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Perinuclear theca protein FNDC8 interacts with CCIN and ACTL7A to ensure proper sperm head shaping during spermiogenesis

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ABSTRACT

Precise shaping of the sperm head is essential for successful fertilization, and defects in this process are frequently associated with severe forms of male infertility. Sperm head morphology is coordinated by several testis-specific structures, including the apical ectoplasmic specialization (ES), manchette, and perinuclear theca (PT)—a dense cytoskeletal layer that envelops the nucleus during spermiogenesis. However, the molecular components and physiological functions of the PT remain incompletely understood. This study identified FNDC8 as a testis-enriched protein primarily localized to the PT. Genetic disruption of *Fndc8* resulted in male infertility characterized by spermatozoa exhibiting acrosome detachment from the nuclear membrane and sperm head surface collapse. FNDC8 interacted with CCIN and ACTL7A during spermiogenesis, and its depletion destabilized both proteins. Together, these results indicate that FNDC8 is integral to the structural integrity of the PT by mediating protein interactions critical for sperm head morphogenesis, implicating *FNDC8* dysfunction as a potential contributor to teratozoospermia in humans.

Keywords: Male Infertility; Perinuclear Theca; FNDC8; Acroplaxome; Spermiogenesis

INTRODUCTION

Spermatozoa function as essential carriers of paternal genetic material during sexual reproduction, consisting of a head that

houses the haploid nucleus and acrosome and a tail that enables motility. Sperm head morphology exhibits pronounced interspecies diversity, shaped by divergent reproductive strategies and evolutionary pressures. For instance, human spermatozoa possess an oval-shaped head (Toshimori, 2009), whereas the sperm of rodents such as mice, rats, and hamsters are characteristically falciform, adopting a hook-like structure (García-Vázquez et al., 2016). These morphological specializations are thought to optimize reproductive success, reflecting adaptive modifications in response to specific reproductive strategies or environments.

Normal sperm head architecture is crucial for successful fertilization, and malformations in head shape are frequently implicated in male infertility (El-Ghobashy & West, 2003; Ye et al., 2024). Head shaping occurs during spermiogenesis, the final phase of spermatid maturation (Kierszenbaum et al., 2003), and is tightly coordinated with acrosome biogenesis, chromatin remodeling, and cytoskeletal dynamics (Kierszenbaum & Tres, 2004). In mice, spermiogenesis is divided into 16 developmental steps, classified primarily by acrosome development (Khawar et al., 2019). As spermatids elongate, chromatin within the nucleus undergoes progressive condensation, accompanied by the replacement of canonical histones with protamines, leading to a marked reduction in head volume (Kierszenbaum et al., 2007). Sertoli cells contribute mechanically to spermatid head remodeling (de Kretser, 1990), forming contractile F-actin rings surrounding

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the apical portion of the elongating spermatid nucleus. These actin-based structures form part of the apical ectoplasmic specialization (ES), a testis-specific adherens junction composed of cell adhesion molecules spanning Sertoli and spermatid membranes, facilitating intimate cell-cell contact and mechanical support (Kopera et al., 2010). Head shaping is orchestrated through the integrated function of the acrosome-acroplaxome-manchette complex (Kierszenbaum & Tres, 2004). The manchette, a transient microtubular-containing structure, is required for head shaping and protein delivery during spermatid elongation (Kierszenbaum et al., 2003). Encasing the spermatid nucleus is the perinuclear theca (PT), a dense cytoskeletal layer unique to mammals (Mújica et al., 2003; Russell et al., 1991; Vogl, 1990). The PT is anatomically subdivided into two contiguous regions: the acroplaxome and the post-acrosomal sheath. The acroplaxome is located between the inner acrosomal and nuclear membranes and comprises a thin, F-actin- and keratin 5-rich cytoplasmic plate that anchors the developing acrosome to the nucleus and spermatid head (Kierszenbaum et al., 2003; Oko & Maravei, 1995; Oko & Sutovsky, 2009). The post-acrosomal sheath lies between the plasma and nuclear membranes.

The PT contains not only conventional cytoskeletal components but also numerous sperm-specific proteins essential for spermiogenesis. The first PT-localized protein, CCIN, was identified in bull and rat sperm in 1987 (Longo et al., 1987), with subsequent studies identifying further PT proteins, such as CYLC1 (Hess et al., 1993), FABP9 (Oko & Morales, 1994), CYCL2 (Hess et al., 1995), CAPZA3 (von Bülow et al., 1997), H2BL1 (Aul & Oko, 2001), ACTRT1 (Heid et al., 2002), ACTRT2 (Heid et al., 2002), PAWP (Aarabi et al., 2014), PLC ζ (Hachem et al., 2017), GSTO2 (Hamilton et al., 2017), and ACTL7A (Xin et al., 2020). More recently, proteomic analyses have expanded the PT protein repertoire to several hundred candidates in mouse and porcine sperm (Zhang et al., 2022a; Zhang et al., 2022b). Despite this growing catalog, the functional relevance of most PT proteins, particularly in regulating sperm head morphogenesis, remains to be elucidated.

The fibronectin type III domain-containing (FNDC) family comprises extracellular matrix-associated proteins characterized by the presence of at least one conserved fibronectin type III domain (Jiang et al., 2022). In humans, this family consists of 11 members: FNDC1, FNDC3A, FNDC3B, FNDC4, FNDC5, FNDC6, interleukin-20 receptor subunit beta (IL20RB), FNDC7, FNDC8, FNDC9, FNDC10, and FNDC11. These proteins have been implicated in diverse cellular processes, including adhesion, migration, development, and proliferation (Lin et al., 2016; Wuensch et al., 2019). In the present study, FNDC8 was identified as a testis-enriched protein localized to the PT. Targeted knockout of *Fndc8* in mice resulted in complete male infertility, attributed to defects in sperm head shaping. Specifically, FNDC8 depletion caused collapse of the sperm head surface during the nuclear condensation stage, coinciding with defective acrosome-nuclear attachment. Mechanistically, FNDC8 interacted with CCIN and ACTL7A—two established PT components—and was required for their stabilization and correct localization. FNDC8 depletion led to compromised acroplaxome integrity and prevented proper anchoring of the acrosome to the nucleus, ultimately leading to sperm head malformation. These findings establish FNDC8 as a critical regulator of PT

organization and sperm head morphogenesis, with potential implications for understanding the molecular basis of male infertility.

MATERIALS AND METHODS

Animals

The *Fndc8* gene (Transcript: ENSMUST00000018988.6), located on chromosome 11, spans 966 bp and comprises four exons. A region within exon 2 was selected as the CRISPR-Cas9 target site. Founder mice harboring *Fndc8* knockout alleles were generated by Rise Mice Biotechnology (China) using co-injection of guide RNA (gRNA) and Cas9 mRNA into fertilized C57BL/6J mouse zygotes. This procedure produced heterozygous mutants carrying a 239 bp deletion. Heterozygotes were intercrossed to generate homozygous knockout (*Fndc8*^{-/-}) and wild-type (WT, *Fndc8*^{+/+}) offspring. Genotyping was performed by polymerase chain reaction (PCR) using specific primers: Forward: 5'-GACATTTCCCTTCAGTCTGGTC-3' and Reverse: 5'-TCAGACGTGTGAGGAGG-3'. The expected amplicon sizes were 243 bp for the mutant allele and 520 bp for the WT allele.

Ethics statement

Ethical approval for all animal procedures was obtained from the Laboratory Animal Welfare and Ethics Committee of Guangzhou Women and Children's Medical Center (Ethics No. RSDW-2024-01391). All surgery was performed under sodium pentobarbital anesthesia, and every effort was made to minimize suffering.

Assessment of fertility

Fertility was evaluated in 2-month-old male mice of each genotype by mating each male with three WT C57BL/6J females aged 6–8 weeks. Vaginal plugs were monitored daily to verify mating. Upon detection of a plug, females were housed individually, and both parturition dates and litter sizes were recorded. Females that failed to deliver within 21 days post-coitus were classified as non-pregnant and euthanized for confirmation.

