

Sperm dysfunction and ciliopathy

Kazuo Inaba¹ · Katsutoshi Mizuno^{1,2}

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Abstract Sperm motility is driven by motile cytoskeletal elements in the tail, called axonemes. The structure of axonemes consists of 9 + 2 microtubules, molecular motors (dyneins), and their regulatory structures. Axonemes are well conserved in motile cilia and flagella through eukaryotic evolution. Deficiency in the axonemal structure causes defects in sperm motility, and often leads to male infertility. It has been known since the 1970s that, in some cases, male infertility is linked with other symptoms or diseases such as Kartagener syndrome. Given that these links are mostly caused by deficiencies in the common components of cilia and flagella, they are called “immotile cilia syndrome” or “primary ciliary dyskinesia,” or more recently, “ciliopathy,” which includes deficiencies in primary and sensory cilia. Here, we review the structure of the sperm flagellum and epithelial cilia in the human body, and discuss how male fertility is linked to ciliopathy.

Keywords Cilia · Ciliopathy · Dynein · Flagella · Sperm · Infertility

Abbreviations

AK	Adenylate kinase
ARMC	Armadillo repeat containing
BB	Basal body
BBS	Bardet–Biedl syndrome

CEP	Centrosomal protein
CCDC	Coiled-coil domain-containing
CP	Central pair apparatus
DC	Docking complex
DNAAF	Dynein axonemal assembly factor
DNAH	Dynein, axonemal, heavy chain
DNAI	Dynein, axonemal, intermediate chain
DNALI	Dynein, axonemal, light intermediate chain
DYNC2	Dynein, cytoplasmic 2
DYX1C1	Dyslexia susceptibility 1 candidate 1
FAP	Flagella-associated protein
FBB	Flagellar basal body
FoxJ1	Forkhead box J1
HC	Heavy chain
HEATR	HEAT-repeat containing
IC	Intermediate chain
IAD	Inner arm dynein
IDA	Inner dynein arm
IFT	Intraflagellar transport
IMT	Intramanchette transport
Iqcg	IQ motif containing G
LC	Light chain
LRRC	Leucine-rich repeat containing
MFN	Mitofusin
MIA	Modifier of inner arms
MKS	McKusick–Kaufman syndrome
MNS1	Meiosis-specific nuclear structural 1
N-DRC	Nexin-dynein regulatory complex
NPHP	Nephronophthisis
OAD	Outer arm dynein
ODA	Outer dynein arm
ODF	Outer dense fiber
OFD	Oral-facial-digital syndrome
PACRG	Parkin co-regulated gene
PCD	Primary ciliary dyskinesia

✉ Kazuo Inaba
kinaba@kurofune.shimoda.tsukuba.ac.jp

¹ Shimoda Marine Research Center, University of Tsukuba, 5-10-1 Shimoda, Shizuoka 415-0025, Japan

² Department of Biological Sciences, Dartmouth College, Hanover, NH 03755, USA

Pcdp1	Primary ciliary dyskinesia protein 1
PCP	Planar cell polarity
PF	Paralyzed flagella
PIH	Protein interacting with HSP90
PKD	Polycystic kidney disease
RFX	Regulatory factor X
RPGR	Retinitis pigmentosa GTPase regulator
RS	Radial spoke
SPAG	Sperm-associated antigen
Spf2	Sperm flagellar protein 2
TXNDC	Thioredoxin domain containing
TZ	Transition zone
VDAC3	Voltage-dependent anion channel 3
XLRP	X-linked retinitis pigmentosa
ZMYND	Zinc-finger, MYND-type containing protein

Introduction

Male infertility is caused by several factors ranging from psychological or behavioral problems to abnormal sperm function. Sperm dysfunction is the most obvious cause of male infertility that can be detected morphologically and physiologically. Sperm dysfunction is observed in several compartments of spermatozoa. In particular, defects in flagella directly affect sperm motility, and often lead to failure of fertilization. During the course of diagnosis of male patients with Kartagener syndrome, an important correlation between sperm dysfunction, bronchitis, and situs inversus was described [1–3]; the patients lacked dynein arms, which serve as molecular motors for motility of cilia and flagella. Because sperm flagella and motile cilia in the human body serve as centers of signal transduction, and have common internal structures for motility called axonemes, defects in the axonemal structure result not only in sperm dysfunction, but also in abnormalities of multiple ciliated cells and organs. Most such defects are caused by genetic mutations of ciliary proteins. Further investigations led to the proposal of the name “immotile-cilia syndrome” or “primary ciliary dyskinesia” (PCD) [4]. More recently, the name “ciliopathy” has been used for the general symptoms caused by disorders of motile, primary and sensory cilia [4, 5]. In this review, we describe the structure and function of the axoneme, and illustrate how abnormalities of the axonemal structure affect the motility of cilia and flagella.

Structure and function of sperm flagella

Spermatozoa are male gametes that are structurally and functionally highly differentiated. Human spermatozoa are composed of a head (acrosome and nucleus) and a tail (a

flagellum, containing a midpiece, principal piece, and end piece) (Fig. 1a) [6–9]. The motile machinery of the flagellum, the axoneme, extends from the basal body (distal centriole) via the transition zone (TZ) and has a $9 + 2$ microtubule structure. Mitochondria helically wrap around both the axoneme and outer dense fibers (ODFs) in the midpiece of the flagellum. In the principal piece, the axonemes are surrounded by ODF and fibrous sheath (FS) (Fig. 1b).

Structure of the axoneme

The axonemes of motile cilia and flagella are supramolecular machinery composed of cytoskeletons and molecular motors [8, 10, 11]. The molecular motors for sperm motility are microtubule-dependent ATPases called dyneins. Dyneins are classified into cytoplasmic and axonemal dyneins, both of which are composed of motor subunits (heavy chains, HCs) and associated subunits (intermediate chains, ICs and light chains, LCs), but are phylogenetically distinct from each other. Axonemal dyneins are observed as “arms,” which are attached to each of nine doublet microtubules (Fig. 1b, c). The sliding of doublet microtubules by axonemal dyneins is the basis of flagellar motility [12]. Outer arm dyneins (OADs) are involved in the increase of flagellar beat frequency and in the propagation of asymmetric flagellar waveforms. Inner arm dyneins (IADs) are responsible for flagellar bend formation and propagation [13]. The activities of axonemal dyneins are regulated by radial spokes (RSs) and by the central pair apparatus (CP) (central pair microtubules and the associated bridge and projections) [14, 15]. The green alga *Chlamydomonas* has been used to generate many useful mutants for studying cilia and flagella, including mutants for the outer and inner dynein arms (*oda* and *ida*) [13, 16] and other substructures (*pf*, paralyzed flagella) [17]. In animals, sperm from marine invertebrates have contributed to our understanding of the molecular architecture of flagella and the mechanism of their motility [18–24].

