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REVIEW

Development of functional spermatozoa in mammalian spermiogenesis

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ABSTRACT

Infertility is a global health problem affecting one in six couples, with 50% of cases attributed to male infertility. Spermatozoa are male gametes, specialized cells that can be divided into two parts: the head and the flagellum. The head contains a vesicle called the acrosome that undergoes exocytosis and the flagellum is a motility apparatus that propels the spermatozoa forward and can be divided into two components, axonemes and accessory structures. For spermatozoa to fertilize oocytes, the acrosome and flagellum must be formed correctly. In this Review, we describe comprehensively how functional spermatozoa develop in mammals during spermiogenesis, including the formation of acrosomes, axonemes and accessory structures by focusing on analyses of mouse models.

KEY WORDS: Acrosome, Flagellum, Knockout mice, Male fertility, Spermatogenesis

Introduction

Spermatozoa develop in the seminiferous tubules of the testes through an organized process called spermatogenesis. During spermatogenesis, spermatogonia produce diploid spermatocytes through mitosis, which undergo meiosis to become haploid round spermatids (Han, 2024). Round spermatids then go through drastic morphological changes in a process called 'spermiogenesis' to become mature spermatozoa. In mice, this can be divided into 16 steps (Russell et al., 1990) (Fig. 1).

We focus here on the development of mature spermatozoa, which have distinct cellular regionalization to perform a variety of functions. Spermatozoa can be divided into two components: a head and a tail-like flagellum (Fig. 2A). Sperm motility is driven by the axoneme-containing flagellum, which allows spermatozoa to travel a long distance in the female reproductive tract. Based on the presence of accessory structures, sperm flagella can be further divided into three parts: midpiece, principal piece and end piece (Fig. 2A). In addition, there is a junctional region between the midpiece and the principal piece called the annulus, and the flagellum is connected to the head by a structure called the head-tail coupling apparatus (HTCA). Sperm heads contain the nucleus and an anterior acrosome, a vesicle of hydrolytic enzymes that are released by exocytosis in an acrosome reaction (reviewed by Hirohashi and Yanagimachi, 2018). The acrosome reaction is important for sperm passage through the zona pellucida (ZP) that surrounds the oocyte and for gamete fusion (Bhakta et al., 2019; Morohoshi et al., 2023). Thus, sperm motility and the acrosome reaction are important for fertilization, and abnormalities in these fertilizing processes are implicated in male infertility (Bhakta et al., 2019; Fesahat et al., 2020; Miyata et al., 2020a; Yogo, 2022).

As it is still difficult to recapitulate spermiogenesis *in vitro* (Ishikura et al., 2021), the functions of genes involved in mammalian spermiogenesis have been mainly analyzed using knockout (KO) mice. In recent years, the development of genome editing technologies has made it possible to create KO mice more efficiently and rapidly, which has dramatically advanced the analyses of spermiogenesis using mouse models (Miyata et al., 2016; Abbasi et al., 2018a; Yogo, 2022). In this Review, we describe how functional, mature spermatozoa develop during mammalian spermiogenesis, discussing the formation of the flagella and acrosome biogenesis by introducing recent progress revealed primarily by the analyses of mouse models (unless otherwise stated).

The flagellum

Sperm axonemes

The main structural component of the sperm flagella is the axoneme. Axonemes consist of a 9+2 microtubule structure with a central pair of singlet microtubules surrounded by nine peripheral microtubule doublets, which is similar to the structure of motile cilia in somatic cells (Inaba, 2011; Viswanadha et al., 2017; Miyata et al., 2020a) (Fig. 2A′). The central pair of microtubules form a complex with proteinous projections, which together form the central apparatus. Between the central and peripheral microtubules are macromolecular structures called radial spokes, and between the peripheral microtubule doublets are macromolecular structures called the nexin-dynein regulatory complexes (N-DRCs). Peripheral microtubule doublets consist of a circular A-tubule with outer dynein arms and inner dynein arms bound to it and an adjacent, incomplete B-tubule (Fig. 2A′). These dynein arms on the A-tubule use ATP energy to slide adjacent peripheral microtubule doublets, causing the flagellum to bend and driving motility.

Axoneme development

Axoneme formation is initiated in the early stages of spermiogenesis (e.g. steps 2-3 in mouse spermiogenesis; Fig. 1) (Russell et al., 1990; Irons and Clermont, 1982b; San Agustin et al., 2015). Axonemes of sperm flagella are thought to be formed by a similar mechanism to ciliary axonemes: the basal structure of axoneme formation is the centrosome, which is composed of distal and proximal centrioles (Avidor-Reiss et al., 2020) (Fig. 2Ba). It is currently unclear how the central pair microtubules is formed; recent studies indicate that katanin, which can sever microtubules, CAMSAPs, which can stabilize the minus end of microtubules, and WDR47, which concentrates CAMSAPs into the axonemal central lumen, are

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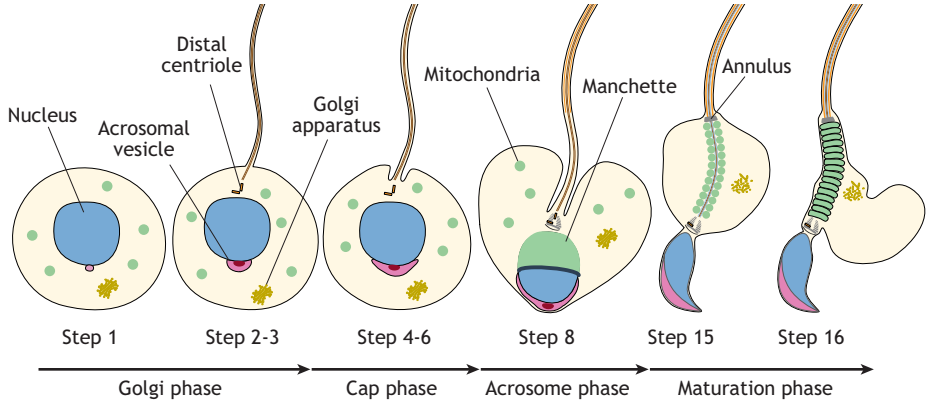

Fig. 1. Overview of spermiogenesis in mice. Drastic morphological changes occur from round spermatids to mature spermatozoa during spermiogenesis that can be divided into 16 steps in mice. At steps 2-3, axonemes elongate from the distal centriole. Acrosomal vesicles localized anterior to the nucleus elongate to form mature acrosomes. Spermiogenesis can also be divided into four phases (Golgi, cap, acrosome and maturation) according to the morphology of the acrosomal vesicle.

important for the initial formation of the central pair microtubules in motile cilia (Liu et al., 2021a). Among these proteins, KO of KATNB1, a regulatory subunit of katanin, causes multiple axonemal defects including missing central pair microtubules in mouse spermatozoa (O'Donnell et al., 2012) (Table 1; microtubule formation). In contrast to the central pair microtubules, it is known that both A-tubules and B-tubules of peripheral microtubule doublets elongate from the distal centriole that is localized nearly perpendicularly to the proximal centriole (Avidor-Reiss et al., 2020). Male mice deficient in proteins that build centrioles, such as CETN1 (Avasthi et al., 2013; Mashiko et al., 2013), CEP78 (Zhu et al., 2023), CEP128 (Zhang et al., 2022b) and CEP131 (Hall et al., 2013) (Table 1; microtubule formation), are infertile because sperm flagella do not form normally and include disruption of the 9+2 microtubule structure.

