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The University of Osaka

Elucidation of the Causes of Male Infertility with a  
Focus on the Sperm Flagellum

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Department of Advanced Pharmaco-Science

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The specific origins of each chapter are as follows.

Introduction and Chapter 1 are mainly adapted from Wang et al., *Andrology*, In press<sup>1</sup>, under the CC-BY 4.0 license. (DOI: 10.1111/andr.70152)

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<https://journals.biologists.com/jcs/article/137/16/jcs261962/361813/MYCBPAP-is-a-central-apparatus-protein-required>)

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## List of Abbreviations

| Abbreviation | Full name                                |
|--------------|------------------------------------------|
| 129          | 129S6/SvEvTac                            |
| 2PN          | Two-pronuclear                           |
| ACTB         | $\beta$ -actin                           |
| BSA          | Bovine serum albumin                     |
| BSG          | BASIGIN                                  |
| CASA         | Computer-assisted sperm analysis         |
| CFAP77       | Cilia and Flagella Associated Protein 77 |
| CFAP91       | Cilia and Flagella Associated Protein 91 |
| DMEM         | Dulbecco's Modified Eagle Medium         |
| DTT          | Dithiothreitol                           |
| FBS          | Fetal bovine serum                       |
| HET          | Heterozygote                             |
| HTCA         | Head-to-tail coupling apparatus          |
| IB           | Immunoblot                               |
| ICC          | Immunocytochemistry                      |
| IF           | Immunofluorescence                       |
| IFT          | Intraflagellar transport                 |
| IHC          | Immunohistochemistry                     |
| IP           | Immunoprecipitation                      |
| KO           | Knockout                                 |
| KSOM         | Potassium simplex optimized medium       |
| MIP          | Microtubule inner protein                |
| MS           | Mass spectrometry                        |
| MYCBPAP      | MYCBP Associated Protein                 |
| NE           | Nuclear envelope                         |
| ORF          | Open reading frame                       |

| Abbreviation | Full name                                                    |
|--------------|--------------------------------------------------------------|
| PAS          | Periodic acid-Schiff                                         |
| PBS          | Phosphate-buffered saline                                    |
| PCR          | Polymerase chain reaction                                    |
| PFA          | Paraformaldehyde                                             |
| PVDF         | Polyvinylidene fluoride                                      |
| RNP          | Ribonucleoprotein                                            |
| RS           | Radial spoke                                                 |
| SD           | Standard deviation                                           |
| SDS          | Sodium dodecyl sulfate                                       |
| SDS-PAGE     | SDS-polyacrylamide gel electrophoresis                       |
| TEM          | Transmission electron microscopy                             |
| TG           | Transgenic                                                   |
| TUNEL        | Terminal deoxynucleotidyl transferase dUTP nick end labeling |
| VAP          | average path velocity                                        |
| VCL          | curvilinear velocity                                         |
| VSL          | straight-line velocity                                       |
| WT           | wild-type                                                    |

## Part I Introduction

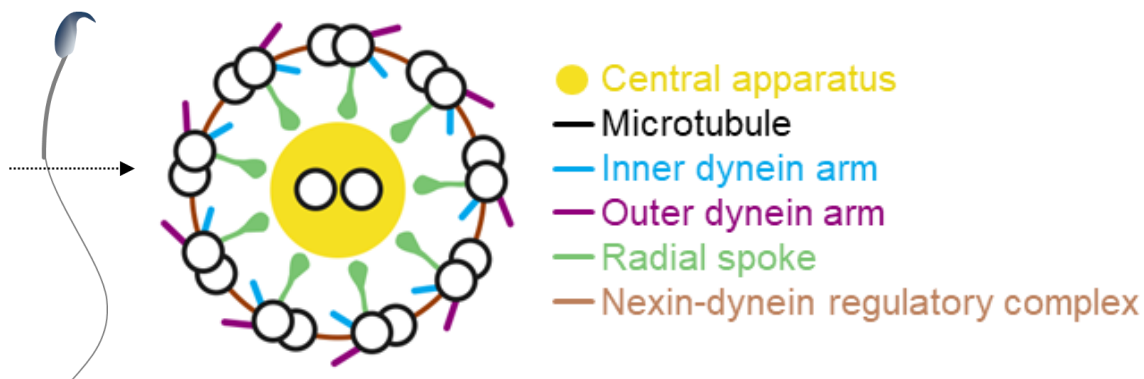
According to recent global estimates, about 17.5% of couples experience infertility at some point in their lives<sup>4</sup>. Male factors account for roughly half of these cases<sup>5</sup>. Although substantial progress has been made in understanding the underlying causes of male infertility, many cases remain unexplained<sup>6</sup>. A common clinical condition of male infertility is asthenozoospermia, which is characterized by markedly reduced motility<sup>7</sup>.

Asthenozoospermia can be seen with or without multiple morphological abnormalities of the sperm flagella, in which sperm tails appear coiled, bent, short, or completely absent<sup>7</sup>. As genetic factors are strongly implicated in these defects, elucidating the molecular roles of the genes involved in sperm flagellum movement and formation is essential for advancing our understanding of male infertility.

Sperm motility is indispensable for male fertility, as spermatozoa must actively migrate through the female reproductive tract to encounter and fertilize the egg<sup>8</sup>. Sperm motility is powered by the flagellum, a whip-like organelle that produces propulsive forces through coordinated bending and beating motions<sup>9</sup>. After entering the female reproductive duct, activation of the sperm-specific calcium channel CatSper leads to an increase in intracellular  $\text{Ca}^{2+}$  concentration<sup>10</sup>. This increase in  $\text{Ca}^{2+}$  levels triggers a transition in sperm flagellar movement from symmetrical to asymmetrical beating, enabling spermatozoa to overcome various physical barriers and ultimately reach and fertilize the egg<sup>11</sup>. This phenomenon is a mammal-specific process known as hyperactivation. Abnormalities in both basal motility and hyperactivation can lead to male infertility.

The core structure responsible for sperm motility is the axoneme, a conserved microtubule-based scaffold shared by cilia and flagella across species<sup>12</sup>. The axoneme exhibits a characteristic '9+2' arrangement, consisting of nine outer microtubule doublets surrounding a central pair of singlet microtubules<sup>13</sup>. Notably, the central pair of singlet microtubules forms a structure with other proteins, known as the central apparatus<sup>14</sup>. Each outer

microtubule doublet is decorated with inner and outer dynein arms that generate bending forces, while radial spokes transmit regulatory signals from the central pair to dyneins<sup>15,16</sup>. In addition, the doublets are interconnected by the nexin-dynein regulatory complex, which contributes to structural stability and coordinated movement<sup>17</sup> (Figure 1). These intricate substructures collectively ensure proper flagellar beating and, consequently, normal sperm motility.



**Figure 1. Schematic representation of the axoneme in cross-section**

The microtubule singlets and their associated structures form the central apparatus, which is surrounded by nine peripheral microtubule doublets. As shown in the figure, the microtubule doublets are decorated with inner dynein arms, outer dynein arms, radial spokes, and the nexin-dynein regulatory complex.

To uncover the roles of axoneme structural proteins in male infertility, we selected proteins that have been reported to localize to the axoneme and are conserved in humans. We used the CRISPR/Cas9 system to generate loss-of-function mouse models for each selected gene and performed phenotypic and molecular analyses. We reported that the microtubule inner protein CFAP77 is responsible for maintaining sperm motility in **Chapter 1**, the central apparatus protein MYCBPAP is essential for sperm tail formation in **Chapter 2**, and together that CFAP91 is indispensable for sperm tail elongation and EFCAB5 is important for sperm hyperactivation in **Chapter 3**. Most importantly, the absence of these proteins in mouse models all results in reduced male fertility, advancing our understanding of human male infertility.

## Part II Main Chapters

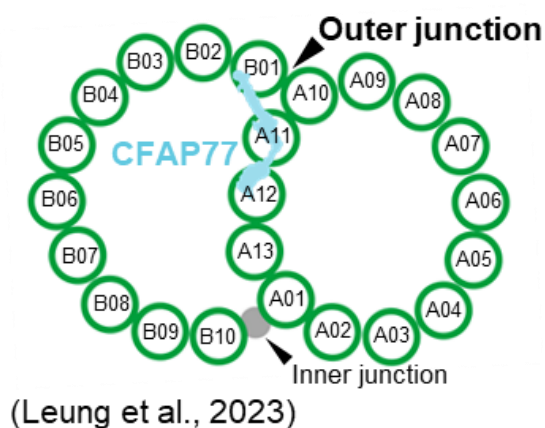
### Chapter 1. Roles of CFAP77 in male reproduction

#### Abstract

The axoneme possesses a conserved '9+2' microtubule arrangement supported by microtubule inner proteins (MIPs) that maintain its stability and function. Although many MIPs have been identified, their physiological roles are poorly understood. Among them, cilia and flagella associated protein 77 (CFAP77) has been characterized as a MIP in multiple species, but its role in mammalian sperm function has not been elucidated. To investigate the function of CFAP77, we generated *Cfap77* knockout (KO) mice using the CRISPR/Cas9 system. Male fertility was evaluated by mating tests, and sperm motility was analyzed using computer-assisted sperm analysis (CASA). Immunoblotting and immunoprecipitation-mass spectrometry were performed to determine the localization and interaction partners of CFAP77. We found that CFAP77 localized to sperm flagella, and *Cfap77* KO males exhibited markedly reduced fertility due to impaired sperm motility despite normal sperm morphology. IP-MS analysis revealed that CFAP77 interacts with the microtubule inner protein TEKTL1, and the absence of CFAP77 led to decreased TEKTL1 levels in spermatozoa. These results demonstrate that CFAP77 is indispensable for normal sperm motility and male fertility, likely by stabilizing other MIPs within the axoneme. Our findings provide new insights into the molecular mechanisms that regulate sperm motility in mammals.

## Introduction

Each microtubule doublet within the axoneme consists of a complete A-tubule and an incomplete B-tubule, which can be further divided into protofilaments A01-A13 and B01-B10, respectively<sup>18</sup> (Figure 2). The connection between the A- and B-tubules is mediated at the inner junction by proteins such as CFAP20 and PACRG<sup>19</sup>, while the outer junction is believed to be stabilized through direct interactions between specific protofilaments<sup>20</sup>. Recent advances in cryo-electron microscopy (cryo-EM) have revolutionized our understanding of the molecular organization of axonemes, leading to the discovery of numerous MIPs<sup>21</sup>.



**Figure 2. Schematic representation of the sperm axonemal microtubules in cross-section**

Each microtubule doublet in the axoneme consists of a complete A-tubule (A01-A13) and an incomplete B-tubule (B01-B10). CFAP77 localizes at the outer junction between the A-tubule and the B-tubule.

MIPs are localized inside the lumen of the microtubule doublets and are thought to provide internal reinforcement that stabilizes the axonemal structure and fine-tunes its mechanical properties<sup>22</sup>. These proteins likely play key roles in maintaining microtubule rigidity, curvature, and resistance to mechanical stress during flagellar beating<sup>23,24</sup>. However, despite these structural insights, the physiological significance of many MIPs remains largely unexplored, especially in the context of mammalian sperm function.

Among the known MIPs, CFAP77 has been identified as a conserved component of the axoneme in several species, including sea urchins, cattle, and mice. Structural analyses using cryo-EM and electron tomography have revealed that CFAP77 localizes to the inner wall of the B-tubule and contacts protofilaments A11, A12, and B01<sup>21</sup> (Figure 2). In bovine spermatozoa, CFAP77 repeats along the microtubule doublets with a periodicity of approximately 16 nm, suggesting its role as a structural stabilizer. In the unicellular organism *Tetrahymena thermophila*, CFAP77 is proposed to be essential for maintaining microtubule doublet stability and supporting ciliary motility<sup>25</sup>. Nevertheless, direct functional evidence of CFAP77 in mammalian spermatozoa is lacking.

In this study, we generated and analyzed *Cfap77* KO mice to elucidate the functional role of CFAP77 in mammalian sperm development and motility. Loss of CFAP77 led to a pronounced reduction in sperm motility and a corresponding decrease in male fertility. Furthermore, biochemical analyses revealed that CFAP77 interacts with several other microtubule-associated proteins, particularly other MIPs, and that the absence of CFAP77 results in the loss or destabilization of some of these components. These findings suggest that CFAP77 plays a crucial role in maintaining the structural integrity of the sperm axoneme and ensuring efficient flagellar beating.

Overall, this study identified CFAP77 as a key axonemal component required for normal sperm motility and male fertility in mice. Given the high degree of evolutionary conservation of CFAP77 across species, it is likely that CFAP77 serves a similar structural and functional role in the human sperm flagellum. Understanding how CFAP77 contributes to axonemal stability will deepen our knowledge of the MIP-dependent mechanisms underlying sperm motility and may provide valuable insights into the molecular basis of human male infertility.

## Materials and Methods

### Experimental animals

All animal experiments were performed in accordance with institutional ethical standards and approved by the Animal Care and Use Committee of the Research Institute for Microbial Diseases, Osaka University (Approval ID: Biken-AP-H30-01 and Biken-AP-R03-01). Mice were purchased from Japan SLC (Shizuoka, Japan) and maintained under specific pathogen-free conditions with unrestricted access to food and water. The animal facility was maintained on a 12-hour light/dark cycle. KO mice were generated using the CRISPR/Cas9 system<sup>26</sup>. Briefly, female B6D2F1 mice were superovulated through intraperitoneal injection of CARD HyperOva (Kyudo, Saga, Japan) and human chorionic gonadotropin (hCG) (ASKA Pharmaceutical, Tokyo, Japan) and mated with wild-type (WT) B6D2F1 males. Two-pronuclear (2PN) zygotes were collected from mated females and subjected to electroporation with a ribonucleoprotein (RNP) complex consisting of Cas9 (#A36497, Thermo Fisher, Waltham, MA), CRISPR RNA (crRNA; Sigma-Aldrich, St. Louis, MO), and trans-activating crRNA (tracrRNA; #TRACRRNA05N-5NMOL, Sigma-Aldrich). The guide RNA sequences used were 5'-ACGGTGGTCGACATCCGTAC-3' and 5'-GAGTCTTAGCGAGGGCTGAC-3'. Polymerase chain reaction (PCR) was carried out using KOD FX Neo DNA polymerase (Toyobo, Osaka, Japan) to confirm the genotype of mice. The primer sequences used in Chapter 1 are listed in Table 1.

Table 1. Primers used in Chapter 1.

| Name | Sequence               |
|------|------------------------|
| KO1  | ctccgaagaactggcggggt   |
| KO2  | ggtgcattagcagtgaggga   |
| WT1  | caagactggcctttcgggtctg |
| WT2  | ccttgctcgatgaggggtgg   |

### Fertility test

To assess fertility, each sexually mature KO or WT male was housed with three 7-week-old females for eight weeks. The presence of vaginal plugs was recorded daily to confirm mating events, and the total number of offspring was counted. After the cohabitation period, the males were removed, and pup production was monitored for an additional three weeks to ensure the completion of all pregnancies initiated during the mating period.

### Histological analysis

Testes and epididymides were fixed in Bouin's solution (16045-1, Polysciences, Warrington, PA) and subsequently processed for paraffin embedding. Sections of 5  $\mu$ m thickness were prepared, treated with 1% periodic acid (Nacalai Tesque, Kyoto, Japan) for 10 minutes, and then incubated with Schiff's reagent (Wako, Osaka, Japan) for 20 minutes. After rinsing thoroughly under running tap water, the sections were counterstained with hematoxylin for 1 minute. Histological images were acquired using an Olympus BX-53 microscope (Tokyo, Japan).

### Immunohistochemistry (IHC)

For immunohistochemistry, tissues were fixed in 4% paraformaldehyde (PFA; Thermo Fisher Scientific, Waltham, MA) and sectioned. The sections were permeabilized using 0.1% Triton X-100 (Nacalai Tesque, Kyoto, Japan) and blocked with 1% bovine serum albumin (BSA; Sigma-Aldrich). Primary antibodies were applied overnight, followed by incubation with secondary antibodies for 2 hours after a brief wash. Nuclei were counterstained with Hoechst 33342 (Thermo Fisher Scientific), and the sections were mounted with Immu-Mount medium (Eprelia, Thermo Fisher Scientific). Fluorescence signals were visualized using an Olympus BX-53 or a Nikon Eclipse Ti C2 confocal microscope (Nikon, Tokyo, Japan). Detailed information on the antibody used in this chapter can be found in Table 2.

### Immunoblotting (IB)

Protein samples prepared in sodium dodecyl sulfate (SDS) buffer were resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE; ATTO, Osaka, Japan) and transferred to polyvinylidene fluoride (PVDF) membranes. After blocking, the membranes were sequentially incubated with primary and secondary antibodies. Protein bands were detected using an Amersham ImageQuant 800 imaging system (Cytiva, Tokyo, Japan). Lysates for sperm head-tail separation and fractionation were prepared using established methods<sup>27</sup>. Detailed information on the antibody used in this chapter can be found in Table 2.

Table 2. Antibodies used in Chapter 1.

| Antigen                  | Origin                   | Identifier | Dilution for IB | Dilution for ICC/IHC |
|--------------------------|--------------------------|------------|-----------------|----------------------|
| CFAP77                   | Atlas antibodies         | HPA021329  | 1/500           | 1/100                |
| Acetylated alpha-tubulin | Sigma-Aldrich            | T7451      | 1/1000          | 1/500                |
| ACTB                     | Abcam                    | ab6276     | 1/2000          | -                    |
| AKAP3                    | Proteintech              | 13907-1-AP | 1/1000          | -                    |
| BASIGIN                  | Santa Cruz Biotechnology | sc-46700   | 1/500           | -                    |
| IZUMO1                   | This lab                 | -          | 1/1000          | -                    |

### Sperm motility analysis

Sperm motility was analyzed using a CEROS II computer-assisted sperm analysis system (Hamilton Thorne Biosciences, Beverly, MA) following established procedures<sup>28</sup>. Cauda epididymal spermatozoa were released into a 100  $\mu$ L drop of TYH medium<sup>29</sup>. Following incubation at 37 °C in a humidified 5% CO<sub>2</sub> atmosphere for 10 minutes and 120 minutes, sperm motility parameters were recorded and analyzed.

### Immunoprecipitation (IP)

For immunoprecipitation, Dynabeads magnetic beads (Thermo Fisher Scientific) were incubated with the desired antibody for 15 minutes at room temperature with gentle mixing. The protein extracts were clarified by centrifugation, transferred to fresh tubes, and incubated with Dynabeads for at least 2 hours at 4 °C with gentle rotation to minimize non-specific

binding. After incubation, the beads were collected using a magnetic rack and washed twice with lysis buffer to remove non-specific proteins. Bound proteins were eluted by resuspending the beads in SDS sample buffer and heating at 70 °C for 10 minutes.

#### Sperm head-tail separation and protein fractionation

Spermatozoa from the cauda epididymis were sonicated to separate the heads and tails. Fractions were purified by centrifugation through 90% Percoll and washed with PBS prior to protein extraction<sup>27</sup>. For protein fractionation, sequential extraction of spermatozoa with Triton X-100 buffer, SDS buffer, and SDS sample buffer yielded Triton-soluble, SDS-soluble, and SDS-resistant fractions, respectively<sup>30</sup>.

#### Mass spectrometry (MS) analysis

Proteins isolated by IP were analyzed using nanocapillary reversed-phase liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS). Samples were separated on a C18 column (IonOpticks, Victoria, Australia) using a nanoLC system (Bruker Daltonics, Billerica, MA) interfaced with a timsTOF Pro mass spectrometer equipped with a CaptiveSpray nano-electrospray ionization source (Bruker Daltonics). The raw spectra were processed using DataAnalysis software (Bruker Daltonics), and peptide identification was performed using MASCOT (Matrix Science, Tokyo, Japan) against the UniProt database. Quantitative assessment and fold-change analysis were conducted using Scaffold 5 software (Proteome Software, Portland, OR).

#### Human sperm sample collection

Human spermatozoa were obtained from healthy donor volunteers. All experimental procedures involving human materials were approved by the Ethics Committee of the Research Institute for Microbial Diseases at the University of Osaka. Human spermatozoa were liquefied by incubation at room temperature for 1 hour and spun down by centrifugation. After a brief wash in PBS, lysis buffer was added to lyse the human spermatozoa.

### Statistical analysis

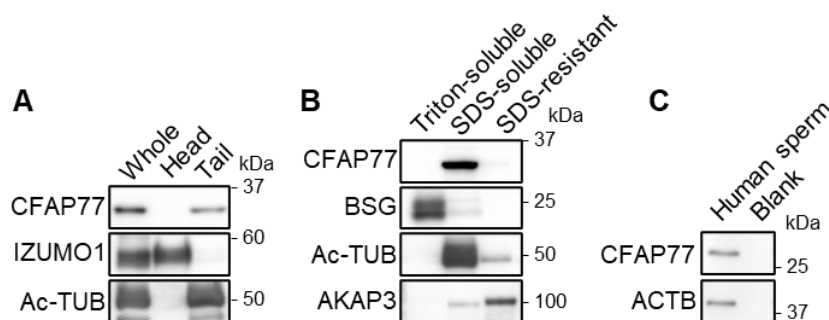
All data are presented as mean  $\pm$  standard deviation (SD). Statistical comparisons were conducted using Student's t-test. Significance levels were set as follows:  $P < 0.05$ ,  $P < 0.01$ , and  $P < 0.001$ .