The molecular composition of axonemal dyneins has been widely examined in the green alga *Chlamydomonas* [25]. Outer arm dyneins (OADs) from *Chlamydomonas* flagella are composed of three heavy chains (HCs), two intermediate chains (ICs), and ten light chains (LCs). Work using sperm of tunicates (a subphylum of marine invertebrates) and sea urchins revealed similar molecular composition of the IADs but not the OADs [26–29]. The OAD from animal sperm has two HCs, two WD-repeat ICs, a unique IC with thioredoxin and nucleoside diphosphate kinase domains [30–32], and regulatory LCs such as Tctex-2 LC [33, 34] and a Ca^{2+} -binding protein named calaxin [35–37] (Fig. 1d). The precise subunits of the outer arm have been described in sea urchins and tunicates, but not in

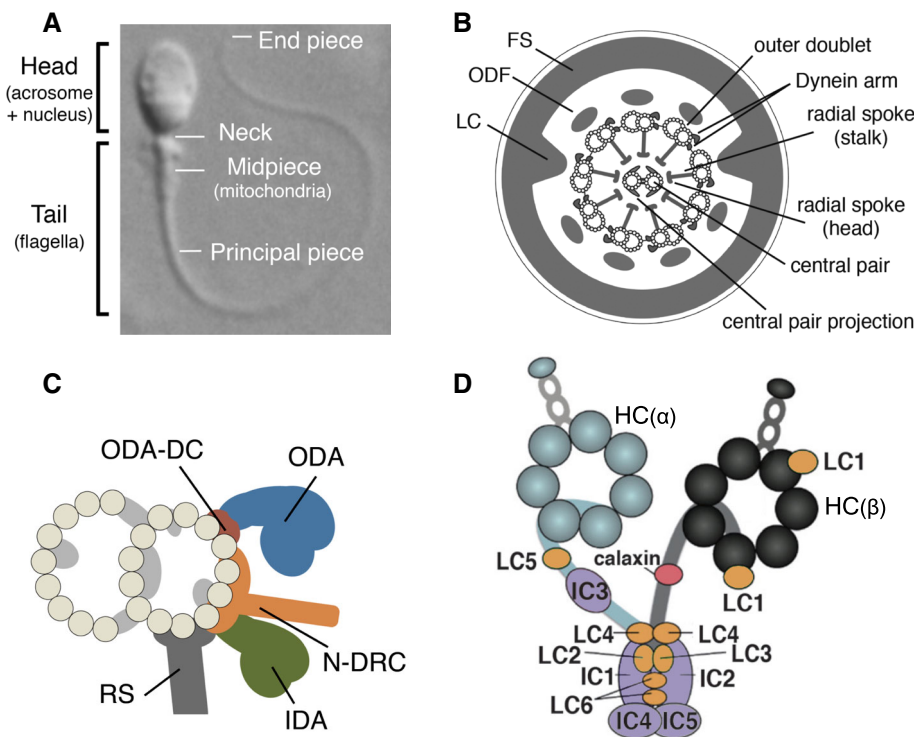


Fig. 1 Structure of sperm flagellum. **a** Differential interference contrast microscopic image of human sperm. The tail is divided into the midpiece, principal piece, and end piece. **b** Internal structure of the flagellar principal piece (cross-section). The central 9 + 2 structure, the axoneme, is the motile machinery of flagella. In mammalian sperm, it is surrounded by accessory structures, outer dense fiber (ODF) and the fibrous sheath (FS). A projection called the longitudinal column (LC) extends from the FS to two outer doublet microtubules (doublet numbers 3 and 7). **c** Detailed substructures of the axonemes. Each doublet microtubule is decorated by an outer

dynein arm (ODA), inner dynein arm (IDA), nexin-dynein regulatory complex (N-DRC), and radial spoke (RS). The ODA docking complex (DC) is anchored at an interval of 24 nm on the microtubule. Each doublet microtubule is decorated by some structures inside the microtubule or between protofilaments. **d** Molecular structure of the ODA from *Ciona* sperm flagella. The ODA is composed of two HCs, five ICs, and six LCs. The HCs are named as α and β , which are orthologs of β - and γ -type HC of *Chlamydomonas* ODA, respectively [8, 28, 37]. Calaxin is a Ca^{2+} sensor of opisthokont ODA that binds to *Ciona* β HC in the presence of Ca^{2+}

human sperm. The ODAs are arranged at 24-nm intervals on each doublet microtubule, mediated by the molecular “ruler” called outer arm dynein docking complex (ODA-DC) [38, 39]. In *Chlamydomonas* flagella, the ODA-DC is composed of three subunits, DC1 (a coiled-coil protein), DC2 (coiled-coil protein), and DC3 (an EF-hand protein). However, orthologs of DC1 and DC3 are not found in animal sperm flagella [28, 40].

Species of the IAD are more heterogeneous than the OAD in *Chlamydomonas*; one is two-headed (two HCs) with three ICs and five LCs, and another six are single-headed (single HC) with other subunits, such as actin, centrin, and a protein called p28 [41]. Only a few studies have been undertaken on the subunit structure of IAD in sperm [42–44]. From the base of IADs, two structures, dynein regulatory complex (DRC) and the nexin link, are observed. Both are now called “N-DRC” and regarded as an integrated substructure. N-DRC is bound to the base of the RS1, RS2 (Fig. 1c) [45–49]. Inside the cylinder of nine doublet microtubules, two types of structures are present in

most motile axonemes: one is RS that extend from the A-tubule of doublet microtubules, and the other is the CP composed of two singlet microtubules (C1 and C2), connected to each other by central bridges and decorated by central pair projections [50].

Each doublet microtubule has several structures between A- and B-tubules and inside the tubule (Fig. 1c, light gray) [47, 51, 52]. The structures between A- and B-tubules are called outer and inner junctions, both of which connect A- and B-tubules and are thought to play roles in the initiation of doublet formation. The structures inside the A- and B-tubules are composed of microtubule inner proteins (MIPs) [51] and the “beak” structure [52]. These structures are bound to the inside wall of microtubules with longitudinal periodicity of 16 and/or 48 nm and are thought to be involved in the assembly and stability of doublet microtubules [51].

The flagellum of mammalian sperm has accessory structures: ODF and FS [6]. These structures mechanically reinforce the axonemes, which is necessary for

spermatozoa to move in highly viscous conditions. Multiple glycolytic enzymes are bound to the fibrous sheath and play important roles in the regulation of sperm motility [53, 54].

Structures of the flagellar base

The basal body (BB) is composed of nine triplet microtubules and is differentiated from the mother centriole (distal centriole) during the cell cycle. When cilia are formed, the mother centriole becomes anchored to the plasma membrane where the cilium starts to extend. Then the mother centriole comes to serve as the BB. Axonemes are nucleated from basal bodies via the TZ. In motile cilia, there are several appendages on the BB and the TZ [55, 56] (Fig. 2a). A striated (or ciliary) rootlet extends from the proximal site of the BB to the cytoplasm and is found in many but not all types of cilia. The basal foot, originated from the subdistal appendage in the mother centriole, directs the orientation of the beating plane [55, 57]. At the distal end of the BB, a structure called the transition fiber (TF), originated from the distal appendage of the mother centriole, links the basal body to the ciliary membranes. The TF serves as a diffusion barrier for ciliary proteins. In the transition region, the Y-link extends from the microtubule to the plasma membrane (Fig. 2a).

