriched kinesin KIF9 is important for progressive motility in mouse spermatozoa. *FASEB J.* 34: 5389–5400. [Medline] [CrossRef]
 42. Morohoshi, A., Miyata, H., Shimada, K., Nozawa, K., Matsumura, T., Yanase, R., Shiba, K., Inaba, K. and Ikawa, M. 2020. Nexin-Dynein regulatory complex component DRC7 but not FBXL13 is required for sperm flagellum formation and male fertility in mice. *PLoS Genet.* 16: e1008585. [Medline] [CrossRef]
 43. Norris, D.P. and Grimes, D.T. 2012. Mouse models of ciliopathies: the state of the art. *Dis. Model. Mech.* 5: 299–312. [Medline] [CrossRef]
 44. O’Gorman, S., Dagenais, N.A., Qian, M. and Marchuk, Y. 1997. Protamine-Cre recombinase transgenes efficiently recombine target sequences in the male germ line of mice, but not in embryonic stem cells. *Proc. Natl. Acad. Sci. USA* 94: 14602–14607. [Medline] [CrossRef]
 45. Oji, A., Noda, T., Fujihara, Y., Miyata, H., Kim, Y.J., Muto, M., Nozawa, K., Matsumura, T., Isotani, A. and Ikawa, M. 2016. CRISPR/Cas9 mediated genome editing in ES cells and its application for chimeric analysis in mice. *Sci. Rep.* 6: 31666. [Medline] [CrossRef]
 46. Olbrich, H., Schmidts, M., Werner, C., Onoufriadis, A., Loges, N.T., Raidt, J., Banki, N.F., Shoemark, A., Burgoyne, T., Al Turki, S., Hurles, M.E., Köhler, G., Schroeder, J., Nürnberg, G., Nürnberg, P., Chung, E.M., Reinhardt, R., Marthin, J.K., Nielsen, K.G., Mitchison, H.M., Omran, H., UK10K Consortium 2012. Recessive HYDIN mutations cause primary ciliary dyskinesia without randomization of left-right body asymmetry. *Am. J. Hum. Genet.* 91: 672–684. [Medline] [CrossRef]
 47. Oura, S., Miyata, H., Noda, T., Shimada, K., Matsumura, T., Morohoshi, A., Isotani, A. and Ikawa, M. 2019. Chimeric analysis with newly established EGFP/DsRed2-tagged ES cells identify HYDIN as essential for spermiogenesis in mice. *Exp. Anim.* 68: 25–34. [Medline] [CrossRef]
 48. Raimondi, A.J., Clark, S.J. and McLone, D.G. 1976. Pathogenesis of aqueductal occlusion in congenital murine hydrocephalus. *J. Neurosurg.* 45: 66–77. [Medline] [CrossRef]
 49. Rupp, G., O’Toole, E. and Porter, M.E. 2001. The Chlamydomonas PF6 locus encodes a large alanine/proline-rich polypeptide that is required for assembly of a central pair projection and regulates flagellar motility. *Mol. Biol. Cell* 12: 739–751. [Medline] [CrossRef]
 50. Sadate-Ngatchou, P.I., Payne, C.J., Dearth, A.T. and Braun,

- R.E. 2008. Cre recombinase activity specific to postnatal, premeiotic male germ cells in transgenic mice. *Genesis* 46: 738–742. [Medline] [CrossRef]
51. 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. [Medline] [CrossRef]
 52. Sha, Y., Liu, W., Wei, X., Zhu, X., Luo, X., Liang, L. and Guo, T. 2019. Biallelic mutations in Sperm flagellum 2 cause human multiple morphological abnormalities of the sperm flagella (MMAF) phenotype. *Clin. Genet.* 96: 385–393. [Medline] [CrossRef]
 53. Shinohara, K., Chen, D., Nishida, T., Misaki, K., Yonemura, S. and Hamada, H. 2015. Absence of Radial Spokes in Mouse Node Cilia Is Required for Rotational Movement but Confers Ultrastructural Instability as a Trade-Off. *Dev. Cell* 35: 236–246. [Medline] [CrossRef]
 54. Sironen, A., Kotaja, N., Mulhern, H., Wyatt, T.A., Sisson, J.H., Pavlik, J.A., Miiluniemi, M., Fleming, M.D. and Lee, L. 2011. Loss of SPEF2 function in mice results in spermatogenesis defects and primary ciliary dyskinesia. *Biol. Reprod.* 85: 690–701. [Medline] [CrossRef]
 55. Sironen, A., Shoemark, A., Patel, M., Loebinger, M.R. and Mitchison, H.M. 2020. Sperm defects in primary ciliary dyskinesia and related causes of male infertility. *Cell. Mol. Life Sci.* 77: 2029–2048. [Medline]
 56. Smith, E.F. and Lefebvre, P.A. 1996. PF16 encodes a protein with armadillo repeats and localizes to a single microtubule of the central apparatus in Chlamydomonas flagella. *J. Cell Biol.* 132: 359–370. [Medline] [CrossRef]