Peripheral microtubules elongated from the distal centriole function as 'rails' for motor proteins that transport molecules necessary for flagellum formation (reviewed by Pleuger et al., 2020; Hibbard et al., 2022). Kinesin motor complexes move from the flagellar base to the tip and dynein motor complexes move from the tip to the base (Pleuger et al., 2020; Hibbard et al., 2022). This process is called intraflagellar transport (IFT), in which IFT-A and IFT-B protein complexes with kinesin or dynein motors transport cargo proteins. Male mice that conditionally lack IFT-A components or IFT-B components in germ cells (except for IFT88) display abnormal sperm flagellum formation, indicating that IFT is essential not only for cilia but also sperm flagellum formation (summarized in Table 1; intraflagellar transport). Proteins constituting axonemal structures, such as outer dynein arms, inner dynein arms, radial spokes, N-DRCs and the central apparatus, are also transported by IFT. Of these structures, outer dynein arms, inner dynein arms and radial spokes are assembled – to some extent – in the cytoplasm before IFT transport (Hibbard et al., 2022; Lechtreck et al., 2022). For example, the pre-assembly of dynein has been studied in *Chlamydomonas* flagella, as well as zebrafish and mammalian cilia, and the chaperones and co-chaperones involved in the cytoplasmic pre-assembly have been identified (reviewed by Desai et al., 2018; Qiu and Roy, 2022). Some of the chaperones for the dynein pre-assembly, such as DNAAF6RT (Dong et al., 2014) and DNAAF5 (Horani et al., 2023), are involved in sperm flagellum formation and motility. In contrast to dynein arms and radial spokes, N-DRCs are likely assembled in a stepwise manner because the N-DRC core proteins, DRC2 and DRC4, are transported and assembled independently in the *Chlamydomonas* flagella (Saravanan et al., 2023). Furthermore, interactome analyses of DRC3 from mouse testes have shown that DRC3 interacts with IFT-B complex proteins, but other DRC proteins have not been detected (Zhou et al., 2023b).

HTCA formation

Axoneme extension from the distal centriole localized on the cell surface causes the plasma membrane to protrude from the cytoplasm (Russell et al., 1990) (Fig. 2Ba,C). The centrioles then migrate and associate with the nuclear envelope to bridge the plasma membrane with the nucleus (Fig. 2Bb,D). The centrioles and nucleus then move toward the cell surface on the opposite side, causing an inward folding of the plasma membrane called cytoplasmic invagination, which allows compartmentalization of the flagellum (Russell et al., 1990) (Fig. 2Bc,D,E). The cytoplasmic invagination disappears as the annulus migrates from the base of the sperm head to the junction between the midpiece and principal piece, and then mitochondrial sheath is formed (Fig. 2Bd,Be,Bf). The disruption of CCDC183 in mice causes an abnormal link between the plasma membrane and nuclear envelope by the centrioles, resulting in the loss of cytoplasmic invagination and collapse of axonemal microtubules (Shimada and Ikawa, 2023).

The connection between the proximal centriole and nucleus leads to the formation of the HTCA or the connecting pieces (reviewed by Wu et al., 2020). Mice lacking proteins that form the HTCA, such as SPATA6 (Yuan et al., 2015), PMFBP1 (Zhu et al., 2018; Sha et al., 2019b), SUN5 (Shang et al., 2017) and CNTLN (Zhang et al., 2021c), display acephalic (headless) spermatozoa syndrome. Mutations in SUN5 (Elkhatib et al., 2017; Shang et al., 2017) and PMFBP1 (Zhu et al., 2018; Sha et al., 2019b) have also been found in infertile human patients with decapitated spermatozoa (Table 1; HTCA formation). These results indicate that an appropriate connection among the nucleus, centrioles and plasma membrane is crucial for spermiogenesis.

Loss-of-function studies of axonemal proteins

The functions of sperm axonemal proteins have been elucidated by analyzing KO mice. Because these proteins are also often present in the axonemes of somatic cilia, KO mice could die before sexual maturity owing to abnormal formation and/or function of cilia. As it is not possible to examine gene function in mature spermatozoa in such cases, methods to avoid lethality have been applied (Miyata et al., 2020a), including the use of conditional KO mice (Kazarian et al., 2018), generating KO mice with mixed genetic backgrounds (McKenzie et al., 2018) and the creation of chimeric mice that possess both wild-type (WT) and KO cells (Oji et al., 2016; Oura et al., 2019). The KO of many axonemal proteins disrupt sperm flagellum formation (summarized in Table 1; axoneme components).

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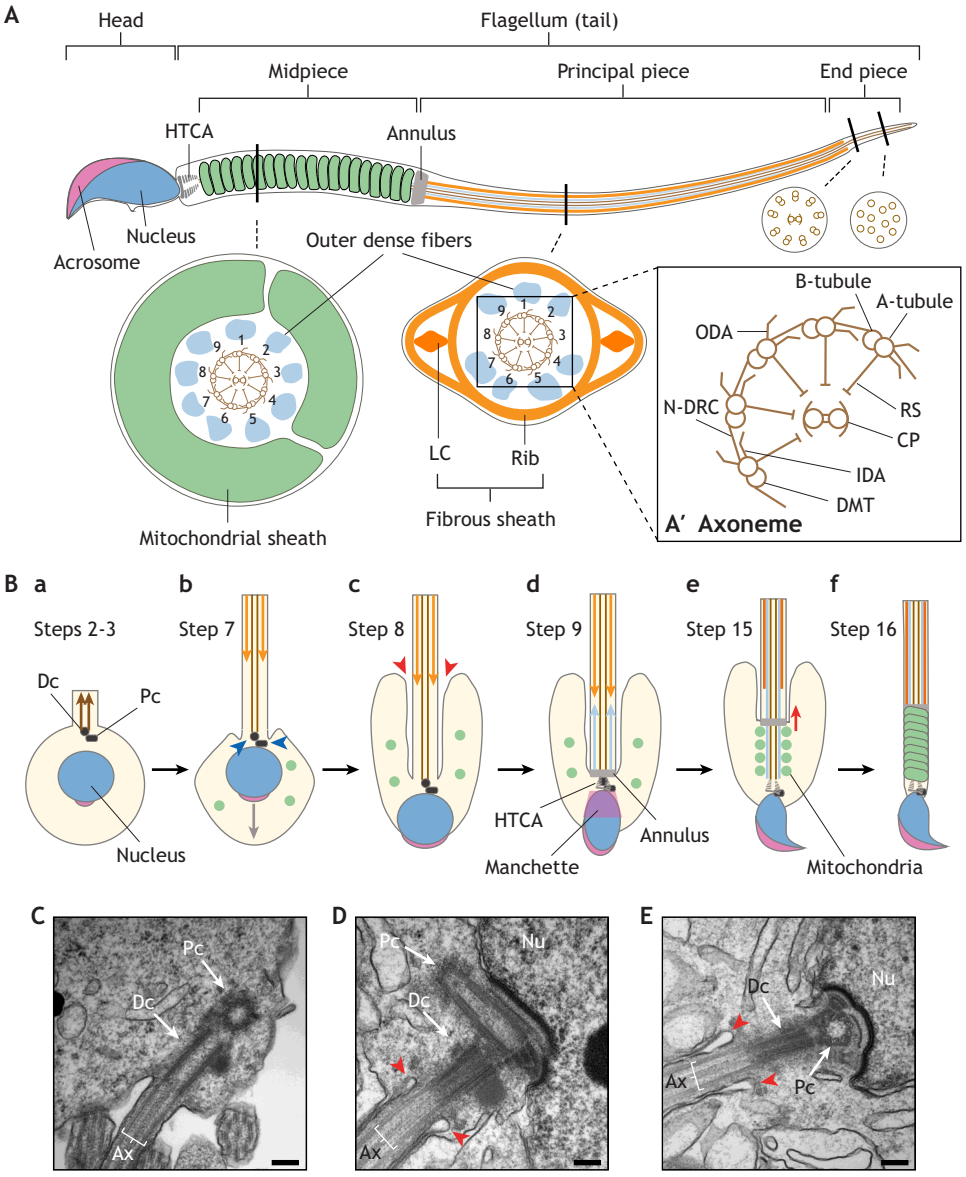

Fig. 2. Mature spermatozoa and flagellum formation. (A) Schematic of mouse mature spermatozoa, including transverse sections of the midpiece, principal piece and end piece. These structures are conserved in human spermatozoa. (A′) Organization of the axoneme. CP, central pair microtubule; DMT, doublet microtubule; HTCA, head-tail coupling apparatus; IDA, inner dynein arm; LC, longitudinal column; N-DRC, nexin-dynein regulatory complex; ODA, outer dynein arm; RS: radial spoke. The numbers 1 to 9 indicate the doublet microtubule numbers. Doublet 1 is defined as the doublet that lies on the line that bisects the central pair. (B) Schematic of flagellum formation. Brown arrows, axoneme elongation; blue arrowheads, centriole bridges between the plasma membrane and the nucleus; orange arrows, fibrous sheath elongation; light blue arrows, outer dense fiber elongation; red arrowheads, cytoplasmic invagination; red arrow, annulus migration. Dc, distal centriole; Pc, proximal centriole. (C) Transmission electron microscopy (TEM) picture of mouse step 2-3 spermatid. Axoneme (Ax) elongates from the distal centriole (Dc). Pc, proximal centriole. (D,E) TEM pictures of mouse step 8 (D) and step 9 (E) spermatids. Pc docks onto the nuclear envelope. Centrioles and nucleus move toward the cell surface on the opposite side, forming cytoplasmic invagination (red arrowheads). Nu, nucleus. TEM pictures were taken as previously described (Shimada et al., 2019). Scale bars: 200 nm.