Vesicles containing ciliary components are sorted by the Golgi network and transported to the base of the cilium (Fig. 3). A complex of Bardet–Biedl syndrome (BBS) proteins, called the BBSome (~450 kDa in molecular mass), is located at the base of a cilium and is involved in

the tethering and transporting of membrane cargos to the cilium in a G-protein (Rab, Ran, and Arf/Arl)-dependent pathway [58, 59]. After passing through the TZ, ciliary components are then delivered by directional movement of intraflagellar transport (IFT) particles. IFT is a bidirectional movement driven by kinesin-2 (anterograde) and cytoplasmic dynein 2 (DYNC2) (retrograde) to transport axonemal and membrane components for construction of cilia and flagella (Fig. 3). Two IFT particles, IFT-B and IFT-A complexes, are specifically involved in anterograde and retrograde transport, respectively [60, 61]. The detailed functions of BBSome, IFT-A or IFT-B in the transport of ciliary components, or their interactions, are not completely understood (Fig. 3).

The mechanism of formation of sperm flagella is considered to be similar to that of ciliogenesis, although some reports have demonstrated that sperm flagella are formed by a mechanism different from that of sensory cilia in *Drosophila* [62, 63]. Both proximal and distal centrioles are present in elongating spermatids, but the distal centriole and its associated structures are degenerated in mature sperm in mouse [64]. Human sperm have intact proximal centrioles, but the distal centrioles are largely degenerated [65]. The neck region of mature spermatozoa, called the connecting piece, is composed of two parts: the capitulum and striated collar (Fig. 2b). The latter is considered to be homologous to the striated rootlets of cilia and connected to the nucleus through the structure called the implantation fossa [6, 66]. The presence of a basal foot was described at least in spermatids, but its presence in mature spermatozoa is not clear.

Fig. 2 Structures at the base of epithelial cilia (a) and sperm flagella (b). AX axoneme, BP basal plate, TZ transition zone, TF transition fiber, BF basal foot, DC distal (mother) centriole, BB basal body, PC proximal (daughter) centriole, SR striated rootlet, MIT mitochondrion, ODF outer dense fiber, SC striated column, CT capitulum

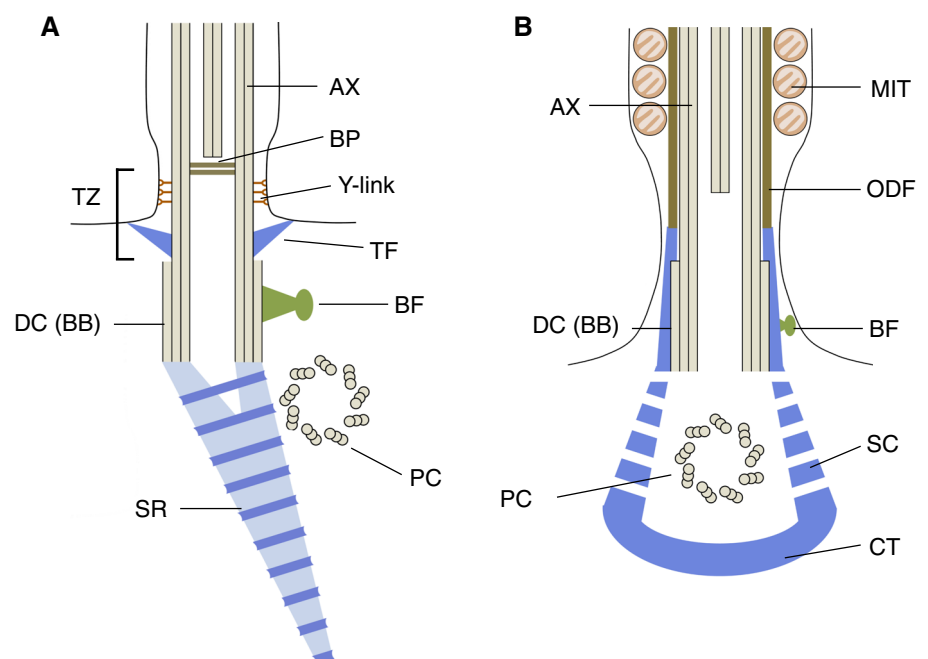
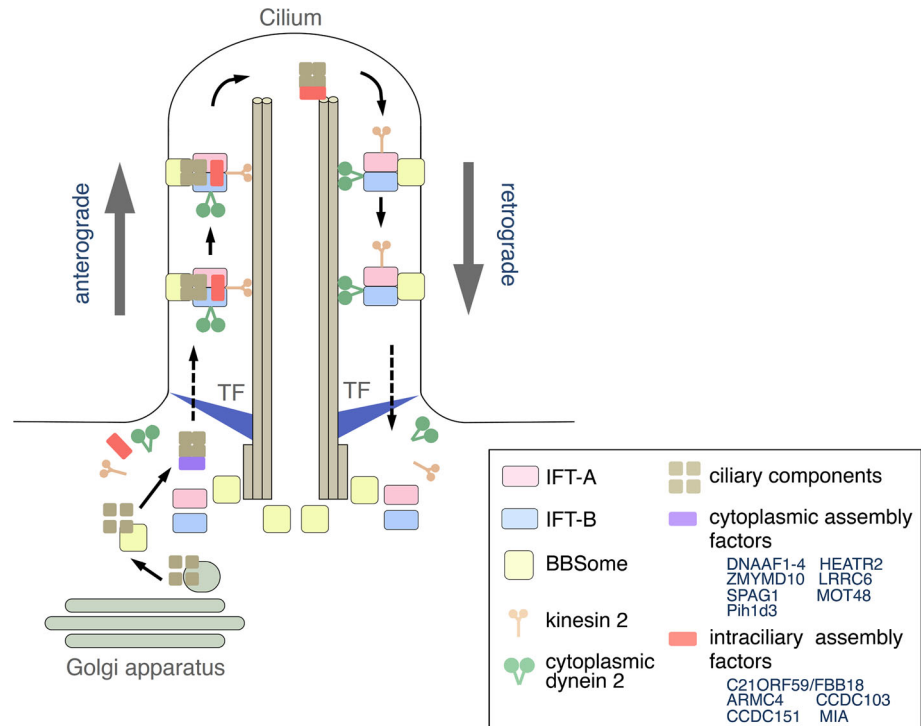


Fig. 3 Simplified diagram of the assembly and transport of axonemal components. Ciliary proteins are first transported to the base of cilium by G-protein- and BBSome-dependent pathway. Substructural components, such as axonemal dyneins, are preassembled in the cytoplasm with the aid of assembly factors (purple square). They are transported by IFT particles (IFT-A + IFT-B) after passing through the transition fiber (TF). IFT is composed of anterograde and retrograde transports, driven by kinesin 2 and cytoplasmic dynein 2, respectively. Other assembly factors (red square) are involved in the intraciliary assembly of axonemal dyneins



Distribution of motile cilia in the human body

The name “ciliated epithelial cells” was originally used for cells bearing motile cilia for generation of water flow. In humans, most such cells are multiciliated (more than one cilium per cell), as seen in nasal and trachea epithelia (airway cilia), the lachrymal sac or Moll’s gland, brain ventricles (ependymal cilia), male efferent ducts, and female oviducts (Fallopian tubes) (Fig. 4). The structures of the axonemes in motile multicilia have a 9 + 2 microtubule lattice with dynein arms, apparently the same as those in sperm flagella, although there are several reports showing minor differences in their components (e.g., [67]).

