

REVIEW ARTICLE

Molecular basis of the morphogenesis of sperm head and tail in mice

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Abstract

Background: The spermatozoon has a complex molecular apparatus necessary for fertilization in its head and flagellum. Recently, numerous genes that are needed to construct the molecular apparatus of spermatozoa have been identified through the analysis of genetically modified mice.

Methods: Based on the literature information, the molecular basis of the morphogenesis of sperm heads and flagella in mice was summarized.

Main findings (Results): The molecular mechanisms of vesicular trafficking and intra-flagellar transport in acrosome and flagellum formation were listed. With the development of cryo-electron tomography and mass spectrometry techniques, the details of the axonemal structure are becoming clearer. The fine structure and the proteins needed to form the central apparatus, outer and inner dynein arms, nexin-dynein regulatory complex, and radial spokes were described. The important components of the formation of the mitochondrial sheath, fibrous sheath, outer dense fiber, and the annulus were also described. The similarities and differences between sperm flagella and *Chlamydomonas* flagella/somatic cell cilia were also discussed.

Conclusion: The molecular mechanism of formation of the sperm head and flagellum has been clarified using the mouse as a model. These studies will help to better understand the diversity of sperm morphology and the causes of male infertility.

KEYWORDS

acrosome, axoneme, flagellum, mouse, spermatogenesis

1 | INTRODUCTION

Spermatozoa are specialized cells that transmit paternal gene information to the next generation. To accomplish the purpose, they have special functional and morphological characteristics. Generally, the spermatozoon has a small head and long flagellum, a hydraulically suitable shape. However, spermatozoon is one of the most taxonomically diverse and rapidly evolving cell types.¹

For example, spermatozoa of brachyuran and *Caenorhabditis elegans* do not have flagella and move by ameboid movement. The house sparrow's spermatozoon has a screw-like-shaped head. Even in mammals, sperm head shape and length vary among species: human and several domestic animal spermatozoa have flattened and rounded (paddle-like) heads, while mouse and rat spermatozoa have falciform heads. Rodents' spermatozoa are relatively longer than primates' spermatozoa in total length. However, the range is

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extensive, 55–105 μm in primates and 35–258 μm in rodents.² The diversity of sperm shape is larger than that of oocyte shape. Why and how this diversity of sperm head and tail arose during evolution is a fascinating question.

In addition to its interest as a basic biological research subject, the study of sperm differentiation is becoming increasingly crucial for elucidating the cause of male infertility. Over 10% of couples suffer from infertility, and about half is attributed to men.³ The primary cause of male infertility is oligoasthenoteratozoospermia (OAT), but the genes responsible for inducing OAT have not been fully elucidated. In mammals, spermatogenesis proceeds in the seminiferous tubule in the testis. Spermatogonia that reside on the basement membrane proliferate through mitosis, and a part of spermatogonia enter the meiosis and become spermatocytes. After two sequential meiosis divisions, spermatocytes become haploid round spermatids. The final differentiation process that round spermatids become spermatozoa is called spermiogenesis. During this period, dynamic morphological alterations are induced, including acrosome

formation, flagellar formation, nuclear shape elongation, and shedding out of the cytoplasm. Spermiogenesis is divided into 16 steps in mice (19 steps in humans) based on the acrosome and nucleus morphological differences. Schematic representation of spermiogenesis is indicated in Figure 1.

During spermatogenesis, numerous genes are expressed in a spatial- and temporal-specific manner.^{4,5} Although these genes are thought to be coordinately involved in sperm differentiation, individual gene functions remain elucidated. Because of the lack of an in vitro experimental system that reproduces mammalian spermiogenesis efficiently, identification of the physiological role of the genes in spermiogenesis has been advanced by studies using knockout mice. Recently, genome editing technology using CRISPR/Cas9 has been developed, and the gene identification necessary for sperm differentiation has been accelerated because of its simplicity and economic advantages. In this review, recently identified critical genes for the mammalian sperm head and flagellum formation as well as their molecular function were summarized.

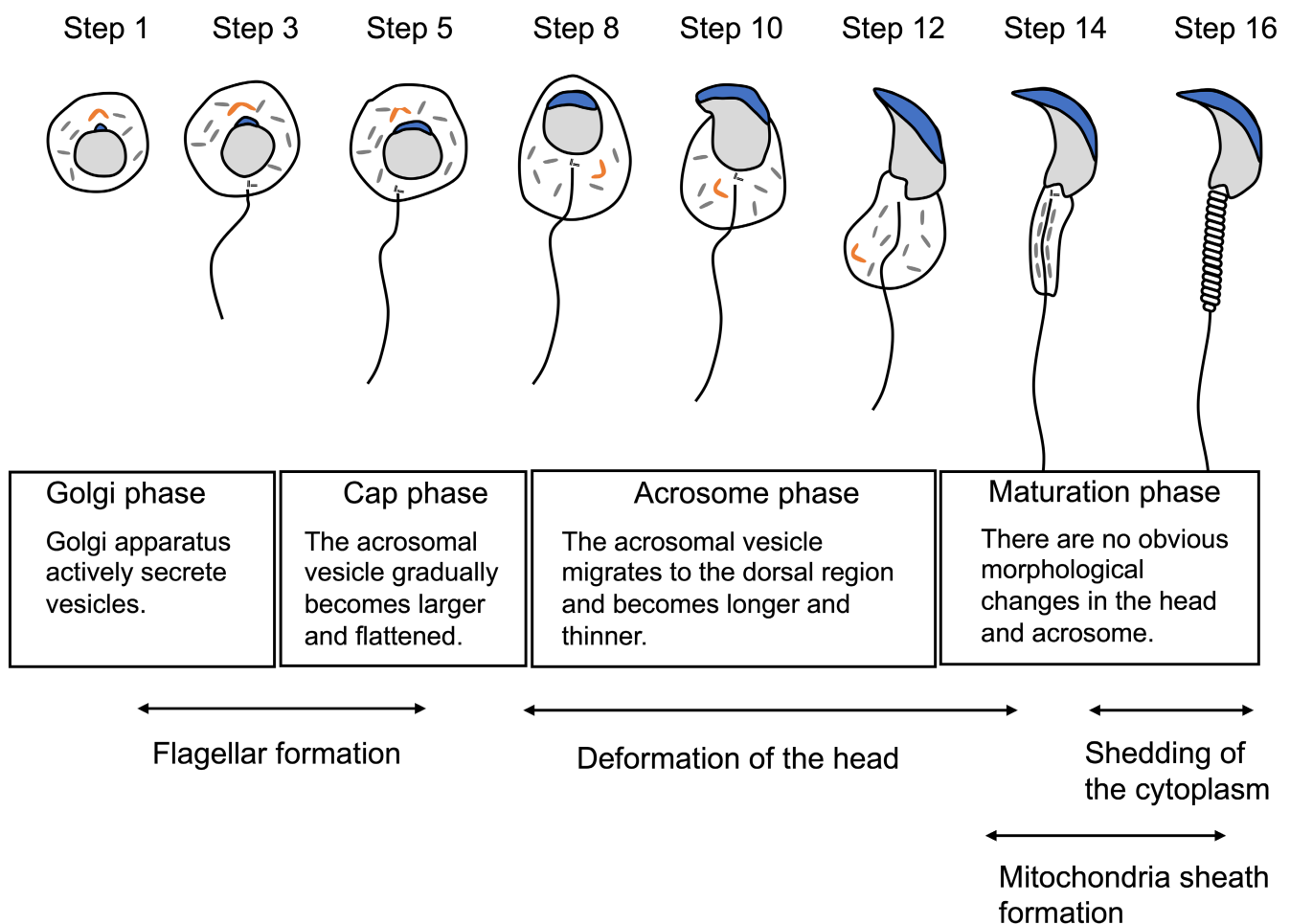


FIGURE 1 Post-meiotic differentiation of mouse spermatozoa. The morphological alteration during post-meiotic differentiation is schematically represented. Acrosome formation is categorized into four phases: Golgi phase (steps 1–3), cap phase (steps 4–7), acrosome phase (steps 8–12), and maturation phase (steps 13–16). The duration of flagellar formation, head deformation, shedding of the cytoplasm, and mitochondrial sheath formation is also indicated

2 | FACTORS AFFECTING THE HEAD FORMATION

A factor determining the sperm head shape is an acrosome formation. When the genes vital for the acrosome formation are mutated, a common phenotype like globozoospermia (a huge and abnormal nuclear shape) is observed. The other essential factor is the manchette. The manchette is a microtubule-based structure that transiently appears in the round and elongating spermatids. The manchette is involved in the head shaping through a sculpting-like function. Deficiency of the gene involved in the manchette formation causes abnormal head formation. In addition to these factors, nuclear condensation by exchanging the histone to protamine is also essential.⁶ In this section, first, the genes involved in acrosome formation were introduced. Since the manchette has a close relationship with the flagellar formation, its function and role in spermiogenesis are described in the next section.

2.1 | Acrosome formation in mice

The acrosome is a lysosome-related organelle formed by fusing vesicles from the Golgi apparatus. The acrosomal formation is divided into four phases in mice: Golgi phase (steps 1–3), cap phase (steps 4–7), acrosome phase (steps 8–12), and maturation phase (steps 13–16) (Figure 1). The Golgi apparatus actively secretes vesicles containing periodic acid-Schiff (pas)-positive proacrosomic granules in the Golgi phase. These vesicles (called proacrosomal vesicles) move by intracellular trafficking toward the nucleus, fuse on the nuclear surface's concave region and form a single large vesicle called an acrosomal vesicle. Trafficking is mediated by clathrin-coated⁷ and coatamer protein I (COPI)-coated vesicles.⁸ The acrosomal vesicle gradually becomes larger and flattened in the cap phase and covers one-third of the nuclear surface at step 8. After step 9, the nucleus gradually elongates, and the head shape changes to falciform. Accompanying the head deformation, the acrosomal vesicle migrates to the dorsal region and becomes longer and thinner. Overall structural and morphological changes in the acrosome and head are completed in step 14.

2.2 | Proteins involved in the membrane budding, trafficking, and tethering/fusion in the acrosome formation

Intracellular vesicular trafficking plays a crucial role in the acrosome formation as described above. Vesicular trafficking involves three steps: membrane budding, trafficking, and tethering/fusion with the target vesicle. The following proteins play pivotal roles in these processes (Figure 2).

SMAP2 is an Arf-activating protein directly involved in membrane budding. When SMAP2 is deleted in mice, the formation of proacrosomal vesicles is disrupted and subsequent acrosome formation

is impaired.⁹ SMAP2 localizes in the trans-Golgi network (TGN) of spermatids and binds to clathrin and the clathrin assembly protein (CALM), which is a protein implicated in clathrin-coated vesicle formation. CALM was not recruited to the TGN in *Smmap2*-deficient mouse spermatids. Additionally, the accumulation of syntaxin2, a component of the SNARE complex, at the acrosome formation site was also impaired.