Antibodies

Rabbit anti-FNDC8 antibody (Dia-an Biotech, China) was used for western blotting (1:1 000) and immunofluorescence (1:100). Mouse anti- α / β -tubulin (ab44928, Abcam, UK), rabbit anti-ODF2 (13972-1-AP, Proteintech, China), and mouse anti-sp56 antibodies (55101, QED, USA) were used for immunofluorescence (1:100). Rabbit anti-CCIN antibody (13972-1-AP, Proteintech, China) was used for western blotting (1:1 000) and immunofluorescence (1:100). Rabbit anti-ACTL7A antibody (17355-1-AP, Proteintech, China) was used for western blotting (1:1 000) and immunofluorescence (1:100). Rabbit anti-ACTRT2 antibody (16992-1-AP, Proteintech, China) was used for western blotting (1:1 000). Rabbit anti-GAPDH antibody (10494-1-AP, Proteintech, China) was used for western blotting (1:5 000). Rabbit anti- α -tubulin antibody (100094-1-AP, Proteintech, China) was used for western blotting (1:2 000). Alexa Fluor 488 conjugate of lectin PNA (1:400, L21409, Thermo Fisher, USA) was used for immunofluorescence. Secondary antibodies included goat anti-rabbit FITC (1:200, ZF-0311, Zhong Shan Jin Qiao, China), goat anti-rabbit TRITC (1:200, ZF-0316, Zhong Shan Jin Qiao, China), goat anti-mouse FITC (1:200, ZF-0312, Zhong Shan Jin Qiao, China), and goat anti-mouse TRITC

(1:200, ZF0313), all from Zhong Shan Jin Qiao, China.

In vitro fertilization

Four-week-old WT female mice were superovulated by intraperitoneal injection with 10 IU of pregnant mare serum gonadotropin (PMSG, 110914564, Ningbo Sansheng, China), followed 46–48 h later by 5 IU of human chorionic gonadotropin (hCG, 110911282, Ningbo Sansheng, China). At 15 h post-hCG injection, oocyte-cumulus complexes were surgically harvested from the ampullae of the oviducts. Spermatozoa were collected from the cauda epididymides of 8-week-old *Fndc8*^{+/+} and *Fndc8*^{-/-} male mice and released into 200 μ L of human tubal fluid (HTF) medium (Sudgen Biotechnology, China). Spermatozoa were capacitated for 1 h at 37°C in a 5% CO₂ incubator. Concurrently, cumulus-free oocytes were cultured in 100 μ L of HTF under mineral oil (M5310, Sigma-Aldrich, USA) at 37°C in a 5% CO₂ atmosphere for *in vitro* fertilization. For insemination, 4 $\times$ 10⁵/mL capacitated sperm were incubated with the ovulated oocytes in HTF for 3 h. Fertilized oocytes were washed in fresh HTF drops to remove abnormal oocytes. All steps were conducted at 37°C on a TPIE-SMZR constant temperature plate (TOKAI HIT, Japan). Successful fertilization was confirmed by the presence of pronuclei at 6 h post-insemination, and the 2-cell embryo development rate was assessed 24 h post-fertilization.

Immunoprecipitation (IP)

Testes were homogenized in ice-cold IP lysis buffer (20 mmol/L HEPES, 150 mmol/L NaCl, 0.5% NP-40, 1 mmol/L DTT, pH 7.3) supplemented with a protease inhibitor cocktail (04693132001, Roche Diagnostics, Switzerland). The lysates were clarified by centrifugation at 12 000 $\times$ g for 30 min at 4°C. The resulting supernatants were incubated overnight with specific antibodies on a shaker at 4°C. Protein A/G-coated magnetic beads were subsequently added and incubated for 4 h at 4°C. After washing three times with lysis buffer, the beads were collected for western blotting or liquid chromatography-tandem mass spectrometry (LC-MS/MS).

Proteomics analysis

Excised gel bands were destained in 50% acetonitrile (v/v), dehydrated in 100% acetonitrile, and subjected to in-gel digestion with trypsin at 37°C overnight. Peptides were extracted, dried, and reconstituted in 2% acetonitrile/0.1% formic acid. Desalting was performed using a Strata-X solid-phase extraction cartridge (8B-S100-AAK, Phenomenex, USA). Tryptic peptides were separated on an EASY-nLC 1200 UPLC system (Thermo Fisher Scientific, USA) and analyzed using an Orbitrap Exploris 480 mass spectrometer equipped with a nano-electrospray ion source, applying an electrospray voltage of 2.3 kV. Both precursor and fragment ions were analyzed using the Orbitrap detector. Full MS scans were performed at a resolution of 60 000 across a 350–1 800 m/z range. MS/MS scans were conducted with a fixed first mass and a resolution of 15 000, with TurboTMT disabled. The top 20 most abundant precursor ions were selected for further MS/MS analysis, with dynamic exclusion set to 20 s. High-energy collision-induced dissociation (HCD) was carried out at a normalized collision energy (NCE) of 28%. The automatic gain control (AGC) target was set to 50%, with a minimum intensity threshold of 5 000 ions/s and a maximum injection time of 200 ms. The acquired MS/MS data were processed using the PD search engine (v.2.4) against the

*Mus_musculus*_10090_SP_20231220_add.fasta database (17 193 entries). Trypsin/P was chosen as the enzyme for cleavage, allowing up to two missed cleavages. Mass tolerance for precursor ions was set to 10 ppm in the initial search, with a tolerance of 0.02 Da for fragment ions. Carbamidomethylation of cysteine was applied as a fixed modification, while acetylation of the protein N-terminal and oxidation of methionine were treated as variable modifications. A peptide score higher than 20 and high confidence were required for peptide identification.

Immunoblotting

Tissue protein extracts were prepared in RIPA buffer containing 1 mmol/L phenyl methyl sulfonyl fluoride (PMSF) and a protease inhibitor cocktail (04693132001, Roche Diagnostics, Switzerland). The mixture was subjected to brief sonication and incubated on ice for 30 min, followed by centrifugation at 12 000 $\times$ g for 15 min at 4°C. Supernatants were collected and separated by electrophoresis and transferred to nitrocellulose membranes via electroblotting. The membranes were incubated in 5% skim milk (232100, Becton, Dickinson and Company, USA) to block non-specific binding, then sequentially incubated with primary and secondary antibodies. Protein signals were detected using the Touch Imager Pro (e-BLOT Life Science, China).

RNA isolation and reverse transcription polymerase chain reaction(RT-qPCR)

Total RNA was extracted from multiple tissues of 8-week-old WT mice using the FastPure Cell/Tissue Total RNA Isolation Kit v2 (RC112-01, Vazyme, China). RNA concentrations were quantified using a NanoDrop nucleic acid analyzer. First-strand cDNA synthesis was performed using the 5 $\times$ PrimeScript RT Master Mix (RR036A, Takara, Japan), following the manufacturer's protocols. Quantitative RT-PCR was conducted on a CFX Real-Time PCR instrument (CFX Opus96, BioRad, USA) using ChamQ Universal SYBR qPCR Master Mix (Q711-02, Vazyme, China). Relative gene expression was calculated using the comparative Δ Ct (cycle threshold) method. Primers used were as follows: *Fndc8*-F: 5'-GCGACGGTATTCTGTAAAGTGG-3', *Fndc8*-R: 5'-GGACAGGGCTTTGGCAGTT-3', β -actin-F: 5'-GGCTGTATTCCCCTCCATCG-3', and β -actin-R: 5'-CCAGTTGGTAACAATGCCATGT-3'.

Epididymal sperm count

The cauda epididymis was isolated from 2-month-old mice and incubated in phosphate-buffered saline (PBS) at 37°C for 15 min to allow sperm release. The suspension was diluted 1:100, and sperm count was determined using a hemocytometer.

Sperm motility assessment by computer-assisted semen analysis

Spermatozoa were extracted from the cauda epididymis of 2-month-old mice by incubation in 1 mL of PBS at 37°C for 15 min. A 10 μ L aliquot of each sperm suspension was used to assess sperm motility. Motile and progressive spermatozoa percentages were quantified using computer-assisted semen analysis (Hamilton CEROS II, USA).