Like airway cilia, some other epithelial tissues in humans have been reported to bear cilia. The epithelium of the middle ear and its connecting Eustachian tube bear multicilia. They have motile 9 + 2 structures and function in mucociliary clearance. A deficiency in motility results in less elimination of bacteria, leading to otitis media [3, 68]. Inner hair cell possesses stereociliary bundles, which are composed of a single kinocilium (monocilium) and 50–200 stereocilia (microvilli) [69]. The kinocilium has a 9 + 0 (lacking CP) or 9 + 2 structure, and seems not to have dynein arms [70]. However, it was reported that porcine kinocilium showed a 9 + 2 structure with ODAs [71]. The axonemal structure of human kinocilium is controversial.

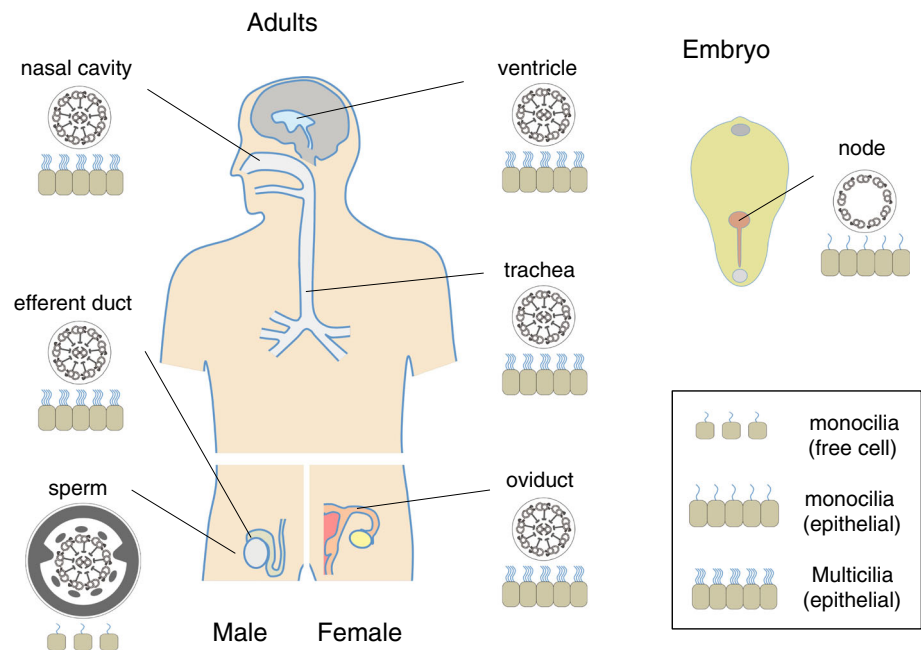
It was thought that the cilia of the follicular epithelium of the human thyroid function in the mixing of the follicular colloid [72]. However, monocilia with a 9 + 0

structure observed in the follicular epithelium of the human thyroid lack dynein arms and appear immotile, although a weak uncoordinated movement was observed [73]. In a follicular carcinoma of the thyroid, a 9 + 2 structure of axonemes was observed, but dynein arms were still absent [73].

Efferent ducts are connected to the epididymis, where a series of events for sperm maturation occur. The epithelial cells of the epididymis bear immotile stereocilia [74]. Transport of sperm through the epididymis is considered to be driven both by hydrostatic pressure and by contraction of smooth muscle. The epithelia of vas deferens also bear immotile stereocilia (long microvilli), but no motile cilia [75, 76]. Apparent motile cilia with a 9 + 2 structure and dynein arms have been observed on the endometrium of the uterine lumen, but often with atypical axonemal structures. The population of ciliated cells varies due to turnover during the menstrual cycle [77, 78]. Movement of cilia is not the only driving force for sperm transport along the endometrium [79]. Other ciliated epithelia may be found in adults, children, growing embryos, and fetuses, but are not completely understood.

Most cells in human tissue possess nonmotile monocilia, called primary cilia, which serve as signaling centers for perception of extracellular signals for regulating cell proliferation and differentiation. Primary cilia possess 9 + 0 axonemes without dynein arms, and function as the antennae of cells, receiving several extracellular signals and transducing them into cells through, for example,

Fig. 4 Distribution of motile cilia in the human body. Tissues or organs with motile cilia are shown with their types (monocilia or multicilia) and internal structures



pathways such as the Hedgehog and Wnt/planar cell polarity (PCP) pathways [80]. For example, loss of ciliary formation results in a disorder of cellular orientation, causing polycystic kidney disease due to nonpolarized cellular alignment. In addition, cilia on sensory organs play critical roles in perception of several stimuli, such as light, mechano- and chemostimuli [80].

Embryos and larvae in many aquatic invertebrates are ciliated for free-swimming. In mammals, embryos develop without motile cilia and implant into the uterine wall. The only motile cilia seen in their early development are those on the node (Fig. 4, right). Nodal cilia have a $9 + 0$ structure with dynein arms [81, 82]. Close observation of the axonemes strongly suggests that nodal cilia possess only outer arm dynein [41]. Nodal cilia are seen at the posterior side of the nodal cells, according to the planar cell polarity. They are tilted posteriorly and show rotational movement. The flow caused by nodal cilia determines left–right asymmetry [83–85]. Two types of cells are found in the node: pit cells and crown cells. The majority of crown cells bear immotile monocilia, which are proposed to have a sensory function. The presence of two types of cilia serves as a basis for the “two-cilia model” for left–right asymmetry [86, 87]. In this model, a leftward flow is caused by cilia on the pit cells and is sensed by immotile cilia on the crown cells. Recent studies have shown that primary cilia are generated in early mouse embryos on nearly all cells in epiblasts (E5.5 and E6.0 embryos), definitive endoderm and node (E8.0), and limb mesenchymes and neural progenitors (E10.5). Trophoblast and extraembryonic endoderm stem cells have no cilia [88].

Cilia become differentiated in developing adult organs, and some of them are considered to play important roles not only in organ function but also in organ differentiation, such as heart development in mouse [89].

Ciliopathy due to deficiencies in transport and assembly of axonemal components

The formation of cilia and flagella initiates from the expression of master genes for ciliary and flagellar formation, such as FoxJ1 and the RFX family of transcription factors (for review, see [90]). Expression of these master genes is followed by maturing of the centriole into the BB, formation of the TZ, anchoring of the basal body to the plasma membrane, and transport of ciliary or flagellar proteins by the BBSome and IFT [91].