## Results

### Section 1: Analyses using a *Cfap77* KO mouse model

#### CFAP77 is associated with sperm axonemes and evolutionarily conserved in mice and humans

To determine the subcellular localization of CFAP77, spermatozoa were separated into head and tail fractions and subjected to immunoblot analysis (Figure 3A). CFAP77 was exclusively detected in the tail fraction. We further fractionated mature spermatozoa into Triton X-100 soluble, SDS-soluble, and SDS-resistant fractions, corresponding to cytoplasmic/membrane-associated proteins, axonemal proteins, and accessory structure proteins, respectively. CFAP77 was present in the SDS-soluble fraction (Figure 3B), suggesting its association with the axonemal compartment. Immunoblotting of human spermatozoa confirmed the presence of CFAP77 in human spermatozoa (Figure 3C).

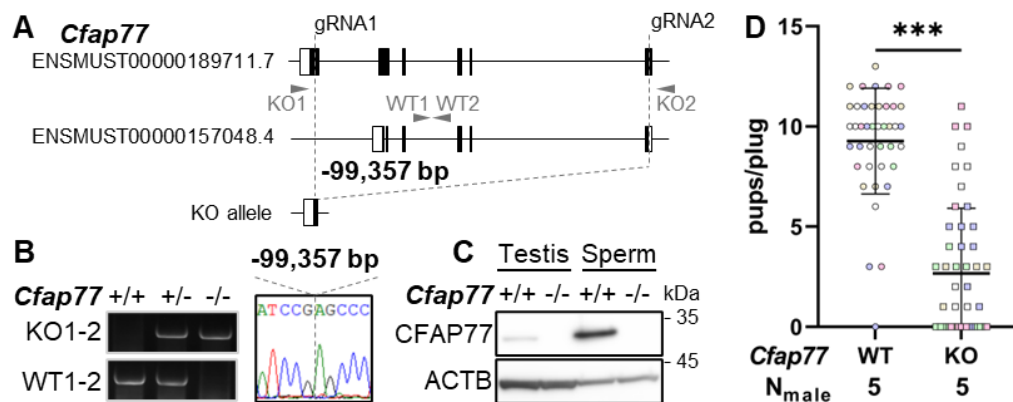


**Figure 3. Evolutionary conservation and localization of CFAP77 in mouse spermatozoa**

(A) Immunoblot analysis of sperm head and tail fractions. IZUMO1 and acetylated tubulin (Ac-TUB) were used as markers to confirm successful separation of heads and tails, respectively. (B) Immunoblotting of subcellular sperm fractions. BASIGIN (BSG), Ac-TUB, and AKAP3 served as markers for proper fractionation into cytoplasmic/membrane, axonemal, and accessory structure components. (C) Immunoblotting of human sperm lysates.  $\beta$ -actin (ACTB) was used as a loading control.

### CFAP77 is essential for male fertility in mice

To examine whether CFAP77 contributes to male reproductive function, *Cfap77* KO mice were produced using the CRISPR/Cas9 system (Figure 4A). Ninety zygotes were electroporated, and 74 two-cell embryos were transferred into three pseudopregnant females. Among the seven pups obtained, one carried a large deletion allele of *Cfap77*. Subsequent breeding of heterozygous and homozygous animals yielded *Cfap77* KO progeny. The KO mice exhibited normal development, behavior, and survival. PCR and Sanger sequencing confirmed a 99,357-bp deletion in the coding region (Figure 4B). Immunoblot analysis further verified the absence of CFAP77 protein in the testis and cauda epididymal spermatozoa of *Cfap77* KO males (Figure 4C). Fertility was evaluated by housing each KO male with WT females. *Cfap77* KO males exhibited subfertility, showing a significant reduction in the number of pups per copulation plug compared to WT males (Figure 4D). These findings indicate that CFAP77 plays a critical role in maintaining normal fertility in males.

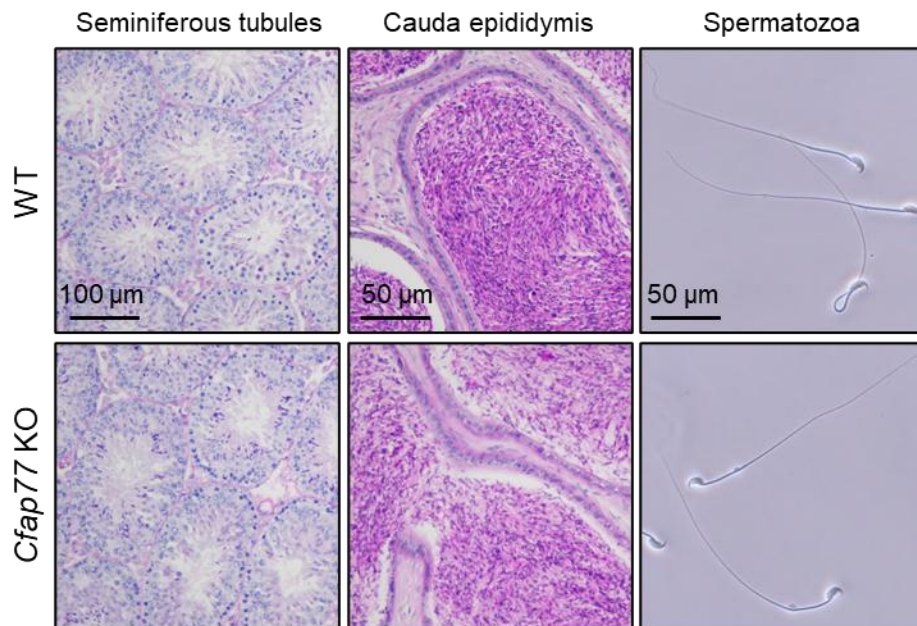


**Figure 4. Generation of *Cfap77* KO mice and fertility evaluation**

(A) Schematic illustration of the *Cfap77* knockout strategy. Two guide RNAs (gRNAs) were designed to target the longest *Cfap77* transcript variant containing two splice isoforms. Arrowheads denote primer positions used for genotyping. (B) Left: PCR genotyping of *Cfap77* mutant mice. Right: Confirmation of the genomic deletion in the *Cfap77* coding region by Sanger sequencing. (C) Immunoblot analysis demonstrating the absence of CFAP77 in testes and spermatozoa of *Cfap77* KO mice. ACTB served as a loading control. (D) Fertility assessment of *Cfap77* KO males. KO males displayed a significant reduction in litter size per mating compared with WT males. Data from individual mice are represented in identical colors. \*\*\*,  $p < 0.001$ .

### Deletion of *Cfap77* impairs sperm motility

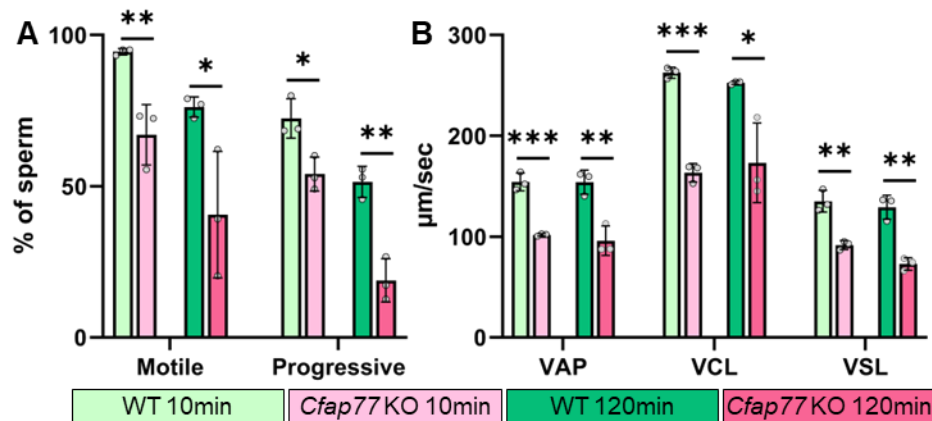
To investigate the cause of reduced fertility, we examined the histological sections of male reproductive organs. The overall architecture of the testis and cauda epididymis appeared normal in *Cfap77* KO mice, and the number of spermatozoa in the cauda epididymis was comparable to that in WT controls (Figure 5). Morphologically, KO spermatozoa were indistinguishable from those of WT males (Figure 5).



**Figure 5. Morphological analyses of reproductive organs and spermatozoa of *Cfap77* KO males**  
Histological analyses of testes and cauda epididymides, along with representative images of cauda epididymal spermatozoa from WT and *Cfap77* KO males.

Given the normal morphology of the reproductive organs and spermatozoa, we next evaluated sperm motility. Spermatozoa undergo characteristic motility changes during capacitation, which is essential for fertilization. We assessed motility after 10 and 120 minutes of incubation in the capacitation medium, representing non-capacitated and capacitated states, respectively. Both the proportion of motile spermatozoa and progressively motile spermatozoa were significantly decreased in the *Cfap77* KO samples at both time points (Figure 6A). Moreover, velocity parameters, including straight-line

velocity (VSL), curvilinear velocity (VCL), and average path velocity (VAP), were all reduced in *Cfap77* KO spermatozoa (Figure 6B), indicating that CFAP77 is required for efficient sperm motility.



**Figure 6. Motility of *Cfap77* KO spermatozoa**

(A) Quantification of total and progressive sperm motility in *Cfap77* KO males. (B) Comparison of sperm motility parameters, including VSL, VCL, and VAP. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ .

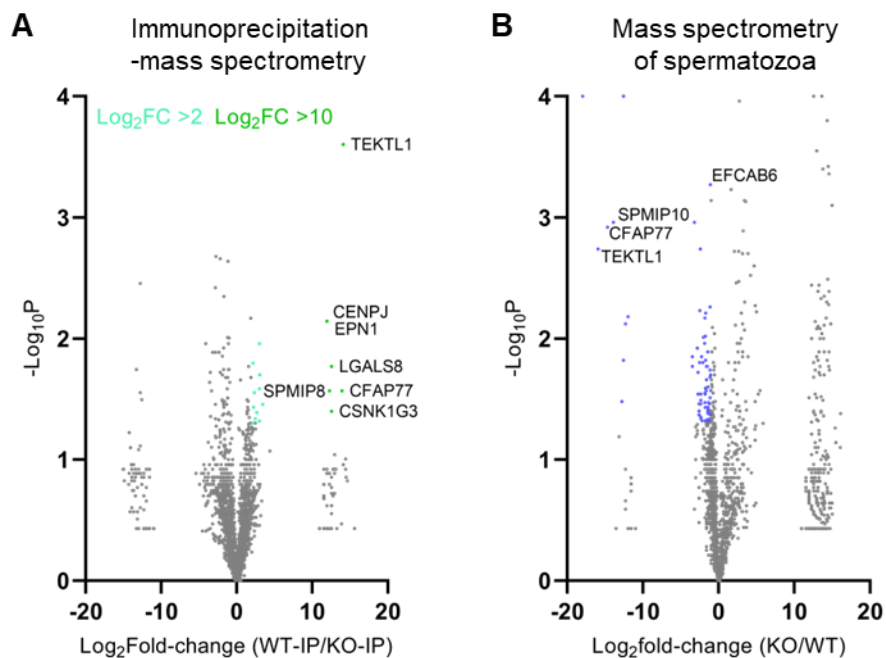
## Section 2: Elucidation of the relationship between CFAP77 and other microtubule inner proteins

### CFAP77 interacts with sperm MIPs and stabilizes their incorporation

To elucidate the molecular role of CFAP77, immunoprecipitation using an anti-CFAP77 antibody, followed by mass spectrometry, was performed on testis lysates. Lysates from *Cfap77* KO testes were used as the negative controls. The analysis identified CFAP77-associated proteins, including the sperm MIPs TEKTL1 and SPMIP8 (Figure 7A), with TEKTL1 showing the strongest enrichment. This interaction aligns with previous cryo-electron microscopy findings<sup>21</sup> showing that TEKTL1 forms filamentous structures binding to A11-A12 protofilaments and CFAP77.

To test whether the incorporation of TEKTL1 into mature sperm tails depends on CFAP77, mass spectrometry was performed on the sperm lysates (Figure 7B). TEKTL1 abundance

was markedly reduced in *Cfap77* KO spermatozoa, along with that of other MIPs, such as EFCAB6 and SPMIP10. These findings indicate that CFAP77 facilitates the stable incorporation of MIPs, particularly TEKTL1, into the sperm flagellum.



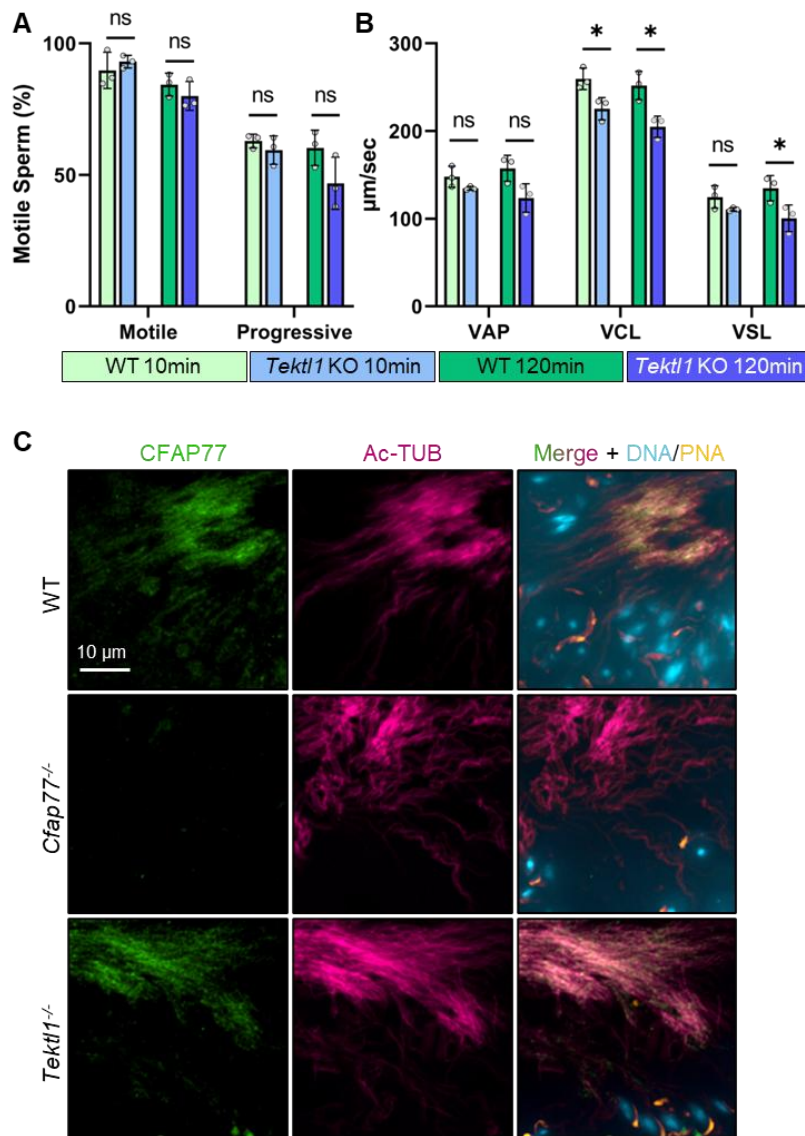
**Figure 7. CFAP77 interacts with TEKTL1**

(A) Immunoprecipitation coupled with mass spectrometry using an anti-CFAP77 antibody. Proteins identified with  $p < 0.05$  were color-coded according to their fold-change relative to *Cfap77* KO. (B) Mass spectrometric comparison of sperm lysates from WT and *Cfap77* KO mice. Proteins significantly reduced in *Cfap77* KO spermatozoa are highlighted in blue.

### TEKTL1 contributes to sperm motility independently of CFAP77

To explore the role of TEKTL1 in sperm function, we examined *Tektl1* knockout mice, which have been previously reported to exhibit normal spermatogenesis and fertility<sup>31</sup>. Despite their fertility, we hypothesized that sperm motility may be subtly affected. Analysis of sperm movement revealed that the absence of TEKTL1 did not alter the proportion of motile or progressively motile spermatozoa (Figure 8A). However, both VCL at 10 and 120 minutes and VSL at 120 minutes of incubation were significantly decreased (Figure 8B). Although VAP also tended to decline, the difference was not statistically significant.

Immunohistochemical staining confirmed the presence of CFAP77 in the flagella of *Tektl1* KO testes (Figure 8C), demonstrating that TEKTL1 influences sperm motility through a mechanism independent of CFAP77. Since the motility defects observed in *Tektl1* KO mice were milder than those in *Cfap77* KO mice, the impaired motility in *Cfap77* KO spermatozoa cannot be solely attributed to the loss of TEKTL1.



**Figure 8. Sperm motility, but not CFAP77 amount, was impaired in *Tektl1* KO spermatozoa**

(A) Quantitative analysis of motile and progressively motile sperm populations in *Tektl1* KO males.

(B) Assessment of VSL, VCL, and VAP in spermatozoa from *Tektl1* KO mice. \*,  $p < 0.05$ ; ns, not significant.

(C) Immunohistochemical detection of CFAP77 in testes from WT, *Cfap77* KO, and *Tektl1* KO mice. CFAP77 signals were present in *Tektl1* KO flagella and co-localized with acetylated tubulin (Ac-TUB).

## Discussion

As an increasing number of MIPs are identified through cryo-electron microscopy (cryo-EM), their potential functions in mammalian male reproduction warrant further investigation. In this study, we confirmed that CFAP77 is present in mature spermatozoa of both mice and humans (Figure 3). CFAP77 likely acts as a MIP in mice, as it was detected in the axoneme-associated protein fraction (Figure 3B).

To clarify the function of CFAP77, we generated *Cfap77* KO mice. These males exhibited a pronounced decline in fertility compared to that of WT males (Figure 4D). Despite this, no apparent morphological abnormalities were detected in the reproductive organs or spermatozoa (Figure 5). However, sperm motility was markedly compromised in *Cfap77* KO males (Figure 6).

To test whether the impaired motility of *Cfap77* KO spermatozoa resulted from the loss of TEKTL1, we examined sperm motility and CFAP77 localization in *Tektl1* KO mice. Absence of TEKTL1 led to a moderate decrease in sperm velocity parameters, but CFAP77 localization was unaffected (Figure 8), suggesting that TEKTL1 contributes to sperm motility independently of CFAP77. Considering TEKTL1's localization within microtubules<sup>21</sup>, it may influence sperm motility by maintaining microtubule rigidity. Since TEKTL1 is absent in *Cfap77* KO spermatozoa, it is plausible that TEKTL1's CFAP77-independent function partially accounts for the motility defects observed in *Cfap77* KO spermatozoa.

In *Trichomonas vaginalis* and *Tetrahymena thermophila*, CFAP77 has been identified as an outer junction protein critical for maintaining axonemal stability<sup>25,32</sup>. In *Tetrahymena*, loss of CFAP77A/B (two orthologs of CFAP77) causes destabilization of the B-tubule, leading to shortened cilia and impaired motility<sup>25</sup>. Consistent with these findings, compromised outer junctions may underlie the reduced motility of *Cfap77* KO spermatozoa. During our

manuscript preparation, another group independently demonstrated that CFAP77 is essential for sperm motility through the stabilization of the B-tubule at the outer junction<sup>33</sup>. Their transmission electron microscopy analysis revealed increased open B-tubules in the axonemes of *Cfap77* KO cilia and spermatozoa, complementing the structural aspects not examined in our study. They also reported complete infertility in *Cfap77* KO males, whereas we observed subfertility in our study. This difference may stem from distinct genetic backgrounds<sup>34,35</sup>, as our experiments used B6D2 hybrid mice, while the other study employed C57BL/6 inbred mice. Hybrid B6D2 mice generally exhibit larger litter sizes than C57BL/6 mice, owing to the contributions of both males and females. B6D2 females produce more oocytes during superovulation<sup>36,37</sup>, and their oocytes show greater resilience to sperm injection and osmotic stress<sup>38</sup>, reflecting higher oocyte quality. On the male side, B6D2 hybrids typically show higher sperm motility than C57BL/6 mice<sup>28,39,40</sup>. Therefore, the differences in sperm motility and oocyte quality between these genetic backgrounds may explain the milder phenotype observed in our study.

In conclusion, our results highlight the critical role of CFAP77 in maintaining sperm motility and male fertility in mice. Given its evolutionary conservation, CFAP77 is likely to fulfill similar roles in human sperm function. Nonetheless, the relevance of CFAP77 variants to human male infertility remains unclear. Continued investigation into MIPs will deepen our understanding of the MIP-dependent mechanisms governing sperm motility.