Protein interacting with C kinase 1 (PICK1) is also essential for acrosome formation.¹⁰ PICK1 is a membrane protein with a BAR domain known to be involved in the budding of vesicles by bending of lipid membrane or sensing membrane curvature.¹¹ PICK1 is localized at vesicles between the TGN and acrosomal vesicle. Additionally, inhibition of vesicular trafficking by brefeldin-A causes the retention of PICK1 at the TGN. Therefore, it is proposed that PICK1 is involved in the vesicular trafficking or membrane budding at TGN. GOPC (Golgi-associated PDZ and coiled-coil motif-containing protein) is a protein important for acrosome formation. GOPC localizes in TGN and proacrosomal vesicles in spermatids. GOPC deletion causes a lack of acrosome formation and globozoospermia.¹² Since PICK1 interacts with GOPC and the expression of GOPC induces PICK1 clustering in 293 cells,¹⁰ GOPC and PICK1 may cooperatively act in the budding or trafficking of vesicles.

AGFG1 (ArfGAP with FG repeats 1, also known as HIV Rev-binding protein HRB) is localized on the cytoplasmic surface of proacrosomic vesicles and plays a crucial role in docking and/or fusion of proacrosomic vesicles.¹³ In *Agfg1*-KO spermatids, proacrosomic vesicles are secreted from the Golgi apparatus but cannot fuse to form an acrosomal vesicle. Moreover, AGFG1 binds to VAMP7, a SNARE protein implicated in vesicle fusion.¹⁴ This result supports the functional AGFG1 importance in membrane fusion.

2.3 | Proteins involved in the anchoring of the acrosomal vesicle

During the development and flattening of the acrosomal vesicle in the cap phase, the acrosomal vesicle requires to be anchored to the nuclear surface. SPACA1 is a membrane protein necessary for the integrity of the structure between the acrosome and nucleus.¹⁵ SPACA1 first appears at step 2 spermatids and is localized in the peripheral region of the acrosomal vesicle at step 7 spermatids and in the equatorial segment in the mature spermatozoa. In *Spaca1*-deficient spermatids, the nuclear plate, a dense lining of the nuclear envelope facing the inner acrosome membrane, is missing, and stretching and thinning of the acrosomal vesicle over the nucleus surface is suppressed. Since acrosome normally develops until step 5 in *Spaca1*-deficient spermatids, SPACA1 is unimportant for vesicle trafficking itself but rather plays a crucial role in the anchoring and/or stabilizing of the acrosomal membrane through the building of a platform of attachment region.¹⁵ In humans, SPACA1 has been indicated to play an indispensable role in acrosomal formation.¹⁶ SPACA1 expression is decreased in patients with globozoospermia and the biallelic loss-of-function

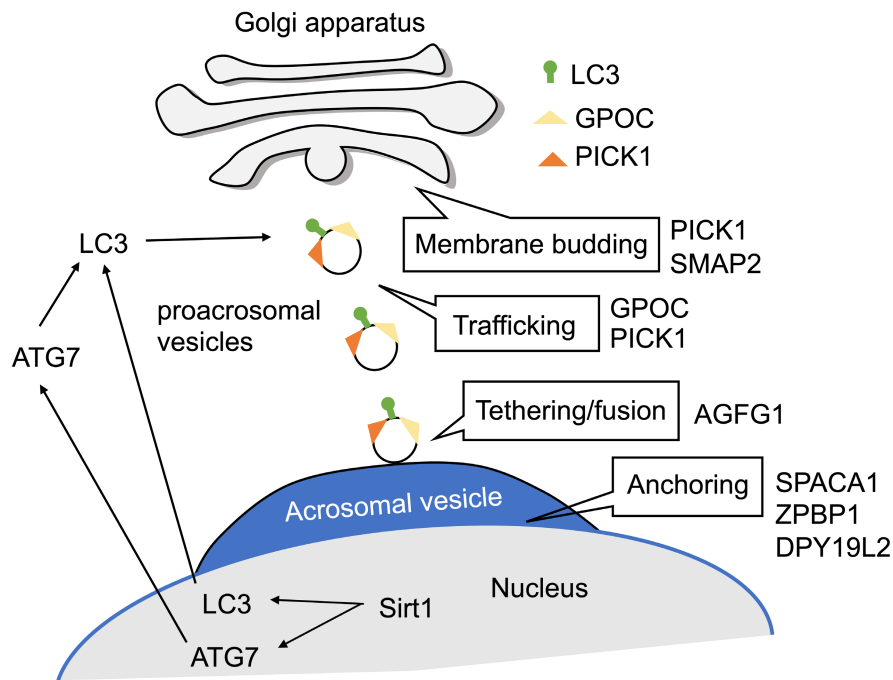


FIGURE 2 Proteins associated with acrosome formation. The acrosome is formed through the following processes: (1) membrane budding from the trans-Golgi network, (2) trafficking of proacrosomal vesicles, (3) tethering/fusion to an acrosomal vesicle, and (4) anchoring of an acrosomal vesicle to the nucleus. Proteins thought to be involved in each process during acrosome formation are shown

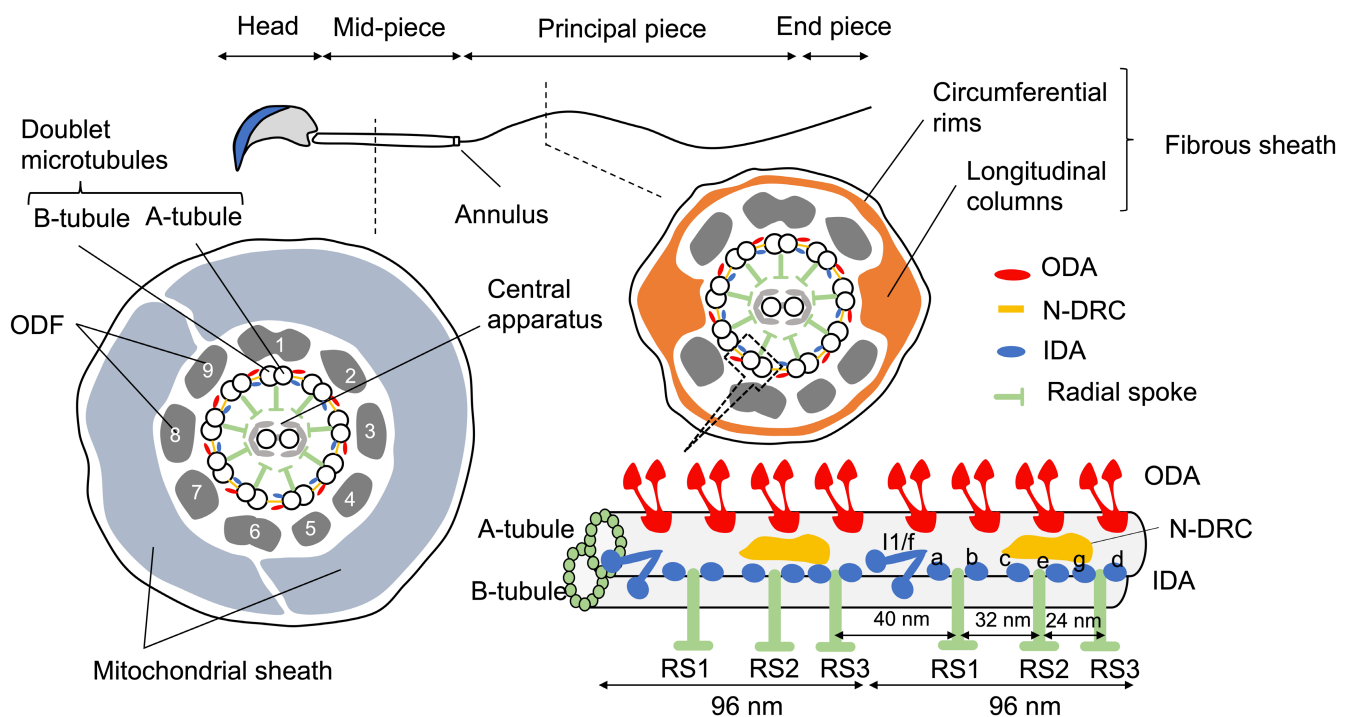


FIGURE 3 The schematic structure of sperm flagella and axoneme in mice. The sperm flagellum is divided into three parts: mid-piece, principal piece, and end piece. The axoneme contains nine outer doublet microtubules, two central pair microtubules, and other components such as the outer dynein arms (ODA), inner dynein arms (IDA), N-DRC, and radial spokes. These components align periodically with the long axis of doublet microtubules. The axoneme is surrounded by a mitochondrial sheath and outer dense fibers (ODF) in the mid-piece and by ODF and fibrous sheath in the principal piece. The annulus is an electron-dense ring-like structure between the mid-piece and the principal piece

mutation of *SPACA1* was identified as the pathogenic variant in a family with globozoospermia. Similar to *Spaca1*-deficient mouse spermatozoa, acrosome-acroplaxome complex formation was impaired.

Zbp1-KO mice indicate a similar phenotype with *Spaca1*-KO mice.¹⁷ ZBP1 (also known as Sp38 or lam38) is a protein localized in the inner acrosome membrane. Deletion of *Zbp1* in mice induces dilated acrosomal vesicle at step 7 round spermatids, and

subsequently, acrosome fragmentation and abnormal head shaping are induced. These results suggest that ZBPB1 is involved in the tethering of acrosomal membrane to the nucleus, similar to SPACA1. However, in contrast to the *Spaca1*-KO spermatids, acroplaxome formation is normally developed in *Zbp1*-KO spermatids, showing that ZBPB1 plays a different role in the tethering of the acrosomal vesicle.

DPY19L2 is also an essential protein for the anchoring of acrosome to the nucleus. *DPY19L2* was first identified as a causative gene of human globozoospermia,¹⁸ and thereafter, its importance is confirmed using KO mice.¹⁹ *Dpy19l2* is predominantly expressed in spermatids and localizes to the inner nuclear membrane facing the acrosomal vesicle. Deletion of *Dpy19l2* causes destabilization of the nuclear dense lamina and the structure linking nuclear membrane and acroplaxome. As a result, the acrosomal vesicle detaches from the nucleus and is eventually removed.