Ciliopathy is caused by recessive mutations in the genes necessary for formation of cilia and flagella. These encode (1) proteins for the gene expression and transport of ciliary and flagellar components, such as those of the BBSome, TZ, and IFT particles; (2) factors for cytoplasmic assembly of axonemal components; and (3) the elements of axonemal structures, such as the subunits of ODAa and IDAs, RSs, and the CP. Defects in these components result in ciliary dysfunction in a wide range of organs, represented by symptoms such as PCD, Kartagener syndrome, BBS, MKS, Meckel–Gruber syndrome, hydrocephalus, PKD, polycystic liver disease, nephronophthisis, Joubert syndrome, Alström syndrome, Jeune asphyxiating thoracic dystrophy, and laterality defects [92]. These diseases are

often demonstrated experimentally using model organisms, such as knockout mice and knockdown embryos of zebrafish.

Two complexes for transport of ciliary proteins

Most of the mutations in the genes for ciliogenesis, i.e., those of the BBSome, TZ, and IFT particles, result in deficiency in ciliary and flagellar formation, causing ciliopathy. The BBSome and IFT are the transport systems of ciliary proteins at the base of the cilium and inside the cilium, respectively. Most mouse mutations in BBS genes are lethal, and the rest of the mutants show obesity, retinal degeneration, cystic kidneys or polydactyly [92–94]. Mutation in BBS2 or BBS4 causes male infertility due to the failure of sperm flagellar formation [95].

Molecular compositions of IFT particles have been studied extensively in *Chlamydomonas* [61]. Deficiency in IFT results in the failure of ciliary formation. The phenotypes of mouse mutants lacking IFT components are similar to those lacking the BBSome components. Mutation of an IFT-B component, IFT172, results in the formation of cystic kidney in zebrafish [96]. Embryos in an IFT-172-knockout mouse show defects in cilia formation, resulting in the failure of brain development [97]. Mice with deletions of the gene encoding another IFT-B component, IFT88/Polaris, develop PKD, hepatic fibrosis, defects in photoreceptor assembly and maintenance, and situs inversus [98, 99]. Mutants lacking some IFT-A components have flagella with bulged membrane at the tips in *Chlamydomonas* [100, 101]. Mutations of IFT-A components in mouse show a phenotype similar to bulged cilia [102]. They connect to abnormalities seen in human diseases, such as short-rib polydactyly syndromes for WDR35 mutation, and Mainzer–Saldino syndrome (MSS) for IFT140 mutation [103, 104].

The transition zone and associated structures

Many proteins related to human ciliopathy are localized to the TZ, a barrier compartment where ciliary proteins are relayed from intracellular to intraciliary transports. Septins are polymerizing GTP-binding proteins with cytoskeletal properties. They play roles in binding and organizing the membrane-cytoskeleton network [105, 106]. The localization of septin 2 at the ciliary base indicates that the septin ring plays a role in the ciliary diffusion barrier to separate the ciliary and cytoplasmic compartments [107]. In mammalian spermatozoa, septin 4 and 12 are reported to be essential for spermatogenesis. In particular, septin 4 is a structural component of the sperm annulus, and septin 4-null mice show abnormal morphology around the sperm neck region [108, 109]. Male patients with mutations in

septin 12 genes show oligoasthenozoospermia or asthenoteratozoospermia. The sperm have a defective annulus with a bent tail [110].

CEP290, also known as NPHP6, is localized to the Y-link in the transition zone and is involved in the assembly of these structures in *Chlamydomonas* [111]. Its mutation causes MKS in humans [112]. There are two other structures in the TZ, namely the basal foot (subdistal appendage) and TF (distal appendage). These are reported to contain ninein/ODF2 and ODF2/CEP164/CEP123, respectively [113–116]. One of the basal foot proteins, ODF2, is necessary for both basal foot formation and orientation of the ciliary beat [117]. ODF2-knockout mice show embryonic lethality [118], in agreement with the observation that ODF2 is a basic component of the mother centriole. ODF2 was originally found as a component of ODF of sperm tail [119]. Analysis of heterozygote mice indicates that ODF2 is essential for ODF formation and its defects result in production of spermatozoa with a bent tail in which one or more doublet microtubules are missing [120]. Several other proteins, such as those in the Tectonic module (TCTN1, TCTN2, and TCTN3) and MKS proteins, are localized to the transition zone and seem to help in ciliary formation and maintenance [91].

Other structures associated with basal bodies are not well understood. Major components of these structures so far identified include SF-assemblin, a principal component of striated microtubule-associated fiber at the basal apparatus of *Chlamydomonas* [121], and rootletin, a coiled-coil protein component of ciliary rootlet [122]. Knockout mice lacking rootletin or its associated small GTPase, RAB28, show retinal disorders, indicating that the ciliary rootlet is especially involved in the ciliary transport in photoreceptors [123, 124].

Cytoplasmic assembly of axonemal components

Substructures in the axoneme, such as ODA/IDA and RSs, are composed of multiple subunits. They need factors for proper assembly and anchoring. Many axonemal proteins are transported to cilia or flagella after preassembly in the cytoplasm. Multiple factors for cytoplasmic assembly of axonemal components have been identified, especially those for OAD and IAD. Ktu (from the name of a gene *kintoun*)/PF13 was the first factor identified for the assembly of dynein arms and is now called DNAAF2 [125]. Both ODA and IDA are partially or completely absent in patients lacking DNAAF2. Respiratory cilia and sperm flagella are immotile in such patients. Some of the patients also show situs inversus. Ktu mutation causes the absence of DNAH5 and DNAI2, especially in the distal region of cilia. DNALI1 (*Chlamydomonas* p28) is also missing, resulting in the partial loss of IDA.

Mutations in DNAAF1/LRRC50/ODA7 cause deficiency in ciliary motility similar to those for DNAAF2 [126, 127]. Axonemes in patients with DNAAF1 mutations show significant loss of both ODAs and IDAs, and the extent is more severe than for DNAAF2 because the mutation in LRRC50/DNAAF1 results in the loss of both DNAH5 and DNAH9 and the IC, DNAI2, affecting the assembly of both proximal (type 1) and distal (type 2) ODAs. Loss of DNAAF3/PF22 results in ciliary defects similar to those for both DNAAF1 and DNAAF2 [128]. All of these assembly factors function together in the assembly of ODAs, but are considered to have distinct roles in the process. In conjunction with studies in *Chlamydomonas*, DNAAF1/ODA7, DNAAF2/PF13, and DNAAF3/PF22 have been shown to function as a chaperone complex for proper folding of the dynein-HCs [128]. DYX1C1 (also named DNAAF4) is also an assembly factor for dynein. DYX1C1/DNAAF4 interacts with DNAAF2 but not with DNAAF1 or DNAAF3 [129].