## Chapter 2. Roles of MYCBPAP in male reproduction

### Abstract

During spermiogenesis, the axoneme extends from the centrosome, which subsequently anchors to the nuclear envelope to initiate tail formation. *Mycbpap*, which is predominantly expressed in the testes of both mice and humans, is evolutionarily conserved with *FAP147* in *Chlamydomonas*. Previous cryo-electron microscopy studies have localized FAP147 to the central apparatus within the flagellar axoneme of *Chlamydomonas*. In this study, we generated *Mycbpap* KO mice to examine its physiological role and found that *Mycbpap* is indispensable for male fertility. Loss of *Mycbpap* disrupted centrosome docking to the nuclear envelope and impaired flagellar assembly. To confirm its functional significance, we produced transgenic mice expressing tagged MYCBPAP, which successfully rescued the infertility phenotype observed in KO males. Further interactome analysis using the transgenic model identified several MYCBPAP-associated proteins, including the central apparatus components CFAP65 and CFAP70 that form part of the C2a projection, as well as centrosomal proteins such as CCP110. Together, these findings reveal that MYCBPAP plays a pivotal role in coordinating centrosome-nuclear envelope attachment and the subsequent development of the sperm tail, providing new insights into the molecular mechanisms underlying sperm biogenesis and male fertility.

## Introduction

The axoneme itself consists of a central pair of microtubule singlets surrounded by nine peripheral microtubule doublets, forming the characteristic ‘9+2’ configuration<sup>13</sup>. Notably, the central pair of microtubules forms an asymmetric complex with proteinaceous projections, known as the central apparatus<sup>41,42</sup>. The overall organization of the central apparatus is evolutionarily conserved from unicellular organisms, such as *Chlamydomonas*, to mammals<sup>43,44</sup>.

During spermiogenesis, haploid round spermatids undergo a series of morphological and structural transformations to become mature spermatozoa. In mice, this process is classified into 16 distinct steps<sup>45</sup>. In the early stages of spermiogenesis, the centrosome, which comprises proximal and distal centrioles, attaches to the plasma membrane<sup>46</sup>. The distal centriole is positioned almost perpendicular to the proximal centriole and serves as a foundation for axonemal elongation. In the mid-phase of spermiogenesis, round spermatids elongate as the flagellum assembles. At approximately step 6, the centrosome migrates and anchors to the nuclear envelope<sup>47</sup>. This centrosome-nuclear envelope docking facilitates the formation of the head-to-tail coupling apparatus (HTCA), and disruption of this process results in male infertility<sup>48</sup>. Importantly, this docking event is unique to spermiogenesis and does not occur during ciliogenesis, leaving many aspects of its underlying mechanism poorly understood<sup>49</sup>.

MYC-binding protein-associated protein (MYCBPAP) has been identified as a testis-enriched protein in humans<sup>50</sup>. Its ortholog in *Chlamydomonas* localizes specifically to the central apparatus, suggesting a conserved role in axonemal structure and function<sup>43</sup>. A recent report confirmed that mouse MYCBPAP localizes to the sperm axoneme central apparatus<sup>44</sup>. However, the role of MYCBPAP in mammalian spermiogenesis and male fertility remains unclear. To elucidate its physiological function, we generated a *Mycbpap* KO mouse model and revealed the crucial role of MYCBPAP during spermiogenesis.

## Materials and Methods

Immunoblotting (IB), immunoprecipitation (IP), mass spectrometry (MS), immunohistochemistry (IHC), Sperm head-tail separation, protein fractionation, fertility test, and statistical analysis

Referring to Chapter 1.

### Experimental animals

The methodology for KO mouse production is described in Chapter 1. For *Mycbpap* KO mouse production, CRISPR/Cas9 genome editing was performed using guide RNAs targeting exon 2 (5'-TCCAGAAAAGAAGCGGGCGA-3') and exon 18 (5'-TAAAGTCTGCAAGCCGGGAC-3').

For transgenic (TG) mouse generation, *Mycbpap* cDNA tagged with a C-terminal 3×FLAG sequence was cloned under the control of the *Clgn* promoter and microinjected into the pronuclei of 2PN zygotes. Following manipulation, all zygotes were cultured in potassium simplex optimized medium<sup>51</sup> (KSOM) until the two-cell stage and transferred into the oviducts of pseudopregnant ICR females. The resulting F0 pups were obtained either by natural delivery or Cesarean section. Genotyping was conducted using PCR and Sanger sequencing to confirm genomic modifications. Primer sequences are indicated in Table 3.

Table 3. Primers used in Chapter 2.

| Name                     | Sequence                  |
|--------------------------|---------------------------|
| KO allele #1             | acctgcacatgcttgtagg       |
| KO allele #2             | cttccttgccactctgttg       |
| WT allele #1             | gtcactgcccgtctctatgg      |
| WT allele #2             | tgacctgcgctcctctctgg      |
| Tg allele #1             | ttgagcgggcccgttgcgactgg   |
| Tg allele #2             | tccttttaatggccaaggc       |
| <i>Mycbpap</i> RT-PCR #1 | ccttcgctgctgttctgtggg     |
| <i>Mycbpap</i> RT-PCR #2 | agtcttctggcgggtgtcca      |
| <i>Actb</i> RT-PCR#1     | catccgtaaagacctctatgccaac |
| <i>Actb</i> RT-PCR#2     | atggagccaccgatccaca       |

### RT-PCR

Total RNA was extracted from various mouse tissues and postnatal testes of C57BL/6J mice using TRIzol reagent (Thermo Fisher). Complementary DNA (cDNA) was synthesized using the SuperScript III First-Strand Synthesis Kit (Thermo Fisher). PCR amplification was performed using KOD Fx Neo DNA Polymerase (Toyobo, Tokyo, Japan). Primer sequences are indicated in Table 3.

### Antibodies

All antibodies used in this Chapter are listed in Table 4.

Table 4. Antibodies used in Chapter 1.

| Antigen                  | Origin                   | Identifier | Dilution for IB | Dilution for ICC/IHC |
|--------------------------|--------------------------|------------|-----------------|----------------------|
| $\alpha$ -Tubulin        | Sigma-Aldrich            | HPA021329  | -               | 1/1000               |
| Acetylated alpha-tubulin | CST                      | 5335       | 1/1000          | 1/500                |
| ACTB                     | Abcam                    | ab6276     | 1/2000          | -                    |
| AKAP4                    | BD Biosciences           | 611564     | 1/1000          | -                    |
| BASIGIN                  | Santa Cruz Biotechnology | sc-46700   | 1/500           | -                    |
| IZUMO1                   | This lab                 | -          | 1/1000          | -                    |
| FLAG                     | Sigma-Aldrich            | F1804      | 1/1000          | -                    |
| CFAP65                   | Atlas                    | HPA055156  | 1/100           | -                    |
| CFAP70                   | Atlas                    | HPA037582  | 1/200           | -                    |
| CCP110                   | Proteintech              | 12780-1-AP | 1/500           | -                    |

### Immunocytochemistry (ICC)

For ICC, spermatids were isolated from seminiferous tubules, air-dried on slides, and fixed with 4% PFA for 10 minutes. ICC slides were then blocked with 3% bovine serum albumin and 10% goat serum in PBS for 1 hour at room temperature, followed by overnight incubation with primary antibodies at 4 °C. After three PBS washes, slides were incubated with Alexa Fluor 488 or 546 secondary antibodies (Thermo Fisher) for 1 hour, counterstained with 0.02% Hoechst 33342 and mounted using Immu-Mount (Fisher Scientific, Pittsburgh, PA). Images were obtained using an Olympus BX53 or a Nikon Eclipse Ti confocal microscope.

#### Transmission Electron Microscopy (TEM)

The testes were perfused and fixed with 4% PFA, post-fixed in 1% glutaraldehyde, followed by 1% osmium tetroxide and 0.5% potassium ferrocyanide. After ethanol dehydration and embedding in epoxy resin, ultrathin sections were stained and imaged using a JEM-1400 Plus electron microscope (JEOL, Tokyo, Japan) at 80 kV, equipped with a Veleta 2K×2K CCD camera (Olympus).

#### TUNEL Assay

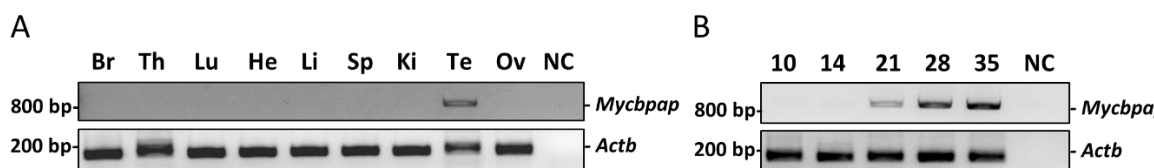
Apoptotic cells were detected in paraffin-embedded testis sections using an In Situ Apoptosis Detection Kit (Takara Bio, Shiga, Japan) according to the manufacturer's protocol. Samples were treated with proteinase K and hydrogen peroxide, labeled with FITC-dUTP, visualized with anti-FITC-HRP and DAB substrate, counterstained with hematoxylin, and observed under an Olympus BX53 microscope.

## Results

### Section 1: Phenotypic analyses on *Mycbpap* KO mice

#### *Mycbpap* shows high expression in the testis during spermiogenesis

To examine the tissue distribution of *Mycbpap* expression, reverse transcription polymerase chain reaction (RT-PCR) was conducted using various mouse organs. The analysis revealed that *Mycbpap* transcripts were predominantly expressed in the testis (Figure 9A). Further RT-PCR using testes from different postnatal stages demonstrated that *Mycbpap* expression began around postnatal day 21 and became highly upregulated by day 28 (Figure 9B), corresponding to the onset of spermiogenesis<sup>52</sup>.



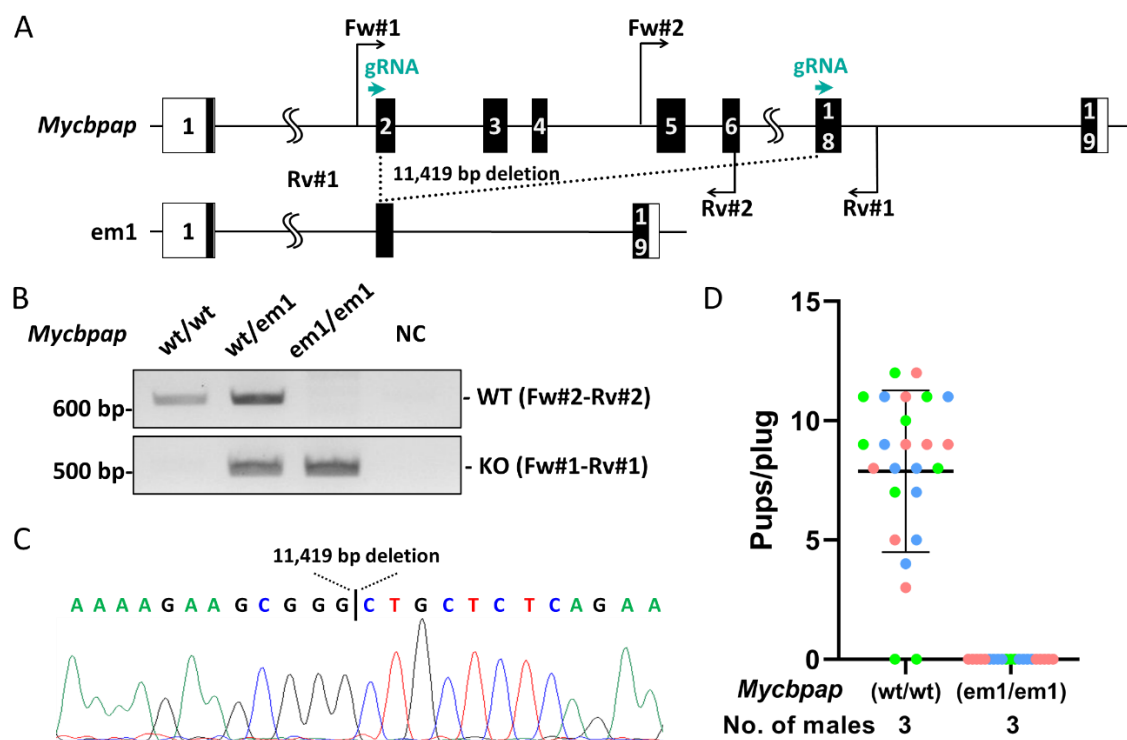
**Figure 9. RT-PCR of *Mycbpap* in mice**

(A) Expression of *Mycbpap* in various mouse tissues analyzed by RT-PCR. *Actb* served as a loading control. Br, brain; Th, thymus; Lu, lung; He, heart; Li, liver; Sp, spleen; Ki, kidney; Te, testis; Ov, ovary; NC, negative control (water). (B) Expression profile of *Mycbpap* in postnatal testes analyzed by RT-PCR. The numbers above indicate postnatal days of male mice. *Actb* was used as a loading control.

#### KO of *Mycbpap* results in male infertility in mice

To investigate the physiological function of MYCBPAP, *Mycbpap* KO mice were generated by deleting a large genomic region within the open reading frame (ORF). This was achieved by electroporating B6D2F1  $\times$  B6D2F1 zygotes with Cas9 and a pair of guide RNAs targeting exons 2 and 18 (Figure 10A). Among 41 zygotes subjected to electroporation, 33 developed into two-cell embryos and were transferred into two pseudopregnant females. Twelve offspring were obtained, ten of which carried extensive deletions. *Mycbpap* KO mice were established by mating founder mice with WT mice, followed by intercrossing of F1 heterozygotes. Genotyping was performed by PCR (Figure

10B), and Sanger sequencing confirmed an 11,419 base pair deletion in the *Mycbpap* locus (Figure 10C). To assess fertility, adult male *Mycbpap* KO mice were housed individually with WT females, and the presence of vaginal plugs was monitored as evidence of copulation. Despite normal mating behavior, no offspring were produced from *Mycbpap* KO males (Figure 10D), demonstrating that MYCBPAP is essential for male fertility in mice.



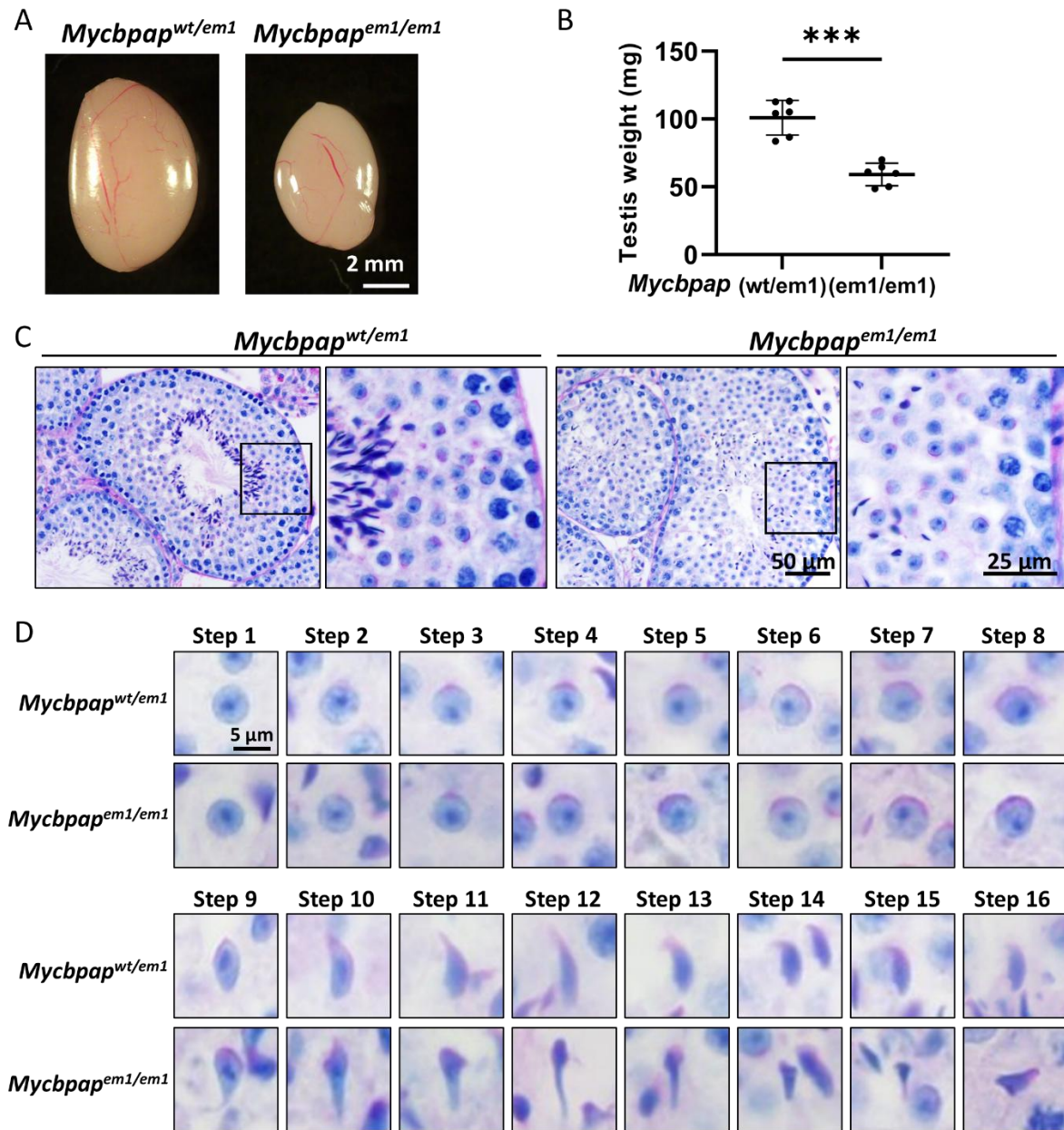
**Figure 10. Generation and mating test of *Mycbpap* KO mice**

(A) Generation of *Mycbpap* KO mice. CRISPR/Cas9 was used to delete exons 2-18 of *Mycbpap* using gRNAs targeting exon 2 and exon 18. (B) Genotyping PCR using primers shown in (A). (C) Deletion of *Mycbpap* confirmed by Sanger sequencing. (D) In vivo fertility test of *Mycbpap* KO males. *Mycbpap* KO males were completely infertile. Data from the same males are indicated by identical colors.

#### Spermiogenesis was defective in *Mycbpap* KO males

To investigate the cause of infertility, we examined the testes of *Mycbpap* KO males. The testes of adult *Mycbpap* KO males were visibly smaller than those of heterozygous controls

(Figure 11A). Testis weight was significantly reduced in *Mycbpap* KO males compared to that in control males (Figure 11B). Because smaller testes often indicate impaired spermatogenesis, we performed histological analyses. In stage VII seminiferous tubules, step 16 spermatids are normally positioned adjacent to step 7 spermatids, with their tails extending toward the lumens. In contrast, *Mycbpap* KO testes exhibited few step 16 spermatids, and their tails were not properly aligned (Figure 11C). To further characterize spermatid morphology, individual spermatids from the testis sections were digitally isolated and aligned according to their developmental steps (Figure 11D). Steps 1-8 appeared normal in *Mycbpap* KO males, but from step 9 onward, spermatids displayed abnormal morphology, culminating in severely misshapen step 16 spermatids (Figure 11D). These results indicate that MYCBPAP is required for normal late spermiogenesis.

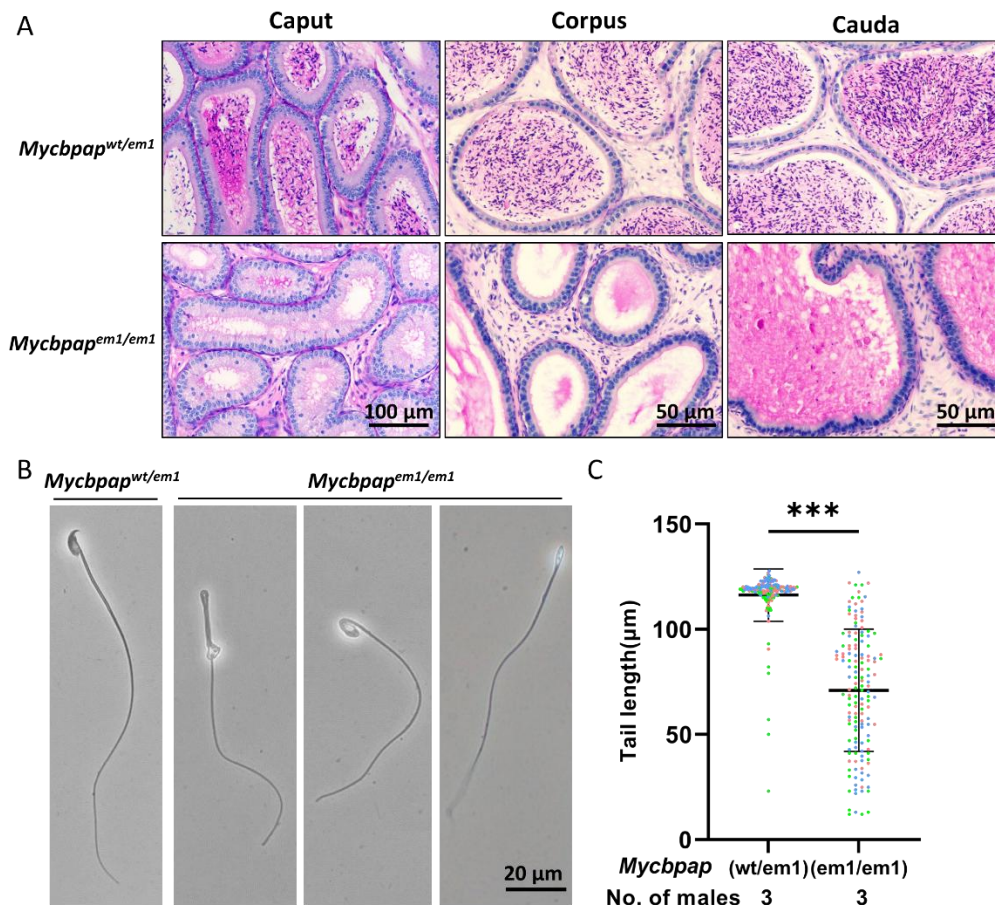


**Figure 11. Abnormal sperm head morphogenesis in *Mycbpap* KO males**

(A) Gross morphology of testes from *Mycbpap* heterozygous and KO males. (B) Testis weight was significantly reduced in *Mycbpap* KO males compared with heterozygotes. \*\*\*,  $p < 0.001$ . (C) Periodic acid-Schiff (PAS) staining of testis sections. Representative seminiferous tubules at stage VII are shown. Higher magnification views of the boxed regions are displayed on the right. (D) Morphological progression of spermatids during spermiogenesis. Abnormal sperm head shaping became evident from step 9 in *Mycbpap* KO males.