Daily sperm production (DSP)

Testes from 8-week-old WT and *Fndc8* knockout (KO) male mice ($n=3$ per genotype) were dissected, weighed, and stored at -20°C until analysis. Testicular DSP was determined (Juma

et al., 2017). Each testis was homogenized in 8 mL of DSP buffer (0.9% (w/v) NaCl and 0.05% (v/v) Triton X-100 in water). A 200 μ L aliquot of lysate was mixed with 200 μ L of 0.04% Trypan Blue (C0040, solarbio, China), and elongated spermatids were counted using a hemocytometer. DSP was calculated by dividing the total number of elongated spermatids per testis by 4.84.

Tissue collection and histological analysis

Two-month-old mice were euthanized, and the testes and caudal epididymides were dissected and fixed in Bouin's fixative (HT10132, Merck, Germany) at 4°C for 24 h. Fixed tissues were dehydrated through a graded ethanol series and embedded in paraffin. For histological examination, 5 μ m sections were cut, mounted onto glass slides, deparaffinized, and subsequently stained with hematoxylin and eosin (H&E) or periodic acid-Schiff (PAS).

Scanning electron microscopy

Cauda epididymides were fixed overnight in 2.5% glutaraldehyde solution, dehydrated in graded ethanol, dried, and coated with gold. Images were acquired and analyzed using a scanning electron microscope (GeminiSEM 300, Zeiss, Germany).

Transmission electron microscopy

Cauda epididymides were dissected and fixed in 2.5% glutaraldehyde in 0.1 mol/L cacodylate buffer at 4°C overnight. After washing with 0.1 mol/L cacodylate buffer, the samples were cut into small blocks (1 mm³) and immersed in 1% OsO₄ for 1 h at 4°C. The samples were then dehydrated through a graded acetone series (50%, 60%, 70%, 80%, 90%, 95%, 100%) and embedded in resin (DDSA, NMA, enhancer, 812). Ultrathin sections were cut, stained with uranyl acetate and lead citrate, and analyzed using a transmission electron microscope (Tcni G2 Spirit, FEI, Czech Republic).

Immunofluorescence

Mouse testes were dissected and immediately fixed in 2% paraformaldehyde (PFA) in 0.05% PBST (PBS containing 0.05% Triton X-100) at room temperature for 5 min. Fixed tissues were placed on glass slides, covered with coverslips, and gently squashed to spread the seminiferous tubules. The slides were flash-frozen in liquid nitrogen and stored at -80°C for further use. For immunostaining, coverslips were removed, and slides were washed with PBS three times, permeabilized with 0.1% Triton X-100 for 10 min, rinsed with PBS, and blocked in 5% bovine serum albumin (BSA, AP0027, Amresco, USA). Primary antibodies were applied and incubated overnight at 4°C, followed by incubation with secondary antibodies. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI), and fluorescent signals were visualized using a Nikon AXR confocal microscope (Japan).

For sperm immunofluorescence, spermatozoa were released by incubating the cauda epididymides in PBS at 37°C for 15 min. The sperm suspension was smeared onto glass slides for morphological and immunostaining analyses. After air drying, spermatozoa were fixed in 4% PFA for 5 min at room temperature, washed three times with PBS, and blocked with 5% BSA for 30 min at room temperature. Primary antibodies were added and incubated overnight at 4°C, followed by secondary antibody incubation. Nuclei were stained with DAPI, and images were captured using a Nikon AXR confocal microscope (Japan).

Phylogenetic analysis and protein sequence alignment

The amino acid sequences of FNDC8 from 12 species were downloaded from the UniProt database. Phylogenetic analysis was conducted using MEGA v.11.0 (Tamura et al., 2021) with the neighbor-joining (NJ) method (Saitou & Nei, 1987). The amino acid sequences of FNDC8 from seven species were downloaded from the NCBI database. Multiple sequence alignment was performed using MEGA v.11.0, and aligned sequences were visualized using the ESPript v.3.0 tool.

Statistical analysis

All experiments were independently repeated at least three times. Data are presented as mean $\pm$ standard deviation (SD). Statistical significance between genotypes was determined using Student's *t*-tests with paired, two-tailed distribution. Significance thresholds were set at **P* < 0.05, ***P* < 0.01, ****P* < 0.001, and *****P* < 0.0001.

RESULTS

Fndc8 knockout leads to male infertility

To identify candidate regulators of sperm head morphogenesis, testis-enriched protein datasets from mice and pigs were analyzed using a previously described high-expression gene enrichment strategy (Chang et al., 2019), in combination with recent proteomic data (Zhang et al., 2022a, 2022b). This analysis yielded 46 proteins (Supplementary Figure S1A). Cross-referencing these candidates with the Mouse Genome Informatics (MGI) database (<https://www.informatics.jax.org/>) revealed that 10 genes were functionally uncharacterized. Among these, *Fndc8* was selected for further investigation. Single-cell RNA sequencing data from the mouse testis (Zhao et al., 2023) showed that *Fndc8* transcripts were predominantly expressed in spermatids, a pattern also observed in human datasets (Supplementary Figure S1B, C). Based on phylogenetic analysis, FNDC8 was highly conserved from reptiles to mammals and restricted to these clades (Figure 1A, B). Tissue-specific expression profiling confirmed that FNDC8 was predominantly expressed in the testis (Figure 1C, D). Temporal expression analysis by immunoblotting demonstrated weak FNDC8 expression at postnatal day 28 (P28), with a marked increase beginning at P35 onward (Figure 1E), coinciding with the elongation phase of spermiogenesis—suggesting a potential role in late-stage spermatid maturation.

To further elucidate the functional role of FNDC8 during spermiogenesis, a CRISPR/Cas9-based strategy was employed to generate *Fndc8* knockout mice by targeting exon 2 (Figure 2A). Genotyping of offspring identified WT (*Fndc8*^{+/+}), heterozygous (*Fndc8*^{+/-}), and homozygous knockout (*Fndc8*^{-/-}) alleles, producing amplicons of 520 bp, 243 bp, or both, respectively (Figure 2B). Immunoblotting of testis and sperm lysates confirmed complete loss of FNDC8 protein in *Fndc8*^{-/-} males (Figure 2C). Functional assessment of reproductive capacity revealed that 2-month-old *Fndc8*^{-/-} males were completely sterile when paired with WT females (Figure 2H), while *Fndc8*^{-/-} females exhibited normal fertility (Figure 2I). *In vitro* fertilization assays further revealed that WT oocytes were unable to develop into two-cell embryos following incubation with spermatozoa from *Fndc8*^{-/-} mice (Figure 2J). Collectively, these findings establish *Fndc8* as a testis-enriched gene essential for male fertility.