2.4 | Autophagy and acrosome formation

Recently accumulated evidence suggests that autophagy-related proteins are involved in acrosome formation. For example, ATG7 is involved in proacrosomal vesicle trafficking or fusion into the acrosomal vesicle in mice.²⁰ When *Atg5* is deleted in germ cell lineage, various morphological abnormalities, such as abnormal head shape and acrosome formation, are induced.²¹ Similarly, the deletion of *Tdrd7*,²² *Tbc1d20*,²³ and *Sirt1*²⁴ also induces abnormal acrosomal biogenesis and misshaped head formation. The regulation of acrosomal formation through these autophagy proteins is considered as follows. *Sirt1* deacetylates ubiquitin-like protein LC3 and E1-like enzyme ATG7. Deacetylated LC3 is transported from the nucleus to the cytosol and activated by ATG7. ATG5 seems to participate in the LC3 activation similar to ATG7. Then, activated LC3 is conjugated to proacrosomal vesicles. LC3 conjugation is necessary to recruit GOPC and PICK1 to proacrosomal vesicles because GOPC and PICK1 in proacrosomal vesicles are greatly decreased in *Atg7*-KO and *Sirt1*-KO spermatids. As described above, GOPC and PICK1 are vital for vesicular trafficking. Thus, the deletion of autophagy-related protein results in abnormal acrosome formation (Figure 2).

3 | THE STRUCTURE OF SPERM FLAGELLA

The schematic structure of mammalian sperm flagella is indicated in Figure 3. Flagella could be divided into three parts: mid-piece, principal piece, and end piece. The electron-dense ring-like structure between the mid-piece and the principal piece is called the annulus. The axoneme is the major flagellar component and comprises nine doublet and two central singlet microtubules (9 + 2 structure). The central pair of microtubules is surrounded by many protein complexes with a protruding structure, which is collectively called the central apparatus. Along the outer doublet microtubules, a number of substructures, including outer dynein arms (ODA), inner dynein

arms (IDA), radial spokes, and nexin-dynein regulatory complexes (N-DRC) are arranged periodically. In the mid-piece and principal piece, the axoneme is surrounded by outer dense fibers (ODF), and further surrounded by a fibrous sheath (FS) in the principal piece. Mitochondria found only in the mid-piece form a mitochondrial sheath (MS) surrounding ODF.

The structure of the flagellar axoneme is highly conserved among many species, from green alga *Chlamydomonas reinhardtii* to humans. Motile cilia present in tracheal epithelial cells, ependymal cells, and oviduct epithelial cells also contain this conserved 9 + 2 axonemal structure. An exception is nodal cilia that has no central apparatus (9 + 0 structure) similar to non-motile cilia (primary cilia). Flagella/motile cilia serve several functions by making the flow of fluids such as progressive motility (sperm), elimination of mucosa (trachea), a nodal flow that determines left-right asymmetry (nodal cilia), and cerebrospinal fluid circulation (ependymal cells). Therefore, the impairment of axonemal structure/function often causes complex symptom such as male infertility, chronic respiratory tract infections, *situs inversus*, and hydrocephalous, called primary ciliary dyskinesia (PCD). This section describes the mechanism of sperm flagellar formation and the genes necessary for constituting each axonemal structure.

3.1 | Flagellar formation and intracellular flagellar transport

Flagella first appear at steps 2–3 round spermatids in mice and rapidly elongate after that²⁵ (Figure 1). Like the primary cilia formation, centrioles act as a base for building the axoneme in spermatozoa.²⁶ The centriole is a barrel-shaped structure comprising nine triplet microtubules and exists as pairs arranged perpendicularly. Nine doublet microtubules are formed using a centriole (distal centriole) as a template. The centrioles are important for proper flagellar formation and male infertility. Refer to a recent review for more detailed centriole function in spermatozoa.²⁶

During the axoneme formation, motor proteins actively move on the microtubules to transport proteins/lipids necessary for axoneme formation to the tip or recover unnecessary materials to the cytosol. This intracellular trafficking is called intraflagellar transport (IFT) (Figure 4). The study of IFT has been actively conducted mainly in *Chlamydomonas* flagella and primary cilia. Two types of motor proteins are involved in IFT: one is kinesin-2, which is responsible for transport from the minus end to the plus end (anterograde transport), and the other is dynein-2, which is responsible for the transport in the opposite direction (retrograde transport).^{27,28} Kinesin and dynein bind to the IFT-B complex and IFT-A complex, respectively, and run on the B- and A-microtubules. IFT-A and IFT-B are large protein complexes comprising many subunits and play a pivotal role in recognizing and loading cargo on the motor proteins. Proteomic analysis has been revealed the proteins that constitute IFT complexes. It is currently believed that IFT-A and IFT-B consist of six subunits and 16 subunits, respectively.²⁹ In addition to IFT

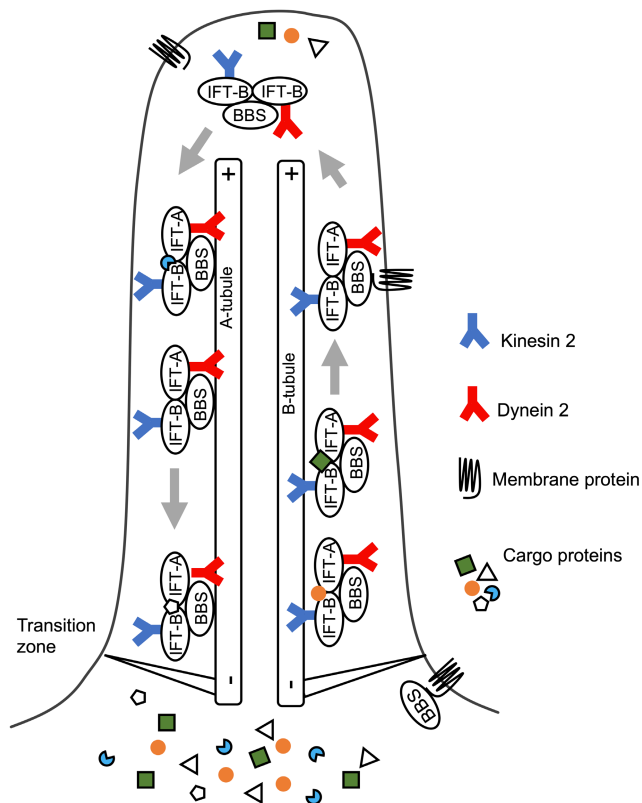


FIGURE 4 Intraflagellar transport. The intraflagellar transport (IFT) is a bidirectional transport system involving the motor proteins (kinesin and dynein), IFT complexes (IFT-A and IFT-B), and BBSome (BBS). Since there are no ribosomes in the flagella and cilia, IFT is important to deliver the proteins necessary for flagellar formation

complexes, Bardet–Biedl syndrome proteins (BBSome) are involved in the IFT. BBSome is a protein complex that comprises eight proteins encoded by the gene responsible for Bardet–Biedl syndrome. BBSome is thought to act as an adaptor between cargo proteins and IFT complexes.³⁰

Deletion of the genes involved in IFT causes male infertility. Table 1 is a list of IFT-related genes and the phenotype of the knockout mice. In numerous cases, whereas the sperm differentiation process of KO is normal until meiosis completion, KO mouse spermatozoa do not have a normal tail and show no motility. The 9 + 2 arrangement of the axoneme is usually disrupted or missing, and the other accessory structures such as ODF, FS, and MS are also disorganized. The number of cauda epididymal spermatozoa is greatly decreased and most of all, KO mice are sterile. Additionally, these KO spermatozoa have abnormal head formation with the aberrant organization of the manchette in common. The manchette is a transient microtubule/F-actin-containing skirt-like structure that surrounds the caudal region of the sperm head (Figure 5). This structure acts as a rail of intracellular trafficking called intramanchette transport (IMT) and affects the change of head shape.³¹ In elongating spermatids, the manchette moves caudally with a constriction of the perinuclear ring, and this zipper-like movement of the manchette

is believed to contribute to the sculpting of the sperm head. Many IFT-related proteins, including KIF3A (kinesin-2 motor protein) have been indicated to localize in the manchette and IFT-related gene KO mice spermatids exhibit abnormal manchette formation. Therefore, IFT machinery would participate in the normal head formation by supporting the manchette formation and function.

Other than IFT or BBS complex subunits, HOOK1, leucine-rich repeats, guanylate kinase-domain containing isoform 1 (LRGUK1), and Rab3-interacting molecule binding protein 3 (RIMBP3) are essential for normal manchette formation. HOOK1 is predominantly expressed in haploid spermatids and localized to the manchette but not to the axoneme.³² The manchette is abnormally formed, and thereby the sperm head is misshaped in HOOK1 mutation mice (*azh/azh*). Recently, HOOK1 has been indicated to act as a dynein activator *in vitro* and *in vivo*.^{33,34} Therefore, HOOK1 would be involved in the IMT by regulating the dynein motor activity. Deletion of *Lrguk1*³⁵ and *Rimbp3*³⁶ also causes abnormal manchette formation, head malformation, and male infertility in mice. LRGUK1 is predominantly expressed in the testis, and its expression increases during spermatogenesis. LRGUK1 interacts with HOOK family proteins (HOOK1–3), RIMBP3, and kinesin light chain 3 (KLC3),³⁷ proposing that these proteins coordinately function in the intracellular transport on the microtubule.

3.2 | Regulator of microtubule polymerization and posttranslational modification

Microtubules are hollow filaments formed by α - and β -tubulin dimer, and their polymerization and degradation are regulated by a number of proteins. KATAN1 is a subunit of microtubule severing protein Katanin and is thought to modulate its severing activity and intracellular localization. KATAN1 is localized to the manchette in mice and humans.^{38,39} Loss-of-function of KATAN1 causes abnormal elongation of the manchette, and the mutant mice exhibit male fertility with impairment of meiotic spindle assembly, flagellar formation, and head shaping.³⁸ The axoneme and ODF are also disorganized in the mutant mouse spermatozoa. Katanin-like 2 (KATNL2), a putative microtubule severing protein, is also important for male fertility in mice. KATNL2 is highly expressed in the testis, and homozygous mutant mice are sterile because of abnormal flagellar and head formation and impaired motility.⁴⁰ Cytoplasmic linker protein of 170 (CLIP170) is a microtubule plus-end tracking protein that regulates microtubule dynamics and mediates cargo loading on the microtubules.⁴¹ CLIP170 is localized to the manchette and centrosome, and CLIP170-KO mice are subfertile and show sperm head abnormality.⁴² However, the number and motility of CLIP 170-KO epididymal spermatozoa is comparable to that of the wild-type, the abnormality of flagella is not prominent compared with other IFT-related gene KO mice.