### Spermatozoa of *Mycbpap* KO males display multiple morphological abnormalities

Histological examination of epididymal sections showed that spermatozoa were abundant in the caput, corpus, and cauda epididymis of control males, whereas they were nearly absent in *Mycbpap* KO males (Figure 12A).



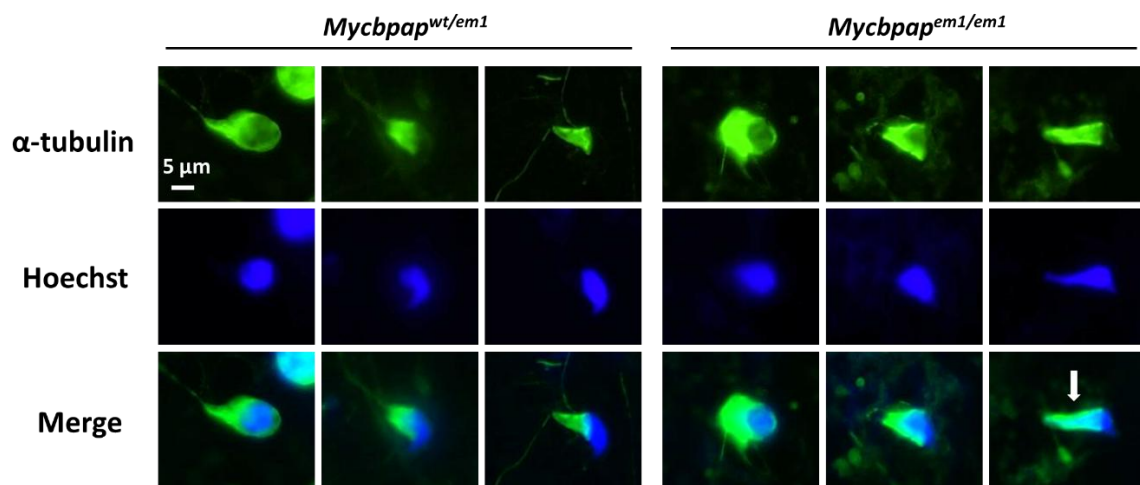
#### **Figure 12. Deletion of *Mycbpap* disrupts sperm tail biogenesis**

(A) PAS-stained sections of caput, corpus, and cauda epididymis from *Mycbpap* heterozygous and KO males. Few sperm heads were observed in the epididymis of *Mycbpap* KO males. (B) Morphology of mature spermatozoa collected from the cauda epididymis of *Mycbpap* heterozygous and KO males. (C) Quantification of sperm tail length from *Mycbpap* heterozygous and KO males. Data from the same individuals are shown in matching colors. \*\*\*,  $p < 0.001$

Furthermore, epididymal spermatozoa from *Mycbpap* KO males exhibited round-headed morphology, consistent with the abnormal spermatids observed in the testis (Figure 12B).

The sperm tails of KO males were also significantly shorter, ranging from approximately 20 to 120  $\mu\text{m}$ , compared to those of the controls (Figure 12C). In addition, spermatozoa from *Mycbpap* KO males were completely immotile. These observations indicate that the infertility of *Mycbpap* KO males is due to severe defects in sperm number, morphology, and motility.

To explore the cause of the abnormal head shape, we examined the manchette, a transient microtubule-based structure that regulates sperm head shaping<sup>53</sup>. Immunostaining of  $\alpha$ -tubulin revealed an abnormally elongated manchette in *Mycbpap* KO spermatids, corresponding to the club-shaped heads observed in testis sections (Figure 13). These findings suggest that both sperm head and tail formation are disrupted in *Mycbpap* KO males, and the elongated manchette likely contributes to the abnormal head morphology.



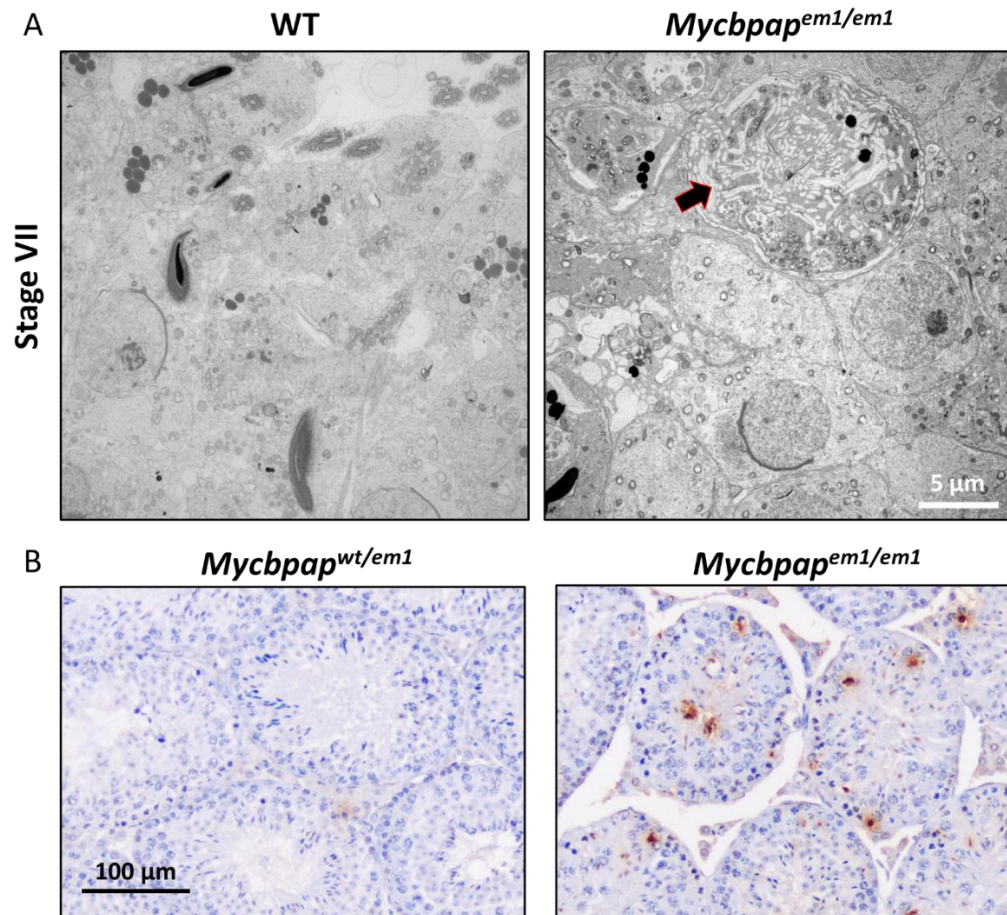
**Figure 13. Deletion of *Mycbpap* disrupts sperm head morphogenesis**

Immunocytochemistry of spermatids. The manchette and nuclei were visualized with anti- $\alpha$ -tubulin antibody (green) and Hoechst 33342 (blue), respectively. The progression of head elongation is shown from left to right. The white arrow indicates an abnormally extended manchette.

#### MYCBPAP is required for centrosome-nuclear envelope docking during spermiogenesis

Transmission electron microscopy (TEM) of *Mycbpap* KO testes revealed the absence of step 16 spermatids, consistent with the histological findings (Figure 14A). Additionally, cytoplasmic condensation and vacuolation, hallmarks of apoptosis<sup>54</sup>, were observed within

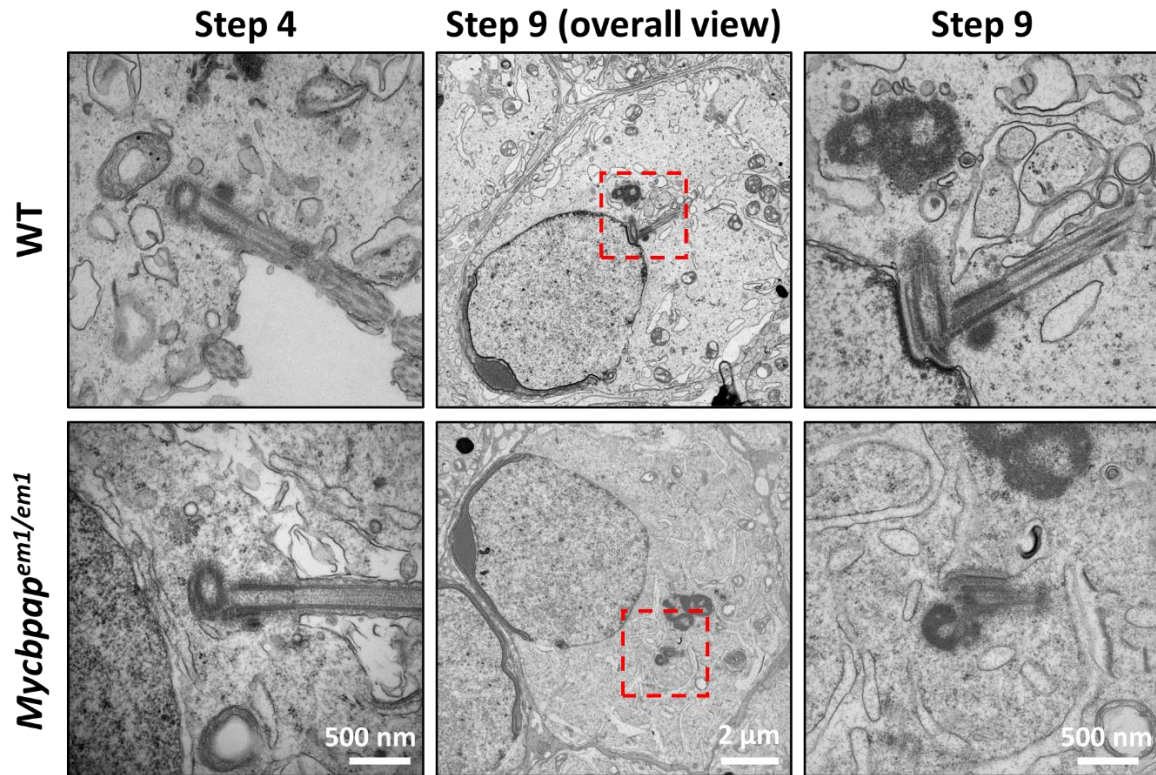
the same tubules. TUNEL staining confirmed that numerous spermatids in *Mycbpap* KO testes were apoptotic (Figure 14B). These results indicate that loss of MYCBPAP leads to spermatid apoptosis during spermiogenesis, contributing to the depletion of mature spermatozoa.



**Figure 14. Ultrastructural analysis reveals apoptotic-like structures in *Mycbpap* KO testes**  
**(A)** TEM images of testes from *Mycbpap* heterozygous and KO males. Apoptotic-like structures (arrow) and condensed cytoplasm with cytoplasmic vacuoles (arrow) were frequently observed in *Mycbpap* KO males. **(B)** Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining of testis sections from *Mycbpap* heterozygous and KO males. TUNEL-positive cells are indicated in brown.

At earlier stages (step 4), axonemal elongation occurred normally in both control and KO spermatids (Figure 15). However, in step 9 spermatids of *Mycbpap* KO males, the centrosome failed to dock onto the nuclear envelope, a process that normally takes place at step 6<sup>55</sup>. These

findings demonstrate that MYCBPAP is essential for centrosome-nuclear envelope docking during spermiogenesis.



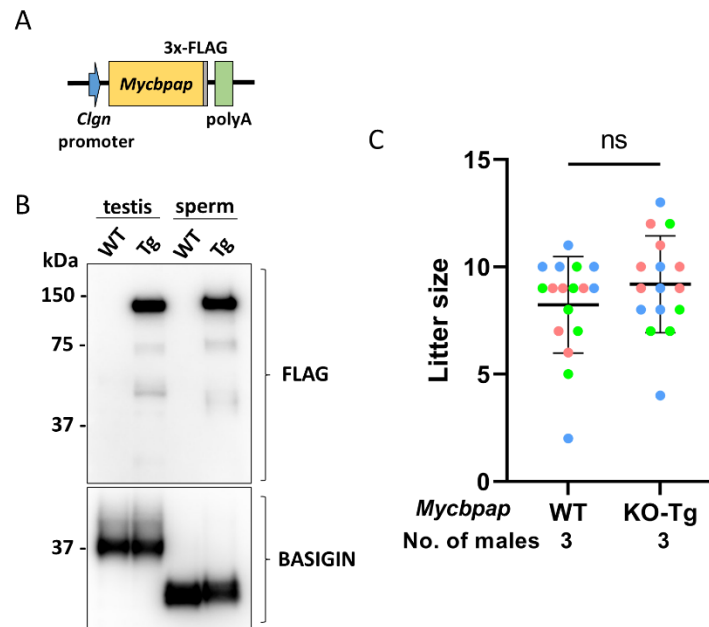
**Figure 15. Ultrastructural analysis reveals defective centrosome docking in *Mycbpap* KO testes**  
TEM images of step 4 and step 9 spermatids. Centrosome docking to the nuclear envelope was disrupted in *Mycbpap* KO males. Higher magnification views of boxed regions in step 9 spermatids are shown on the right.

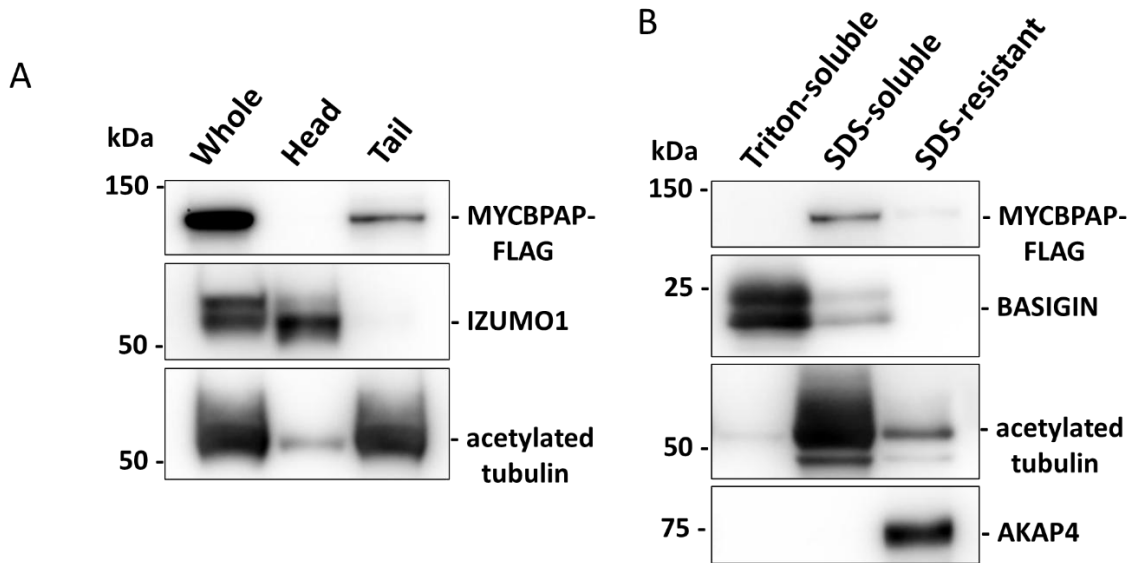
## Section 2: Analyses using a *Mycbpap* transgenic mouse line

### A *Mycbpap* transgene driven by the *Clgn* promoter rescues male infertility in *Mycbpap* KO mice

To examine MYCBPAP localization and function, transgenic mice expressing *Mycbpap* cDNA fused with a C-terminal 3×FLAG tag under the control of the testis-specific *Clgn* promoter<sup>56</sup> were generated (Figure 16A). Successful generation of *Mycbpap* TG mice was

confirmed by immunoblotting, which detected clear FLAG-tagged bands in both testis and sperm lysates (Figure 16B). When *Mycbpap* KO males carrying the transgene were mated with WT females, fertility was restored, and litter sizes were comparable to those of WT males (Figure 16C).





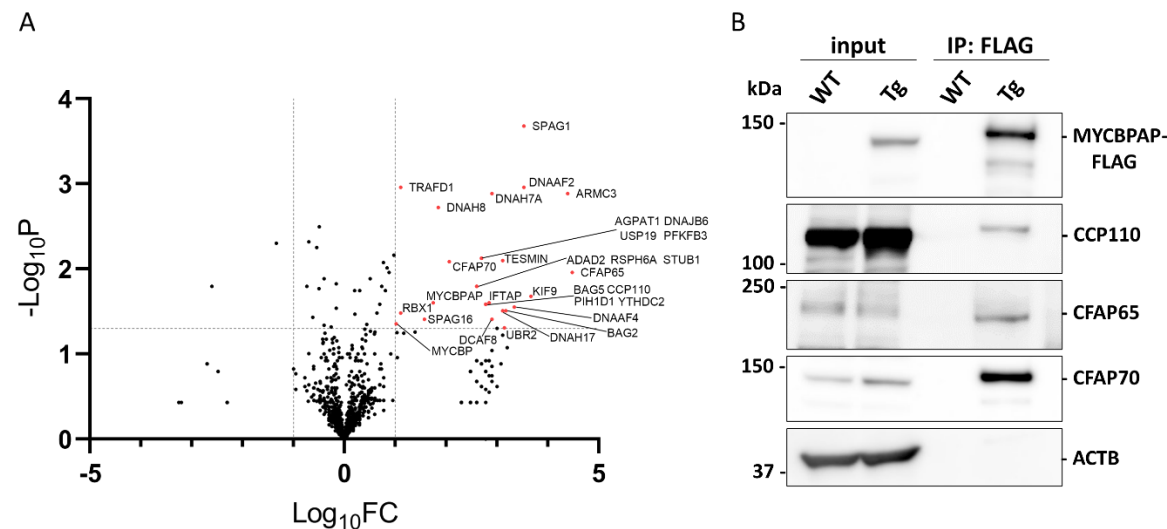
**Figure 17. MYCBPAP is an axoneme-associated protein**

(A) Immunoblotting of head-tail separated spermatozoa from *Mycbpap* KO Tg cauda epididymis. IZUMO1 and acetylated tubulin were used as markers for sperm heads and tails, respectively. (B) Fractionation of spermatozoa from *Mycbpap* KO Tg cauda epididymis. BASIGIN, acetylated tubulin, and AKAP4 were used as markers for Triton-soluble, SDS-soluble, and SDS-resistant fractions, respectively.

#### MYCBPAP interacts with central apparatus proteins

To identify MYCBPAP-interacting proteins, immunoprecipitation (IP) using anti-FLAG antibody was performed with testis lysates from *Mycbpap* KO Tg mice. Mass spectrometry identified 30 interacting proteins (Figure 18A), including CFAP65, CFAP70, SPAG16, KIF9, and MYCBP, orthologs of central apparatus proteins characterized in *Chlamydomonas*. Co-immunoprecipitation and immunoblotting confirmed the interaction of MYCBPAP with CFAP65 and CFAP70 (Figure 18B), suggesting that MYCBPAP is a component of the central apparatus. Cryo-electron microscopy (Cryo-EM) of the *Chlamydomonas* central apparatus has shown that orthologs of MYCBPAP, CFAP65, CFAP70, SPAG16, and MYCBP localize to the C2a projection, whereas KIF9 is associated with the adjacent C2c projection<sup>43,57</sup>. These findings suggest that mouse MYCBPAP is likely to be a component of the C2a projection. Interestingly, MYCBPAP also interacted

with the centrosomal protein CCP110<sup>58,59</sup> (Figure 18). This suggests that MYCBPAP may fulfill the function of centrosome-nuclear envelope docking through CCP110.



**Figure 18. MYCBPAP interacts with central apparatus components**

(A) Mass spectrometry analysis comparing IP products from *Mycbpap* KO Tg and WT testes. Proteins significantly enriched in *Mycbpap* KO Tg testes are shown in red ( $p < 0.05$ , fold-change  $> 10$ ). (B) Immunoblotting of IP products from *Mycbpap* KO Tg and WT testes. ACTB served as a loading control.