Among these, PCDP1 (also known as CFAP221; Lee et al., 2008), SPEF2 (Sironen et al., 2011), HYDIN (Lechtreck et al., 2008; Oura et al., 2019), DRC7 (Morohoshi et al., 2020) and DRC9 (IQCG; Li et al., 2014) are thought to be present in both flagella and cilia, but KO mice of these proteins exhibit only abnormal sperm flagellum formation and no overt abnormalities in cilia formation, indicating that the mechanisms controlling the formation of sperm flagella and cilia are different. Indeed, recent analyses using cryo-electron tomography (cryo-ET) have revealed some such differences (Leung et al., 2021; Chen et al., 2023). For example, both cilia and sperm flagella have three types of radial spokes, RS1, RS2 and RS3, which are repeatedly arranged at intervals of 96 nm along the long axis of the axoneme (Fig. 3A). In mammalian spermatozoa, there are barrel-shaped structures associated with RS1 and a bridge connecting RS2 and RS3 that have not been found in ciliary axonemes (Leung et al., 2021; Chen et al., 2023), which may be a reason for the phenotypic differences. Alternatively, sperm flagella contain accessory structures that are not present in cilia (discussed below). It is possible that sperm flagella show more serious defects than cilia because of incorrect interactions between axonemes and these sperm-specific flagellar structures. Other possibilities include the presence of multiple isoforms for one gene or the evolution of new genes, which may lead to the compensation of gene functions in cilia. Further studies using cryo-ET are expected to reveal differences in axonemal structures among various species and cell types, which may lead to a better understanding of their formation and different motility patterns.

Although it has often been reported that axonemal protein-disrupted mice show abnormal sperm flagellum formation, there are also some KO mice that show defects in sperm motility but not in overt flagellum formation. For example, some axonemal proteins [e.g. DNAH1 (Neesen et al., 2001), DNAH3 (Meng et al., 2024), KIF9 (Miyata et al., 2020b), TCTE1 (Castaneda et al., 2017), LRRC23 (Zhang et al., 2021b; Hwang et al., 2023), IQUB (Zhang et al., 2022c) and SPAG16L (PF20; Zhang et al., 2006), described in Table 1; axoneme components] are essential for motility regulation, but no overt abnormalities have been found in flagellum morphology. The lack of obvious abnormalities in flagellum formation after disruption of these axonemal proteins may be due to their localization

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Table 1. Summary of factors required for mouse sperm axoneme formation.

Role	Protein	Loss-of-function phenotype (mouse)	Reference(s)
Microtubule formation			
Sever microtubules	KATNB1	Abnormal sperm flagellum formation; disordered meiosis	O'Donnell et al., 2012
Centriole component	CEP78	Abnormal sperm flagellum formation; associated with male infertility in human	Zhu et al., 2023
	CEP128	Abnormal sperm flagellum formation; associated with cryptozoospermia (virtually no spermatozoa) in human	Zhang et al., 2022b
	CEP131	Abnormal sperm flagellum formation	Hall et al., 2013
	CETN1	Abnormal sperm flagellum formation; centriole rearrangement failure	Avasthi et al., 2013; Mashiko et al., 2013
Intraflagellar transport			
IFT-A component	IFT140	Abnormal sperm flagellum formation; associated with oligoasthenoteratozoospermia in human	Zhang et al., 2018; Wang et al., 2019
IFT-B component	IFT20	Abnormal sperm flagellum formation	Zhang et al., 2016
	IFT25	Abnormal sperm flagellum formation	Liu et al., 2017b
	IFT27	Abnormal sperm flagellum formation	Zhang et al., 2017
	IFT74	Abnormal sperm flagellum formation; associated with MMAF in human	Shi et al., 2019; Lorès et al., 2021
	IFT81	Abnormal sperm flagellum formation	Qu et al., 2020
	IFT88	Abnormal sperm flagellum formation	San Agustin et al., 2015
	IFT172	Abnormal sperm flagellum formation	Zhang et al., 2020b
Dynein pre-assembly	DNAAF5	Sperm motility defects; absence of dynein arms; associated with PCD in human	Horani et al., 2023
	DNAAF6RT	Sperm motility defects; absence of dynein arms	Dong et al., 2014
Flagellar compartmentalization			
Link between the plasma membrane and nucleus	CCDC183	Abnormal sperm flagellum formation; collapse of axonemal microtubules	Shimada and Ikawa, 2023
HTCA formation			
HTCA component	CNTLN	Decapitated spermatozoa	Zhang et al., 2021c
	PMFBP1	Decapitated spermatozoa; associated with acephalic spermatozoa syndrome in human	Sha et al., 2019b; Zhu et al., 2018
	SPATA6	Decapitated spermatozoa	Yuan et al., 2015
	SUN5	Decapitated spermatozoa; associated with acephalic spermatozoa syndrome in human	Shang et al., 2017; Elkhatib et al., 2017
Ornithine decarboxylase inhibitor	OAZ3	Decapitated spermatozoa	Tokuhiro et al., 2009
Axoneme component			
Outer dynein arm heavy chain	DNAH8	Abnormal sperm flagellum formation; associated with MMAF in human	Liu et al., 2020b
	DNAH17	Abnormal sperm flagellum formation; associated with MMAF in human	Whitfield et al., 2019; Zhang et al., 2020a
Inner dynein arm heavy chain	DNAH1	Sperm motility defects; associated with MMAF in human	Neesen et al., 2001; Ben Khelifa et al., 2014
	DNAH2	Abnormal sperm flagellum formation; associated with MMAF in human	Hwang et al., 2021
	DNAH3	Abnormal sperm flagellum formation; associated with MMAF in human	Meng et al., 2024
	DNAH10	Abnormal sperm flagellum formation; associated with MMAF in human	Tu et al., 2021b
Central apparatus	CFAP54	Abnormal sperm flagellum formation; associated with PCD in human	McKenzie et al., 2015; Zhao et al., 2023
	CFAP69	Abnormal sperm flagellum formation; associated with MMAF in human	Dong et al., 2018; He et al., 2019
	HYDIN	Abnormal sperm flagellum formation; associated with PCD in human	Oura et al., 2019; Olbrich et al., 2012
	KIF9	Sperm motility defects; associated with asthenozoospermia in human	Miyata et al., 2020b; Meng et al., 2023
	PCDP1 (CFAP221)	Abnormal sperm flagellum formation	Lee et al., 2008
	SPAG6	Abnormal sperm flagellum formation; associated with MMAF in human	Sapiro et al., 2002; Xu et al., 2022
	SPAG16L (PF20)	Sperm motility defects	Zhang et al., 2006
	SPAG17	Abnormal sperm flagellum formation	Kazarian et al., 2018
	SPEF2	Abnormal sperm flagellum formation; associated with MMAF in human	Sironen et al., 2011; Liu et al., 2019a, 2020a; Sha et al., 2019a
N-DRC	DRC1	Abnormal sperm flagellum formation; associated with PCD in human	Zhang et al., 2021a; Wirschell et al., 2013; Lei et al., 2022

Continued

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Table 1. Continued

Role	Protein	Loss-of-function phenotype (mouse)	Reference(s)
	DRC3	Abnormal sperm flagellum formation; associated with MMAF in human	Zhou et al., 2023b
	DRC7	Abnormal sperm flagellum formation	Morohoshi et al., 2020
	DRC9 (IQCG)	Abnormal sperm flagellum formation	Li et al., 2014
	TCTE1	Sperm motility defects; associated with asthenozoospermia in human	Castaneda et al., 2017; Zhou et al., 2022a
Radial spoke	CFAP61	Abnormal sperm flagellum formation; associated with MMAF in human	Liu et al., 2021b
	DNAJB13	Abnormal sperm flagellum formation; associated with PCD in human	Oji et al., 2016; El Khouri et al., 2016
	IQUB	Sperm motility defects; associated with asthenozoospermia in human	Zhang et al., 2022c, 2023b
	LRRC23	Sperm motility defects; associated with asthenozoospermia in human	Zhang et al., 2021b; Hwang et al., 2023
	NME5	Abnormal sperm flagellum formation; associated with PCD in human	Vogel et al., 2012; Cho et al., 2020
	RSPH1	Abnormal sperm flagellum formation; associated with PCD in human	Tokuhiro et al., 2008; Kott et al., 2013; Vanaken et al., 2017
	RSPH6A	Abnormal sperm flagellum formation	Abbasi et al., 2018b

Relevance to human pathology is indicated where relevant. IFT, intraflagellar transport; MMAF, multiple morphological abnormalities of the flagella; PCD, primary ciliary dyskinesia.

to the periphery rather than constituting the core of the macromolecular structures. It is also possible that these axonemal proteins have a regulatory role and not a structural role.