Factors for cytoplasmic dynein assembly other than DNAAF1–4 have been reported. HEATR2 (recently called DNAAF5) interacts with DNAI2, but not with other DNAAFs or HSPs. It seems to play a role different from those of DNAAF1–4 at an early stage of dynein pre-assembly in the cytoplasm [130]. LRRC6 has structural and functional similarities to DNAAF1; its knockout mouse lacks both OAD and IAD [131]. LRRC6 interacts with another assembly factor, ZMYND10 [132, 133]. SPAG1 is also a cytoplasmic assembly factor for both ODA and IDA in respiratory cilia, similar to the function of DNAAF1, DNAAF3, LRRC6, and ZMYND10. SPAG1 mutant cilia lack the IDA component DNALI1 in the axoneme, suggesting further involvement of the assembly factors with the IAD [134]. In *Chlamydomonas*, two assembly factors for IADs are known. One is *Chlamydomonas* MOT48 (IDA10), which, like DNAAF2, contains a PIH domain, and is involved in the assembly of IAD species b, c, and d [135]. The other is the MIA complex [MIA1 (FAP100) plus MIA2 (FAP73)], which is located at the distal part of the IAD f/I1 and is involved in the assembly of this dynein [136].

Ciliopathy due to mutations in axonemal components

Mutations in axonemal components often result in the loss of substructure of the axoneme, leading to the dysfunction of motility in most cases. The spectrum of ciliopathy depends on how important the substructure is to the ciliary function of a tissue. For example, many patients or mutants lacking proteins of the RS/CP, such as HYDIN [137] and RSPH4 [138], suffer from defects in the motility of

epithelial cilia but not from laterality defects because nodal cilia have 9 + 0 structures with no RS/CP (Fig. 4).

ODA and IDA

Approximately 70–80 % of PCD cases are caused by defects of ODA [139, 140]. OAD is involved in the elevation of ciliary beat frequency [19], force generation for penetration of sperm through the zona pellucida [141], metachronal synchrony of motile cilia [142], and propagation of asymmetric waveforms of sperm during chemotaxis [36]. The mutations in OAD reported so far include those in HCs (DNAH5, DNAH11), ICs (DNAI1, DNAI2, NME8), a LC (DNAL1), and a component of the DC (coiled-coil domain containing protein 114, CCDC114; DC2 in *Chlamydomonas*) (Table 1). Compared with OAD, there are few mutants showing a lack of IAD components; these include DNAH7 (only in knockout mouse [143]) and DNALI1 [144]. Mouse mutants lacking Dnah7 show deficiency in ciliary and flagellar motility, but no apparent difference is found in the ultrastructure of OAD/IAD [143]. One interesting report is that mice lacking the gene for DNA polymerase lambda (Dpcd/poll) show situs inversus totalis and defects in motility of ependymal and respiratory cilia. The knockout male mice are infertile due to sperm immotility, but the female mice are fertile. Ultrastructures of cilia from $\lambda^{-/-}$ mice show a lack of IADs but not of OADs [145]. It is not known how mutation of $\lambda^{-/-}$ leads to the loss of IADs. The human ortholog (DPCD) was tested in 51 PCD patients (15 with IDA defects), and no mutation in the *dpcd* locus was discovered [146].

Heterogeneity in the HCs of OAD

Several studies indicate that animal and *Chlamydomonas* flagella contain two and three HCs, respectively, in the OAD [11, 37]. From their molecular substructures, these are referred to as two-headed or three-headed dyneins. Unlike the heterogeneous IAD HCs, OAD had been thought to be present as a single form in an organism, lined along the doublet microtubule at intervals of 24 nm. However, recent studies suggest heterogeneities of OADs. Respiratory cilia in humans contain two types of OAD: type 1 OAD contains only DNAH5 (ortholog of *Chlamydomonas* ODA γ) and is present at the proximal region of cilia; type 2 OAD contains both DNAH5 and DNAH9 (ortholog of *Chlamydomonas* ODA β) at the distal part of the cilia [147]. In sperm, however, DNAH9 is present along the entire length of the flagellum, whereas DNAH5 is only present in the proximal part of the flagellum. The motility defects in DNAH5-mutated patients also differ between respiratory cilia and sperm flagella [147]. It is not clear whether type 1 OAD is present as a single-headed dynein or a two-headed dynein with another unknown HC.

Table 1 List of genes for human ciliopathy affecting specific axonemal structures and sperm motility

Human gene	Protein	Localization	<i>Chlamydomonas</i> protein	Defect	References
<i>DNAH5</i>	Dynein heavy chain	ODA	γ HC	ODA	[200]
<i>DNAH11</i>	Dynein heavy chain (lrd dynein)	Putative ODA	Similar to β HC	n.d.	[149, 201]
<i>DNAH7</i>	Dynein heavy chain	Putative IDA	DHC5 (β HC)	IDA, ODF ^a	[143, 202]
<i>DNAI1</i>	Dynein intermediate chain	ODA	IC1/IC78	ODA	[203]
<i>DNAI2</i>	Dynein intermediate chain	ODA	IC2/IC69	ODA	[204]
<i>DNAL1</i>	Dynein light chain	ODA	LC1	ODA ^a	[205]
<i>TXNDC3/NME8</i>	Dynein intermediate chain	ODA	n.d.	ODA	[206]
<i>TCTE3</i>	Dynein light chain	ODA	Tctex2	ODA	[207]
<i>DNALI1</i>	Dynein light chain	IDA	p28	IDA	[144]
<i>CCDC114</i>	Coiled-coil protein	ODA	DCC2	ODA	[153]
<i>ARMC4</i>	Armado repeat containing protein 4	ODA	n.d.	ODA	[160]
<i>CCDC151</i>	Coiled-coil domain containing 151	ODA	ODA10	ODA	[208]
<i>CCDC103</i>	Coiled-coil protein	ODA	CCDC103	ODA	[161, 163]
<i>RSPH1</i>	Radial spoke head protein	RS	RSP1	RS, CP	[131]
<i>RSPH9</i>	Radial spoke protein	RS	RSP9	RS, CP	[138]
<i>RSPH4A</i>	Radial spoke protein	RS	RSP4	RS, CP	[138]
<i>HYDIN</i>	Hydin (gene causing hydrocephalus)	CP (C2b)	Hydin	CP	[137, 209]
<i>Spag6</i>	Sperm antigen 6	CP	PF16	CP ^a	[210]
<i>Spag16L</i>	Sperm antigen 16	CP	PF20	CP ^a	[175]
<i>SPAG17</i>	Sperm antigen 17	CP (C1)	PF6	CP	[211]
<i>Ak7</i>	Adenylate kinase 7	CP	n.d.	CP, DMT	[212]
<i>HSF-1</i>	Heat shock transcription factor 1	CP	n.d.	CP, DMT	[213]
<i>Pacrg</i>	Parkin co-regulated gene product	DMT	PACRG	DMT	[172]
<i>TECT2</i>	Tektin 2	DMT	p58 ^b	IDA, DMT	[214]
<i>HEATR2/ DNAAF5</i>	HEAT-repeat containing 2	Cytoplasm	Cre03.g162400.t1.1 ^c	ODA	[130]
<i>LRRC6</i>	Leucine-rich repeat containing 6	Cytoplasm	Cre17.g739850.t1.2 ^c	ODA, IDA	[131, 215]
<i>DNAAF3</i>	Dynein axonemal assembly factor 3	Cytoplasm	PF22	ODA, IDA	[128]
<i>ZMYND10</i>	Zinc finger, MYND-type containing 10	Cytoplasm	Cre08.g384628.t1.1 ^c	ODA, IDA	[216]
<i>DNAAF4</i>	Dynein axonemal assembly factor 4 (DYX1C1)	Cytoplasm	Cre11.g467560.t1.1 ^c	ODA, IDA	[129]
<i>SPAG1</i>	Sperm antigen 1	Cytoplasm	Cre16.g682251.t1.1 ^c	ODA, IDA	[134]
<i>C21ORF59</i>	Chromosome 21, ORF59	Flagellar matrix	FBB18	ODA, IDA	[163]
<i>DNAAF1</i>	Dynein axonemal assembly factor 1 (LRRC50)	Cytoplasm	ODA7	ODA, IDA	[126, 127]
<i>DNAAF2</i>	Dynein axonemal assembly factor 2 (KTU)	Cytoplasm	PF13	ODA, IDA	[125]
<i>Pih1d3</i>	PIH1 domain-containing protein	Cytoplasm	n.d.	ODA and IDA	[187]
<i>CCDC39</i>	Coiled-coil domain containing 39	AX	FAP59	IDA, DMT	[167]
<i>CCDC40</i>	Coiled-coil domain containing 40	Cilia/cytoplasm	Cre05.g238270.t1.1 ^c	CP, IDA	[168]
<i>CCDC164</i>	Coiled-coil domain containing 164	N-DRC	DRC1	N-DRC	[169]
<i>CCDC65</i>	Coiled-coil domain containing 65	N-DRC	FAP250	N-DRC	[163]