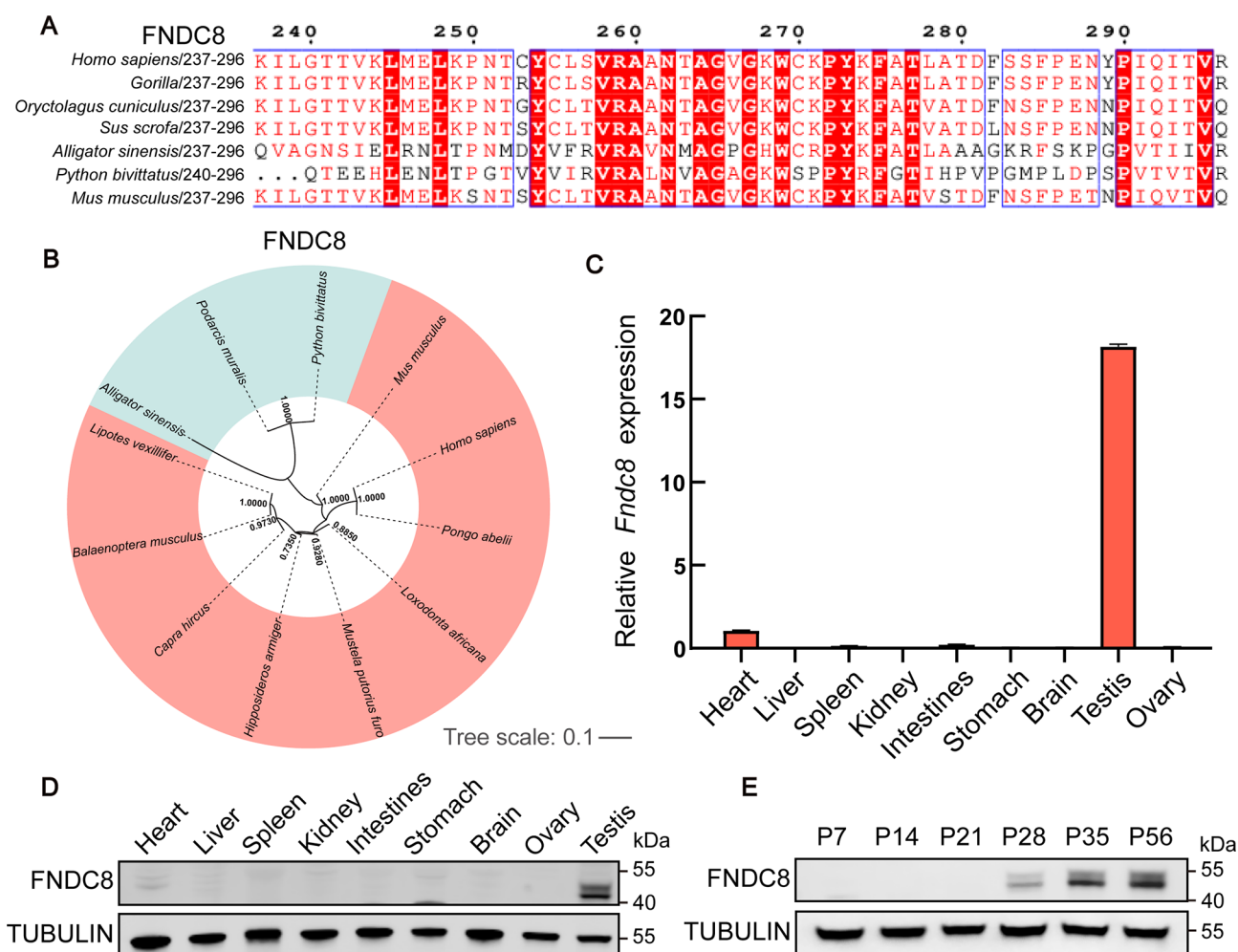


Figure 1 FNDc8 is an evolutionarily conserved protein highly expressed in the testis

A: Sequence identity analysis of FNDc8 proteins from different species. B: Phylogenetic tree of FNDc8 across species. C: RT-qPCR analysis of *Fndc8* mRNA expression in the heart, liver, spleen, kidney, intestines, stomach, brain, testis, and ovary of adult mice. D: Expression pattern analysis of FNDc8 in different tissues of 2-month-old adult mice based on western blotting, showing predominant expression in the testis. Tubulin served as the loading control. E: Expression pattern analysis of FNDc8 in the testis at different ages based on western blotting, showing expression began at P28. Tubulin served as the loading control.

***Fndc8* knockout induces a teratozoospermia-like phenotype**

To investigate the basis of male infertility in *Fndc8*^{-/-} mice, both gross and histological architecture of the testes were examined. Testis size appeared unaffected by *Fndc8* deletion (Figure 2D, F), although the testis-to-body weight ratio was slightly altered in *Fndc8*^{-/-} mice (Figure 2E, G). Histological analysis using H&E staining revealed a substantial reduction in luminal sperm content during spermiation within the seminiferous tubules of *Fndc8*^{-/-} mice compared to *Fndc8*^{+/+} mice (Figure 3A, black arrow). Quantification of DSP confirmed a significant decrease in *Fndc8*^{-/-} males relative to *Fndc8*^{+/+} males (Figure 3B). H&E-stained sections of the caudal epididymis further revealed diminished sperm content and the presence of vacuolated sperm heads in *Fndc8*^{-/-} mice (Figure 3C, black arrow). Direct sperm counts from the cauda epididymis confirmed a pronounced reduction in sperm number (Figure 3D), along with a notable reduction in motility and progressive movement (Figure 3E, F). To assess the underlying structural defects contributing to impaired motility, spermatozoa were co-immunostained with ODF2 and α/β -tubulin—markers of outer dense fibers and the axonemal “9+2” microtubule configuration (Lehti & Sironen, 2016; Zhao

et al., 2018; Zhu et al., 2022). Immunofluorescence analysis showed distinctly abnormal signals for ODF2 and α/β -tubulin in *Fndc8*^{-/-} spermatozoa (Figure 3G). Furthermore, TEM analysis of sperm flagella confirmed abnormalities in the “9+2” microtubule structure, indicating disorganization of the sperm flagellum (Figure 3H).

To investigate the cause of the observed sperm head defects, immunofluorescent staining was performed using sp56, a marker of the acrosomal outer membrane (Cohen & Wassarman, 2001). While *Fndc8*^{+/+} spermatozoa displayed the typical hook-shaped acrosome morphology, *Fndc8*^{-/-} sperm exhibited irregular and fragmented sp56 signals, often accompanied by prominent head vacuolization (Figure 3I). Quantitative analysis revealed that approximately 93% of *Fndc8*^{-/-} sperm exhibited vacuoles within the head region (Figure 3J). SEM revealed clear subsidence on the sperm head surface in *Fndc8*^{-/-} mice, which was corroborated by TEM imaging showing concave deformation (Figure 3K, red asterisks). These results suggest that surface collapse of the sperm head and defective acrosome attachment are major contributors to the teratozoospermia-like phenotype observed in *Fndc8*^{-/-} males.