Identifying new axonemal proteins

Biochemical and genetic analyses have been the main approaches to identify the proteins that compose the axonemes of cilia and flagella (Inaba, 2011; Viswanadha et al., 2017; Miyata et al., 2020a). Recently, cryo-electron microscopy (cryo-EM) combined with artificial intelligence modeling has made great progress in identifying the proteins that make up the axoneme, as well as understanding their localization and interaction (Ma et al., 2019; Gui et al., 2021, 2022b; Han et al., 2022). This strategy has also been used in mammalian spermatozoa to identify new proteins that comprise the inner structure of the peripheral microtubule including sperm-specific proteins (Gui et al., 2022a; Leung et al., 2023; Zhou et al., 2023a); for example, these studies show that the A-tubules of mammalian spermatozoa are packed with bundles of tektin, which is more filled with proteins than that of mammalian respiratory cilia, indicating that molecular components of the axoneme are different between sperm flagella and somatic cilia.

Axonemal proteins in human pathology

Mutations in genes encoding axonemal proteins can lead to male infertility in humans as well. Primary ciliary dyskinesia (PCD) is a genetic disease in which motile cilia do not form or function properly, and is associated with bronchiectasis, chronic sinusitis, situs inversus and male infertility. Several causative genes have been identified for PCD, some of which encode axonemal proteins (described in Table 1; axoneme components). However, there are cases in which mutations are found in genes encoding axonemal proteins but only male infertility is observed. These patients may have defects in sperm flagellum formation, which is a condition called multiple morphological abnormalities of the flagella (MMAF), a subtype of asthenoteratozoospermia (a condition characterized by reduced sperm motility with malformed morphology) (described in Table 1; axoneme components). In addition, asthenozoospermia (a condition characterized by reduced sperm motility without malformed morphology) could also be caused by mutations in genes encoding axonemal proteins (described in Table 1; axoneme components).

Summary

Except for the HTCA formation that is sperm-specific, the proteins involved in the axoneme formation of sperm flagella are similar to those of somatic cilia, including centrioles, IFT, dynein pre-assembly and axonemal components. However, as mentioned above, KO of axonemal proteins present in both sperm flagella and somatic cilia often results in impaired flagellum formation in spermatozoa, in contrast to the absence of obvious morphological abnormalities in cilia. In spermatozoa, there may be a quality control mechanism that prevents further formation if the axonemes are abnormal (Abbasi et al., 2018b). Alternatively, it is possible that axonemes may be fully formed in spermatozoa but unstable and disintegrate. For example, unlike cilia, sperm centrioles need to dock to the nucleus (Fig. 2B), and this process may fail due to abnormal axoneme structure, resulting in the collapse of axonemes (Shimada and Ikawa, 2023). Detailed analysis is needed to determine the extent to which axonemes are formed in KO spermatozoa. Infertile patients with disrupted flagellum structures have been found (Table 1); elucidating the mechanism of axoneme formation with the help of gene manipulation in mice and cryo-EM will clarify the etiology of human infertility.

Accessory structures

The axoneme of the sperm flagellum is accompanied by distinct accessory structures. The midpiece possesses a structure called the mitochondrial sheath, in which mitochondria wrap around the axoneme and elastic fibers called outer dense fibers (ODFs) in a helical pattern (Figs 2A,3B). The principal piece is accompanied by a structure called the fibrous sheath (Figs 2A,3C). At the boundary between the midpiece and principal piece is a ring-like structure called the annulus, which is thought to act as a diffusion barrier between the two compartments (Kwitny et al., 2010). Meanwhile, in the end piece, there are no accessory structures and the axonemal 9+2 structure is not preserved in the most distal part, with the peripheral doublet microtubules transitioning into singlets (Figs 2A,3D) (Zabeo et al., 2019; Leung et al., 2021). In addition, the ODFs exist parallel to each peripheral microtubule, beginning from the HTCA and extending to the principal piece (Figs 2A,3C). These accessory structures are thought to be important for providing elasticity to the flagellum and for regulating sperm motility (Lehti and

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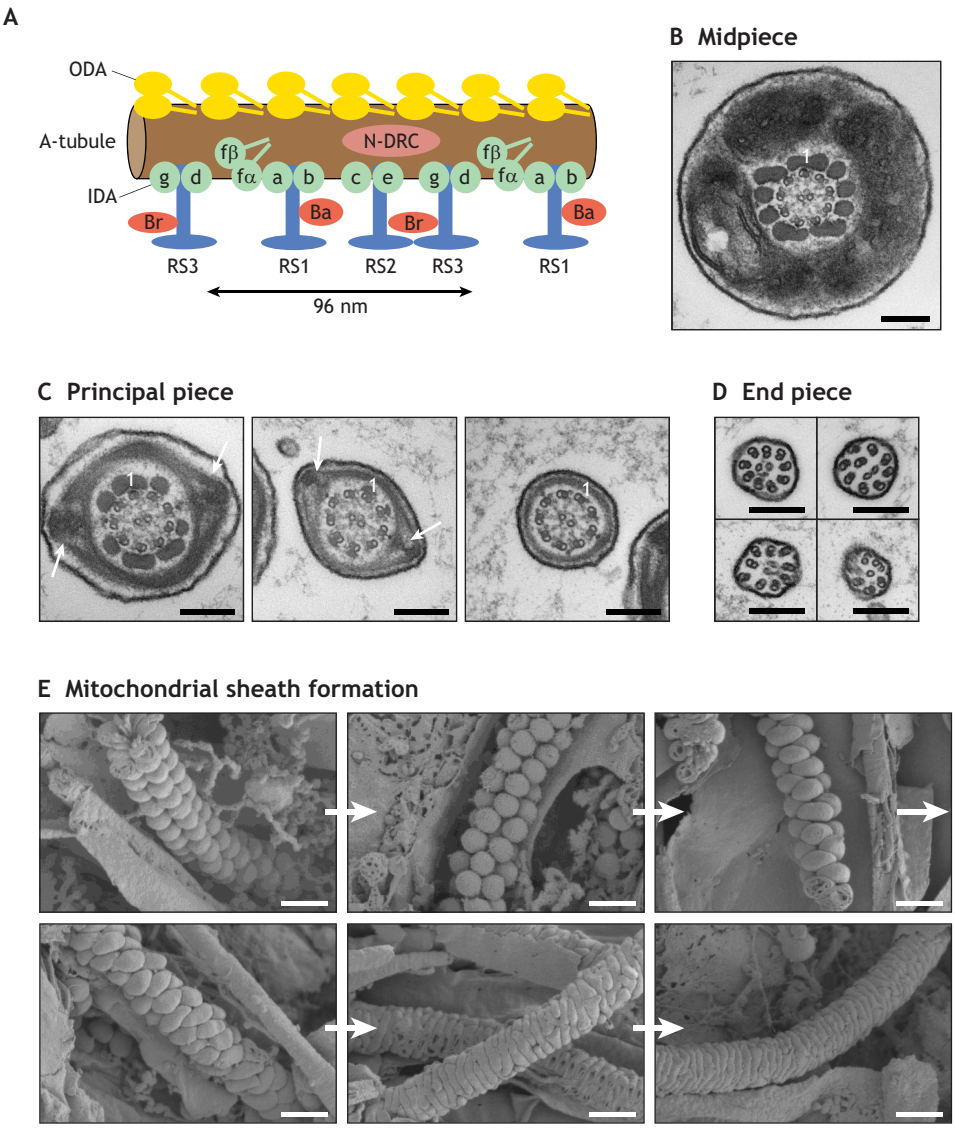

Fig. 3. Sperm flagellum. (A) Schematic of radial spokes (RSs) in sperm axoneme. RS1, RS2 and RS3 are repeatedly arranged at intervals of 96 nm along the long axis. Ba, a barrel-shaped structure associated with RS1; Br, a bridge connecting RS2 and RS3. (B) Transmission electron microscopy (TEM) picture of midpiece in mature spermatozoa. Doublet 1 is marked. (C) TEM pictures of principal pieces in mature spermatozoa. Cross sections are shown from the proximal region (left) to the distal region (right). The number of ODFs decreases from the proximal region to the distal region. White arrows indicate longitudinal columns. Doublet 1 is marked. (D) TEM pictures of end pieces in mature spermatozoa. (E) Formation of the mitochondrial sheath in the testis observed using scanning electron microscopy (SEM). Spherical mitochondria align around the flagellum and change their shape to crescent-like mitochondria (upper panels). Mitochondria then continue to elongate to form the mitochondrial sheath (lower panels). TEM and SEM pictures were taken as described previously (Shimada et al., 2019). Scale bars: 200 nm (B-D); 1 µm (E).