Tubulin undergoes various posttranslational modifications, such as tyrosination acetylation, glycylation, polyglutamylation, which affects the dynamics and protein interaction of microtubules.⁴¹

TABLE 1 Effect of deletion of IFT-related genes on male infertility and sperm differentiation and motility

Name	Phenotype of KO					References
	conventional or conditional	Fertility	Number of sperm	Motility	Morphology of flagella	
IFT20	Germ cell-specific KO	Adult: infertile (6 weeks: subfertile)	<1/10 of wild type	Faintly motile	Short, kinked, disrupted 9+2 structure, lacking ODF	Round and swollen head ¹⁸²
IFT25	Germ cell-specific KO	Infertile	<1/10 of wild type	Immotile	Short, bent, or branched	Round and other misshaped heads ¹⁸³
IFT27	Germ cell-specific KO	Infertile	<1/10 of wild type	Faintly motile	Short and bend tail	Round and other misshaped heads ¹⁸⁴
IFT74	Germ cell-specific KO	Infertile	<1/10 of wild type	Faintly motile	Short tail	Abnormally shaped heads ¹⁸⁵
IFT81	Germ cell-specific KO	Infertile	<1/10 of wild type	Faintly motile	Short tail, some are kinked. Disorganization of ODF and 9+2 structure.	Abnormally shaped heads ¹⁸⁶
IFT88	Insertional mutation	Infertile	<1/100 of wild type	Immotile	Lack or very short and misshaped	Abnormally shaped heads ²⁵
IFT140	Germ cell-specific KO	Infertile	<1/10 of wild type	Faintly motile (<10%)	Short and/or bent	Amorphous heads ¹⁸⁷
IFT172	Germ cell-specific KO	Subfertile	<1/10 of wild type	Motile but reduced	Bent tail. Vacuoles are present in some sperm.	Abnormal manchette and head formation ¹⁸⁸
BBS1	Mutation knock in	Infertile	N.D.	N.D.	The flagella are absent in the seminiferous tubule lumens.	N.D. ¹⁸⁹ ¹⁹⁰
BBS2	Conventional	Infertile	N.D.	N.D.	The flagella are absent in the seminiferous tubule lumens.	N.D. ¹⁹¹
BBS3	Conventional	Infertile	N.D.	N.D.	The flagella are absent in the seminiferous tubule lumens.	N.D. ¹⁹²
BBS4	Conventional	Infertile	N.D.	N.D.	The flagella are absent in the seminiferous tubule lumens.	N.D. ¹⁹³
BBS6	Conventional	Infertile	N.D.	N.D.	The flagella are absent in the seminiferous tubule lumens.	N.D. ¹⁹⁴
BBS7	Conventional	Infertile	N.D.	N.D.	Coiled tail, disorganized 9+2 structure and ODF	N.D. ¹⁹⁵
KIF3A	Germ cell-specific KO	Infertile	Significantly reduced	N.D.	Lack of the tail.	Abnormally elongated head ¹⁹⁶

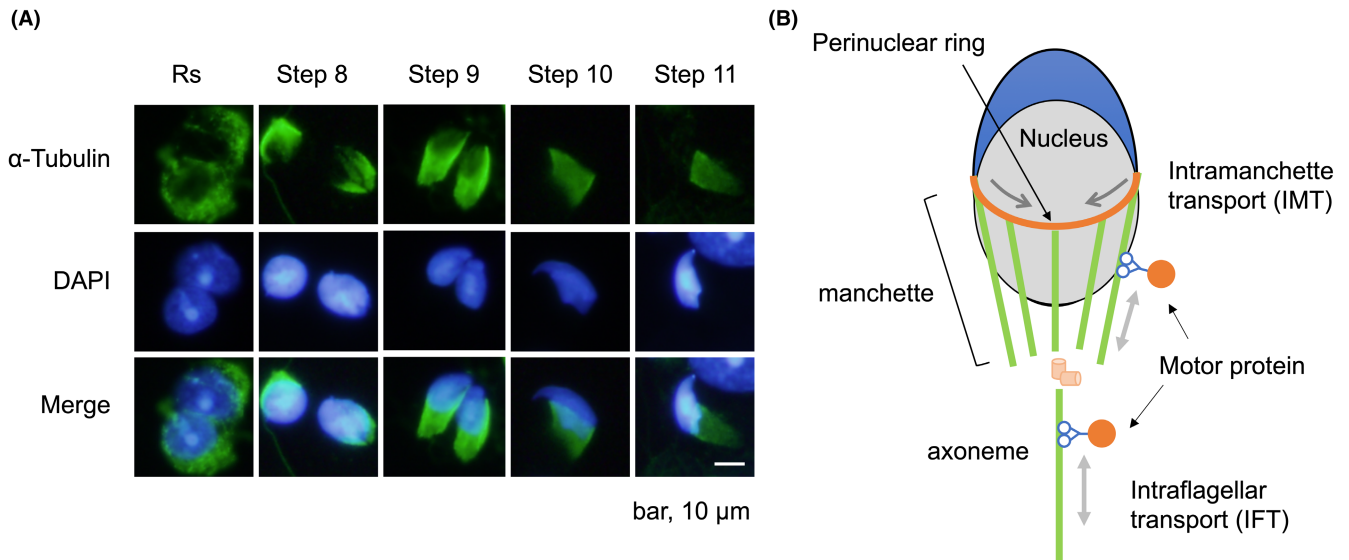


FIGURE 5 Formation of manchette in elongating spermatids. (A) Manchette was visualized using immunofluorescence staining of alpha-tubulin (a marker for the manchette, green) in various steps of mouse spermatids. The nucleus was stained using 4',6-diamidino-2-phenylindole (DAPI, blue). This figure was reproduced from the paper by Okitsu et al.⁵⁹ published under the Creative Commons Attribution 4.0 International license (CC-BY). Rs: round spermatids. (B) Schematic representation of intramanchette transport (IMT). IFT and IMT share similar mechanistic constitutions, motor proteins, IFT complexes, and BBSomes. Manchette is also involved in head morphogenesis

Additionally, since cilia and flagella are hot spots of post-translation modifications of tubulin, the lack of the modification enzymes affects motile cilia function and formation of the sperm head and flagella.⁴³ Polyglutamylation of microtubules plays a critical role in spermiogenesis. Deleting tubulin polyglutamylase *Ttll1* (tubulin-tyrosine ligase-like 1) causes decreased polyglutamylation and sperm defect with shortened tail in mice.⁴⁴ Similarly, *Ttll9*-KO male mice are infertile and have defects in sperm motility with abnormal beating patterns.⁴⁵ Interestingly, the reduction of polyglutamylation occurs preferentially on doublet 5 in *Ttll9*-KO spermatozoa. Additionally, the disappearance of the distal region of doublet 7 is frequently observed. Since it has been indicated that nine outer doublet microtubules are heterogeneous in structure and biochemical qualities, the above results suggest that *TLL9* is involved in the formation of heterogeneous polyglutamylation patterns and thereby maintains the normal structure and bending of flagella. However, excessive glutamylation is detrimental to spermatogenesis. Microtubule deglutamylase *Ccp5*-deficient male mice are infertile because of abnormal manchette and formation,^{46,47} showing that appropriate microtubule glutamylation is necessary for normal microtubule function during spermatogenesis.

Recently, Gadadhar et al.⁴⁸ reported that tubulin glycylation regulates axonemal dynein activity and sperm motility in mice. When two key enzymes for microtubule glycylation, *Ttll3* and *Ttll8*, are deficient, sperm motility decreases due to the changed flagellar beating pattern, while differentiation, number, and morphology of sperm are normal. Further investigation using cryo-electron tomography showed that the conformation of dynein on the axoneme is perturbed in *Ttll3/Ttll8* double-KO spermatozoa, while the overall structure of the axoneme is unaffected. The beating pattern of motile cilia in the ependymal cells of the brain is unaffected, suggesting that the importance of tubulin glycylation differs among cell types.

Acetylated tubulins are rich in cilia and flagella and are often used as a marker. In mammals, a major enzyme for tubulin acetylation is α TAT1 (*Atat1*), and, actually, acetylation of microtubule was abrogated in most of the tissues, including the tests in *Atat1*-KO mice.⁴⁹ Although knockdown study using RPE-hTERT cells indicated that α TAT1 is needed for the primary cilia assembly,⁵⁰ *Atat1*-KO mice were viable and developed normally, and any structural or functional defects in the somatic cells, which normally express a high level of acetylated tubulin were observed.⁴⁹ However, although the phenotype was mild, male fertility and sperm motility were decreased, suggesting that the acetylation of tubulin is necessary for appropriate flagellar motion.⁴⁹ Histone deacetylase 6 (*HADC6*), which acts as a tubulin deacetylase, is highly expressed in the testis. However, *Hdac6*-KO male mice were fertile, and there was no obvious spermatogenesis defect, despite the increased acetylation of tubulin.⁵¹ In contrast, it has been indicated that HDAC6 inhibitor could modulate sperm motility parameters⁵² and that HDAC6 significantly reduced in asthenozoospermia in humans.⁵³ Further studies will be required to clarify the function of HDAC6 in sperm motility.

4 | PROTEINS COMPOSE THE AXONEMAL STRUCTURE

4.1 | Central apparatus

In the center of the axoneme, central pair of microtubules (C1 and C2) and numerous structural components attaching the microtubules can be observed in electron microscopic analysis. These components contain a bridge connecting C1 and C2 and projection proteins (C1a, C1b, C1c, C1d, C1e, C1f, C2a, C2b, C2c, C2d, and C2e).

They are huge protein complexes and periodically aligned longitudinally (Figure 6). The identification of the proteins that comprise central apparatus has been led by studies using *Chlamydomonas*. Particularly, the development of analysis using mass spectrometry and cryo-electron microscopy accelerates the identification of protein components and their localization in the flagella. Until a few years ago, only about 22 proteins were known as central apparatus projection proteins in the flagella, but recently, a comprehensive protein analysis using wild-type and mutant *Chlamydomonas* lacking central apparatus identified new 44 proteins constituting the central apparatus.⁵⁴ Of these, 13 have homologs in humans and mice, and among them, the following genes have been genetically identified to be vital for male fertility or sperm flagellar formation: *Spag16*,^{55,56} *Spag17*,⁵⁷ *Spag6*,⁵⁸ *Dlec1*,⁵⁹ *Spef2*,⁶⁰ *Cfap69*,⁶¹ *Lrguk*,³⁵ *Cfap54*,⁶² *Cfap221*,⁶³ and *Hydin*.⁶⁴ The deletion of these genes induces a

considerable decrease in sperm number and structural/functional abnormalities of the flagella, such as a shortened tail, lacking central apparatus, malformation of other flagellar components and/or motility defects. Additionally, abnormal manchette and head morphology are often simultaneously observed.^{35,57,59,61,64} Supporting this, these proteins have been revealed to interact with IFT/IMT-related proteins and/or to localize at the manchette. For example, SPEF2 interacts with IFT20⁶⁵ and dynein 1⁶⁶ and localize to the manchette. As mentioned earlier, LRGUK1 interacts with HOOK family proteins, RIMBP3, and KLC3 and plays an important role in the organization of manchette. Thus, some central apparatus proteins may be involved in the formation of axoneme and in the formation and function of the manchette in mice.