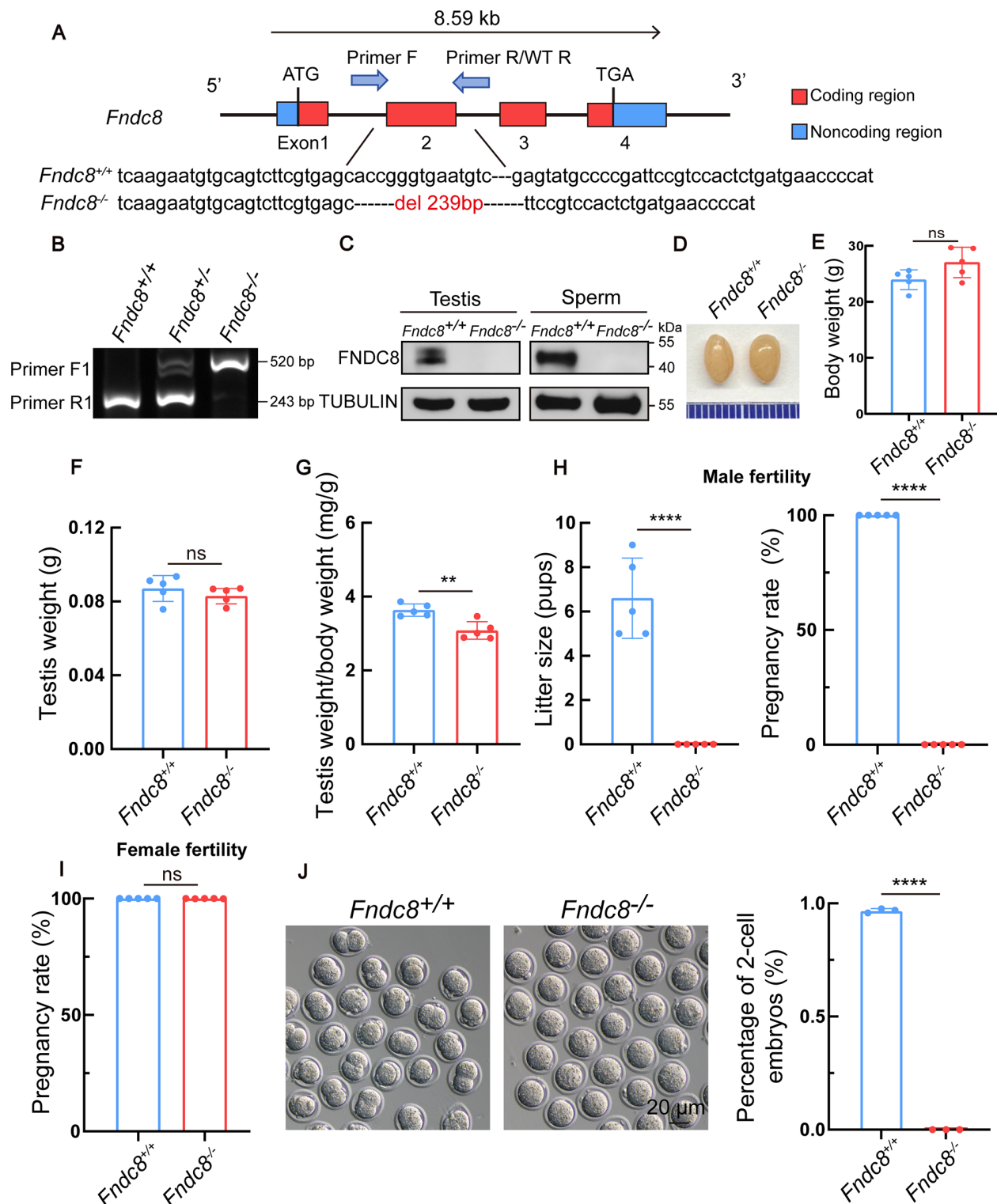


Figure 2 *Fndc8* knockout leads to male infertility in mice

A: Generation of *Fndc8* knockout mice using CRISPR-Cas9 technology. B: Genotyping of *Fndc8* knockout mice. C: Knockout efficiency was determined by western blotting using testis and sperm protein lysates. Tubulin served as the loading control. D: Testis size of *Fndc8*^{+/+} and *Fndc8*^{-/-} mice. E: Body weights of *Fndc8*^{+/+} and *Fndc8*^{-/-} male mice ($n=5$ independent experiments). Data are presented as mean $\pm$ SD. ns: Not significant. F: Testis weights of *Fndc8*^{+/+} and *Fndc8*^{-/-} male mice ($n=5$ independent experiments). Data are presented as mean $\pm$ SD. ns: Not significant. G: Ratio of testis weight to body weight in *Fndc8*^{+/+} and *Fndc8*^{-/-} male mice ($n=5$ independent experiments). Data are presented as mean $\pm$ SD. **: $P<0.01$. H: Litter size of *Fndc8*^{+/+} and *Fndc8*^{-/-} male mice at 2 months and pregnancy rates of WT female mice after mating with 2-month-old *Fndc8*^{+/+} and *Fndc8*^{-/-} male mice ($n=5$ independent experiments). Data are presented as mean $\pm$ SD, ****: $P<0.0001$. I: Pregnancy rates of *Fndc8*^{+/+} and *Fndc8*^{-/-} female mice at 2 months ($n=5$ independent experiments). Data are presented as mean $\pm$ SD. ns: Not significant. J: Percentage of two-cell stage embryos from *Fndc8*^{+/+} and *Fndc8*^{-/-} groups. *Fndc8*^{+/+}, 96.79% $\pm$ 0.90% ($n=3$ independent experiments, total oocytes=155); *Fndc8*^{-/-}, 0% ($n=3$ independent experiments, total oocytes=156). Data are presented as the mean $\pm$ SD, ****: $P<0.0001$.

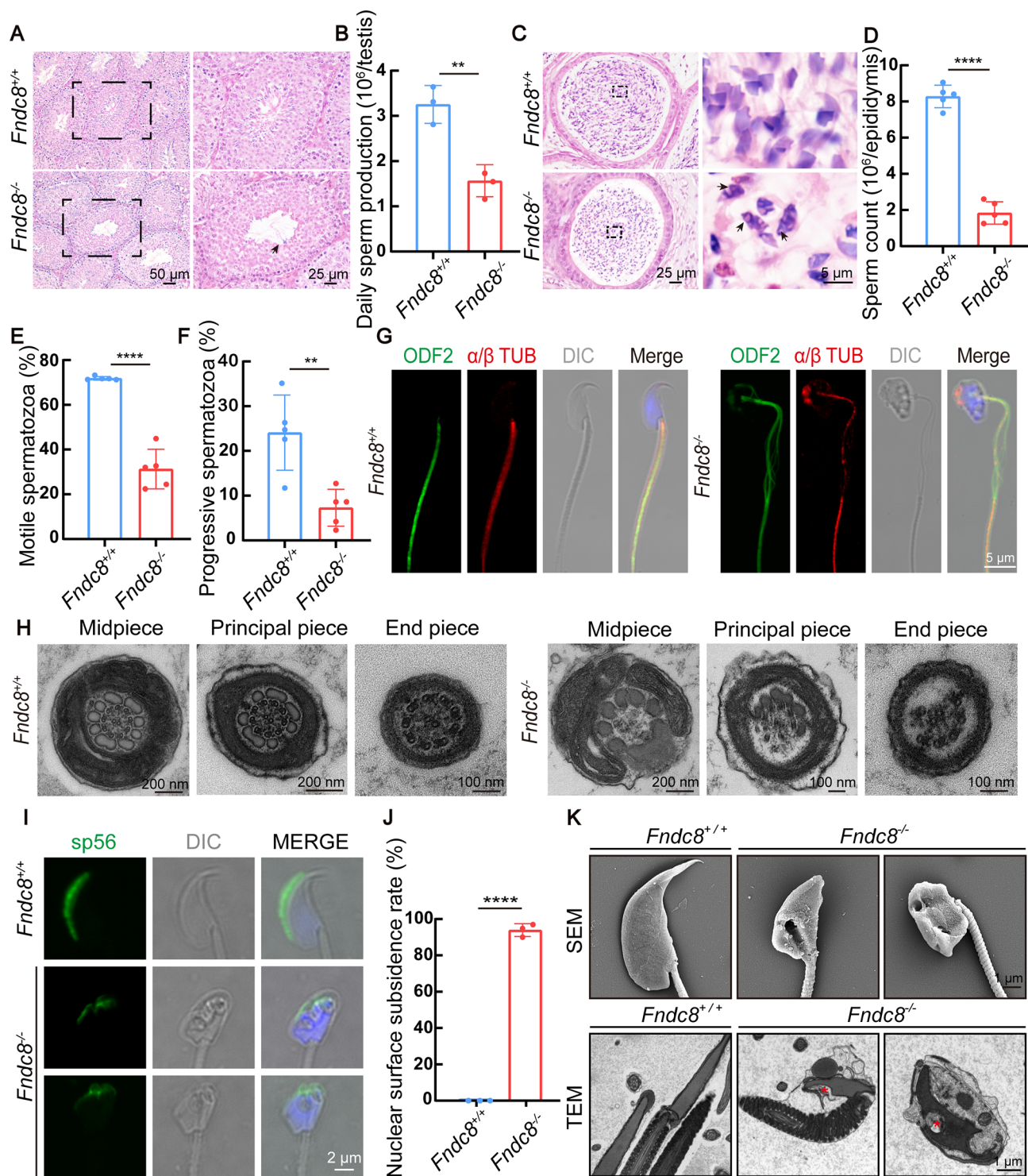


Figure 3 Loss of *Fndc8* results in sperm nuclear surface subsidence

A: H&E-stained testis sections from *Fndc8*^{+/+} and *Fndc8*^{-/-} male mice. Black arrows indicate a reduction in sperm accumulation within seminiferous tubules. B: Daily sperm production per testis for *Fndc8*^{+/+} and *Fndc8*^{-/-} male mice. (n=5 independent experiments). Data are presented as mean $\pm$ SD. **: $P < 0.01$. C: H&E-stained cauda epididymis sections from *Fndc8*^{+/+} and *Fndc8*^{-/-} male mice. Black arrows indicate sperm with vacuoles in sperm heads. D: Analysis of sperm counts in *Fndc8*^{+/+} and *Fndc8*^{-/-} male mice. Mature spermatozoa were released from unilateral cauda epididymis and dispersed in PBS. Sperm counts were measured by hemocytometers (n=5 independent experiments). Data are presented as mean $\pm$ SD. ****: $P < 0.0001$. E, F: Analyses of sperm motility and progressive motility of *Fndc8*^{+/+} and *Fndc8*^{-/-} male mice (n=5 independent experiments). Data are presented as mean $\pm$ SD. ****: $P < 0.0001$. **: $P < 0.01$. G: Immunofluorescence staining of ODF2 (green) and α -tubulin (red) in *Fndc8*^{+/+} and *Fndc8*^{-/-} spermatozoa. Nuclei were counterstained with DAPI (blue). H: TEM analysis of cross-sections of *Fndc8*^{+/+} and *Fndc8*^{-/-} sperm flagellum. I: Immunofluorescence staining of sp56 in *Fndc8*^{+/+} and *Fndc8*^{-/-} spermatozoa. Nuclei were counterstained with DAPI (blue). J: Analyses of sperm nuclear surface subsidence rate of *Fndc8*^{+/+} and *Fndc8*^{-/-} male mice (n=5 independent experiments). Data are presented as mean $\pm$ SD. ****: $P < 0.0001$. K: SEM and TEM analysis of spermatozoa from cauda epididymis of *Fndc8*^{+/+} and *Fndc8*^{-/-} male mice. Red asterisks indicate vacuoles in sperm head.