## Discussion

MYCBPAP was previously reported as a testis-specific binding partner of MYCBP; however, its function during spermatogenesis remains largely unexplored. To investigate the physiological role of *Mycbpap*, we generated *Mycbpap* KO mice. *Mycbpap* KO males were completely infertile (Figure 10D), whereas female KOs exhibited normal fertility. Phenotypic analyses revealed multiple abnormalities in *Mycbpap* KO males, including a markedly reduced number of spermatozoa in the epididymis, immotile spermatozoa, shortened sperm tails, and abnormally shaped sperm heads (Figure 12). Transmission electron microscopy further revealed that *Mycbpap* KO spermatids failed to properly dock the centrosome to the nuclear envelope (Figure 15), suggesting that MYCBPAP plays a crucial role in centrosome-nuclear envelope docking during spermiogenesis.

To elucidate the molecular network of MYCBPAP, we performed interactome analyses using the testes of transgenic mice expressing FLAG-tagged MYCBPAP. The identified interactome contained several central apparatus proteins, including CFAP65, KIF9, CFAP70, SPAG16, and MYCBP (Figure 18). These proteins are evolutionarily conserved in *Chlamydomonas reinhardtii* as FAP65, KLP1, FAP70, PF20, and FAP174. Notably, MYCBPAP is conserved as FAP147 in *Chlamydomonas*, where cryo-electron microscopy and biochemical studies have shown that FAP147 localizes to the C2a projection of the central apparatus<sup>43,57,60</sup>, forming a structural axis with FAP65 and FAP70. The similarity between the MYCBPAP interactome in mice and the FAP147 complex in *Chlamydomonas* suggests that the role of MYCBPAP in the C2a projection is evolutionarily conserved.

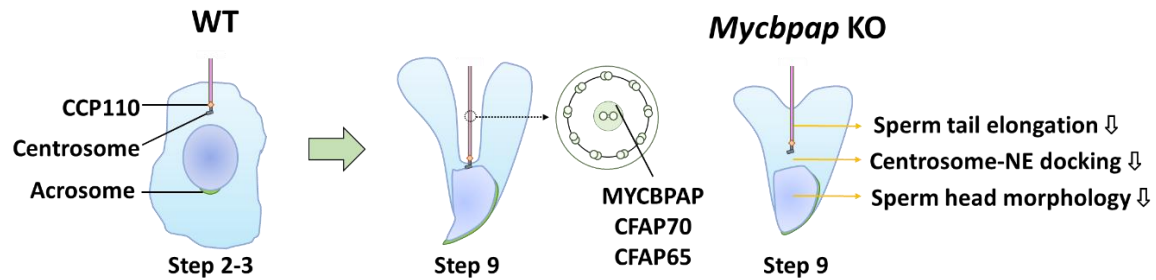
In mice, loss of *Cfap70* results in a reduced number of spermatozoa in the cauda epididymis<sup>61</sup>, while *Cfap65* KO leads to defective sperm head morphogenesis<sup>62</sup>. Both *Cfap65* and *Cfap70* KOs exhibited abnormal sperm tail formation, similar to the phenotype observed in *Mycbpap* KO males. Taken together, these findings indicate that MYCBPAP is a component of the central apparatus that interacts with CFAP65 and CFAP70, thereby contributing to proper

sperm tail biogenesis. The abnormal tail formation observed in *Mycbpap* KO mice may subsequently disrupt centrosome-nuclear envelope docking and the organization of the manchette. As a consequence, intra-manchette transport (IMT), which plays a crucial role in transporting molecules along the manchette<sup>63</sup>, may also be disrupted. Given that multiple KO studies on proteins affecting IMT have also resulted in apoptosis<sup>64,65</sup>, we suspect the apoptotic spermatids observed in *Mycbpap* KO males may be a secondary effect stemming from an abnormal manchette structure.

Our TEM analyses confirmed that MYCBPAP is essential for centrosome-nuclear envelope docking during spermiogenesis. Although we could not definitively determine whether MYCBPAP localizes directly to the centrosome due to antibody limitations, the interactome data provided insights into possible partners involved in this process. Among the MYCBPAP interactors, CFAP65, KIF9, and CCP110 are of particular interest. CCDC108, the *Xenopus* ortholog of CFAP65, has been shown to regulate the apical trafficking of centrioles during ciliary cell differentiation<sup>66</sup>. KIF9 localizes to axonemes and basal bodies in multiciliated cells, and its loss impairs motility in both human airway cilia and mouse sperm flagella<sup>67,68</sup>. CCP110, which localizes near centrioles in human spermatozoa, is essential for centriole maturation and acts as a negative regulator of ciliogenesis. Dysregulation of CCP110 results in abnormal ciliary outgrowth<sup>58</sup>, and its activity must be tightly controlled both spatially and temporally<sup>69</sup>. The interaction between MYCBPAP and CCP110 identified in this study may contribute to the spatial regulation of CCP110 during spermiogenesis, while its temporal control is likely governed by the ubiquitin-proteasome system<sup>70,71</sup>. These observations suggest that MYCBPAP cooperates with centrosome-associated proteins to achieve accurate centrosome-nuclear envelope docking.

In conclusion, our study demonstrates that MYCBPAP is indispensable for centrosome-nuclear envelope docking and sperm tail biogenesis, and its loss leads to complete male infertility in mice (Figure 19). Several variants in MYCBPAP predicted to cause loss of function are listed in the Genome Aggregation Database<sup>72</sup> (gnomAD), including a stop-gain

variant (p.Cys58Ter) with an allele frequency of  $4.94 \times 10^{-4}$ . These findings suggest that MYCBPAP dysfunction may underlie certain cases of human male infertility. Further investigations into MYCBPAP function will enhance our understanding of centrosome-nuclear envelope docking and flagellar assembly during spermiogenesis.



**Figure 19. Summary diagram of MYCBPAP function**

MYCBPAP is essential for sperm tail elongation, head morphogenesis, and centrosome-nuclear envelope (NE) docking.

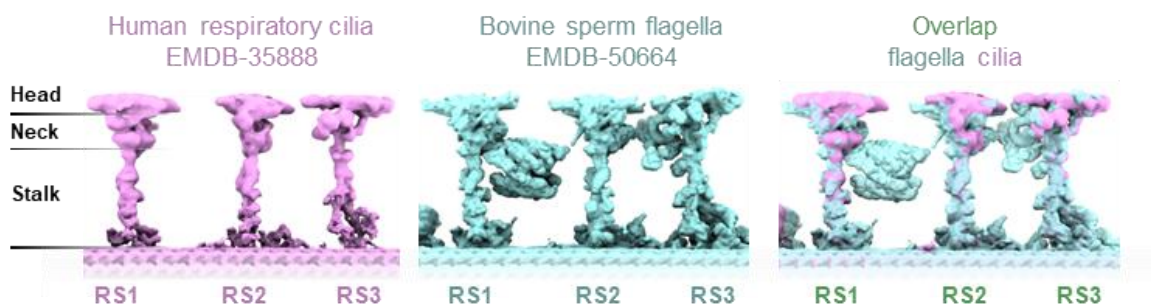
### Chapter 3. Roles of CFAP91 and its proximate protein EFCAB5 in male reproduction

#### Abstract

Radial spokes (RSs) are conserved multiprotein complexes anchored to axonemal microtubule doublets and play a critical role in regulating the motility of both cilia and sperm flagella. CFAP91, a component of the RS3 complex, has been implicated in human male infertility; however, its molecular function remains largely unclear. In this study, we showed that *Cfap91* KO mice displayed defective sperm flagellum formation and complete male infertility. Using a transgenic rescue model expressing FLAG- and BioID2-tagged CFAP91, we found that CFAP91 immunoprecipitated with RS3-associated proteins CFAP251 and LRRC23, both of which failed to localize properly in *Cfap91* KO spermatozoa. Furthermore, proximity labeling in mature spermatozoa identified EFCAB5 as a sperm-specific CFAP91-proximal component. We demonstrated that *Efcab5* KO males exhibited significantly reduced sperm motility and fertility. Together, these findings establish CFAP91 as a critical scaffolding factor required for RS3 assembly and identify EFCAB5 as a sperm-specific regulator of flagellar movement, providing new insights into the molecular architecture underlying sperm motility and its effects on male fertility.

## Introduction

Radial spokes (RSs) are T-shaped structures that extend from each of the nine outer doublet microtubules toward the central pair<sup>73</sup>. Generally, RSs play a crucial role in controlling motility in both cilia and flagella<sup>74</sup>, and structural defects in RSs have been associated with male infertility<sup>75,76</sup>. Each of the three RSs (RS1, RS2, and RS3) is composed of multiple proteins that form distinct configurations. These structures can be divided into head, neck, and stalk domains based on their spatial organization<sup>77</sup> (Figure 20). Along the doublet microtubules, RS1, RS2, and RS3 are arranged in a repeating 96 nm periodic pattern<sup>78</sup>. Interestingly, RS3 is evolutionarily less conserved across species and structurally more diverse between cell types. For example, in *Chlamydomonas reinhardtii*, RS3 exists as a truncated form<sup>15</sup>. Moreover, a bridging structure connecting RS2 to RS3 has been observed exclusively in sperm axonemes but not in other known ciliated cell types<sup>79</sup> (Figure 20).



**Figure 20. Structure of radial spokes**

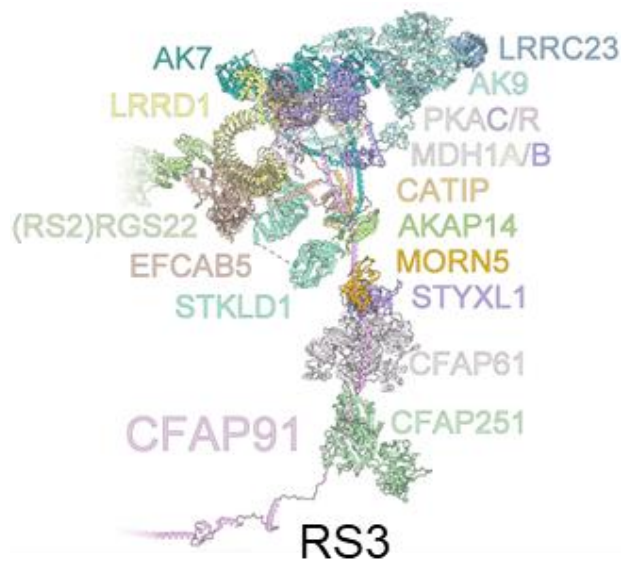
Cryo-EM images of radial spokes of human respiratory cilia and bovine sperm flagella in longitudinal view.

During the final phase of spermatogenesis, spermiogenesis, the axoneme assembles from the centrosome and subsequently anchors to the haploid sperm nucleus<sup>80</sup>. In mice, spermiogenesis is divided into 16 distinct steps, with axoneme formation occurring during the early stages. Axonemal elongation depends heavily on intraflagellar transport (IFT), a system of large protein complexes that facilitates bidirectional cargo movement within cilia and flagella<sup>81</sup>. From step 8 onward, a skirt-like structure, called the manchette, appears at the

caudal side of the sperm nucleus. This structure contributes to the sculpting of the posterior region of the sperm head<sup>49</sup>. Disruptions in axoneme assembly or manchette formation can lead to male infertility<sup>82</sup>.

Previous studies have identified biallelic variants in Cilia and Flagella Associated Protein 91 (CFAP91) among infertile men presenting with severe astheno-teratozoospermia<sup>83</sup>, which is characterized by reduced sperm motility and abnormal morphology. However, the molecular mechanism by which CFAP91 regulates sperm flagellar biogenesis remains poorly understood. In *Chlamydomonas reinhardtii*, the CFAP91 ortholog FAP91 forms a complex with FAP251 (ortholog of CFAP251), and CFAP251 is required for proper RS3 assembly in *Tetrahymena thermophila*<sup>84</sup>. Recent studies integrating structural modeling and cryo-electron microscopy have localized CFAP91 to RS3, where it interacts with CFAP251 in both mouse ependymal and human respiratory cilia<sup>85,86</sup>.

High-resolution analyses of bovine sperm axonemes revealed that the N-terminal region of CFAP91 traverses from the RS2 base to the RS3 base through the base plate of the nexin-dynein regulatory complex (N-DRC), intertwining with the protofilament formed by CCDC39/40. Conversely, the C-terminal region reaches from the RS3 base to the RS3 neck<sup>87</sup>



**Figure 21. Atomic model of Bovine sperm RS3**  
The atomic model was adapted from previous work<sup>87</sup>.

(Figure 21). In this study, we applied a combination of cell biological, proteomic, and genetic approaches to uncover that the loss of CFAP91 causes the mislocalization of its interacting partners and disrupts the elongation of the sperm tail. Moreover, proximity labeling in mature sperm identified EFCAB5 as a sperm-specific protein associated with CFAP91, and deletion of *Efcab5* led to defective sperm motility.

## Materials and Methods

Immunoblotting (IB), immunoprecipitation (IP), mass spectrometry (MS), immunohistochemistry (IHC), Sperm head-tail separation, protein fractionation, fertility test, and statistical analysis

Referring to Chapter 1.

### Experimental animals

The methodology of KO mouse production can be found in Chapter 1. For *Cfap91* KO mouse production, CRISPR/Cas9 genome editing was performed using guide RNA (5'-CAAACAAAGACCCCATACAC-3') and (5'-TTGACATTCAACGAGTGTCT-3'). For *Efcab5* KO mouse production, CRISPR/Cas9 genome editing was performed using guide RNA (5'-TATGCTGGATATGACTACAC-3') and (5'-AGTTGCAACACACCGAGTGT-3'). The methodology of TG mouse production is described in Chapter 2. Primer sequences are indicated in Table 5.

Table 5. Primers used in Chapter 2.

| Primer set name      | Sequence #1               | Sequence #2                          |
|----------------------|---------------------------|--------------------------------------|
| Genotyping F1 - R1   | GAAAGCGGTGCCTGGGCTTAT     | TTGGAAGCCGAGCCTGAGAGA                |
| Genotyping F2 - R2   | TGAGCGAGACCATGCACAGG      | TGCGGGTCTCCTAGAGGCAAA                |
| Genotyping F3 - R3   | TTGAGCGGGCCGCTTGCGCACTGG  | GCCACACCAGCCACCACCTTCTG              |
| Genotyping F4 - R4   | CCTTCCTGCGGCTTGTTCTCT     | GTGTCCATCGCAGCTTGAAGC                |
| Genotyping F5 - R5   | TGTGTGCGAGCGATGGTCAG      | GGCCTACTGTGACTCCCCCATT               |
| Genotyping F6 - R6   | GGGCAGTTGGTTTCTCTTTGTTGC  | TCCTGTGGGTTTTAGGACCGC                |
| <i>Cfap91</i> RT-PCR | AGCCAGACAGTGACCATCCA      | CTAGCCTTCTCCCTCATCCTG                |
| <i>Efcab5</i> RT-PCR | GAAGTGGGACAATGATGGCTCAGG  | TTAAAGTTTCTCTCGGATTGATCT<br>TTCTGAGG |
| <i>Actb</i> RT-PCR   | CATCCGTAAAGACCTCTATGCCAAC | ATGGAGCCACCGATCCACA                  |

### RT-PCR

The methodology is described in Chapter 1. Primer sequences are indicated in Table 5.

## Antibodies

All antibodies used in this Chapter are listed in Table 6.

Table 6. Antibodies used in Chapter 1.

| Antigen            | Identifier | Source                            | Dilution                                        |
|--------------------|------------|-----------------------------------|-------------------------------------------------|
| Acetylated tubulin | T7451      | Sigma-Aldrich                     | 1: 1000 for IB, 1: 500 for IF                   |
| Acetylated tubulin | D20G3      | Cell Signaling Technology         | 1: 1000 for IB, 1: 500 for IF                   |
| ACTB               | ab6276     | Abcam                             | 1: 2000 for IB                                  |
| AKAP3              | 13907-1-AP | Proteintech                       | 1: 1000 for IB                                  |
| AKAP4              | 611564     | BD Biosciences                    | 1: 1000 for IB                                  |
| Alpha-tubulin      | T6074      | Sigma-Aldrich                     | 1: 1000 for IB                                  |
| BASIGIN            | -          | This lab                          | 1: 1000 for IB                                  |
| CFAP251            | HPA040005  | Atlas Antibodies                  | 1: 200 for IB, 1: 50 for IF                     |
| EFCAB5             | -          | This study                        | 1: 200 for IB                                   |
| EFCAB5 (human)     | 24296-1-AP | Proteintech                       | 1: 100 for IB                                   |
| EGFP               | -          | This lab                          | 1: 1000 for IB                                  |
| FLAG               | PM020      | MBL                               | 1: 1000 for IB, 1: 500 for IF                   |
| FLAG               | F1804      | Sigma-Aldrich                     | 1: 1000 for IB, 1: 500 for IF, 2 $\mu$ L for IP |
| HA                 | M180-3     | MBL                               | 1: 1000 for IB                                  |
| IFT140             | 17460-1-AP | Proteintech                       | 1: 500 for IB                                   |
| IZUMO1             | 64-125     | This lab                          | 1: 1000 for IB                                  |
| LRRC23             | -          | This lab                          | 1: 200 for IB, 1: 50 for IF                     |
| PA                 | NZ-1       | FUJIFILM Wako Pure Chemical Corp. | 1: 1000 for IB                                  |
| RGS22              | PA5-104095 | Invitrogen                        | 1: 1000 for IB                                  |
| RSPH6A             | -          | This lab                          | 1:1000 for IB                                   |
| RSPH9              | HPA031703  | Atlas Antibodies                  | 1:200 for IB                                    |

## Immunocytochemistry (ICC) and Transmission Electron Microscopy (TEM)

The methodology is described in Chapter 2.

## Imaging of the manchette using *EGFP-Tuba3a* transgenic (TG) male mice

Seminiferous tubules were isolated from *EGFP-Tuba3a* TG males by carefully removing the tunica albuginea. The tubules were gently pipetted to detach Leydig cells and then lightly

squeezed with fine forceps to release spermatids into phosphate-buffered saline (PBS). The collected spermatids were stained with 0.1% Hoechst 33342 for nuclear visualization and imaged using an Olympus BX-53 fluorescence microscope.

#### Cell culture and transfection

HEK293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich) and 1% penicillin-streptomycin (Thermo Fisher Scientific) at 37 °C in a humidified atmosphere containing 5% CO<sub>2</sub>. Cells were subcultured every three to four days. Transfection was performed using the calcium phosphate-DNA co-precipitation method when the cells reached 70-80% confluency. Cells were harvested 24 hours post-transfection for subsequent experiments.

#### Proximity labeling of cauda epididymal spermatozoa

Proximity labeling was performed according to previous reports<sup>88,89</sup>. Spermatozoa were collected from the cauda epididymis into TYH medium supplemented with 250 µM biotin (Sigma-Aldrich) and incubated at 37 °C under 5% CO<sub>2</sub> for 16 hours. After incubation, spermatozoa were washed twice with PBS. Spermatozoa were lysed in SDS lysis buffer [50 mM Tris (pH 7.4), 500 mM NaCl, 0.4% SDS, 1 mM dithiothreitol (DTT), and 1× protease inhibitor cocktail] for 1 hour at 4 °C. Triton X-100 was then added to the lysate to a final concentration of 2%, followed by vigorous vortexing for 2 minutes. Subsequently, 50 mM Tris (pH 7.4) was added to adjust the final Triton X-100 concentration to 1%. Samples were briefly vortexed and centrifuged at 15,000 × g for 20 minutes, and the supernatants were collected. The supernatants were incubated with Pierce Streptavidin Magnetic Beads (Thermo Fisher Scientific) overnight at 4 °C to capture biotinylated proteins. After incubation, the beads were washed three times using the wash buffer described in the referenced study. Proteins bound to the beads were eluted by incubating the beads with 2× SDS sample buffer at 95 °C for 5 minutes. The eluates were analyzed using immunoblotting and MS.

### Structural analysis

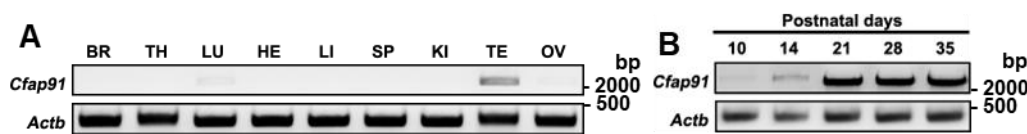
Cryo-electron microscopy (cryo-EM) density maps and fitted atomic models were visualized and analyzed using UCSF ChimeraX<sup>90</sup>.

## Results

### Section 1: Phenotypic analyses on *Cfap91* KO mice

#### RT-PCR of mouse *Cfap91*

To determine the tissue-specific expression profile of *Cfap91*, reverse transcription polymerase chain reaction (RT-PCR) was performed on multiple adult mouse tissues. *Cfap91* exhibited predominant expression in the testis, with weak expression in the lung (Figure 22A). RT-PCR analysis of developing testes from postnatal days 10 to 35 demonstrated that *Cfap91* expression began on postnatal day 14 (Figure 22B), which coincided with the first appearance of spermatocytes during the initial wave of spermatogenesis.