Sironen, 2017). Here, we describe the development of the annulus and ODFs before describing the formation of the midpiece and principal piece. Molecules involved in the formation of the end piece are poorly understood and are not discussed further in the following sections.

Annulus

The annulus emerges at step 9 of mouse spermiogenesis and is first located at the boundary between the flagellar compartment and head cytoplasm, near the HTCA (Fig. 2Bd) (Yuan et al., 2015). As spermiogenesis progresses, the annulus migrates distally along the flagellum (Fig. 2Be,Bf) (Guan et al., 2009). The fibrous sheath of the future principal piece is present distal from the annulus, and mitochondria assemble proximally from the annulus to form the mitochondrial sheath of the midpiece.

The annulus is a septin cytoskeletal structure that is composed of SEPTIN2, SEPTIN4, SEPTIN6, SEPTIN7 and SEPTIN12 (Kissel et al., 2005; Ihara et al., 2005; Kuo et al., 2015). KO of SEPTIN4 or loss of the GTP binding ability of SEPTIN12 results in a disorganized annulus and subsequent abnormal flagellar bending at the annulus region (Table 2; annulus) (Kissel et al., 2005; Ihara et al., 2005; Kuo et al., 2015). Recently, it has been shown that the CBY3/CIBAR1 complex positions the annulus by maintaining the plasma membrane rigidity. KO of CBY3 results in the annulus migration over the fibrous sheath into the principal piece (Hoque et al., 2024).

Outer dense fibers

During steps 8-9 of mouse spermiogenesis, the ODFs elongate from the HTCA in the sperm cytoplasm toward the distal end of the spermatozoa (Fig. 2Bd) (Irons and Clermont, 1982b; Oko and Clermont, 1989). During the elongation, nine ODFs extend in parallel to each peripheral microtubule; nine ODFs are observed in the midpiece (Fig. 3B), but ODF numbers 3 and 8 are shorter than the other seven ODFs (Vera et al., 1984) and are rarely observed in the principal piece (Figs 2A, 3C). This may be owing to the presence of longitudinal columns (LCs) of the fibrous sheath (see below) outside the position of ODF numbers 3 and 8 (Fig. 2A) (Zhao et al., 2018). The remaining seven ODFs also disappear in the middle of the principal piece; when transverse sections of the principal piece are observed by transmission electron microscopy (TEM), different ODF numbers can be observed ranging from a flagellum with nine ODFs to

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Table 2. Summary of factors required for mouse accessory structure formation

Role	Protein	Loss-of-function phenotype (mouse)	Reference(s)
Annulus			
Annulus component	SEPTIN4	Disorganized annulus and subsequent abnormal flagellar bending at the annulus region; associated with asthenoteratozoospermia in human	Kissel et al., 2005; Ihara et al., 2005; Wang et al., 2022
	SEPTIN12	Disorganized annulus and subsequent abnormal flagellar bending at the annulus region (D197N mutation impairing GTP binding); associated with disorganized annulus and asthenozoospermia in human	Kuo et al., 2015, 2012
Annulus positioning	CBY3	Annulus migration over the fibrous sheath into the principal piece	Hoque et al., 2024
Outer dense fiber			
ODF component	ODF1	Detachment of the sperm heads and abnormal morphology of the ODF and the mitochondria sheath	Yang et al., 2012
	ODF2	Embryonic lethal; heterozygous infertility; a heterozygous missense mutation was found in MMAF	Salmon et al., 2006; Ito et al., 2019; Zhu et al., 2022
Midpiece			
Mitochondrial sheath formation	ARMC12	Abnormal mitochondrial sheath formation, sperm motility defects, bent flagella; associated with multiple midpiece defects in human	Shimada et al., 2021; Liu et al., 2023c
	GK2	Abnormal mitochondrial sheath formation, sperm motility defects, bent flagella	Chen et al., 2017; Shimada et al., 2019
	MISFA	Abnormal mitochondrial sheath formation, sperm motility defects, bent flagella	Mise et al., 2022
	TBC1D21	Abnormal mitochondrial sheath formation, sperm motility defects, bent flagella	Shimada et al., 2021; Wang et al., 2020a; Chen et al., 2022
	VDAC3	Abnormal mitochondrial sheath formation, sperm motility defects, bent flagella	Mise et al., 2022
Principal piece			
Glycolytic enzyme	GAPDHS	Sperm motility defects	Miki et al., 2004
Fibrous sheath component	AKAP3	Fibrous sheath abnormalities especially in rib formation; associated with asthenoteratozoospermia in human	Xu et al., 2020; Liu et al., 2023a
	AKAP4 (AKAP82)	Loss of fibrous sheath; associated with MMAF in human	Miki et al., 2002; Zhang et al., 2022a
	CABYR	Fibrous sheath abnormalities especially in rib formation	Young et al., 2016
	FSIP2	Abnormal formation of fibrous sheath; associated with MMAF in human	Fang et al., 2021; Martinez et al., 2018; Liu et al., 2019b
	ROPN1 and ROPN1L	Double knockout spermatozoa show impaired motility and reduced amount of AKAP3	Fiedler et al., 2013
Fibrous sheath formation	DNALI1	Abnormal asymmetric positioning of two fibrous sheath longitudinal columns; associated with asymmetrical development of sperm fibrous sheath and asthenozoospermia in human	Wu et al., 2023
	ENO4	Fibrous sheath abnormalities; associated with asthenozoospermia and abnormal sperm morphology in human	Nakamura et al., 2013; Nawaz et al., 2024
	IRGC	Fibrous sheath abnormalities	Kaneda et al., 2022

Relevance to human pathology is indicated where relevant. MMAF, multiple morphological abnormalities of the flagella.

a flagellum with none depending on the position. Additionally, the flagellum thins as the principal piece moves towards the distal end (Fig. 3C).

The major ODF proteins are ODF1 and ODF2 (Table 2; outer dense fiber) (Schalles et al., 1998). Mice lacking ODF1 are infertile due to the detachment of the sperm heads, as well as abnormal morphology of the ODF and the mitochondria sheath (Yang et al., 2012). In contrast, *Odf2* KO mice show embryonic lethality (Salmon et al., 2006), whereas heterozygous mice created with a different KO strategy exhibit male infertility due to sperm head-neck separation (Ito et al., 2019). Acephalic spermatozoa shown in *Odf1* and *Odf2* mutant mice are consistent with the fact that ODFs elongate from the HTCA. A heterozygous missense mutation in *ODF2* was found in an MMAF infertile patient (Zhu et al., 2022).

Midpiece

During step 15 of mouse spermiogenesis, as the annulus migrates distally from the base of the sperm head (see above) (Guan et al., 2009), spherical mitochondria assemble in the future midpiece region (Fig. 2Be,Bf) (Otani et al., 1988). Mitochondria assemble outside the axoneme and the ODF, and form a coiled mitochondrial sheath by elongating laterally to the flagellum (Fig. 3E) (Otani et al., 1988; Ho and Wey, 2007). ARMC12 acts as an adhesion factor between the mitochondria during mitochondrial sheath formation (Shimada et al., 2021). ARMC12 is involved in the mitochondrial adhesion by interacting with mitochondrial proteins GK2, TBC1D21, VDAC2 and VDAC3; both VDAC2 and VDAC3 bind to two sperm-specific polypeptides, Kastor and Polluks, which are encoded by a single locus *Misfa* (Shimada et al., 2019, 2021; Wang et al., 2020a; Chen et al., 2017, 2022; Mise et al., 2022). KO of *Armc12*, *Gk2*, *Tbc1d21*, *Vdac3* or *Misfa* in mice results in abnormal mitochondrial sheath formation, impaired sperm motility and infertility (Table 2; midpiece) (Shimada et al., 2019, 2021; Wang et al., 2020a; Chen et al., 2017, 2022; Mise et al., 2022). These KO mouse models also exhibit abnormally bent flagella, suggesting that the mitochondrial sheath is important to maintain the flagellar morphology. In addition to the structural role, the mitochondrial sheath is thought to be necessary as a site to

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produce ATP required for sperm motility; however, this idea requires further investigation because it is also suggested that ATP synthesis by oxidative phosphorylation may be less significant in mouse sperm motility (Mukai and Okuno, 2004; Shimada et al., 2024).