Although overall axoneme and central apparatus structure is conserved among species, phenotypic differences have been observed

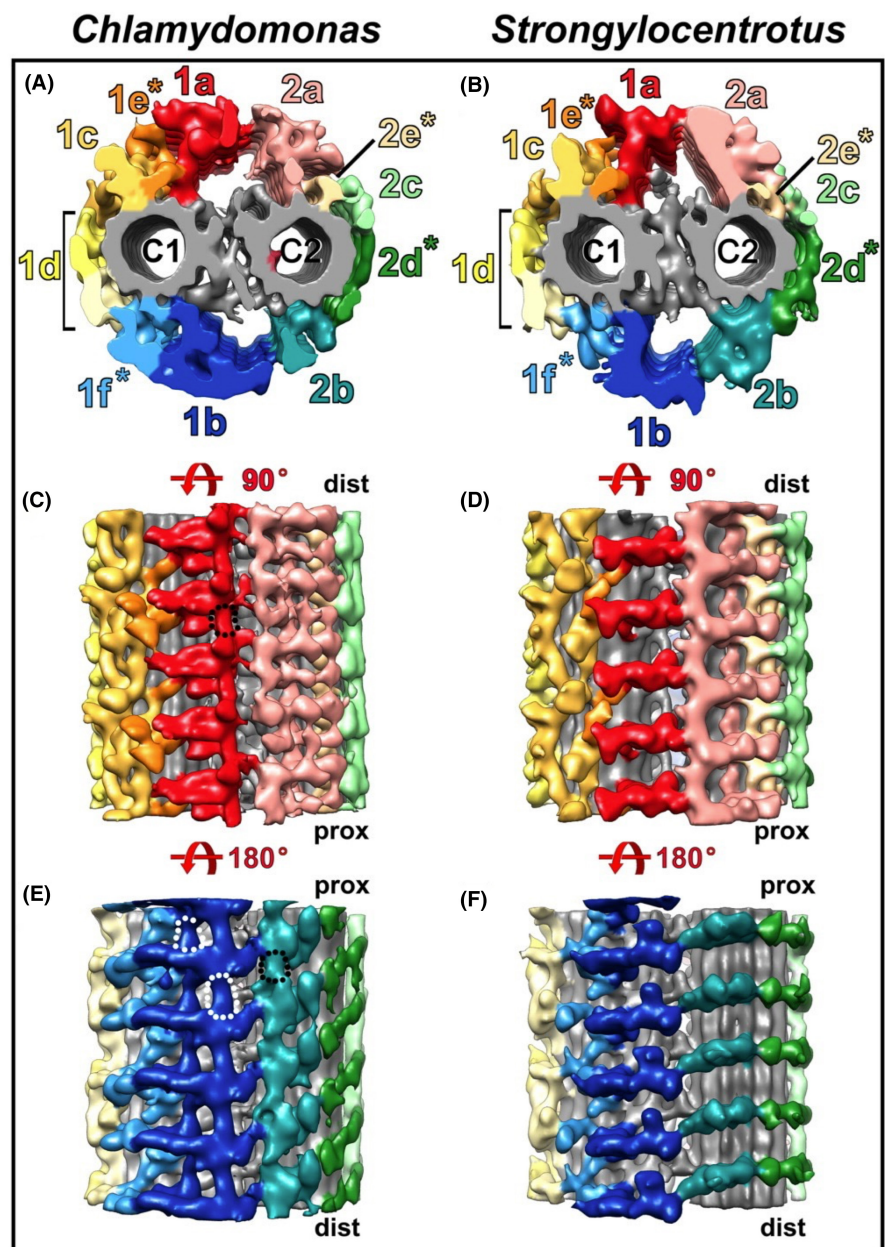


FIGURE 6 Ultrastructure of the central apparatus of the axoneme. Ultrastructure of axoneme central apparatus in *Chlamydomonas* (A, C, and E) and *Strongylocentrotus* (B, D, and F) via cryo-electron tomography. A pair of microtubules (C1 and C2, gray) and projection proteins (colored) are indicated. (A and B) Cross-sectional and (C–F) side views. The overall structure of the central apparatus is similar, despite the evolutionary distance between these species. This figure is reproduced from a paper reported by Blanca I. Carbajal-González et al.¹⁹⁷ with permission from Wiley Periodicals, Inc. Copyright © 2012 Wiley Periodicals, Inc

between *Chlamydomonas* and mice when homologous genes are deleted/mutated. The flagella of *PF20* (a *Chlamydomonas* homolog of *Spag16*) mutants are comparable in length to wild-type but paralyzed and lacked central apparatus.⁶⁷ On the other hand, *Spag16L*-specific KO mice (SPAG16 have two isoforms, SPAG16L and SPAG16S; since SPAG16L is localized in the central apparatus, but SPAG16S is localized in the nucleus, SPAG16L is considered to function in the flagella) have motile spermatozoa and no-ultrastructural abnormality in the flagella, while the number of spermatozoa and motility is reduced.⁵⁶ Similarly, the flagella of *fap81* (a homolog of *Dlec1*) *Chlamydomonas* mutants lack C1e and C1c projections but have a normal flagellar length and can swim but slowly.⁵⁴ However, the flagellar formation of *Dlec1*-KO mouse sperm is severely impaired. The number of epididymal spermatozoa is greatly decreased and the motility was utterly lost in *Dlec1*-KO spermatozoa.⁵⁹ Although the mechanism by which this phenotypic difference arises is unclear, considering numerous proteins that are localized in the central apparatus in *Chlamydomonas* are not conserved in mammals, the molecular composition and ultrastructure of the central apparatus may differ. A detailed analysis of the constituent molecules and structure of the mammalian central apparatus will be necessary in the future.

4.2 | Radial spoke

The radial spoke is a T-shaped structure protruding from the A-tubule of the doublet microtubule toward the center (Figure 3). There are three types of T-shape structures (RS1, RS2, and RS3), and they are arranged in the order at regular intervals along the long axis. The intervals are 32 nm (S1–S2), 24 nm (S2–S3), and 40 nm (S3–S1), which are multiples of 8 nm for the tubulin molecule, causing a total repeat structure of 96 nm.⁶⁸ *Chlamydomonas* RS3 is small compared with mammals (R3S). These RSs have three parts, head, neck, and stalk. The RSs are in contact with the central apparatus at the head part. RS1 and RS2 have similar structures overall, suggesting that they share a common molecular composition. Moreover, structural differences have been observed at the base of each RS, including RS3, suggesting that different molecules function at the base in each RS.

Two-dimensional electrophoresis analyses using wild-type and *Chlamydomonas* mutants lacking radial spoke have shown at least 23 proteins (RSP1–RSP23) in the radial spokes.^{69,70} Further, the studies using cryo-electron tomography, genetics, and biochemical analysis in *Chlamydomonas* proposed the model that RSP1, 4, 6, 9, and 10 are localized in the head, RSP2, 16, and 23 are in the neck, and RSP3, 5, 8, 11–15, and 17–22 are in the stalk.⁷¹ Among 23 radial spoke proteins in *Chlamydomonas*, 15 homologous proteins are found in mammals.⁷² Of these 15 genes, *Rsph6a*,⁷³ *Lrrc23* (a mouse homolog of *Rsp15*),⁷⁴ and *Rsph1*^{75,76} are important for male fertility in mice, and mutation of *RSPH3* have indicated to cause male infertility in humans.⁷⁷

Spermatogenic phenotypes vary in each mutant mouse. *Rsph6a*-deficient mouse sperm tail is short and shows no motility.⁷³ Spermatogenesis normally proceeds until round spermatids, but

spermatozoa were significantly decreased. Although the mechanism is unclear, the abnormal elongation of the manchette and nuclear was also observed in elongating spermatids. Ultrastructural analysis of *Rsph6a*-KO testis indicated that the 9 + 2 structures are disorganized in the axoneme surrounded by MS but not in the axonemes not surrounded by MS, proposing that the formation of the axoneme properly proceeded but is disrupted before the MS is formed. Deletion of the *Rsph1* gene in mice also results in a similar phenotype with *Rsph6a*-KO mice. Post-meiotic sperm differentiation is disrupted, and sperm tail formation is retarded. Degenerate spermatids are phagocytosed by the Sertoli cells, and spermatozoa are rarely observed in cauda epididymis.⁷⁵ In contrast, *Lrrc23*-KO mice can produce mature spermatozoa comparable to wild-type mice. *Lrrc23*-KO sperm tail length seems normal, but the radial spokes are disorganized.⁷⁴ The motility was significantly decreased compared with control spermatozoa, whereas the beating motion itself was observed. These results suggest that each radial spoke protein plays a distinct role in sperm flagellar formation/function.

Rsph6a and *Lrrc23* are predominantly expressed in the testis, and deletion of these genes in mice does not seem to cause obvious functional abnormality in motile cilia in other tissues. In contrast, mutations of *RSPH1*, *RSPH3*, *RSPH4A*, and *RSPH9* are found to be associated with PCD in humans.^{76–78} Intriguingly, radial spokes are critical for the motile cilia's motility pattern (planer motion). For example, the deletion of *Rsph4* in mice causes the lack of radial spokes in the trachea and ependymal cilia and a change in the motility pattern from planer motion to clockwise rotation motion.⁷⁹ Mutation of *RSPH1* also induces a loss of radial spoke head (and occasionally central apparatus) and the change in motility from planar to rotational motion.⁷⁶ The nodal cilium, which indicates a rotational motion, has no central apparatus and radial spoke. Additionally, eel sperm flagella do not have radial spokes and indicate rotational movement. These results suggest that the presence or absence of radial spokes determines the motility pattern.