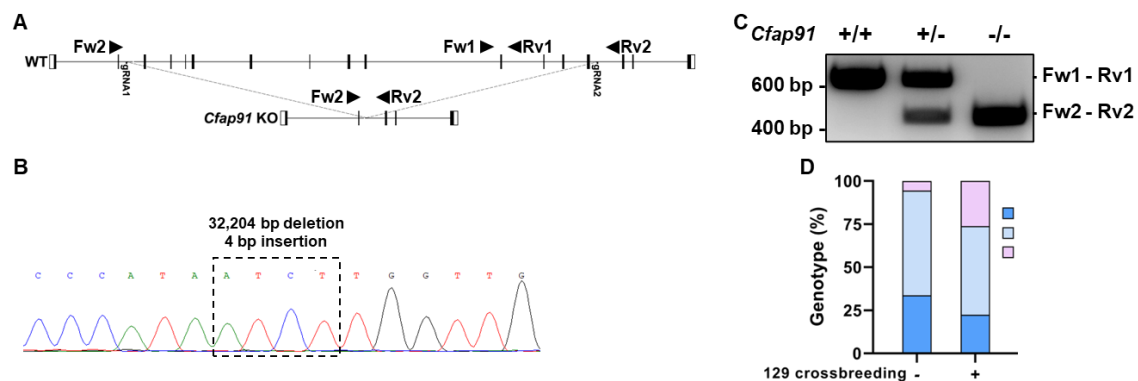
**Figure 22. RT-PCR of *Cfap91***

Expression analysis of *Cfap91* by RT-PCR using cDNA from multiple adult mouse tissues (A) and from postnatal testes (B). *Actb* served as the loading control. Br, brain; Th, thymus; Lu, lung; He, heart; Li, liver; Sp, spleen; Ki, kidney; Te, testis; Ov, ovary.

#### Generation of *Cfap91* KO mouse

To elucidate the function of *Cfap91* in spermatogenesis, we generated *Cfap91* KO mice. Cas9 and two guide RNAs (gRNAs) targeting the *Cfap91* genomic region were electroporated into 64 zygotes obtained from B6D2F1 × B6D2F1 mating (Figure 23A). After transferring 62 two-cell embryos into pseudopregnant females, we obtained eight pups, six of which carried large deletions in *Cfap91*. *Cfap91* mutant F0 mice were crossed with WT B6D2F1 mice to produce heterozygous (HET) F1 mice, which were intercrossed to obtain *Cfap91* KO mice. Sequence analysis revealed a 32,204 bp deletion and a 4 bp insertion in the *Cfap91* locus (Figure 23B), which was confirmed by genomic PCR (Figure 23C). Similar to KO studies of other axonemal proteins, *Cfap91* KO mice were not obtained at Mendelian ratios; only 5.6% of pups from HET × HET mating (5 of 89) were homozygous mutants (Figure 23D), and all

died before 8 weeks of age. Since mutations in other *Cfap* genes have been associated with hydrocephalus<sup>91,92</sup>, we hypothesized that *Cfap91* KO mice on the B6D2 background might exhibit a similar phenotype. As mice on the 129S6/SvEvTac (129) background are less susceptible to hydrocephalus<sup>93</sup>, we crossed B6D2 *Cfap91* HET mice with 129 WT mice. From this B6D2/129 background, we obtained *Cfap91* KO mice at Mendelian ratios (28 of 107 pups; 26.2%) (Figure 23D). Therefore, *Cfap91* KO mice on the B6D2/129 background were used for subsequent analyses.

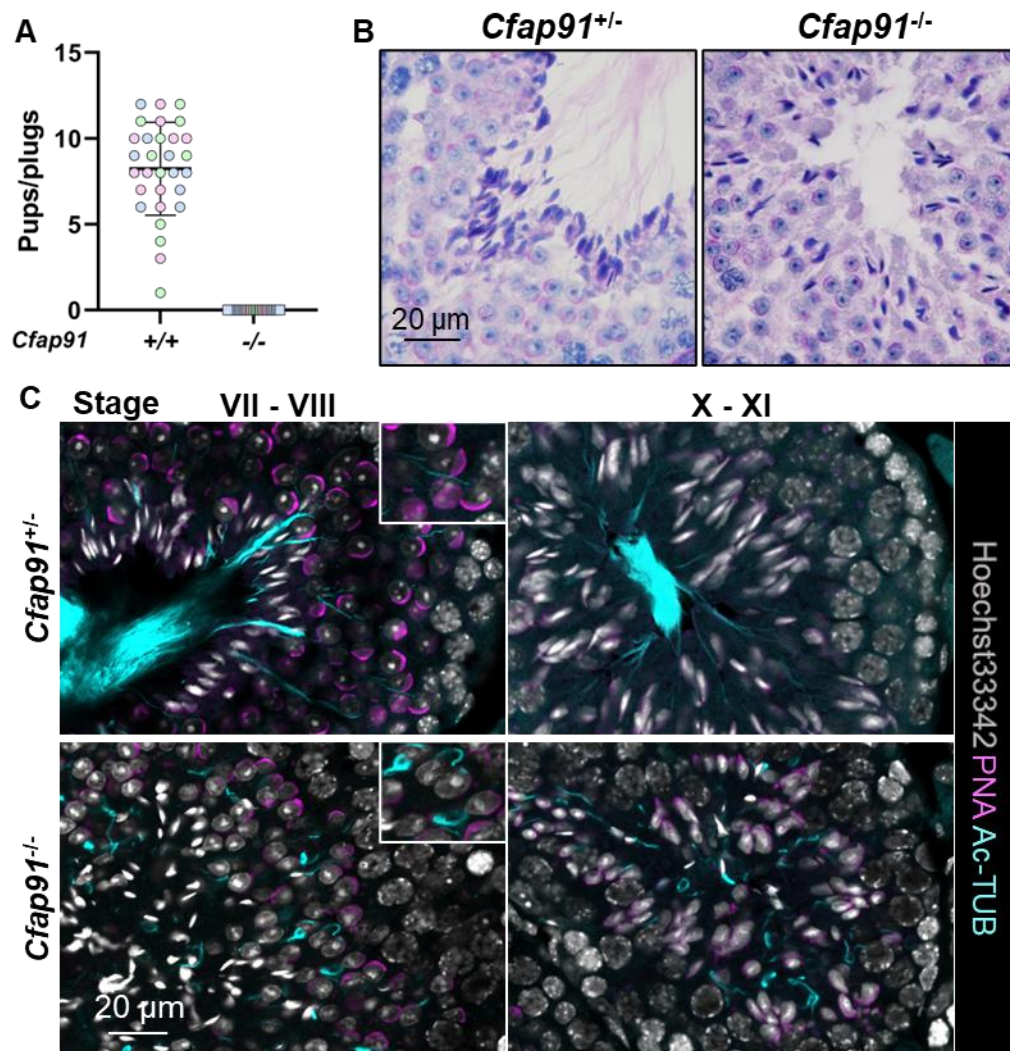


**Figure 23. Generation of *Cfap91* KO males**

(A) Schematic representation of the *Cfap91* KO strategy. (B) Confirmation of a large genomic deletion in *Cfap91* by Sanger sequencing. (C) Genomic PCR analysis of *Cfap91* mutant mice using the primers indicated in (A). (D) Genotypic ratio of *Cfap91* WT, HET, and KO offspring obtained from intercrosses of *Cfap91* HET mice in B6D2 or B6D2/129 backgrounds.

### Ablation of *Cfap91* results in male infertility accompanied by defective spermiogenesis

To assess fertility, *Cfap91* KO males were caged with WT females. None of the *Cfap91* KO males produced offspring in the three independent mating trials (Figure 24A). Histological examination revealed that in *Cfap91* HET testes, elongated spermatids were aligned toward the lumen of the seminiferous tubules at stages VII-VIII, whereas sperm tails were absent in *Cfap91* KO testes (Figure 24B). Immunohistochemistry using anti-acetylated tubulin confirmed shortened sperm tails in *Cfap91* KO testes (Figure 24C). These observations indicate that *Cfap91* KO disrupts spermiogenesis.



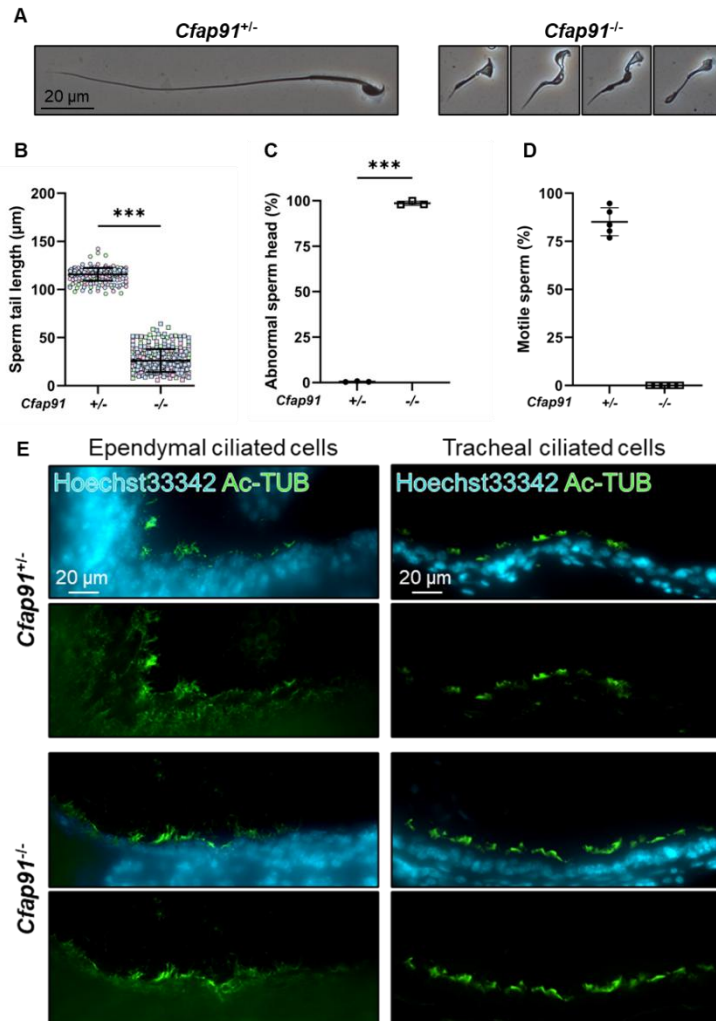
**Figure 24. *Cfap91* KO males exhibited abnormal sperm tail biogenesis**

(A) Mating test of *Cfap91* WT and KO males. Data from each male are individually color-coded. (B) Testicular sections of stage VII-VIII seminiferous tubules from *Cfap91* HET and KO males. (C) Immunohistochemistry of stage VII-VIII, and X-XI testicular seminiferous tubules from *Cfap91* HET and KO males.

#### *Cfap91* KO males exhibit oligoasthenoteratozoospermia

Next, we examined the epididymis and spermatozoa of *Cfap91* KO males. *Cfap91* KO spermatozoa exhibited abnormal morphology (Figure 25A), including shorter tails (Figure 25B), misshapen heads (Figure 25C), and complete immotility (Figure 25D). Because motile cilia share a similar axonemal structure with sperm flagella, we examined ependymal and

tracheal motile cilia using anti-acetylated tubulin staining. No apparent differences were observed between *Cfap91* HET and KO mice (Figure 25E), suggesting that ciliary elongation was preserved in *Cfap91* KO mice despite the disrupted sperm flagellum formation.



**Figure 25. Shortened spermatozoa but normal cilia were seen in *Cfap91* KO males**

(A) Cauda epididymal spermatozoa from *Cfap91* HET and KO males.

(B) Tail length of cauda epididymal spermatozoa from *Cfap91* HET and KO males. Data from each male are individually color-coded.

(C) Ratio of spermatozoa with abnormal heads in *Cfap91* HET and KO males.

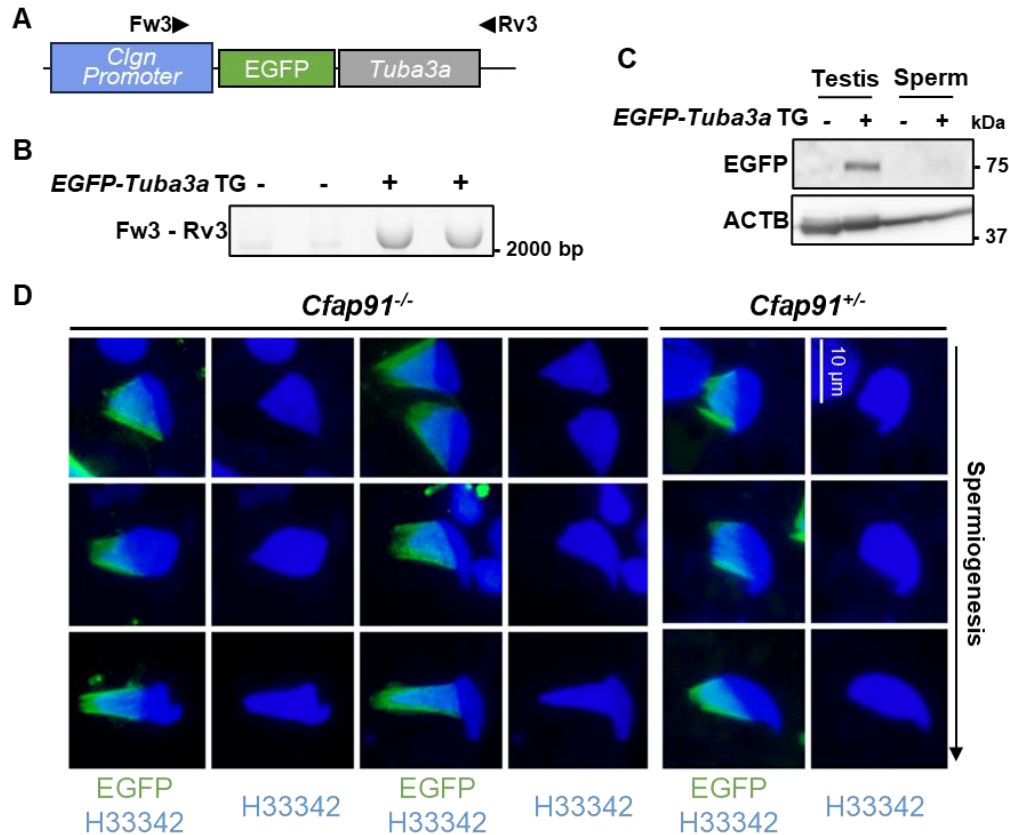
(D) Ratio of motile spermatozoa from the cauda epididymis of *Cfap91* HET and KO males.

(E) Immunohistochemistry on sections of mouse brain and trachea from *Cfap91* HET and KO males.

\*\*\*,  $p < 0.001$ .

Because *Cfap91* KO mice show abnormal sperm heads and manchette-mediated sperm head morphogenesis could cause such a defect, we attempted to visualize the manchette. To achieve this, we generated TG mice expressing EGFP-TUBA3A under the testis-specific *Clgn* promoter (Figure 26A). The integration of the transgene was verified using genomic PCR (Figure 26B), and EGFP-TUBA3A expression was confirmed by immunoblotting in testes but not in mature spermatozoa (Figure 26C). *EGFP-Tuba3a* TG mice were crossed

with *Cfap91* mutant mice to examine the manchette morphology in *Cfap91* KO spermatids (Figure 26D). In early elongating spermatids, manchettes appeared normal; however, at later stages, *Cfap91* KO spermatids displayed elongated manchettes and abnormal head shapes, indicating that aberrant manchette elongation likely caused head deformation.

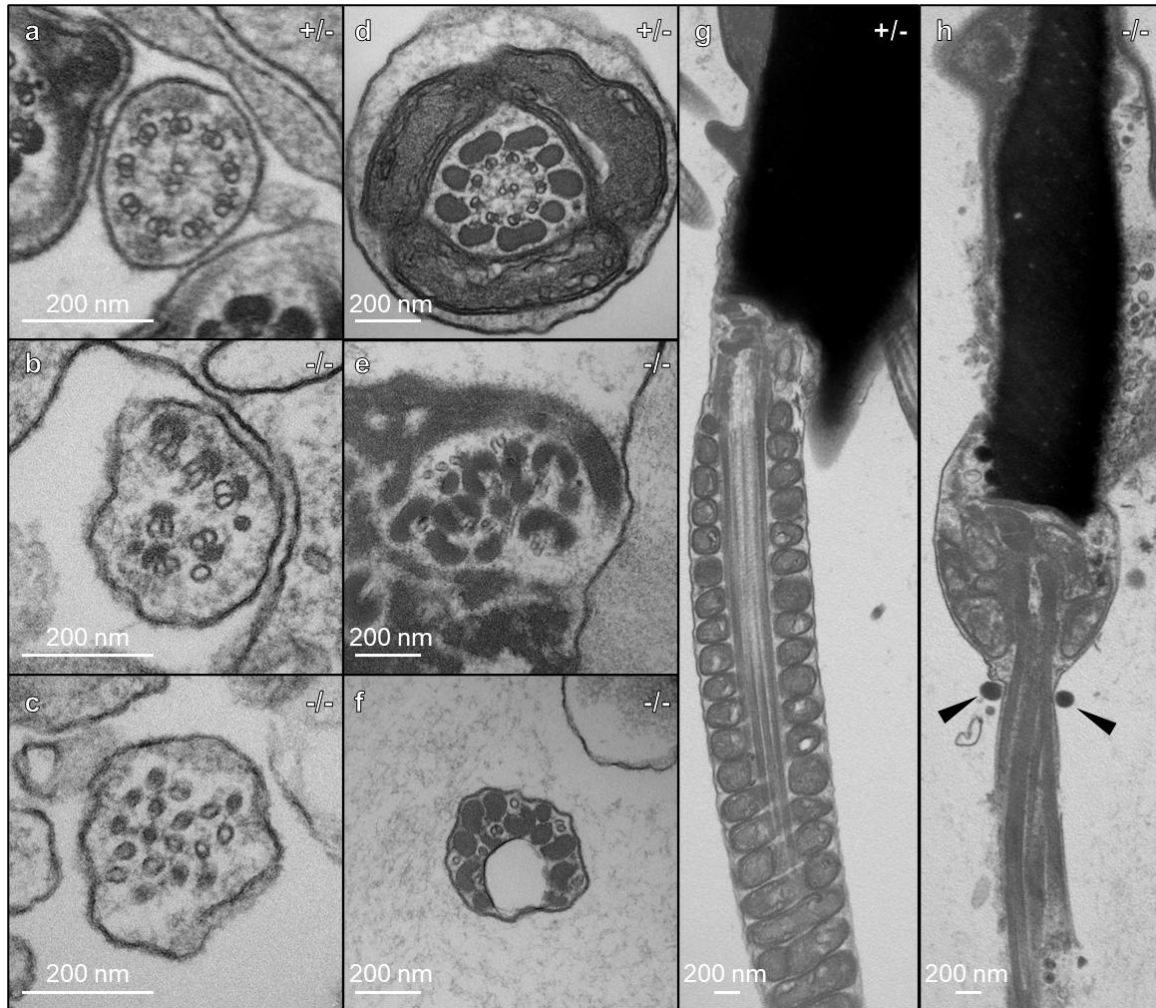


**Figure 26. Observation of manchette in *Cfap91* KO males**

(A) Schematic representation of the EGFP-Tuba3a construct under the control of the *Clgn* promoter. (B) Genomic PCR using specific primers confirmed the transgene. (C) Immunoblot analysis showing EGFP expression in the testes and cauda epididymal spermatozoa of wild-type and *EGFP-Tuba3a* transgenic males, with ACTB serving as a loading control. (D) Visualization of manchette microtubules in *EGFP-Tuba3a* TG mice carrying *Cfap91* heterozygous or knockout alleles.

TEM revealed structural defects in both testicular spermatids and cauda epididymal spermatozoa of *Cfap91* KO males (Figure 27). In testes, *Cfap91* KO spermatids showed disrupted microtubule organization (Figure 27A-C). In cauda spermatozoa, the axoneme, mitochondrial sheath, outer dense fibers, and fibrous sheath were all disordered (Figure 27D-

H). The annulus, which normally migrates distally during late spermiogenesis to demarcate the midpiece-principal piece junction<sup>46</sup>, failed to reach its proper position in *Cfap91* KO spermatozoa (Figure 27H). Together, these findings demonstrate that *Cfap91* deficiency leads to oligoasthenoteratozoospermia and male infertility.