Principal piece

The fibrous sheath present in the principal piece surrounds the axonemes and ODFs (Figs 2A, 3C); it is divided into two LCs and closely arrayed semicircular ribs (Eddy et al., 2003). The fibrous sheath anlagen appear at the distal end of the axoneme as soon as axonemal elongation begins (Irons and Clermont, 1982a) and extend from distal toward the proximal end (Fig. 2Bb) (Oko and Clermont, 1989). The formation of principal piece is well studied in rats (Irons and Clermont, 1982a; Oko and Clermont, 1989). Therefore, we introduce the formation of the principal piece in rats and corresponding steps in mice. In rats, two thin LCs appear on the distal side of the axoneme in step 2-3 spermatids (corresponding steps 2-3 in mice) and extend until step 10 (corresponding step 10 in mice) (Fig. 2B). During steps 11-15 in rat spermatids (corresponding steps 11-13 in mice), ribs begin to appear and bind to the LCs, finally completing the fibrous sheath formation at step 15 spermatids (corresponding step 14 in mice) (Irons and Clermont, 1982a; Oko and Clermont, 1989).

The fibrous sheath has an insoluble keratin-like structure (Bedford and Calvin, 1974; O'Brien and Bellvé, 1980) and is thought to be related to flagellar flexibility and movement (Eddy et al., 2003). The fibrous sheath also anchors glycolytic enzymes. For example, GAPDHS is localized to the fibrous sheath ribs (Tanii et al., 2007), and the disruption of *Gapdhs* in mice induces a low amount of ATP supplied by glycolysis in spermatozoa, resulting in decreased sperm motility and infertility (Miki et al., 2004). AKAP4 (AKAP82) is a major protein of the fibrous sheath (Moss et al., 1997) and its deletion in mice causes almost complete loss of the fibrous sheath, impaired sperm motility and sterility (Miki et al., 2002). There are also other KO mouse lines that show abnormal fibrous sheath structures (summarized in Table 2; principal piece). For example, AKAP3 and CABYR constitute the fibrous sheath and deletion of these proteins causes major abnormalities especially in rib formation, but not in LC formation (Young et al., 2016; Xu et al., 2020). Furthermore, KO of IRGC, a testis-enriched immunity related GTPase, results in abnormal organization of both LCs and ribs, and reduced sperm motility in mice (Kaneda et al., 2022). More recently, KO of DNALI1, a component of dynein motors, has shown abnormal asymmetric positioning of two LCs, suggesting that DNALI1 controls the formation of two LCs in the correct symmetric position (Wu et al., 2023). Mutations in *AKAP3*, *AKAP4* and *DNALI1* have been reported in infertile patients with disrupted flagellar structures and impaired sperm motility (Liu et al., 2023a; Zhang et al., 2022a; Wu et al., 2023).

Summary

In contrast to the axoneme formation of sperm flagella, which is similar to that of somatic cilia, accessory structures are sperm-specific, and little was known about the mechanism regulating the formation. Analyses of KO mice have now revealed a large number of proteins that are important in the formation of accessory structures, but further analyses are needed to understand their interactions and regulatory mechanisms. For example, several proteins involved in the mitochondrial sheath formation have been identified, including ARMC12. Transient expression of ARMC12 in cultured cells results in mitochondrial aggregation, suggesting that ARMC12 has a role in mitochondrial adhesion by interacting with mitochondrial proteins (Shimada et al., 2021). However, it is unknown how the adhered mitochondria change their morphology to form highly packed mitochondrial sheaths (Fig. 3E). The involvement of GK2, TBC1D21, VDAC2, VDAC3 or MISFA, which interact with ARMC12, is suggested (Shimada et al., 2021, 2019; Wang et al., 2020a; Chen et al., 2017, 2022; Mise et al., 2022), but further analyses are needed to determine how this protein complex is involved in the mitochondrial sheath formation. It is also unclear how ODF1 and ODF2, the major components of ODF, and AKAP4, the major component of fibrous sheath, form their structures and how their formation is spatiotemporally regulated. Recently, DNALI1 has been shown to be involved in the symmetric localization of two LCs in the fibrous sheath (Wu et al., 2023). Further analyses of DNALI1 may provide insight into the spatial control mechanisms of the LC formation.

Sperm head

Sperm heads consist of a nucleus and an acrosome located anterior to the nucleus (Fig. 2A). During spermiogenesis, the nucleus is condensed. One of the major processes of nuclear condensation is the replacement of histones with protamines that are cysteine-rich for disulfide bond formation (reviewed by Okada, 2022). The manchette, a sperm-specific and microtubule-based structure surrounding the caudal region of sperm heads (Fig. 4), is also involved in nuclear condensation in the posterior side (reviewed by Lehti and Sironen, 2016). On the anterior side, nuclear condensation is associated with the acrosome formation, which is anchored to the nuclear membrane by a cytoskeletal structure called the acroplaxome (Fig. 4). Here, we focus on proteins involved in the acrosome formation.

The acrosome

As mentioned earlier, the acrosome is a vesicle that undergoes exocytosis for the acrosome reaction. It is thought that enzymes during the acrosome reaction dissolve the ZP allowing spermatozoa to reach the oocyte. Acrosin is the major proteolytic enzyme localized in the acrosome (Klemm et al., 1991), but mouse spermatozoa lacking acrosin do not show impaired ZP penetration or reduced fertility (Table 3; acrosome reaction) (Baba et al., 1994). However, disruption of acrosin in hamsters results in infertility due to impaired ZP penetration, indicating that acrosin is the enzyme dissolving the ZP – at least in hamsters (Hirose et al., 2020). In mice, physical thrusting by sperm motility may contribute more significantly to the ZP penetration, allowing spermatozoa to pass through the ZP even when acrosin is disrupted.

The acrosome reaction is important for not only ZP penetration but also sperm fusion with oocytes because IZUMO1, a sperm transmembrane protein essential for sperm-oocyte fusion (Inoue et al., 2005), is localized in the inner and outer acrosomal membrane and is only exposed in the equatorial segment (where the fusion takes place) after the acrosome reaction (Satouh et al., 2012). The acrosome reaction is impaired in mice lacking FER1L5, a C2 domain-containing ferlin protein expressed predominantly in the testis (Morohoshi et al., 2023). Although the motility of *Fer1l5* KO spermatozoa is comparable with that of WT spermatozoa, the ZP penetration and sperm-oocyte fusion are impaired in *Fer1l5* KO mice, confirming that the acrosome reaction is important for both processes. As the acrosome reaction is essential for ZP penetration and sperm-oocyte fusion, abnormal acrosome formation could lead to male infertility, a condition called globozoospermia (reviewed by Fesahat et al., 2020). Globozoospermia is often associated with abnormal midpiece morphology (Battaglia et al., 1997), suggesting