4.3 | Nexin-dynein regulatory complex

To make the coordinated beating waveform, dynein regulation at distinct parts of cilia/flagella is necessary. Because *Chlamydomonas* mutants lacking the central apparatus or radial spoke usually had paralyzed flagella, initially, their structure is considered to regulate the dynein activity. However, after that some *Chlamydomonas* mutants that rescue flagella paralysis without repairing of radial spoke/central apparatus have been found,⁸⁰ the existence of a dynein regulator [dynein regulatory complex (DRC)] has been proposed. Apart from this, a filamentous structure that connects neighboring doublet microtubules, called nexin, was found using an electron microscopic analysis. Based on its structural features, it was speculated that nexin might function to restrict the sliding of microtubules to a certain extent: however, the molecular basis remained unclear. Subsequent analysis using high-resolution cryo-electron tomography showed that DRC is the only structure connecting the neighboring microtubule doublet other than

the ODA and IDA. Therefore, DRC is found to be the nexin itself, it is called the N-DRC.⁸¹ To date, 11 proteins are subunits of N-DRC using two-dimensional electrophoresis analysis and immunoprecipitation-mass spectrometric analysis in *Chlamydomonas*. All these proteins are conserved in humans, and the evolutionary conservation is higher than the radial spokes and central apparatus. Eight of eleven proteins have coiled-coil domain and/or leucine-rich repeat and make complex through the interaction of these domains.⁸²

In the past few years, there has been rapid progress in the functional analysis of N-DRC genes. Deletion or mutation of *Drc1*,⁸³ *Lrrc48* (*Drc3*),⁸⁴ *Tcte1* (*Drc5*),⁸⁵ *Drc7*,⁸⁶ and *lqcg* (*Drc9*)⁸⁷ has been shown to cause male infertility in mice. Additionally, mutation of *DRC1*,⁸³ *CCDC65* (*DRC2*),^{88,89} and *GAS8* (*DRC4*)⁹⁰ was found to be associated with PCD in humans. In contrast, *Fbxl13* (*Drc6*) is unlikely to be vital for male fertility and motile ciliary function.⁸⁶ *Drc1*-, *Drc7*-, and *lqcg*-KO mice indicate similar phenotypes. The number and motility of spermatozoa are significantly reduced while sperm differentiation until meiosis completion is normal. The flagella are coiled or short, and the 9 + 2 structure is disorganized. Usually, the misshaped head is accompanied in these mutant mice. The phenotype of *Tecte1*(*Dec5*)-KO mice differs from that of *Drc1*-, *Drc7*-, and *lqcg*-KO. Namely, the motility of *Tcte1*-KO spermatozoa is significantly reduced whereas no morphological abnormalities including N-DRC were found. Additionally, the expression of glycolytic enzymes such as hexokinase 2 is low, and a significant reduction in ATP was found in *Tcte1*-deficient spermatozoa. These results suggest that TCTE1 is needed to produce the energy source for motility but not for the formation of N-DRC. In contrast to the essentiality of the genes in sperm function, no severe defects of morphology and function of motile cilia are observed in *Drc1*-, *Tcte1*(*DRC5*)-, *Drc7*-, and *lqcg* (*Drc9*)-KO mice. Therefore, these genes would function specifically in the regulation/formation of sperm flagella.

Recent cryo-electron tomographic analysis revealed the N-DRC structure in *Chlamydomonas*.⁹¹ The study suggested that DRC1/2/4 form the N-DRC backbone that serves as a scaffold, and DRC3/5/6/7/8/11 are attached to the backbone and interact with other axonemal complexes. Biochemical protein interaction analyses support this structural model.^{83,86,92} Additionally, these reports also indicate that the lack of one N-DRC subunit affects the expression of other subunits. For example, the expression of DRC2, LRRC48 (*DRC3*), and *GAS8* (*DRC4*) is reduced in *DRC1* mutant mice.⁸³ The expression of DRC1, DRC3, DRC5, and DRC11 is reduced in *Chlamydomonas* *DRC2* mutant.⁹² Similarly, DRC7 interacts with DRC3, *GAS8* (*DRC4*), and TCTE1 (*DRC5*), and the expression of those proteins are decreased in *Drc7*-KO spermatozoa.⁸⁶ These results suggest that the formation of N-DRC needs the interdependent interaction of each subunit, at least in part.

4.4 | Outer dynein arm and inner dynein arm

Dynein is a motor protein that binds to microtubules and moves toward the minus end. There are two types of dynein, cytoplasmic

dynein and axonemal dynein. Cytoplasmic dynein is involved in the intracellular transport of protein/lipid to the target site in the cell. The intracellular transport by dynein was recently reviewed.⁹³ The axonemal dynein is involved in the making of bends in flagella (Figure 7). One end of the dynein binds to the A-tubule stably, and the other domain binds neighboring B-tubule transiently. Then, ATP-dependent structural change induces the sliding of the doublet tubule. Since dynein moves only to the minus end, symmetrical activation/inhibition of dynein on nine doublet microtubules is necessary to make flagellar bending. Namely, during one side of the dynein of the axoneme is activated, the opposite side of the dynein must be inactivated. Additionally, to create the wave-like flagellar beating, the tail's asymmetric activation/deactivation state must move continuously caudally. The molecular mechanism of this highly coordinated regulation is still a mystery.

In the axoneme, there are two types of dynein arms, the ODA and the IDA, and four ODAs and seven IDAs [one double-headed type (I1/f) as well as six monomeric types (a, b, c, d, e, and g)] exist in the 96-nm repeat structure of the long axis (Figure 3). In addition to these major dyneins, some minor dyneins have been identified in *Chlamydomonas* flagella, although it is lesser extent.⁹⁴ ODA and I1/f IDA are large protein complexes composed of two heavy chains (three in *Chlamydomonas* ODA), many intermediate chains, and light chains. Analysis of *Chlamydomonas* mutants showed that ODA and IDA have distinct roles; ODA is involved in regulating beat frequency and generation of force,^{95,96} and IDA is involved in controlling the size and shape of the bend.⁹⁷

There are 16 dynein heavy chain genes in humans. All genes encode over 500 kDa proteins with a conserved primary structure containing a tail domain, AAA+ domains, coiled-coil domains (Figure 7). The tail domain comprises nine helices and is involved in the oligomerization and binding to intermediate and light chains (Figure 7). In the axonemal dynein, this domain is also involved in binding to the A-tubule. Downstream of the tail domain, there are six AAA+ domains. These domains form a ring-like structure called "head" (Figure 7). Two coiled-coil domains exist between the fourth and fifth AAA+ domain, and a microtubule-binding domain exists between the coiled-coil domains. The coiled-coil and microtubule-binding domains form "stalk" of dynein heavy chain (Figure 7).

The mutations of ODA and IDA-related genes are associated with PCD.^{98,99} Some dynein heavy chains are found to play a role specifically in spermatozoa. For example, bi-allelic mutation of *DNAH10* (an IDA heavy chain) induces male infertility with multiple morphological abnormalities in the flagella (MMAF), but the infertile patients do not exhibit PCD symptoms, such as lung disease, sinusitis, and *situs inversus*.¹⁰⁰ Similar results have been obtained in mutation or deletion of *DNAH1*,^{101,102} *DNAH2*,¹⁰³ *DNAH8*,¹⁰⁴ and *DNAH17*.^{105,106} These gene mutations cause sperm abnormalities such as short or coiled-tail, disorganized axoneme, and decreased motility but do not exhibit other obviously clinical PCD manifestations. Conversely, not all PCD patients indicate male infertility. Mutation of dynein axonemal heavy chain *DNAH5* and *DNAH11* causes PCD but some fertile patients.⁹⁹ These results suggest that

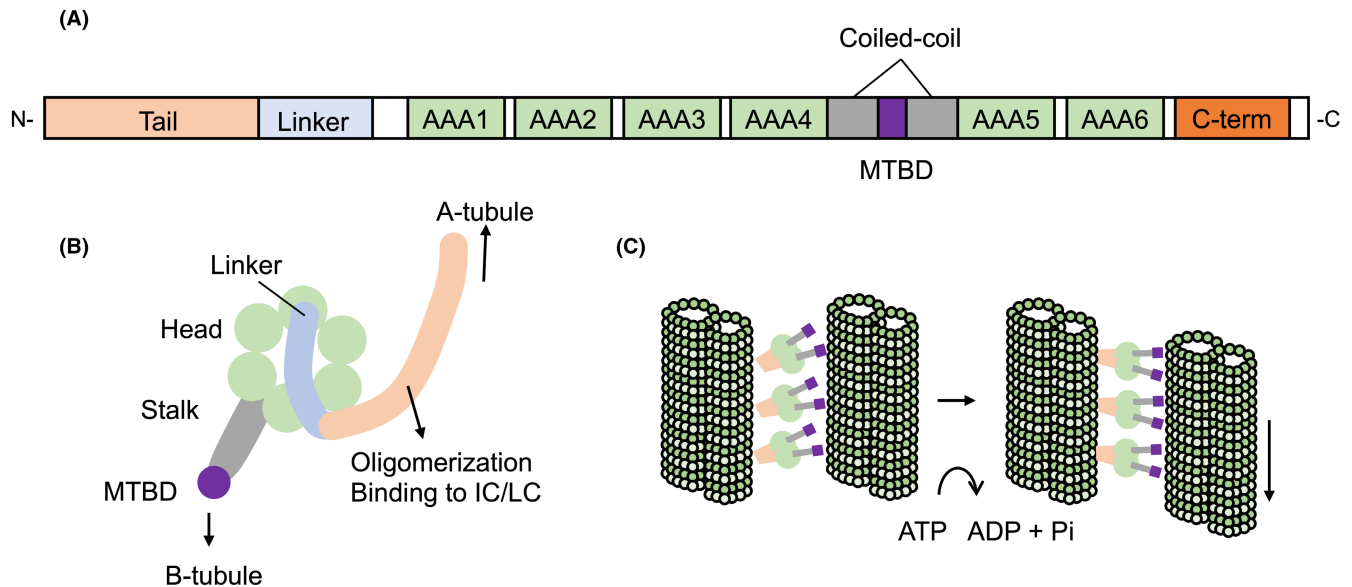


FIGURE 7 Structure of dynein protein. (A) Primary structure of dynein. Dynein contains a tail domain, linker domain, 6 ATPases associated with diverse cellular activities (AAA+) domains, two coil-coil domains, and microtubule-binding domains (MTBD). (B) Schematic representations of the tertiary structure of dynein. The tail domain is involved in oligomerization and binding to intermediate chains (IC), light chains (LC), and A-tubule. MTBD binds to the neighboring B-tubule transiently. (C) ATP hydrolysis induces conformational changes in dynein and causes the microtubules to slide; this structural change leads to flagellar bending

the composition of dyneins (and other axonemal components) differs between somatic motile cilia and sperm flagella. Supporting this, the expressions of *DNAH8* and *DNAH17* increase during spermatogenesis and indicate predominant expression in the testis.⁹⁹ Moreover, individual dynein heavy chain motors exhibit discrete rates of microtubule translocation and ATPase activity,¹⁰⁷ and beating patterns clearly vary between somatic cell cilia and sperm flagella. Therefore, the difference in the composition of dyneins may generate a distinct beating pattern in cilia and flagella.