 57. Smith, E.F. and Lefebvre, P.A. 1997. The role of central apparatus components in flagellar motility and microtubule assembly. *Cell Motil. Cytoskeleton* 38: 1–8. [Medline] [CrossRef]
 58. Smith, E.F. and Lefebvre, P.A. 1997. PF20 gene product contains WD repeats and localizes to the intermicrotubule bridges in Chlamydomonas flagella. *Mol. Biol. Cell* 8: 455–467. [Medline] [CrossRef]
 59. 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. [Medline] [CrossRef]
 60. Teves, M.E., Zhang, Z., Costanzo, R.M., Henderson, S.C., Corwin, F.D., Zweit, J., Sundaresan, G., Subler, M., Salloum, F.N., Rubin, B.K. and Strauss, J.F. 3rd. 2013. Sperm-associated antigen-17 gene is essential for motile cilia function and neonatal survival. *Am. J. Respir. Cell Mol. Biol.* 48: 765–772. [Medline] [CrossRef]
 61. Tokuhiro, K., Hirose, M., Miyagawa, Y., Tsujimura, A., Irie, S., Isotani, A., Okabe, M., Toyama, Y., Ito, C., Toshimori, K., Takeda, K., Oshio, S., Tainaka, H., Tsuchida, J., Okuyama, A., Nishimune, Y. and Tanaka, H. 2008. Meichroacidin containing the membrane occupation and recognition nexus motif is essential for spermatozoa morphogenesis. *J. Biol. Chem.* 283: 19039–19048. [Medline] [CrossRef]
 62. Tokuhiro, K., Satouh, Y., Nozawa, K., Isotani, A., Fujihara, Y., Hirashima, Y., Matsumura, H., Takumi, K., Miyano, T., Okabe, M., Benham, A.M. and Ikawa, M. 2015. Calreticulin is required for development of the cumulus oocyte complex and female fertility. *Sci. Rep.* 5: 14254. [Medline] [CrossRef]
 63. Tu, C., Nie, H., Meng, L., Wang, W., Li, H., Yuan, S., Cheng, D., He, W., Liu, G., Du, J., Gong, F., Lu, G., Lin, G., Zhang, Q. and Tan, Y.Q. 2020. Novel mutations in SPEF2 causing different defects between flagella and cilia bridge: the phenotypic link between MMAF and PCD. *Hum. Genet.* 139: 257–271. [Medline] [CrossRef]
 64. Viswanadha, R., Sale, W.S. and Porter, M.E. 2017. Ciliary Motility: Regulation of Axonemal Dynein Motors. *Cold Spring Harb. Perspect. Biol.* 9: a018325. [Medline] [CrossRef]
 65. Wang, H., Yang, H., Shivalila, C.S., Dawlaty, M.M., Cheng, A.W., Zhang, F. and Jaenisch, R. 2013. One-step generation of mice carrying mutations in multiple genes by CRISPR/Cas-mediated genome engineering. *Cell* 153: 910–918. [Medline] [CrossRef]
 66. Yoke, H., Ueno, H., Narita, A., Sakai, T., Horiuchi, K., Shingyoji, C., Hamada, H. and Shinohara, K. 2020. Rsp4a is essential for the triplet radial spoke head assembly of the mouse motile cilia. *PLoS Genet.* 16: e1008664. [Medline] [CrossRef]
 67. Yokoyama, R., O'toole, E., Ghosh, S. and Mitchell, D.R. 2004. Regulation of flagellar dynein activity by a central pair kinesin. *Proc. Natl. Acad. Sci. USA* 101: 17398–17403. [Medline] [CrossRef]
 68. Young, S.A., Miyata, H., Satouh, Y., Kato, H., Nozawa, K., Isotani, A., Aitken, R.J., Baker, M.A. and Ikawa, M. 2015. CRISPR/Cas9-Mediated Rapid Generation of Multiple Mouse Lines Identified Ccdc63 as Essential for Spermiogenesis. *Int. J. Mol. Sci.* 16: 24732–24750. [Medline] [CrossRef]
 69. Zhang, H. and Mitchell, D.R. 2004. Cpc1, a Chlamydomonas central pair protein with an adenylate kinase domain. *J. Cell Sci.* 117: 4179–4188. [Medline] [CrossRef]
 70. Zhang, Z., Kostetskii, I., Moss, S.B., Jones, B.H., Ho, C., Wang, H., Kishida, T., Gerton, G.L., Radice, G.L. and Strauss, J.F. 3rd. 2004. Haploinsufficiency for the murine orthologue of Chlamydomonas PF20 disrupts spermatogenesis. *Proc. Natl. Acad. Sci. USA* 101: 12946–12951. [Medline] [CrossRef]
 71. Zhang, Z., Kostetskii, I., Tang, W., Haig-Ladewig, L., Sapiro, R., Wei, Z., Patel, A.M., Bennett, J., Gerton, G.L., Moss, S.B., Radice, G.L. and Strauss, J.F. 3rd. 2006. Deficiency of SPAG16L causes male infertility associated with impaired sperm motility. *Biol. Reprod.* 74: 751–759. [Medline] [CrossRef]
 72. 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. [Medline] [CrossRef]
 73. Zhao, L., Hou, Y., Picariello, T., Craigie, B. and Witman, G.B. 2019. Proteome of the central apparatus of a ciliary axoneme. *J. Cell Biol.* 218: 2051–2070. [Medline] [CrossRef]