Head surface collapse initiates during late nuclear condensation

To determine the developmental timing of nuclear surface collapse in *Fndc8*^{-/-} spermatids, PAS staining was performed to stage seminiferous tubules in testicular sections from *Fndc8*^{+/+} and *Fndc8*^{-/-} males. In mouse testes, seminiferous epithelium is organized into 12 cyclic stages (I–XII) defined by cellular associations (Oakberg, 1956), and spermiogenesis is further subdivided into 16 morphological steps (steps 1–16) that characterize post-meiotic differentiation (Endo et al., 2017). While the overall progression of spermatogenesis appeared unperturbed in *Fndc8*^{-/-} testes, aberrant head shaping was specifically observed beginning at stage IX (Figure 4A, red asterisks). Further analysis across the 16 steps of spermiogenesis revealed no overt differences in acrosome or nuclear morphology during steps 1–8. However, by steps 9–10, corresponding to the onset of nuclear elongation, *Fndc8*^{-/-} spermatids exhibited malformed head contours compared to *Fndc8*^{+/+} mice. Additionally, step 9 spermatids from *Fndc8*^{-/-} mice frequently contained prominent nuclear vacuoles (Figure 4B).

To further investigate acrosome-nucleus dynamics, acrosomal membranes were visualized using fluorescently conjugated PNA, which specifically labels the acrosomal matrix (Nakata et al., 2015). From step 9 onward, *Fndc8*^{-/-} spermatids displayed clear separation between the acrosome and nuclear membrane, with the formation of a gap between the two structures (Figure 4C, red asterisks). As differentiation proceeded, subsidence on the sperm head surface became more pronounced, with vacuoles progressively increasing in number and the acrosome gradually adopting an irregular shape. TEM confirmed these defects at the ultrastructural level. Starting from step 9, *Fndc8*^{-/-} spermatids exhibited acrosomal detachment from the nuclear surface and marked depression of the nuclear contour (Figure 4D). Collectively, these findings indicate that loss of FNDC8 disrupts nuclear shaping during the late stages of spermiogenesis, specifically during the nuclear condensation phase, resulting in head surface collapse and acrosomal dislocation.

FNDC8 localizes to the PT during spermatid maturation

To further investigate the spatial distribution and function of FNDC8 during spermatogenesis, immunofluorescence analysis was conducted using an anti-FNDC8 antibody. Results revealed a distinct signal in *Fndc8*^{+/+} spermatids, specifically localized to the PT. During the elongation stage, FNDC8 localized predominantly to the acroplaxome. As nuclear shaping progressed, the signal gradually shifted toward the post-acrosomal region of the PT. In fully differentiated spermatozoa from the cauda epididymis, FNDC8 remained confined to the PT, with signal enrichment notably concentrated in the post-acrosomal region. This spatial pattern supports a role for FNDC8 in structural remodeling during late spermiogenesis. Notably, no immunofluorescence signal was detected in *Fndc8*^{-/-} spermatozoa, confirming the specificity of the anti-FNDC8 antibody staining (Figure 5A).

To further refine the subcellular localization of FNDC8, dual immunostaining was performed on mature spermatozoa using antibodies against FNDC8 and sp56. The FNDC8 signal was positioned beneath the outer acrosomal membrane, consistent with its integration into the PT (Figure 5B). This observation aligns with prior proteomic studies identifying FNDC8 as a putative PT component (Zhang et al., 2022b), suggesting a

compartment-specific function during late spermiogenesis.

FNDC8 interacts with CCIN and ACTL7A during spermiogenesis

To define the molecular partners of FNDC8 within the PT, IP-MS was performed on mouse testis lysates using an anti-FNDC8 antibody (Figure 6A, B; Supplementary Table S1). This analysis delineated the FNDC8 interactome, providing insights into its potential function in spermatid maturation. Co-immunoprecipitation (co-IP) assays confirmed the physical interactions between FNDC8 and two key PT proteins, CCIN and ACTL7A (Figure 6C). Notably, loss of FNDC8 affected the protein abundance of both CCIN and ACTL7A in spermatozoa (Figure 6D, E). CCIN is a structural scaffold protein essential for organizing the perinuclear theca and facilitating proper sperm head shaping. It mediates acrosome-nucleus linkage by bridging the inner acrosomal membrane and the nuclear envelope, forming a continuous acrosome-theca-nucleus complex (Fan et al., 2022; Zhang et al., 2022b). To examine the impact of FNDC8 deficiency on spermatozoa head shaping, immunofluorescence staining was performed on epididymal spermatozoa from *Fndc8*^{+/+} and *Fndc8*^{-/-} mice, co-staining with PNA and anti-CCIN to label the acrosome and PT, respectively. In *Fndc8*^{+/+} spermatozoa, CCIN was localized to both the acroplaxome and post-acrosomal regions, forming a discrete layer beneath the PNA-labeled acrosome. In contrast, *Fndc8*^{-/-} spermatozoa exhibited diffuse and mislocalized CCIN signals, particularly in the acroplaxome, where its organization was disrupted. This was accompanied by irregular PNA staining, indicating loss of acrosome-nucleus structural integrity (Figure 6F).

ACTL7A, another essential PT protein, is required for acrosome biogenesis and mediates the tethering of the acrosomal outer membrane to the nucleus. It is essential for the proper migration of acrosomal granules and the maintenance of F-actin structures during spermatid elongation. Loss of ACTL7A disrupts acrosome formation and leads to impaired male fertility (Ferrer et al., 2023; Xin et al., 2020). To investigate the spatial relationship between FNDC8 and ACTL7A, mature spermatozoa from *Fndc8*^{+/+} and *Fndc8*^{-/-} mice were immunostained with anti-ACTL7A antibody and PNA. In *Fndc8*^{+/+} spermatozoa, ACTL7A was localized to the PT. However, in *Fndc8*^{-/-} spermatozoa, ACTL7A displayed a fragmented and disorganized distribution, with loss of signal in the post-acrosomal region and spatial separation from the PNA-labeled acrosome. Acrosomal morphology was likewise abnormal, with detachment from the nuclear surface and shape distortion (Figure 6G). These findings indicate that FNDC8 functions as a stabilizing factor for CCIN and ACTL7A, ensuring their correct localization and maintenance within the PT. Loss of FNDC8 destabilizes this protein network, resulting in defective acrosome-nucleus attachment during the acrosome phase of spermiogenesis. As differentiation progresses, disorganization of the acroplaxome leads to structural collapse of the sperm head nucleus, manifesting as subsidence and impaired head shaping (Figure 7).