4.5 | Mitochondrial sheath

ATP, the energy source of cells, is produced by glycolysis and oxidative phosphorylation. The balance between these two pathways is changed during spermatogenesis: the glycolytic pathway is dominant in spermatogonia, while the oxidative phosphorylation pathway is dominant in spermatocytes and spermatids. This reflects the difference in the availability of glucose, i.e., while the spermatogonia localized at the basal membrane can receive glucose from blood, spermatocytes and spermatids localized within the blood–testis barrier cannot receive glucose and instead use metabolites, such as lactate, supplied by Sertoli cells as carbohydrates.^{108,109} Supporting this, mutation of a gene involved in mitochondrial function frequently causes spermatogenic failure at the spermatocyte stage.¹⁰⁹

The number, shape, and localization of mitochondria are dynamically altered during spermatogenesis. Mitochondria in spermatogonia are relatively small but elongate during meiosis and again fragment in spermatids. In the later spermiogenesis stage,

mitochondria were aligned at mid-piece and the MS was formed. In this process, first, spherical mitochondria are lined up regularly at mid-piece and attached on the ODF, forming four dextral helical arrays. Next, mitochondria elongate laterally and become crescent-shaped. Subsequently, mitochondria continue to elongate, interlock with multiple adjacent mitochondria, and finally wrap around the mid-piece to form mitochondria sheath.¹¹⁰ These dynamic changes of mitochondria must need a sophisticated system. Recently, many genes were identified as essential for MS formation in mice. Since the abnormal MS formation is often observed as a secondary effect of other axonemal defects, only molecules localized or closely associated with the mitochondria are picked up.

The morphology of mitochondria is dynamically regulated by fission and fusion. This regulation is necessary for cell homeostasis and its dysregulation is associated with diseases such as neurogenic disorder, cardiovascular diseases, and cancer.¹¹¹ Varuzhanyan et al¹¹² have reported that mitochondrial fission factor (Mff) plays a crucial role in the organization of MS and male fertility in mice. Mitochondria of *Mffst* homozygous mutant spermatozoa are swollen and sparsely localized at the mid-piece. The abnormally elongated and constricted mitochondria were already observed in the round spermatid stage, suggesting that the impairment of MS formation is caused by the defect of fission that occurs earlier. Mitophagy is another mechanism that controls the abundance and quality of mitochondria. Mitophagy is a selective mitochondrial degradation by autophagy and regulates mitochondria's number and physiological function by eliminating dysfunctional or excess mitochondria.¹¹³ When mitophagy is inhibited by ablation of the *Fis1* gene, mitochondria are aberrantly accumulated in spermatids and sperm differentiation after step 5 is impaired,¹¹⁴ showing that mitophagy is critical for spermatogenesis.

Armadillo repeat-containing 12 (ARMC12) and TBC1 domain family member 21 (TBC1D21) are essential for mitochondrial elongation after alignment at mid-piece in mice.^{115,116} In *Armc12*-KO mice, although spherical mitochondria are normally arranged on mid-piece, their elongation and interlocking with adjacent mitochondria are impaired. As a result, a disorganized MS is formed, and sperm motility is decreased. Because of these defects, *Armc12*-KO male mice are sterile. *Tbc1d21*-KO mice indicate a similar phenotype with *Armc12*-KO.^{115,116} TBC1D21 was found to interact with ARMC12 and is necessary for interacting ARMC12 with VDAC2 and VDAC3.¹¹⁵ VDAC3 is a voltage-dependent anion channel localized at mitochondria and is essential for MS formation and male fertility.¹¹⁷ Therefore, these cooperative interactions may be necessary for proper MS formation.

Glycerol kinase-like protein 1 (Gyk1) and glycerol kinase 2 (Gk2) also play a pivotal role in the alignment of crescent-shape mitochondria. Gyk1 and Gk2 are members of glycerol kinase family proteins but have no kinase activity. Meanwhile, both proteins can interact with phospholipase D6 (PLD6) and cause PLD6-dependent phosphatidic acid production and mitochondrial clustering in HEK293 cells. Since mitochondrial dynamics are closely associated with phosphatidic acid, these results suggest that Gyk1 and Gk2 regulate MS formation through PLD6.

Other than the above proteins, vacuolar protein sorting 13 homolog a (VPS13A) and testis-specific gene antigen 10 (TSGA10) are reported to be essential in the MS formation in mice.^{118,119} Although the number and overall morphology of spermatozoa in a homozygous mutation of *Vps13a* and heterozygous mutation of *Tsga10* are normal, ultrastructural abnormalities of the mitochondria are observed. The motility of those spermatozoa was significantly decreased, albeit ATP concentration is comparable to control mouse. The mechanism by which mutation of the genes induces aberrant MS formation remains to be elucidated. However, since TSGA10 interacts with ODF2, an ODF protein, TSGA10 is predicted to act as a glue that connects mitochondria and ODF. Interestingly, the phenotype of the gene mutation differs between mice and humans. For example, although the mutation of human VPS13A causes chorea-acanthocytosis, reduction in fertility has not been observed generally in human male chorea-acanthocytosis patients. *Tsga10* is haploinsufficient for male infertility in mice, whereas biallelic mutation of *TSGA10* is found in patients of male infertility in humans.^{120,121} These data may show the structural or functional difference in the MS between mice and humans.

4.6 | Outer dense fiber

In mammalian spermatozoa, the axoneme is surrounded by filamentous accessory structures, such as ODFs and FS. These structures ensure elasticity, shear resistance, and structural integrity of spermatozoa. ODFs and FS exist in spermatozoa but not in other motile cilia. ODF comprises nine fibers in the mid-piece, but two (#3 and #8) are not extended to the principal piece and are replaced with

longitudinal columns of the FS (Figure 3). Two-dimensional polyacrylamide gel electrophoresis analysis showed that ODF comprises 14 proteins at least.¹²² To date, the following proteins are found to be ODF localized proteins: ODF1,¹²³ ODF2,¹²³ ODF3 (firstly reported as Shippo 1),^{124,125} ODF4 (firstly reported as Oppo1),¹²⁶ SPAG5,¹²⁷ Tekt4,¹²⁸ VDAC2,¹²⁹ VDAC3,¹²⁹ TPX-1,¹³⁰ and PMFBP1 (firstly reported as ODF3,¹²⁴ also known as sperm-tail-associated protein¹³¹). The role of the following proteins in sperm morphology and/or motility has been investigated using a genetic approach.

ODF1 is specifically expressed in the testis and localized not only in ODF but also in connecting pieces of spermatozoa.¹²³ *Odf1*-KO spermatozoa show ODF and MS disorganization, decapitation, and decreased motility. Because of these sperm defects, *Odf1*-KO male mice are sterile.¹³² Reduced ODF1 expression in patients with male infertility has been reported, and the abnormality of the connecting piece was observed in the patient spermatozoa.¹³³ ODF2 is a major component of ODF and is predominantly expressed in the testis,¹³⁴ while ODF2 has also been shown to be localized in the centrosome of somatic cells.¹³⁵ The phenotype of *Odf2*-KO mice in male fertility and sperm morphology/motility is different between the mice lines used. Salmon et al¹³⁶ reported that homozygous *Odf2*-KO mice die at the preimplantation period and heterozygous mutants normally produce spermatozoa without any defects. On the other hand, Tarnasky et al¹³⁷ and Ito et al¹³⁸ reported that spermatozoa from heterozygous mutant mice exhibit abnormal flagellar bending, decreased motility, or neck-mid-piece separation using different *Odf2*-KO mice lines. Additionally, in the analysis using germ-line-specific *Odf2*-KO mice, while heterozygous mice spermatozoa did not show any defect, decreased motility and morphological abnormalities of ODF were observed in homozygous mutant spermatozoa.¹³⁹ One reason for these differences may be attributed to the expression of an isoform transcribed by alternative splicing, but a more detailed analysis is required. ODF3 and ODF4 proteins are enriched in spermatozoa. Although the physiological role of *Odf3* and *Odf4* in sperm differentiation and function has not been reported, the data that *Odf4*-deficient male mouse is infertile is registered in The International Mouse Phenotyping Consortium database. Elucidation of ODF4 function in spermatozoa is expected to be clarified in the near future.

Tektins are evolutionally conserved filament-forming alpha-helical coiled-coil proteins. Tektins are abundantly expressed in spermatozoa and in somatic cells with motile cilia. In sea urchin spermatozoa, all tektin family proteins (Tektin A-Tektin C) are localized in axonemal microtubules, centrioles, and basal bodies.^{140,141} Recently, Gui et al¹⁴² have shown that tektin bundles are localized in the A-tubule lumen in bovine tracheal cilia using cryo-EM. In mammalian spermatozoa, tektins are localized outside microtubules: TEKT1; acrosome, flagella, centrosome and the caudal end of the head,^{143,144} TEKT2; surface to the ODF,¹⁴⁵ TEKT3; the surface of mitochondria,¹⁴⁶ TEKT4; ODF and neck region,¹²⁸ TEKT5; the inner side of the MS.¹⁴⁷ Among these, *Tekt2*-, *Tekt3*-, and *Tekt4*-KO mice are produced. In *Tekt2*-KO mouse spermatozoa, abnormal tail bending and loss of IDA structure is observed. Sperm motility was reduced, and KO male mice are

sterile.¹⁴⁸ *Tekt3*-KO spermatozoa indicated abnormal tail bending and decreased motility, but *Tektin 3*-KO male mice are fertile.¹⁴⁹ Although deficiency of *Tekt4* does not affect male fertility on a 129:B6 background, the deletion on 129 background results in subfertility.¹⁵⁰ In the line of the latter mice, sperm motility is significantly reduced, whereas flagellar ultrastructure is grossly unaltered.

SPAG5 is identified as ODF1 interacting protein.¹⁵¹ Both SPAG5 and ODF1 have leucine zipper domains and interact through this domain. SPAG5 is localized to the endoplasmic reticulum and microtubules in somatic cells but to ODF in spermatozoa.¹⁵² Deletion of *Spag5* does not affect male fertility in mice,¹⁵² suggesting that another protein(s) compensate for SPAG5 function.

PMFBP1 is firstly reported as ODF3 by Petersen et al.¹²⁴ They identified this protein by cDNA expression screening using an antibody obtained by immunization of biochemically isolated ODF and confirmed localization in the ODF using immunoelectron microscopy. However, other groups reported that PMFBP1 is localized at head-tail junctions and that mutation or deletion of PMFBP1 causes acephalic spermatozoa in mice and humans.^{153,154} Therefore, even if PMFBP1 is localized to the ODF, it may play a more crucial role in the structural stability of head-tail junctions.