ODA outer dynein arm, IDA inner dynein arm, RS radial spoke, CP central apparatus, DMT doublet microtubule, N-DRC nexin-dynein regulatory complex, AX axoneme, ODF outer dense fiber, n.d. not defined

^a Observed in mouse

^b p58 is a homolog of tektin B1 (tektin 2) [217]

^c ID of the genes showing highest similarity in the *Chlamydomonas reinhardtii* genome (database v5.5)

Another interesting HC is DNAH11 (lrd). This HC was originally discussed in mouse, as it was first identified as the left-right dynein (product of *lrd* gene), which is

mutated in inversus viscerum mouse. lrd shows high similarity to the β -type HC of OAD and is specifically expressed in monocilia of the embryonic node [148].

DNAH11-null male mice are fertile, and their tracheal cilia and sperm flagella show no apparent deficiency in ultrastructure and motility. Unlike in mice, human patients with mutations in the ortholog of DNAH11 show situs inversus and also immotile respiratory cilia [149, 150]. Knowledge of the precise composition of the OAD with respect to the HCs in nodal cilia would be critical to elucidate the mechanism of the unique phenotype of DNAH11 mutants.

Notes on the differences between sperm and *Chlamydomonas*

Chlamydomonas is an excellent model organism for studying the molecular composition and function of cilia and flagella. However, difference in the usage of axonemal proteins has been observed between *Chlamydomonas* and animal sperm [28, 37, 151]. The composition of the ODA-DC is likely different between sperm and *Chlamydomonas* flagella [40]. DC2 is present in multiple forms in sperm of the ascidian *Ciona intestinalis* and humans. Studies on the molecular composition of the OAD in *C. intestinalis* indicate the presence of multiple forms of DC2 as IC4 and IC5 and a protein similar to DC2, Axp66.0 [40, 152]. Orthologs of DC1 and DC3 are not found in the *Ciona* or human genomes [40]. Male fertility was not greatly affected among human individuals carrying CCDC114 (DC2) mutations [153]. Although it has not been elucidated why, the redundancy of DC2 might compensate for the mutation of CCDC114 in sperm flagella. Similarly, knowledge has been accumulating showing that some axonemal components are specific to metazoans and do not exist in *Chlamydomonas*. For example, a calcium sensor for the OAD, calaxin, is specific to metazoan species [35, 37]. The calcium sensor for OAD in *Chlamydomonas* is dynein LC4, which is present in most bikont species [37]. Other examples of metazoan-specific axonemal components include TXNDC3, an IC of the OAD, and RS proteins CMUB116, AK58, and NDK/DM44 [154].

Factors for intraciliary docking and regulation of OAD

Recent studies in *Chlamydomonas* suggest that ODA10 (CCDC151 in humans), ODA5 (homolog of a DC2-like protein CCDC63), and ODA8 (LRRC56 in humans) form an accessory complex of the ODA-DC [153, 155, 156]. The complex is associated with an adenylate kinase with sequence similarity to human cytosolic AK1 and AK5 [155]. ODA8 is localized to both the flagellar matrix and cytoplasm, similar to IFT components [157]. Thus, this accessory complex might be involved in IFT-dependent assembly of the OAD before docking onto the doublet microtubules. Oda16/Wdr69 (WD-repeat domain 65) is also an adaptor of

the OAD for IFT [158]; Oda16 directly interacts with a component of IFT-B, IFT46 [159], suggesting a role for IFT46 as a cargo-specific adaptor between OAD and IFT-B.

Other nonintegral components necessary for intraciliary docking of OADs have been reported. ARMC4 is a protein localized at the base of cilia and flagella. Patients with mutations in ARMC4 show reduced numbers of ODAs. ARMC4 interacts with DNAI2 and is considered to be involved in the specific targeting and anchoring of OAD [160]. CCDC103 binds tightly to microtubules and appears to have a role in attachment of OAD [161, 162]. C21orf59/FBB18 is a flagellar matrix protein and participates in dynein arm assembly. Loss of C21orf59/FBB18 in humans causes deficiency of both ODAs and IDAs [163].

Deficiency in other axonemal substructures

Because axonemes contain several nondynein structures for direct or indirect regulation of ciliary motors, mutations in the proteins of these structures may cause ciliopathy. RS and the CP are located in the center of 9 + 2 axonemes (Fig. 1b, c) and are involved in the activation or inactivation of dyneins on a specific doublet microtubule, resulting in the determination of the ciliary beating plane [14, 15, 164, 165]. Mutations in the genes for RS/CP leading to ciliopathy include RS proteins (RSPH1, RSPH4A, and RSPH9) and those in the CP (HYDIN and SPAG17) (Table 1). The DRC is located near RS2 and is connected to the nexin link (Fig. 1c) [46, 48, 166]. This “N-DRC” (nexin-DRC, Fig. 1c) is related to ciliopathy by defects in the regulation of ciliary motility. From studies in *Chlamydomonas*, this N-DRC structure is thought potentially important in the regulation of IADs. Recent genetic and ultrastructural studies have identified the components of the N-DRC, its associated proteins such as CCDC39, CCDC40, CCDC65, and DRC1 (CCDC164), and their connections with ciliopathy [163, 167–169].

In some cases, genetic diseases apparently unrelated to the function of axonemal dyneins cause abnormality in ciliary structure and motility. Mutations of RPGR cause XLRP. Several defects in axonemal structure and motility are observed in XLRP patients, including missing dynein arms and disorganization of ciliary orientation [132]. OFD1 is a coiled-coil protein with a LisH domain, located at the base of the cilium [170]. Respiratory cilia with mutated OFD1 are motile with normal beat frequency, but show uncoordinated beating.