DISCUSSION

Spermiogenesis relies on precise cytoskeletal remodeling to orchestrate nuclear reshaping, acrosome assembly, and protein trafficking essential for sperm maturation (Pereira et al., 2019; Pleuger et al., 2020). The PT, a structural scaffold encasing the spermatozoa nucleus, plays a critical

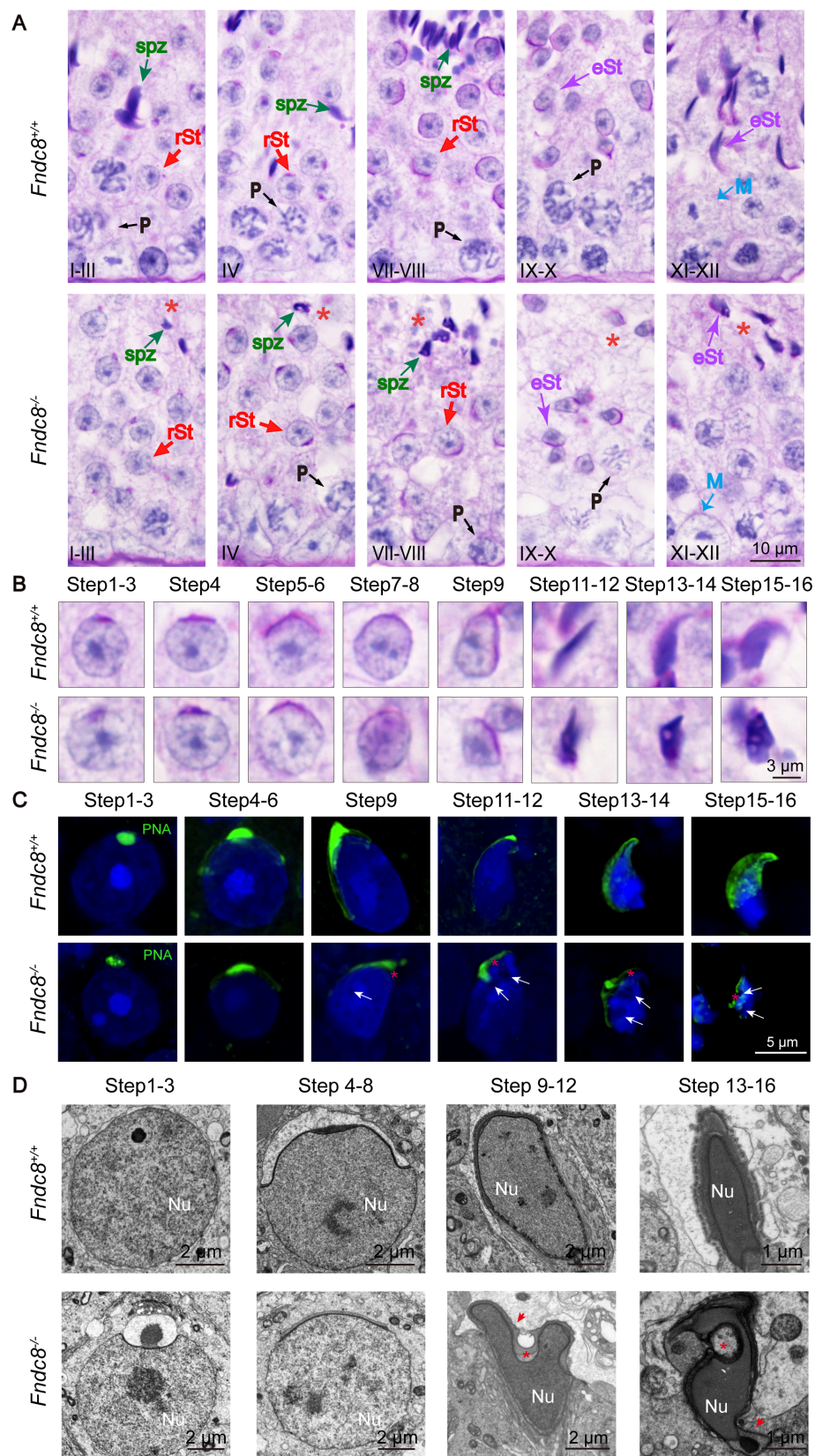


Figure 4 Sperm head subsidence occurs at late stage nuclear condensation

A: PAS-stained testis sections from *Fndc8*^{+/+} and *Fndc8*^{-/-} male mice. P, pachytene spermatocyte; M, meiotic spermatocyte; rSt, round spermatid; eSt, elongating spermatid; spz, spermatozoa; Red asterisks indicate sperm with head morphogenesis abnormalities. B: PAS-stained testis sections from *Fndc8*^{+/+} and *Fndc8*^{-/-}, showing spermatid morphology in different steps. C: Fluorescence staining of PNA in spermatids at different steps in *Fndc8*^{+/+} and *Fndc8*^{-/-} testis sections. Nuclei were counterstained with DAPI (blue). White arrows indicate vacuoles in sperm head. Red asterisks indicate gap between acrosome and nuclear membrane. D: TEM analysis of *Fndc8*^{+/+} and *Fndc8*^{-/-} spermatids in different steps. Red arrows indicate detached acrosomes. Red asterisks indicate vacuoles in sperm head.

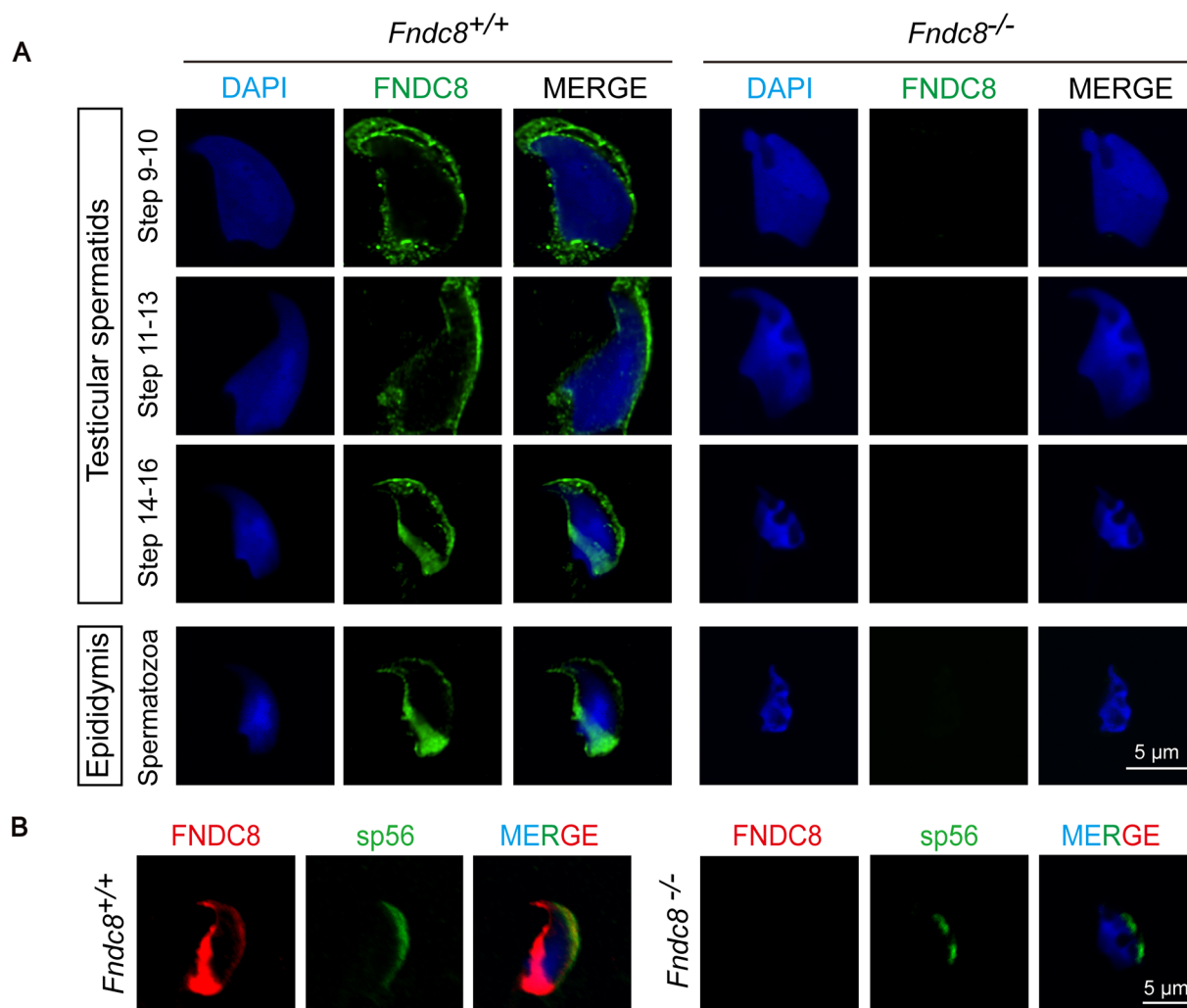


Figure 5 FNDC8 localizes in the perinuclear theca (PT)