4.7 | Fibrous sheath

FS is an electron-dense structure surrounding ODF in the principal piece and formed at steps 14–16 in mice. FS comprises two symmetrical fibers called longitudinal columns and the circumferential rims that link the longitudinal columns (Figure 3). To date, A-kinase anchoring protein (AKAP) 4, AKAP3, TKAP-80, Rhoophilin, Ropporin (ROPN1), glyceraldehyde 3-phosphate dehydrogenase-S (GAPDS), type 1 hexokinase-S (germ-cell specific Hk1 splice variants), CABYR, FSCB, and Sp17 (sperm protein 17) are reported to be localized in the FS. However, similar to the ODF, how these proteins comprising the filamentous structure is largely unknown.

AKAP3 and AKAP4 are major structural FS components and are expressed specifically in post-meiotic male germ cells. These AKAPs are predicted to be involved in the signal transduction through a binding and recruitment of protein kinase A regulatory subunits I and II. Additionally, AKAP3 and AKAP4 act as a scaffold for CABYR, ROPN1, and Sp17 that have a domain with high similarity of protein kinase A regulatory subunits II. AKAP4 is critical for FS development.¹⁵⁵ FS formation in the *Akap4*-KO spermatozoa is incomplete, and nascent longitudinal columns as well as thin circumferential rims have been observed. The length of the principal piece is short, and the expression of other component proteins in FS such as GAPDS and AKAP3 is reduced in *Akap4*-KO. Thus, *Akap4*-KO mice show reduced motility and fertility.¹⁵⁵ AKAP4 mutation has been associated with human male infertility.¹⁵⁶ The loss-of-function missense variant of AKAP4 causes abnormal sperm flagella development and male infertility with decreased expression of QRICH2¹⁵⁶ which plays an important role in sperm flagellar development.¹⁵⁷ AKAP3 also plays a pivotal role in sperm flagellar morphology and male fertility.¹⁵⁸

Akap3-deficient mice are sterile, and their spermatozoa barely exhibit any motility. Longitudinal columns of FS are present but not for circumferential rims in *Akap3*-KO spermatozoa. The global protein expression pattern was changed in *Akap3*-KO spermatozoa, but it was different with *Akap4*-KO spermatozoa. These results show that AKAP3 and AKAP4 play a distinct role in sperm tail formation.

ROPN1 (Ropporin 1) and ROPN1L (ROPN1-like protein, also known as ASP) are R2D2 (protein kinase A RII like) proteins and bind with AKAP3.¹⁵⁹ Although both single KO of *Ropn1* or *Ropn1l* gene did not affect sperm morphology, double-KO mice spermatozoa had obvious structural defects in principal piece of spermatozoa.¹⁶⁰ DKO spermatozoa are immotile, and DKO male mice are infertile. Since the expression of ROPN1L is increased in *Ropn1*-KO spermatozoa, a compensation mechanism may exist in these proteins. Rhoophilin1 is an effector of Rho small GTPase and localized in the FS of spermatozoa.¹⁶¹ *Rhoophilin1* is highly expressed in the testis and binds to ROPN1. It has been reported that *Rhoophilin1*-deficient mice have defects in podocyte cytoskeletal architecture and renal filtration¹⁶²; however, the reproductive phenotype was not evaluated, unfortunately.

CABYR was identified as a testis-specific calcium ion binding protein localized in the FS in human spermatozoa. Deletion of *Cabyr* in mice causes reduced sperm motility and male infertility. *Cabyr*-KO spermatozoa have structural defects in the FS and doublet microtubule.¹⁶³ Because CABYR becomes tyrosine- and serine/threonine phosphorylated during capacitation and Ca²⁺ plays critical roles in capacitation, CABYR is predicted to be involved in capacitation. However, *Cabyr*-KO spermatozoa exhibited normal PKA activity and protein tyrosine phosphorylation during capacitation, suggesting that CABYR is unnecessary for the PKA signaling pathway, including the activation of protein tyrosine kinases.

4.8 | Annulus

The annulus is an electron-dense ring-like structure existing between the mid-piece and principle piece. The annulus is first observed at the flagella's basement in round spermatids and migrates caudally along with the axoneme as differentiation proceeds. Septins are essential proteins that form the annulus. Septins are evolutionally conserved genes in eukaryotes from yeast to humans. Thirteen members have been found in humans, and all members have conserved GTP-binding domains. Septins can form a multimeric complex comprising two or more different subunits. This complex is organized into a higher-order structure such as filament, lattice, or ring, depending on the cell types and conditions. Septins are associated membrane and microtubule filaments and are involved in cytokinesis, vesicle trafficking, or subcellular compartmentalization of membrane in somatic cells.^{164,165}

In mammalian spermatozoa, at least six members of septins (SEPT2, SEPT4, SEPT6, SEPT7, SEPT12, and SEPT14) express.^{166,167} Although SEPT4 and SEPT7 are localized only at the annulus, other members are localized at the annulus and at the post acrosome region, neck, and/or mid-piece.^{166,167} Among the family genes, *Sept4*

and *Sept12* are essential for male fertility in mice.¹⁶⁸⁻¹⁷⁰ *Septin4*-deficient mice were normally born and showed no obvious gross abnormality, but male mice are infertile. Although the overall sperm differentiation seems to be normal, *Septin4*-KO cauda epididymal spermatozoa exhibit 180° bend at the mid-piece and principal piece-junction and lack motility. *Septin4*-KO spermatozoa also show structural defects such as lack of the annulus, abnormal mitochondria alignment, formation defects of FS.^{168,169} Additionally, basigin, a membrane protein localized to the principal piece of spermatozoa, is dislocalized and diffused in the entire sperm region. This result suggests that the annulus acts as a diffusion barrier that compartmentalized membrane protein into a specific region. Haploinsufficiency of *Sept12* causes male infertility because of impaired spermatogenesis in mice.¹⁷⁰ The chimeric mice produced by introducing ES cells having a hemizygous deletion of *Septin12* into a blastocyst could not produce ES cells-derived offspring. The spermatozoa of the chimeric mice have decreased motility and morphological defects in the acrosome and tail.¹⁷⁰ Defects of the annulus or impaired expression of septin have been observed in the spermatozoa of infertile men.^{167,169,171,172} An association has been found between mutations in the septin genes and male infertility and/or sperm disorder.¹⁷³⁻¹⁷⁵

Other than septins, some transporters play a critical role in the integrity of the annulus structure. For example, *Slc26a8* (also known as TAT1, testis anion transporter 1) is specifically localized to the annulus and deletion of *Slc26a8* causes structural defects in the annulus and mitochondria.¹⁷⁶ The deletion of *Slc22a14*, a member of organic anion/cation transporter, induces disorganization of the annulus of spermatozoa in mice.¹⁷⁷ Whether the structural defect of the annulus in these KO mice is a direct effect of the deletion of the genes or not is unclear; however, the clarification of the specific roles of these transporters in the formation of the annulus may help elucidate the molecular mechanism of the formation of the annulus or stability of septin complex.

5 | OSMOTIC-CELL SWELLING

When the spermatozoa are exposed to hypo-osmotic conditions, the sperm tail morphology changes to characteristic shapes such as hairpin-like (mice) or coiled-like (humans), depending on the species. This change is caused by cell swelling caused by the permeation of water into the cytoplasm and is called "osmotic-cell swelling." Therefore, if the function of transporters involved in the transport of water or solute, similar abnormal flagellar bending is occasionally induced. For example, disruption of aquaporin3 (*Aqp3*), a putative water efflux channel expressed in spermatozoa, causes the typical hairpin-like flagellar bending.¹⁷⁸ Similarly, disruption of *Atp1a4* (Na, K-ATPase α 4),¹⁷⁹ *Slo3*,^{180,181} and *Slc22a14*¹⁷⁷ also result in similar abnormal flagellar bending. These spermatozoa showed decreased motility and fertility, whereas sperm production of KO mice was normal. The tissue region where flagellar bending occurs is different in each KO mouse, although not all KO mice have been analyzed. In *Aqp3*-KO mice, spermatozoa obtained from the uterus after coitus show abnormal flagellar

bending, whereas cauda epididymal spermatozoa show normal morphology. In *Slc22a14*-KO mice, the tail morphology of spermatozoa in the testis, caput, and corpus epididymis is normal, but abnormal flagellar bending is observed in cauda epididymal spermatozoa. These may indicate the region in which the function of each transporter is required in spermatozoa. In fact, the osmotic pressure of the uterine cavity (~310 mOsm) is lower than cauda epididymis (~420 mOsm).¹⁷⁸ Additionally, the microenvironment of the epididymis (e.g. amino acid composition of luminal fluid) vary in the caput, corpus, and cauda epididymis. Therefore, spermatozoa probably require adjusting the osmotic pressure by the transport of solute/water during movement in the male and female reproductive tract.

6 | CONCLUSION

Sperm head and flagellum formation is a highly coordinated process involving numerous proteins expressed in a spatial- and temporal-specific manner. As described in this review, many genes that are essential for sperm head and flagellum formation have been identified over the past two decades using a genetic approach in mice. Additionally, the development and spread of new generation sequencing techniques are pushing the identification of human gene mutation associated with globozoospermia, asthenozoospermia, and MMAF. The identification of genes involved in spermatogenesis will continue to progress rapidly in the future.

Meanwhile, several questions have been raised by the progress of studies in this field, one of which is how is the axoneme built up. The axoneme comprises many tremendous protein complexes aligned periodically along the long axis of microtubules. Where is the complex made, and how does the complex recognize the position to be localized? Is there an order to transport the parts of the axoneme on microtubules? The second question is the diversity of the axonemal molecular architecture and the relevance to the flagellum/cilium wave motion. As mentioned above, the overall sperm axonemal 9 + 2 structure is shared using motile cilia and is highly conserved among species. However, the beating motions differ among sperm flagella, *Chlamydomonas* flagella, and motile cilia. Additionally, the phenotype of homologous gene deletion/mutation in flagellar motility differs between *Chlamydomonas* and mice, and the deletion of some genes affects only sperm flagella formation/function, but not for motile cilia. Although the proteins specifically expressed in spermatozoa probably contribute to the characteristic sperm motility, the mechanism that generates the specific wave motion in each flagellum/cilium largely remains to be elucidated. The third question is how the difference in sperm morphology is made. As described in the Introduction, the rodent sperm head is generally falciform-like, but that of humans is paddle-like. Additionally, sperm tail length varies even in the same rodent. How is the length of the tail determined? To date, numerous genes essential for sperm tail formation have been found, but the gene affecting tail length without affecting the structure has not been found. Since the axoneme contains periodically aligned

structures and the length seems to be determined strictly in the same species, the mechanism measuring length or counting the number of the periodic structure possibly exist. To understand this question, it will be necessary to study spermatogenesis at the molecular level in a wider range of animal species.

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CONFLICTS OF INTEREST

The author declared that there are no conflicts of interest with the contents of this article. Because this is a review, this article does not contain any studies with human and animal subjects performed by the author.

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