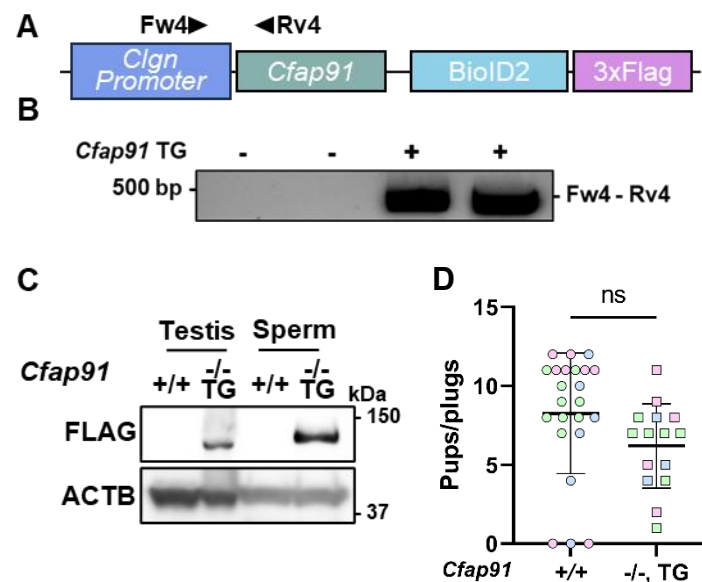
**Figure 27. TEM analysis of *Cfap91* KO spermatozoa**

(A-F) Transmission electron microscopy images showing cross-sections of spermatids from the testes (A-C) and spermatozoa from the cauda epididymis (D-F) of *Cfap91* HET and KO males. (G and H) Longitudinal sections of cauda epididymal spermatozoa from *Cfap91* HET and KO males. The genotype of each sample is indicated in the upper right corner of each panel. Abnormal positioning of the proximal annulus observed in *Cfap91* KO spermatozoa is marked with black arrowheads.

## Section 2: Identification of axonemal protein EFCAB5 using a *Cfap91* transgenic mouse line

### *Cfap91* transgene rescues the infertility of *Cfap91* KO males

To confirm that the infertility observed in *Cfap91* KO males resulted specifically from the loss of *Cfap91* and not from unrelated factors, we generated *Cfap91* TG mice. The transgene encoded CFAP91 tagged with BioID2 and 3×FLAG at its C-terminus, driven by the testis-specific *Clgn* promoter (Figure 28A). The genotypes of *Cfap91* TG mice were confirmed by genomic PCR (Figure 28B), and CFAP91-BioID2-3×FLAG protein expression was verified by immunoblotting (Figure 28C). Furthermore, *Cfap91* KO TG males exhibited normal fertility, equivalent to that of WT males (Figure 28D). These results demonstrate that the infertility and oligo-astheno-teratozoospermia observed in *Cfap91* KO males were specifically caused by the absence of CFAP91.

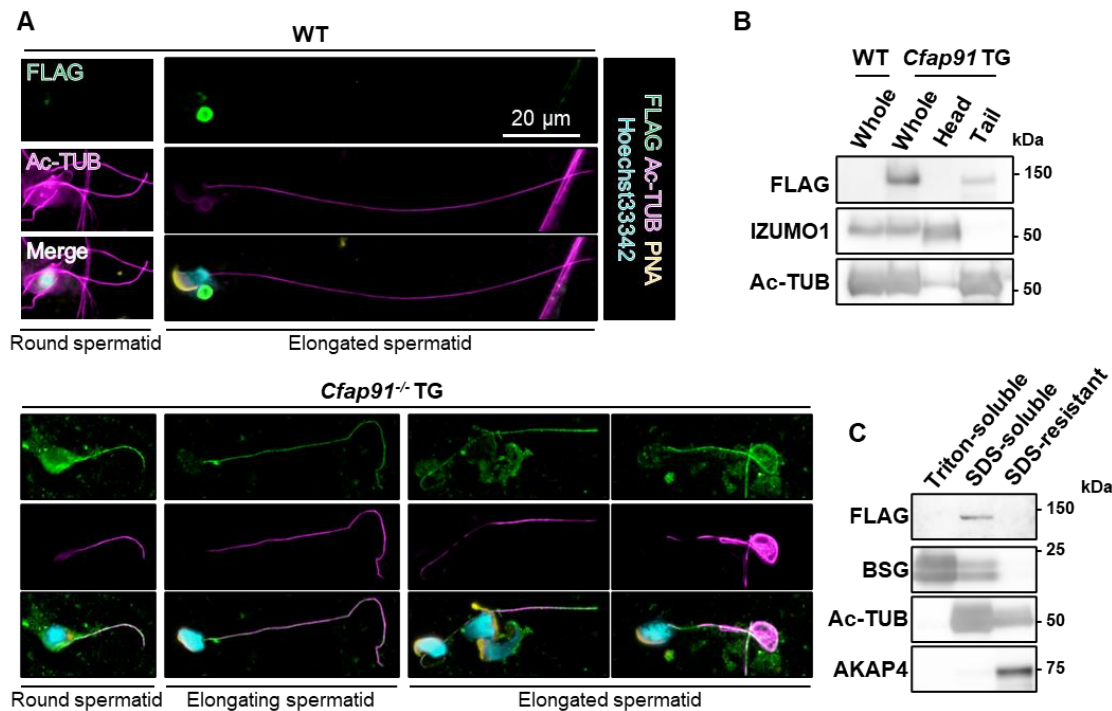


**Figure 28. Generation and mating test of *Cfap91* KO TG males**

(A) Schematic representation of the *Cfap91*-BioID2-3×FLAG transgene construct. (B) Genomic PCR validation of *Cfap91* TG mice. (C) Immunoblotting of CFAP91-BioID2-3×FLAG using an anti-FLAG antibody on lysates from testes and cauda epididymal spermatozoa of WT and *Cfap91* KO TG males. (D) Fertility assessment of *Cfap91* WT and KO TG males. Data from each male are individually color-coded. ns, not significant.

### CFAP91 localizes to sperm tails and associates with axonemal proteins

To determine the subcellular localization of CFAP91, we performed immunocytochemistry on isolated germ cells. Immunocytochemistry results revealed that CFAP91 localizes to both the cytoplasm and developing sperm tails during the round spermatid stage, while cytoplasmic signals diminished as spermatids elongated (Figure 29A), suggesting that CFAP91 is transported from the cytoplasm to the sperm tail during spermiogenesis. Consistently, immunoblotting of sperm head-tail-separated lysates showed that CFAP91 was present exclusively in the tail fraction (Figure 29B). To identify the specific compartmental association, sperm tails were fractionated into Triton X-100-soluble (transmembrane/cytosolic), SDS-soluble (axonemal), and SDS-resistant (outer dense fiber/fibrous sheath) fractions. CFAP91 was enriched in the SDS-soluble fraction (Figure 29C), indicating that it associates primarily with the axoneme.

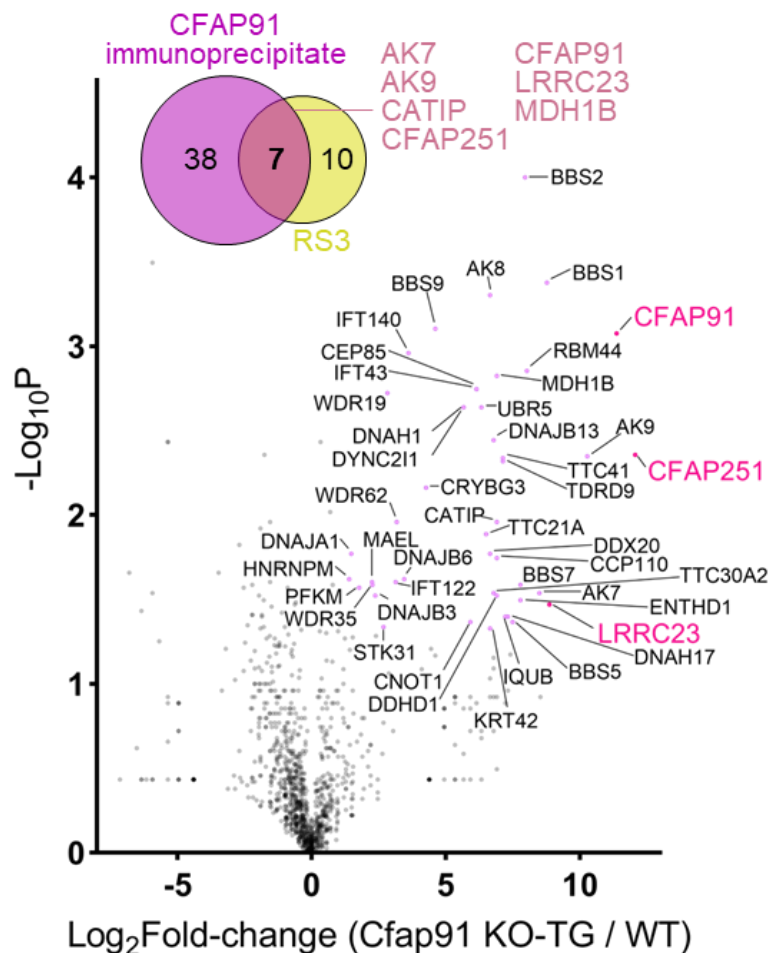


**Figure 29. Localization of *Cfap91* in spermatozoa**

(A) Immunocytochemical detection of *CFAP91-BioID2-3xFLAG* in spermatids. (B) Immunoblotting of separated sperm head and tail fractions. IZUMO1 and acetylated tubulin (Ac-TUB) were used as markers for the head and tail fractions, respectively. (C) Immunoblotting of fractionated sperm lysates. BASIGIN, Ac-TUB, and AKAP4 were used as markers for the triton-soluble, SDS-soluble, and SDS-resistant fractions, respectively.

### CFAP91 interacts with RS3 proteins during spermiogenesis

To identify proteins associated with CFAP91, we performed immunoprecipitation-mass spectrometry on testis lysates from *Cfap91* KO TG males using an anti-FLAG antibody. Forty-five proteins were significantly enriched in the CFAP91 immunoprecipitates (Figure 30). Among them, CFAP91, AK7, AK9, CATIP, CFAP251, LRRC23, and MDH1B are known RS3-associated proteins in mammalian sperm<sup>87</sup>, consistent with CFAP91 localization in the RS3 region.

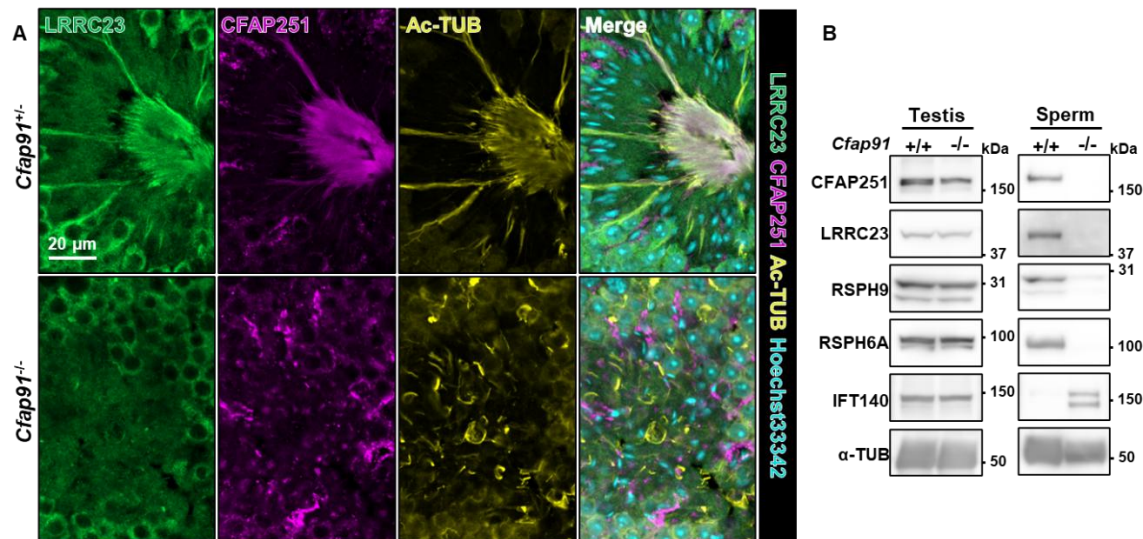


**Figure 30. Interactome of CFAP91 mapped by IP-MS**

Volcano plot showing the results of IP-MS analysis using an anti-FLAG antibody. Proteins exhibiting a fold change > 2 and a P-value < 0.05 in *Cfap91* KO TG males compared with WT males are considered significantly upregulated and are highlighted in magenta.

### CFAP91-interacting proteins could not be localized to spermatozoa in the absence of CFAP91

We next examined how the absence of CFAP91 affects these RS3 proteins. In *Cfap91* KO testes, CFAP251 and LRRC23 failed to localize to sperm tails (Figure 31A). Although their total protein levels were unchanged in *Cfap91* KO testes, both were markedly reduced in *Cfap91* KO spermatozoa (Figure 31B). Furthermore, IFT140, a marker of ongoing flagellar elongation, persisted abnormally in *Cfap91* KO spermatozoa but not in WT spermatozoa, suggesting that tail formation was arrested in the absence of CFAP91. Collectively, these results indicate that CFAP91 is required for proper RS3 protein localization during spermiogenesis.



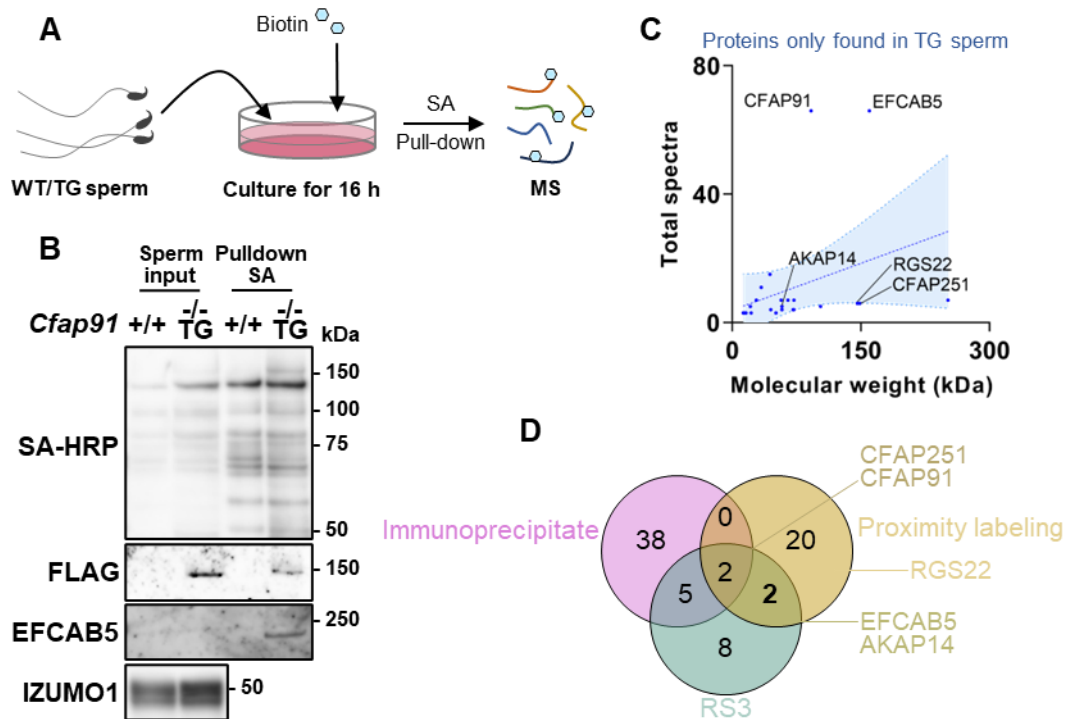
**Figure 31. RS3 proteins were mis-localized in *Cfap91* KO males**

(A) Immunohistochemical analysis of testicular sections from *Cfap91* HET and KO males. (B) Immunoblotting of testes and cauda epididymal spermatozoa from WT and *Cfap91* KO males, with  $\alpha$ -tubulin ( $\alpha$ -TUB) used as a loading control.

### Proximity labeling identifies EFCAB5 as a sperm-specific RS3 protein

Studying protein interactions of axonemal components in mature spermatozoa is challenging, as harsh lysis buffers often disrupt native interactions<sup>94</sup>. To overcome this, we used proximity labeling in *Cfap91* KO TG spermatozoa expressing CFAP91-BioID2-3 $\times$ FLAG. BioID2 catalyzes the biotinylation of nearby proteins within a ~10 nm radius in

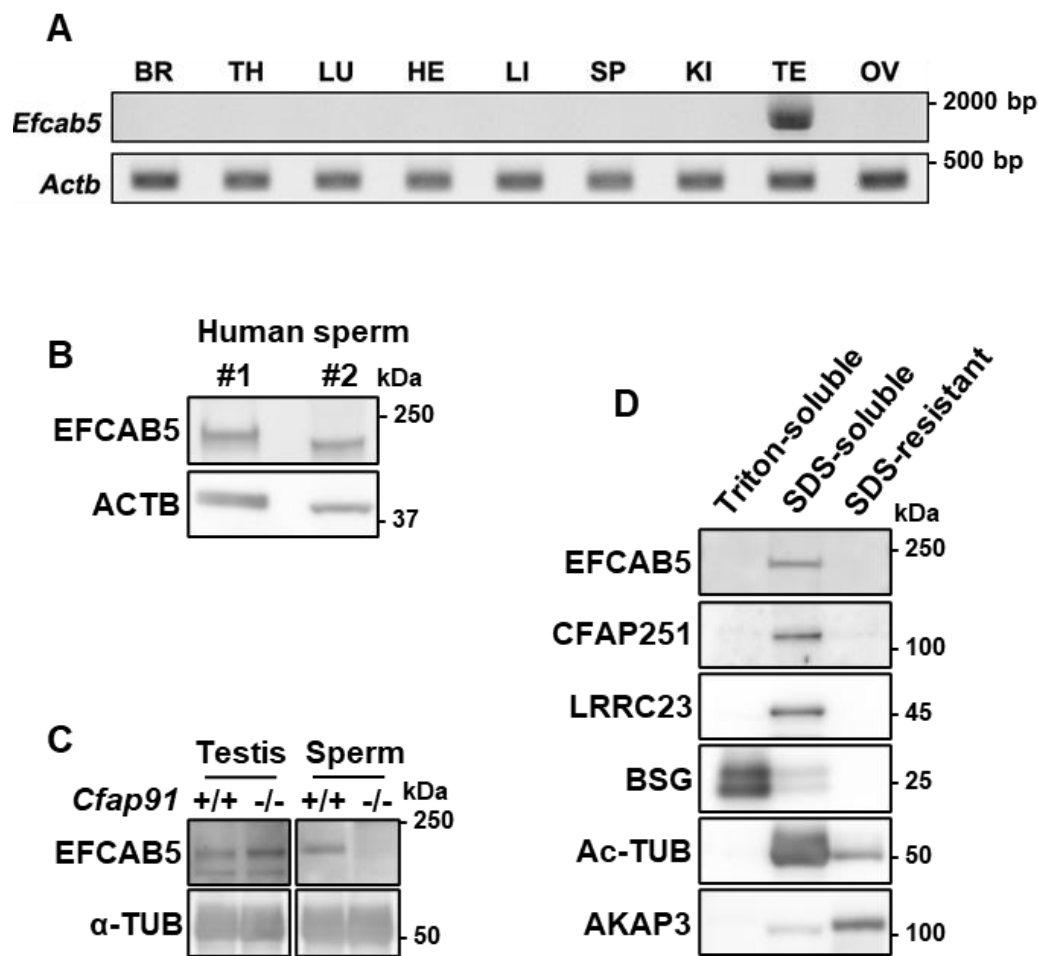
the presence of biotin<sup>88</sup>. Mature spermatozoa from *Cfap91* KO TG cauda epididymis were incubated with biotin for 16 hours, lysed, and biotinylated proteins were pulled down using streptavidin and analyzed by MS (Figure 32A). Immunoblotting confirmed the self-biotinylation of CFAP91 (Figure 32B), validating this assay. Twenty-four proteins were uniquely detected in the streptavidin pull-down assay of *Cfap91* KO TG spermatozoa, with CFAP91 and EFCAB5 showing the highest enrichment (Figure 32C). EFCAB5 co-purified with streptavidin in mature spermatozoa, as confirmed by immunoblotting (Figure 32B). Proximity labeling also identified two RS3 proteins (CFAP251 and AKAP14) and one RS1/2 protein (RGS22), the latter of which was predicted to interact with EFCAB5 within the RS2-RS3 bridge region (Figure 32D).



**Figure 32. Proximity labeling identified EFCAB5 as a CFAP91-proximate protein**

(A) Schematic illustration of the BioID2 approach applied to mature spermatozoa. Spermatozoa from WT and *Cfap91* KO TG males were incubated in biotin-supplemented medium for 16 hours. Biotinylated proteins were then isolated using streptavidin (SA) pull-down following lysis. (B) Immunoblot analysis of biotinylated proteins from WT and *Cfap91* KO TG spermatozoa. (C) Molecular weight and total spectra of 24 proteins identified in *Cfap91* KO TG spermatozoa, with CFAP91 and EFCAB5 showing the highest peptide counts. (D) Venn diagram comparing CFAP91 immunoprecipitates, proteins identified by proximity labeling, and known sperm RS3 proteins.