Mutations that affect sperm flagella but not cilia

Because the structure of axonemes and the mechanism of their formation are quite similar between somatic cilia and sperm flagella, many deficiencies lead to ciliopathy

including male infertility. However, there are some cases of patients or animal mutants showing different phenotypes between somatic cilia and sperm flagella. This seems to be due to differences in the axonemal components [67], the presence of unique accessory structures in sperm, and degeneration of the basal body and its appendages during spermatogenesis.

Manchette-dependent flagellar formation

Parkin and *PACRG* are genes located on mouse chromosome 17, and their deletions cause “quaking” mice, and male infertility [171, 172]. Studies in *Chlamydomonas* and *Trypanosoma* revealed that *PACRG* binds to EFHC1/RIB72 in the A-tubule of a doublet microtubule [54, 173, 174]. In mice, *PACRG* is associated with MEIG1, which was identified as a binding partner of a CP protein SPAG16 (PF20) [175]. MEIG1-knockout mice show male infertility, but no other phenotypes for ciliopathy are observed [176–178]. Further studies suggest that MEIG1/*PACRG* plays a critical role in the control of the structure called “manchette” [179], a microtubule-based structure transiently formed during spermiogenesis. This structure is thought to play roles in the significant morphological changes of the nucleus and in flagellar formation. Manchette-dependent protein transport, called IMT, is proposed to be essential in these changes [180].

Another example showing a possible role of manchette in the formation of sperm flagella is *Iqcg* [181]. *Iqcg* is highly expressed in ciliary tissues including trachea, oviduct, and testis, and its knockout male produces immotile sperm with highly disorganized axonemal structures [182]. However, the ciliary structures in the trachea and oviduct appear normal in the knockout mice. This raises the possibility that the deficiency in sperm flagella is caused by a spermatogenesis-specific process for flagellar formation, such as IMT.

CP proteins *Pcdp1* and *Spef2*

Testis- or sperm-specific phenotypes are also seen in the knockout mice of another two CP proteins. One is *Pcdp1*, whose knockout results in hydrocephalus, male infertility, and slight defects in ciliary movement in trachea [183]. Studies in *Chlamydomonas* demonstrate that the *Pcdp1* homolog, FAP221, is a CP protein that binds to calmodulin and is localized to the C1d projection [184]. Interestingly, sperm from *Pcdp1*-null mice lack flagella, although respiratory epithelial cilia are apparently normal [183]. This suggests that *Pcdp1* has a function specific to the formation of sperm flagella.

A similar phenotype with regard to ciliary structure is observed in the knockout mice of *Spef2* [185]. Although

the respiratory cilia in the knockout mice are normal in ultrastructure but have lower beat frequency, their sperm show shortened flagella with a disorganized axoneme and accessory structures. *Spef2* is a homolog of *Chlamydomonas* CP protein Cpc1, which has an EF-hand domain and a conserved adenylate kinase domain, and is suggested to function in ATP regeneration [186]. The fact that *Spef2* interacts with an IFT-B component, IFT20 in mouse testis [185], further suggests a specific function of CP proteins, like that of *Pcdp1*, in the formation of sperm flagella.

A candidate for a sperm-specific assembly factor: *Pih1d3*

Two proteins for assembly of axonemal dynein, DNAAF2 and MOT48, are known to possess PIH domains. Another PIH protein, TWISTER, has a function in motile cilia in zebrafish [96]. A *Twister*-like gene, *Pih1d3*, is specifically expressed in mouse testis. Knockout of this gene causes a defect in flagellar motility, although cilia of the respiratory tract and ependymal cells are normal in their motility and ultrastructure [187]. Most of the *Pih1d3*-null mice lack both OADs and IADs in sperm flagella, and some show disorganized axonemes lacking a few doublet microtubules. *Pih1d3* interacts with DNAI2, Hsp70, and Hsp90, but not with any of the DNAAFs, suggesting the involvement of this protein in a distinct process of dynein assembly in spermatogenesis. It is possible that *Pih1d3* is involved in assembly coupled with a testis-specific transport pathway, such as IMT. Alternatively, *Pih1d3* may play a role in the assembly of axonemal dyneins mediated by a sperm-specific component of the OAD and IAD [67].

The mitochondria sheath

The development of helical configuration of mitochondria is well coordinated with the elongation of the sperm tail [6, 188–190]. MFNs are the mediators for mitochondrial fusion. MFN2 is located on the mitochondrial outer membrane and participates in the fusion, maintenance, and operation of the mitochondrial network [191]. Loss of function of Fzo (fuzzy onions), the *Drosophila* homolog of MFN1 and MFN2, results in male sterility [192]. Recently, MFN2 was reported to form a complex with MNS1 in murine spermatogenic cells [193]. MFN2 is localized in the midpiece of spermatozoa, whereas MNS1 is present throughout the sperm tail. Knockout of MNS1 results in situs inversus, hydrocephalus, and partial loss of OADs in tracheal cilia, resulting in a motility defect. Interestingly, MNS1-null sperm have abnormal and short flagella with disruption of outer doublet microtubules and the ODF [194]. These observations imply common mechanisms of axonemal formation between sperm and epithelia and an

additional mitochondria/ODF-dependent mechanism specific to axonemal formation in sperm flagella. It is hypothesized that a MNS1/MFN2 complex and ODF2 are involved in anchoring the mitochondria in the midpiece [193]. The involvement of mitochondria in the formation of axonemes is also suggested in mice lacking VDAC3, which show male infertility with structural loss of doublet microtubules from the sperm flagellar axonemes, but no structural abnormality in trachea cilia [195].

Conclusions

The connection between male infertility and ciliopathy was first disclosed by the observation of a common ultrastructural abnormality in sperm flagella and epithelial cilia in patients with Kartagener syndrome [1, 2, 196]. However, it also became evident that mutations in the proteins, such as Spef2 and Pih1d3, result in sperm dysfunction but not in ciliary defects of epithelia, suggesting a sperm-specific mechanism leading to immotile flagella. Considering fertility, males are more affected by deficiencies of axonemal components than females, as seen in cases where females with Kartagener syndrome with dyneinless cilia were fertile [197, 198]. This further indicates a unique functional aspect of sperm flagella. In addition, some patients with typical clinical symptoms of Kartagener syndrome show normal sperm motility with normal axonemal ultrastructure [5, 199], suggesting some axonemal proteins whose deletion cannot be detected by electron microscopy but critically affects the flagellar motility.

Knockout mice are powerful models for studying ciliopathy. However, in most cases, loss of axonemal dyneins results in embryonic lethality. This is not compatible with the symptoms of human ciliopathy, in which patients are alive and show fertility, making it possible to carry out family studies. Further experiments are necessary with mouse models, including analyses with extensive *N*-ethyl-*N*-nitrosourea mutants or dominant-negative mutants, and the engineering of human point mutations in mice [94]. Studies using *Chlamydomonas* and marine invertebrates also shed light on the molecular structure and mechanism of cilia and flagella, and on the genetic diagnosis of ciliopathy.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Human/animal studies This article does not contain any studies with human or animal subjects performed by any of the authors.

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