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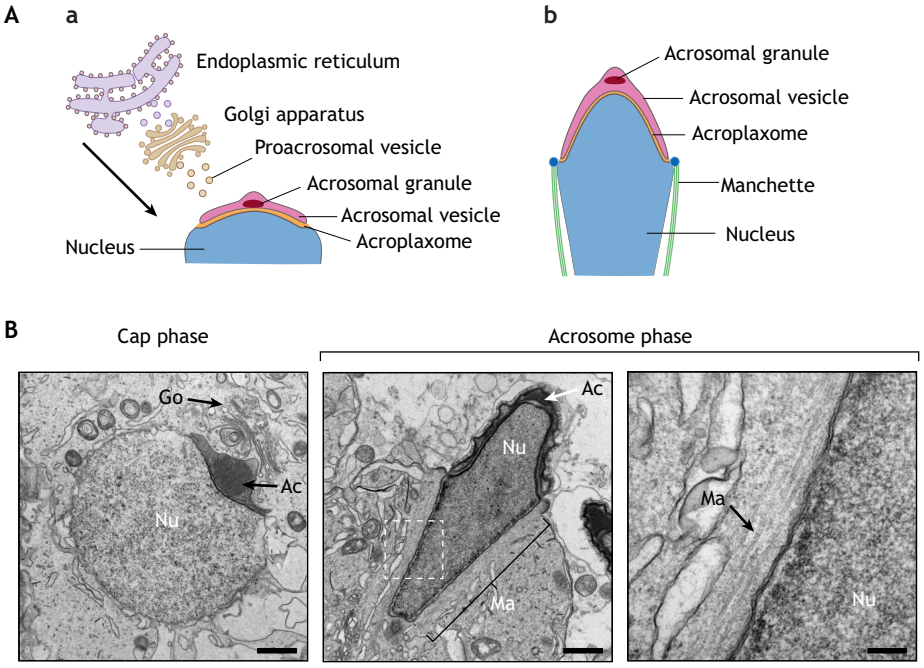

Fig. 4. Acrosome formation. (A) Schematic of the acrosome formation at the cap phase (Aa) and acrosome phase (Ab). Proacrosomal granules derived from the Golgi apparatus fuse to form and enlarge the acrosomal vesicle. The acrosomal vesicle is attached to the nucleus via the acroplaxome. Manchette, localized in the caudal region of sperm heads, is involved in nuclear elongation. (B) Transmission electron microscopy (TEM) pictures of the acrosome formation at the cap phase and the acrosome phase. Ac, acrosomal vesicle; Go, Golgi apparatus; Ma, manchette; Nu, nucleus. Right panel is magnification of white dashed boxed area in center panel. TEM pictures were taken as previously described (Shimada et al., 2019). Scale bars: 1 µm (left and center); 200 nm (right).

a link between the acrosome formation and mitochondrial sheath formation.

Acrosome formation

Acrosome formation proceeds along four phases corresponding to the steps of mouse spermiogenesis: Golgi (steps 1-3), cap (steps 4-7), acrosome (steps 8-12) and maturation (steps 13-16) (Leblond and Clermont, 1952; Clermont and Leblond, 1955) (Fig. 1). During the Golgi phase, Golgi-derived proacrosomal vesicles fuse to form a single large vesicle located next to the nucleus. During the cap phase, the acrosomal vesicle enlarges by fusion of additional Golgi-derived proacrosomal vesicles and spreads over the surface of the nucleus to form a cap-like structure (Fig. 4Aa,B). As the nucleus condenses and elongates during the acrosome phase, the acrosomal vesicle also elongates (Fig. 4Ab,B). Finally, during the maturation phase, the nucleus and acrosomal vesicle elongate further, completing the acrosome formation. Here, we describe proteins involved in the major processes of acrosome formation (summarized in Table 3; acrosome biosynthesis).

Budding, transport and fusion of proacrosomal vesicles

The first step of acrosome biogenesis is the budding of proacrosomal vesicles from the Golgi apparatus during the Golgi phase. These Golgi-derived proacrosomal vesicles fuse to form a single large vesicle located next to the nucleus (Fig. 4Aa). Golgi-localized proteins that are essential for acrosome formation include SMAP2 (Funaki et al., 2013), PICK1 (Xiao et al., 2009) and GOPC (Yao et al., 2002) (see Table 3; acrosome biosynthesis). SMAP2 is an Arf GTPase-activating protein that binds to clathrin and clathrin-assembly proteins, and facilitates the formation of clathrin-coated vesicles (Natsume et al., 2006). Deletion of SMAP2 in mice results in an abnormal increase in the diameter of proacrosomal vesicles and disrupted acrosome formation (Funaki et al., 2013). PICK1 is a peripheral membrane protein with a BAR (Bin/amphiphysin/Rvs) domain that increases membrane curvature for vesicle formation (Peter et al., 2004). PICK1 and GOPC co-localize in the Golgi apparatus, and loss of either protein results in abnormal acrosome formation (Xiao et al., 2009; Yao et al., 2002). PICK1 and GOPC are also localized to proacrosomal vesicles and are suggested to be involved in the transport of proacrosomal vesicles as well.

Proacrosomal vesicles transported from the Golgi apparatus then fuse to form and enlarge the acrosomal vesicle adjacent to the nucleus at the Golgi and cap phases (Fig. 4Aa). HRB (AGFG1), which is localized to the surface of the proacrosomal vesicle and the acrosomal vesicle, is required for proacrosomal vesicle fusion, with loss of *Hrb* resulting in abnormal acrosome formation (Kang-Decker et al., 2001). In addition, proteins involved in autophagy play roles in the transport and fusion of proacrosomal vesicles. For example, testis-specific KO of ATG7, which is involved in autophagosome formation, results in impaired transport and fusion of proacrosomal vesicles (Wang et al., 2014). Deletion of other autophagy-associated proteins [e.g. TBC1D20 (Sidjanin et al., 2016), SIRT1 (Liu et al., 2017a), TDRD7 (Tu et al., 2021a), PFN3 (Umer et al., 2021) and NRDP1 (RNF41; Luo et al., 2023); described in Table 3; acrosome biosynthesis] also result in defective acrosome formation. As the acrosome contains hydrolytic enzymes and has an acidic pH (Nakanishi et al., 2001), the acrosome has been considered a lysosome-related organelle (Bilekova et al., 2023). The involvement of autophagy-related proteins in acrosome formation suggests that it may be similar to autolysosome formation.

As these KO mouse analyses indicate, budding, transport and fusion of proacrosomal vesicles are important for proper acrosome formation. Deletion of these factors tends to result in the absence or deterioration of acrosome formation, whereas phenotypes in which the acrosome is abnormally enlarged have been reported as well. For example, deletion of FAM71F1 (GARIN1B) that binds to RAB2A/2B, small GTPases, results in an abnormally enlarged acrosome (Morohoshi et al., 2021). In addition, deletion of the BTBD18-targeted pachytene piRNA cluster in mice results in outgrowth of the acrosome (Choi et al., 2021). These results suggest that there is also a negative regulatory mechanism that prevents an oversupply of proacrosomal vesicles to the developing acrosomal vesicle.

Association of acrosomal vesicle and nucleus

The acrosomal vesicle needs to remain associated with the nucleus during all the phases of the acrosome formation. The acrosomal inner

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Table 3. Summary of factors required for mouse acrosome formation