RT-PCR revealed that *Efcab5* was predominantly expressed in the mouse testis (Figure 33A). EFCAB5 was also detected in human spermatozoa (Figure 33B) but was absent in *Cfap91* KO spermatozoa (Figure 33C). Fractionation analysis of WT spermatozoa revealed that EFCAB5, CFAP251, LRRC23, and CFAP91 all localized to the axonemal (SDS-soluble) compartment (Figure 33D). Together, these results identify EFCAB5 as a sperm-specific RS3 component that associates with CFAP91 and other RS3 proteins in mice.



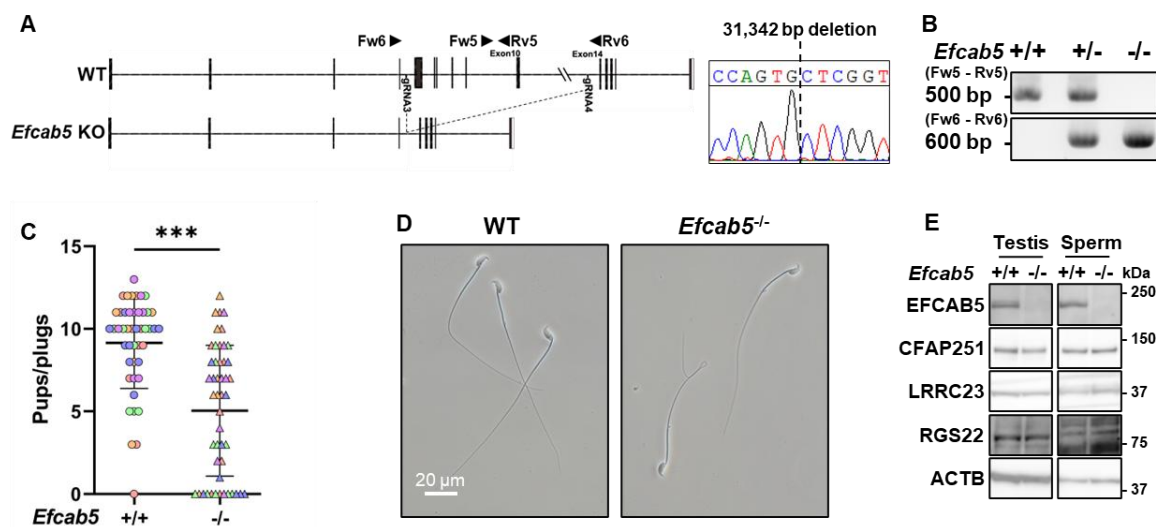
**Figure 33. EFCAB5 is an axoneme associated protein**

(A) RT-PCR analysis of *Efcab5* using cDNA from various mouse organs, with *Actb* serving as a loading control. (B) Immunoblot analysis of EFCAB5 in human spermatozoa. (C) Immunoblotting of testes and cauda epididymal spermatozoa from WT and *Cfap91* KO males using an anti-EFCAB5 antibody. Alpha-tubulin ( $\alpha$ -TUB) was used as a loading control. (D) Fractionation analysis of WT spermatozoa, showing that EFCAB5, LRRC23, and CFAP251 are present in the SDS-soluble fraction.

### Section 3: Phenotypic analysis on *Efcab5* KO mice

#### EFCAB5 functions in male fertility

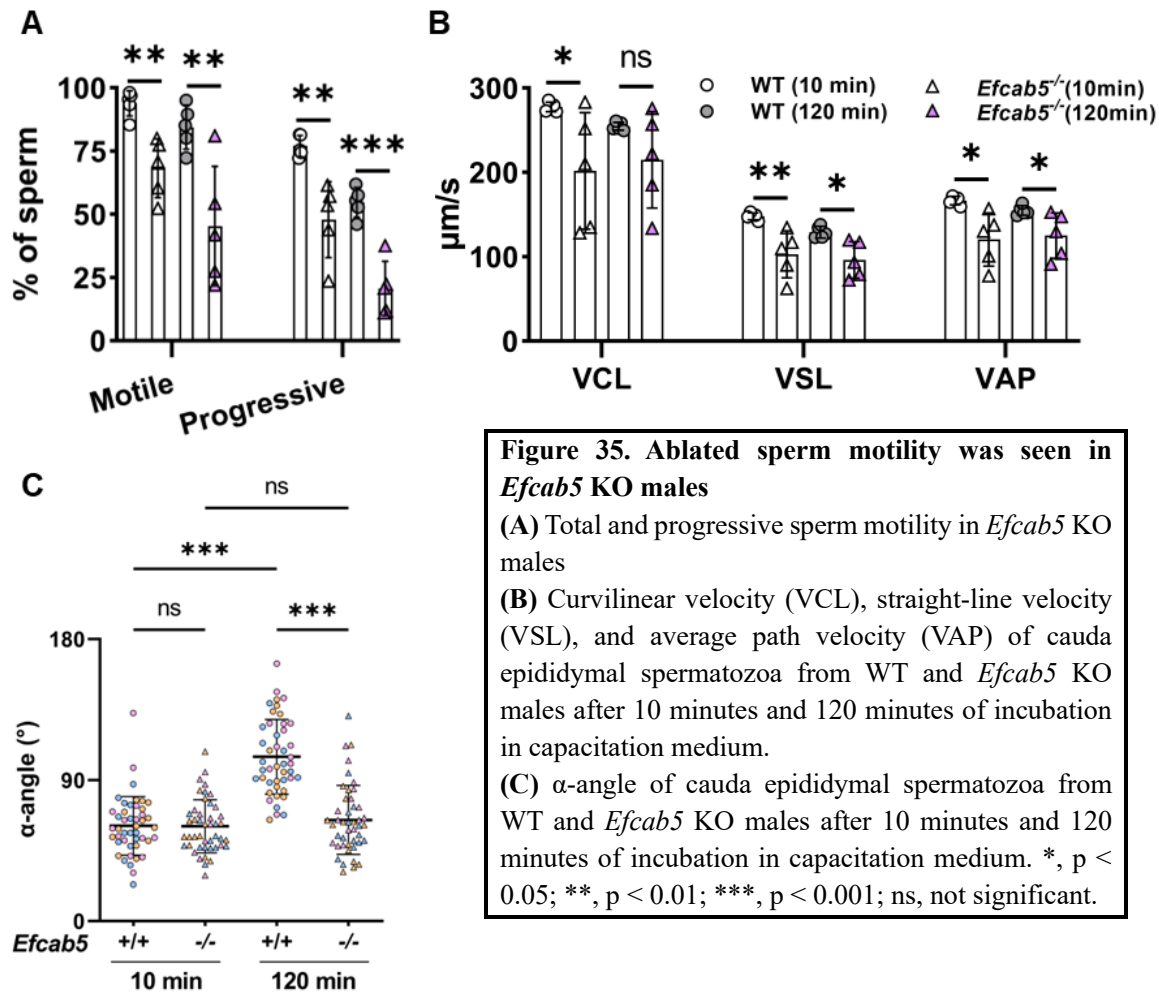
To assess the role of *Efcab5* in male fertility, we generated *Efcab5* KO mice. Cas9 and two guide RNAs (gRNAs) targeting the *Efcab5* genomic region were electroporated into 64 zygotes derived from B6D2F1  $\times$  B6D2F1 mating (Figure 34A). Fifty-six two-cell embryos were subsequently transferred to the oviducts of two pseudopregnant females, resulting in 13 offspring. Among them, two carried large deletions within the *Efcab5* locus. Through breeding of the founder animals, *Efcab5* KO mice were established, and the targeted deletion was confirmed by genomic PCR (Figure 34B). Male *Efcab5* KO mice exhibited slightly reduced fertility in mating tests (Figure 34C). However, no apparent abnormalities were observed in the morphology of mature spermatozoa (Figure 34D). In contrast to *Cfap91* KO males, CFAP251 and LRRC23 were still detectable in both testes and spermatozoa of *Efcab5* KO males (Figure 34E).



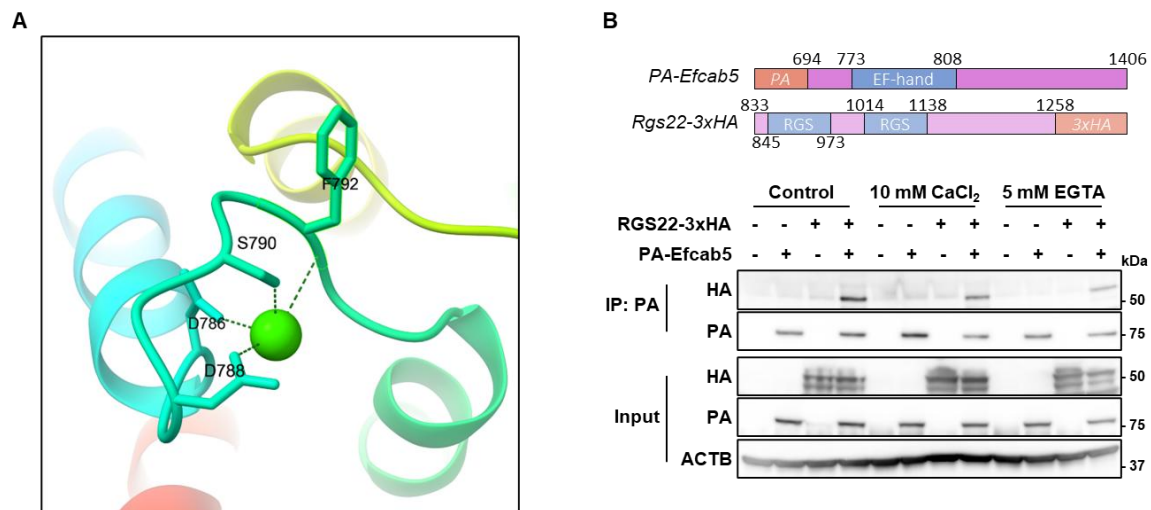
**Figure 34. KO of *Efcab5* resulted in normal sperm tail formation**

(A) Schematic diagram of the *Efcab5* KO strategy. (B) Genomic PCR of *Efcab5* mutant mice using the primers shown in (A). (C) Mating of WT and *Efcab5* KO males. Each male is color-coded individually. \*\*\*,  $p < 0.001$ . (D) Phase-contrast images of cauda epididymal spermatozoa from WT and *Efcab5* KO males. (E) Immunoblot analysis of testes and cauda epididymal spermatozoa from WT and *Efcab5* KO males using antibodies against EFCAB5, CFAP251, LRRC23, and RGS22.

Analysis of sperm motility revealed that *Efcab5* KO spermatozoa displayed significantly lower motility after both 10 minutes and 120 minutes of incubation in the capacitation medium (Figure 35A). In addition, VCL, VSL, and VAP were all reduced after 10 minutes of incubation, and VSL and VAP remained decreased after 120 minutes when compared with WT spermatozoa (Figure 35B). To evaluate the pattern of flagellar movement, we measured the maximal bending angle of flagella relative to the sperm head ( $\alpha$ -angle), which is a quantitative index of hyperactivation<sup>95</sup>. As previously reported,  $\alpha$ -angles of WT spermatozoa increased after 2 hours of incubation in capacitation medium compared with 10 minutes. However, this increase was absent in *Efcab5* KO spermatozoa (Figure 35C), indicating that the reduced fertility of *Efcab5* KO males is attributable to abnormal flagellar motility.



Given that EFCAB5 localizes within the sperm-specific RS2-RS3 bridge<sup>87</sup>, we propose that it contributes to the specialized regulation of sperm flagellar movement through this structure. In bovine spermatozoa, the RS2-RS3 bridge is composed of EFCAB5 and RGS22, and it has been suggested that EFCAB5 binding induces conformational changes in RGS22. To test this possibility, we examined the amount and localization of RGS22 in *Efcab5* KO testes but found no apparent abnormalities (Figure 34E). EFCAB proteins typically contain one or more EF-hand  $\text{Ca}^{2+}$ -binding domains, and AlphaFold<sup>96</sup> predictions indicated that EFCAB5 retains  $\text{Ca}^{2+}$ -binding capacity within this domain (Figure 36A). Because  $\text{Ca}^{2+}$  influx occurs during capacitation to regulate hyperactivation<sup>97,98</sup>, we next investigated whether  $\text{Ca}^{2+}$  influences the EFCAB5-RGS22 interaction. EFCAB5 and RGS22 were co-expressed in HEK293T cells, followed by immunoprecipitation in the presence or absence of  $\text{Ca}^{2+}$ . RGS22 consistently co-immunoprecipitated with EFCAB5, regardless of the  $\text{Ca}^{2+}$  concentration (Figure 36B), indicating that their interaction is  $\text{Ca}^{2+}$ -independent.



**Figure 36. Interaction of EFCAB5 with RGS22 is independent of  $\text{Ca}^{2+}$  concentration**

**(A)** The structure of the EFCAB5- $\text{Ca}^{2+}$  complex was predicted using AlphaFold, yielding a predicted template modeling (pTM) score of 0.75 and an interface pTM (ipTM) score of 0.84. **(B)** Predicted interacting regions of EFCAB5 and RGS22 were cloned and heterologously expressed in HEK293T cells. Lysates were treated with either EGTA or  $\text{CaCl}_2$ , followed by immunoprecipitation using an anti-PA antibody and immunoblotting of the IP product. RGS refers to the regulation of the G-protein signaling domain, and EF-hand refers to the calcium-binding EF-hand domain.

## Discussion

Human male patients lacking functional CFAP91 have been reported to be infertile<sup>83</sup>. However, the precise role of *Cfap91* in mammalian spermatogenesis remains unclear. In this study, we generated *Cfap91* KO mice using the CRISPR/Cas9 system and demonstrated that *Cfap91* is indispensable for murine male fertility (Figure 24A). *Cfap91* KO males exhibited abnormal elongation of the sperm flagellum (Figure 24C), resulting in shortened and immotile sperm tails (Figure 25A-D). Ultrastructural analyses of both testicular and cauda epididymal spermatozoa revealed severe disruption of axonemal integrity in *Cfap91* KO males (Figure 27). Because flagellum formation was defective in KO spermatozoa, we could not directly assess whether CFAP91 contributes to the regulation of flagellar motility. Nevertheless, disruption of *Cfap91* orthologs in *Chlamydomonas reinhardtii*, *Trypanosoma brucei*, and *Tetrahymena thermophila* results in reduced flagellar motility<sup>83,84,99</sup>, suggesting that CFAP91 may have an evolutionarily conserved function in motility regulation.

To visualize the manchette structure during spermatid development, we established *EGFP-Tuba3a* TG mice and applied this tool to *Cfap91* KO males. As expected, EGFP-TUBA3A fluorescence marked the manchette, enabling us to demonstrate that abnormal sperm head morphology in *Cfap91* KO males is associated with defective manchette formation (Figure 26D). Interestingly, previous studies have reported that the loss of certain axonemal proteins not localized to the manchette can still lead to abnormal head shaping<sup>76,100</sup>, suggesting that manchette defects may arise secondarily from impaired flagellum biogenesis rather than from the direct involvement of CFAP91 in manchette elongation. Notably, EGFP-TUBA3A signals were absent from the flagella (Figure 26C), even though TUBA3A is a known component of both the flagellar doublet microtubules and manchette<sup>101,102</sup>. This may be due to N-terminal EGFP fusion interfering with its incorporation into microtubules. Overall, the EGFP-Tuba3a TG line provides a valuable tool for studying manchette-mediated head morphogenesis.

To elucidate the molecular function of CFAP91, we generated *Cfap91* TG mice expressing FLAG- and BioID2-tagged CFAP91 and conducted immunoprecipitation-mass spectrometry. Forty-five proteins were significantly enriched in CFAP91 immunoprecipitates (Figure 30), among which CFAP251 and LRRC23 drew particular attention. Orthologous CFAP91-CFAP251 interactions have been reported in *C. reinhardtii* and *T. thermophila* and are conserved in mouse ependymal and human respiratory cilia<sup>85,86,103,104</sup>. These findings suggest that the positioning of CFAP251 at the base of RS3 is evolutionarily conserved from protists to mammals. In humans, CFAP251 is reduced in spermatozoa of patients with pathogenic CFAP91 variants<sup>83</sup>, and CFAP251-deficient males show shortened sperm tails<sup>105</sup>, underscoring its importance in flagellum formation. Our results revealed that CFAP251 localization was disrupted in *Cfap91* KO spermatozoa (Figure 31A), indicating that CFAP91 is essential for incorporating CFAP251 into the developing axoneme and, consequently, for proper flagellum elongation. LRRC23, another CFAP91-associated RS3 component, is known to localize at the RS3 head and to depend on the C-terminal region of CFAP91 for its positioning<sup>75,87</sup> (Figure 21). Considering the existing evidence, CFAP91 and CFAP251 are likely important for core RS3 complex formation and flagellum elongation, whereas LRRC23 is added later to fine-tune motility.

Proximity labeling using CFAP91-BioID2 in mature spermatozoa further identified EFCAB5 as a CFAP91-proximal protein with high peptide coverage (Figure 32C). Consistent with our findings, a recently published atomic model of bovine sperm RS3 also positioned EFCAB5 within the RS3 region<sup>87</sup> (Figure 21). Although EFCAB5 was detected in our IP-MS analysis of *Cfap91* TG testes, its enrichment was not statistically significant, possibly due to the heterogeneous composition of testicular cell types and the limited solubility of mature axonemal complexes. Given that EFCAB5 is absent from early-diverging eukaryotes<sup>106</sup>, it is plausible that this protein was evolutionarily incorporated into the RS3 structure to support sperm-specific motility regulation. In HEK293T cells, co-immunoprecipitation confirmed that EFCAB5 interacts with RGS22, an RS2 component, independent of Ca<sup>2+</sup> (Figure 36).

This  $\text{Ca}^{2+}$ -independent EFCAB5-RGS22 bridge may serve as a structural and regulatory link between RS2 and RS3, ensuring the coordinated movement of sperm flagella.

In summary, our study establishes CFAP91 as an essential RS3 component required for sperm tail biogenesis through the recruitment of CFAP251 during axoneme assembly. We also introduced the EGFP-Tuba3a TG mouse as a useful tool for studying manchette dynamics and demonstrated the utility of proximity labeling in mature spermatozoa for identifying structural proteins resistant to conventional extraction methods. Finally, our findings identify EFCAB5 as a sperm-specific RS3 protein that regulates motility, thereby broadening our understanding of axonemal specialization and the molecular basis of male infertility.

## **Part III Conclusion**

### **Chapter 1: CFAP77 Chapter 1. Roles of CFAP77 in male reproduction**

Our findings demonstrated that CFAP77 plays a critical role in maintaining sperm motility and male fertility in mice. Loss of CFAP77 resulted in reduced progressive motility and impaired sperm function despite apparently normal sperm structure. The interaction of CFAP77 with the microtubule inner protein TEKTL1 suggests that CFAP77 contributes to the structural stabilization or regulatory tuning of the axoneme. Collectively, CFAP77 emerged as an evolutionarily conserved component required for efficient flagellar motion and fertilization competence in mammals.

### **Chapter 2. Roles of MYCBPAP in male reproduction**

We established MYCBPAP as a key factor in sperm tail formation and male fertility. MYCBPAP localized to the developing axoneme, and its loss led to severely defective flagellar assembly and infertility, which were rescued by transgenic expression of FLAG-tagged MYCBPAP. Proteomic analyses further revealed interactions between MYCBPAP and components essential for axonemal organization. These findings identified MYCBPAP as an indispensable regulator of sperm flagellum biogenesis and provided new insights into the molecular mechanisms underlying spermatogenic ciliogenesis.

### **Chapter 3. Roles of CFAP91 and its proximate protein EFCAB5 in male reproduction**

We identified CFAP91 as an essential RS3-associated protein that drives sperm tail biogenesis and male fertility. CFAP91 recruited CFAP251 into the assembling axoneme and contributes to the construction of the RS3, while its loss disrupted sperm tail elongation and motility. Through proximity labeling, we further uncovered EFCAB5 as a sperm-specific

RS3 protein that bridges RS2-RS3, refining our understanding of the regulatory network controlling flagellar motility. Together, these findings elucidated how CFAP91 and its associated proteins orchestrate axonemal assembly and sperm function.

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## Part V Publications

### First-Author Publications

Wang, H., Iida-Norita, R., Mashiko, D., Pham, A. H., Miyata, H., & Ikawa, M. (2024). Golgi associated RAB2 interactor protein family contributes to murine male fertility to various extents by assuring correct morphogenesis of sperm heads. *PLoS genetics*, 20(6), e1011337.

Wang, H., Kobayashi, H., Shimada, K., Oura, S., Oyama, Y., Kitakaze, H., Noda, T., Yabuta, N., Miyata, H., & Ikawa, M. (2024). MYCBPAP is a central apparatus protein required for centrosome-nuclear envelope docking and sperm tail biogenesis in mice. *Journal of cell science*, 137(16), jcs261962.

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Wang, H., Pham, A. H., Luo, M., Ikawa, M., & Miyata, H. (2025). Microtubule Inner Protein CFAP77 Contributes to Sperm Motility and Male Fertility in Mice. *Andrology*, In press.

### Co-Authored publications

Kawaguchi, K., Yokoyama, S., Wang, H., & Asano, S. (2024). Inhibition of ezrin phosphorylation by NSC305787 attenuates procaterol-stimulated ciliary beating in airway cilia. *Biochemical and biophysical research communications*, 703, 149685.

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