Role	Protein	Loss-of-function phenotype (mouse)	Reference(s)
Acrosome reaction			
Proteolytic enzyme	ACR (acrosin)	Unimpaired male fertility; infertility in hamsters due to impaired ZP penetration	Baba et al., 1994; Hirose et al., 2020
Exocytosis	FER1L5	Impaired acrosome reaction, ZP penetration and sperm-oocyte fusion	Morohoshi et al., 2023
Acrosome biosynthesis			
Endoplasmic reticulum	GBA2	Abnormal acrosome formation; abnormal accumulation of glycolipids	Yildiz et al., 2006
	HSP90B1	Abnormal acrosome formation	Audouard and Christians, 2011
	PDCL2	Abnormal acrosome formation	Fujihara et al., 2023
	SEC23IP	Abnormal acrosome formation	Arimitsu et al., 2011
Formation of proacrosomal vesicles from the Golgi	GOPC	Abnormal acrosome formation	Yao et al., 2002
	PFN4	Abnormal acrosome formation; impairment in manchette formation	Umer et al., 2022
	PICK1	Abnormal acrosome formation; associated with globozoospermia in human	Xiao et al., 2009; Liu et al., 2010
	SMAP2	Abnormal increase in the diameter of proacrosomal vesicles and disrupted acrosome formation	Funaki et al., 2013
Fusion of proacrosomal vesicles	AU040320	Abnormal acrosome formation	Guidi et al., 2018
	ELAPOR1	Abnormal acrosome formation	Bilekova et al., 2023
	GOLGA2	Abnormal acrosome formation	Han et al., 2017
	HRB (AGFG1)	Abnormal acrosome formation	Kang-Decker et al., 2001
	TRIP11	Abnormal acrosome formation; reduced amount of IFT20	Wang et al., 2020b
	VPS54	Abnormal acrosome formation; UBPy-positive endosomes remain scattered	Paiardi et al., 2011
Autophagy and vesicle transport	ATG7	Impaired transport and fusion of proacrosomal vesicles; disrupted autophagic flux	Wang et al., 2014
	NRDP1 (RNF41)	Abnormal acrosome formation; impaired expression and/or localization of lysosomal or mitochondrial proteins	Luo et al., 2023
	PFN3	Abnormal acrosome formation; disrupted autophagic flux; impairment in manchette formation	Umer et al., 2021
	SIRT1	Abnormal acrosome formation; disrupted autophagic flux	Liu et al., 2017a
	TBC1D20	Abnormal acrosome formation; disrupted autophagic flux	Sidjanin et al., 2016
	TDRD7	Abnormal acrosome formation; disrupted autophagic flux; associated with nonobstructive azoospermia in human	Tu et al., 2021a; Tan et al., 2019
Adhesion of acrosomal vesicles to the nucleus (acrosomal vesicles)	SPACA1	Abnormal acrosome formation; associated with globozoospermia in human	Fujihara et al., 2012; Chen et al., 2021
	ZPBP1	Abnormal acrosome formation	Lin et al., 2007
Adhesion of acrosomal vesicles to the nucleus (acroplaxome)	ACTL7A	Abnormal acrosome formation; loss of F-actin in the acroplaxome; associated with globozoospermia in human	Zhou et al., 2022b; Ferrer et al., 2023; Zhang et al., 2023a; Xin et al., 2020
	ACTRT1	Abnormal acrosome formation; loosened acroplaxome structure; associated with globozoospermia in human	Zhang et al., 2022e, 2024
	ACTL9	Abnormal acrosome formation; associated with globozoospermia in human	Dai et al., 2021
	CCIN (calicin)	Loosened acroplaxome structure; nuclear surface subsidence; deformed acrosome	Zhang et al., 2022d
	CYLC1	Abnormal acrosome formation; associated with deformed acrosomes in human	Schneider et al., 2023; Jin et al., 2024
	CYLC2	Abnormal acrosome formation; impairment in manchette formation	Schneider et al., 2023
Adhesion of acrosomal vesicles to the nucleus (nucleus)	CSNK2A2	Abnormal acrosome formation; indented nucleus	Xu et al., 1999
	DPY19L2	Abnormal acrosome formation; destabilization of both the nuclear dense lamina and the junction between the acroplaxome and the nuclear envelope; associated with globozoospermia in human	Pierre et al., 2012; Koscinski et al., 2011
	FAM209	Abnormal acrosome formation	Castaneda et al., 2021
	SPATA46	Abnormal acrosome formation	Chen et al., 2016
Others	BTBD18-targeted pachytene piRNA cluster	Enlarged acrosome; upregulation of GOLGA2 transcript and protein	Choi et al., 2021
	COPS5	Abnormal acrosome formation; reduced sperm numbers	Huang et al., 2020
	FAM71F1 (GARIN1B)	Enlarged acrosome	Morohoshi et al., 2021
	FAM71F2 (GARIN1A)	Slightly enlarged acrosome	Morohoshi et al., 2021

Relevance to human pathology is indicated where relevant. ZP, zona pellucida.

membrane and nuclear membranes are associated via an F-actin and keratin-containing structure called the acroplaxome, a structure found between the acrosomal membrane and the nuclear membrane (reviewed by Kierszenbaum et al., 2003) (Fig. 4A). Proteins related to this association, such as SPACA1 (Fujihara et al., 2012) and ZPBP1 (Lin et al., 2007), have been found by analyzing KO mice (see Table 3; acrosome biosynthesis). For example, SPACA1 is a single transmembrane protein that localizes to the acrosomal inner membrane and is thought to be involved in this association. When SPACA1 is disrupted, acrosomal vesicles are formed normally up to the Golgi stage of spermiogenesis, but subsequent elongation and thinning of acrosomal vesicles are impaired, and eventually the acrosomal vesicles degenerate and disappear (Fujihara et al., 2012). This might be due to the lack of close association between the acrosomal and nuclear membranes in the acroplaxome.

ACTL7A, a protein that can bind to SPACA1 (Chen et al., 2021), is expressed predominantly in the testis and localizes to the acroplaxome (Boëda et al., 2011). Disruption of *Actl7a* results in loss of F-actin from the acroplaxome and peeling of acrosomal vesicles from the nucleus, leading to abnormal acrosome formation (Zhou et al., 2022b; Ferrer et al., 2023; Zhang et al., 2023a). As mice lacking acroplaxome proteins ACTRT1 (Zhang et al., 2022e) and ACTL9 (Dai et al., 2021) also show detachment of acrosomal vesicles, the formation and maintenance of F-actin by these actin-related proteins may be involved in the adhesion of acrosomal vesicles to the nucleus in the acroplaxome (Table 3; acrosome biosynthesis). As with ACTL7A, calicin is thought to interact with SPACA1 (Zhang et al., 2022d). Loss of calicin results in a loosened acroplaxome structure, suggesting that calicin is also involved in the adhesion of acrosomal vesicles to the nucleus (Zhang et al., 2022d).

Proteins localized to the nucleus and involved in adhesion have also been found: DPY19L2, which localizes to the inner nuclear membrane, is involved in the adhesion of the acroplaxome to the nucleus. Deletion of *Dpy19l2* destabilizes the nuclear dense lamina, as well as the junction between the acroplaxome and the nuclear envelope, causing acrosomal vesicles to detach from the nuclear membrane (Pierre et al., 2012). Similarly, deletion of FAM209, which interacts with DPY19L2 (Castaneda et al., 2021), and SPATA46, which localizes to the nuclear membrane (Chen et al., 2016), show disrupted acrosome formation, suggesting that DPY19L2, FAM209 and SPATA46 work as a bridge between the nuclear membrane and the acroplaxome (Table 3; acrosome biosynthesis).

Summary

A number of proteins involved in acrosome formation have been identified in mice, including proteins related to vesicle trafficking, autophagy and actin cytoskeleton as well as sperm-specific unique proteins. Among these, mutations in *ACTL7A* (Xin et al., 2020), *ACTL9* (Dai et al., 2021), *DPY19L2* (Koscinski et al., 2011), *PICK1* (Liu et al., 2010) and *SPACA1* (Chen et al., 2021) have been found in infertile patients with globozoospermia, suggesting that the mechanisms regulating its formation are similar between mice and humans, although the morphology of the acrosome differs. Spermatozoa from globozoospermia patients with mutations in *ACTL7A* or *ACTL9* cannot fertilize oocytes by normal intracytoplasmic sperm injection because the amount and localization of PLCZ1, a sperm-borne oocyte activation factor localized in the post-acrosomal region (Hachem et al., 2017; Nozawa et al., 2018), is abnormal. Although oocytes cannot be activated by injecting these spermatozoa, sperm injection with subsequent artificial oocyte activation by increasing intracellular calcium (Ca^{2+}) concentrations with ionophores or strontium is successful in producing offspring (Xin et al., 2020; Dai et al., 2021). Identification of genes involved in acrosome formation and understanding their regulatory mechanisms are expected to lead to elucidating the etiology of globozoospermia and effective treatment methods.

Conclusion

Here, we review how functional spermatozoa develop during spermiogenesis by focusing on analyses with KO mice. As described above, the advent of genome editing technology has led to the discovery of a significant number of genes important for spermiogenesis in mice. However, what is described here does not include detailed discussion of all steps of spermiogenesis, such as nuclear condensation (reviewed by Okada, 2022), manchette formation (reviewed by Lehti and Sironen, 2016) and spermiation [the release of mature spermatids into the lumen of the seminiferous tubule (Shimada et al., 2023; reviewed by O'Donnell et al., 2011)], which are also essential for spermiogenesis. Further analyses using KO mice will continue to uncover genes important for spermiogenesis and male fertility, which will advance our understanding of the etiology of human infertility. However, there are species specific differences. For example, SSX1, a primate-specific gene, is absent in mice but essential for human fertility (Liu et al., 2023b). Therefore, it is also important to examine the functions of genes in mammals other than mice, especially primates. As it is difficult to generate genetically modified primates, it is expected that further development will be made in gene manipulation techniques, such as injecting viral vectors into the testes (Ikawa et al., 2002; Watanabe et al., 2018) as used in the SSX1 study (Liu et al., 2023b), or generating mature spermatozoa with *in vitro* culture systems (Sato et al., 2011; Komeya et al., 2018).

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Competing interests

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