A: Immunofluorescence staining of FNDC8 in spermatids at different steps in *Fndc8*^{+/+} and *Fndc8*^{-/-} testis sections. Nuclei were counterstained with DAPI (blue). B: Immunofluorescence staining of FNDC8 and sp56 in *Fndc8*^{+/+} and *Fndc8*^{-/-} epididymal spermatozoa. Nuclei were counterstained with DAPI (blue).

architectural and regulatory role in this process, yet its physiological significance remains incompletely understood due to the absence of genetically modified models targeting PT components. FNDC8, a testis-enriched protein (Wheeler et al., 2001), was identified here as a PT-localized factor indispensable for male fertility. Notably, knockout of *Fndc8* resulted in complete sterility, driven by profound morphological abnormalities in multiple spermatozoa structures. FNDC8 exhibited dynamic spatiotemporal localization during spermiogenesis, initially enriched in the acroplaxome and gradually repositioned to the post-acrosomal region during elongation. This dynamic localization pattern is similar to that of other PT-enriched proteins such as CPβ3/CPα3 and CCIN (Lécuyer et al., 2000; Paranko et al., 1988; Zhang et al., 2022b). Mechanistically, the earliest phenotypic defects emerged at step 9 of spermiogenesis, when acrosome detachment from the nuclear surface became evident. In mature *Fndc8*^{-/-} spermatozoa, these defects culminated in acrosomal displacement and pronounced nuclear subsidence, implicating FNDC8 as a structural stabilizer of the acrosome-nucleus interface essential for maintaining head integrity during late spermatid maturation. MS-based profiling of the PT identified several highly abundant candidate proteins, among which FNDC8 was found to interact specifically with CCIN and

ACTL7A, but not with ACTRT2. Although prior studies have reported interactions among ACTRT2, CCIN, and ACTL7A (Zhang et al., 2022b, 2022c), the lack of detectable association between FNDC8 and ACTRT2 suggests that FNDC8 may participate in a distinct protein subnetwork within the PT. CCIN, a core structural component of the acroplaxome, has been implicated as a scaffolding hub that anchors acrosomal and nuclear membranes through interactions with a broad array of partners. Disruption of CCIN impairs acrosomal tethering and compromises sperm head shaping (Zhang et al., 2022b). Additional CCIN-associated proteins—such as DPY19L2 and FAM209—are essential for coupling the acroplaxome to the nuclear lamina, with their loss shown to destabilize this linkage (Pierre et al., 2012; Castaneda et al., 2021). Similarly, deletion of PARP11 or SPATA46 perturbs the nuclear envelope and acroplaxome architecture, resulting in malformations of the sperm head (Meyer-Ficca et al., 2015; Chen et al., 2016). These findings are consistent with the phenotypes observed in *Fndc8*^{-/-} mice spermatozoa, which exhibited nuclear vacuolization and sperm head surface subsidence beginning in the elongation stage, along with abnormal dispersion of CCIN. These defects suggest that FNDC8 interacts with CCIN to maintain PT integrity and withstand the mechanical forces required for

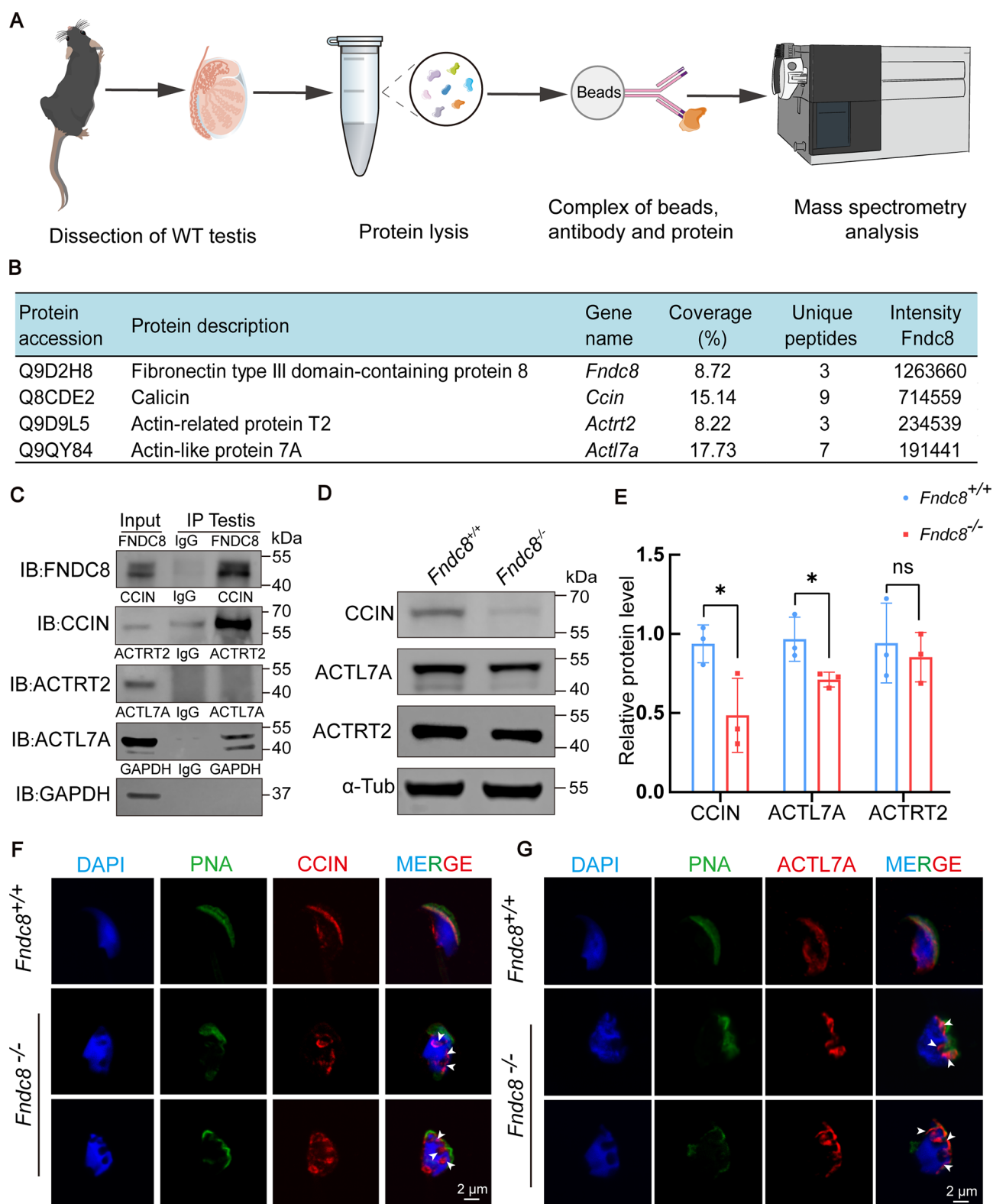


Figure 6 FNDc8 interacts with CCIN and ACTL7A to maintain PT integrity

A: Schematic overview of experimental procedure used to identify FNDc8-interacting proteins in mouse testis via immunoprecipitation (IP) and mass spectrometry (MS). B: List of high-confidence FNDc8-enriched proteins identified by co-immunoprecipitation (Co-IP) in the testis. C: Co-IP validation of interactions between endogenous FNDc8 and PT-associated proteins CCIN, ACTRT2, and ACTL7A in mouse testis. D: Quantification of relative protein levels ($n=3$ independent experiments). Data are presented as mean $\pm$ SD. ns: Not significant. *: $P<0.05$. E: Western blotting analysis showing protein levels of CCIN, ACTL7A, and ACTRT2 in the testes of *Fndc8*^{+/+} and *Fndc8*^{-/-} mice. α -Tub served as the loading control. F: Immunofluorescence staining with PNA (green) and antibodies against CCIN (red) in epididymal spermatozoa from *Fndc8*^{+/+} and *Fndc8*^{-/-} mice. Nuclei were counterstained with DAPI (blue). White arrows indicate CCIN mislocalization area. G: Immunofluorescence staining with PNA (green) and antibodies against ACTL7A (red) in epididymal spermatozoa from *Fndc8*^{+/+} and *Fndc8*^{-/-} mice. Nuclei were counterstained with DAPI (blue). White arrows indicate ACTL7A mislocalization area.

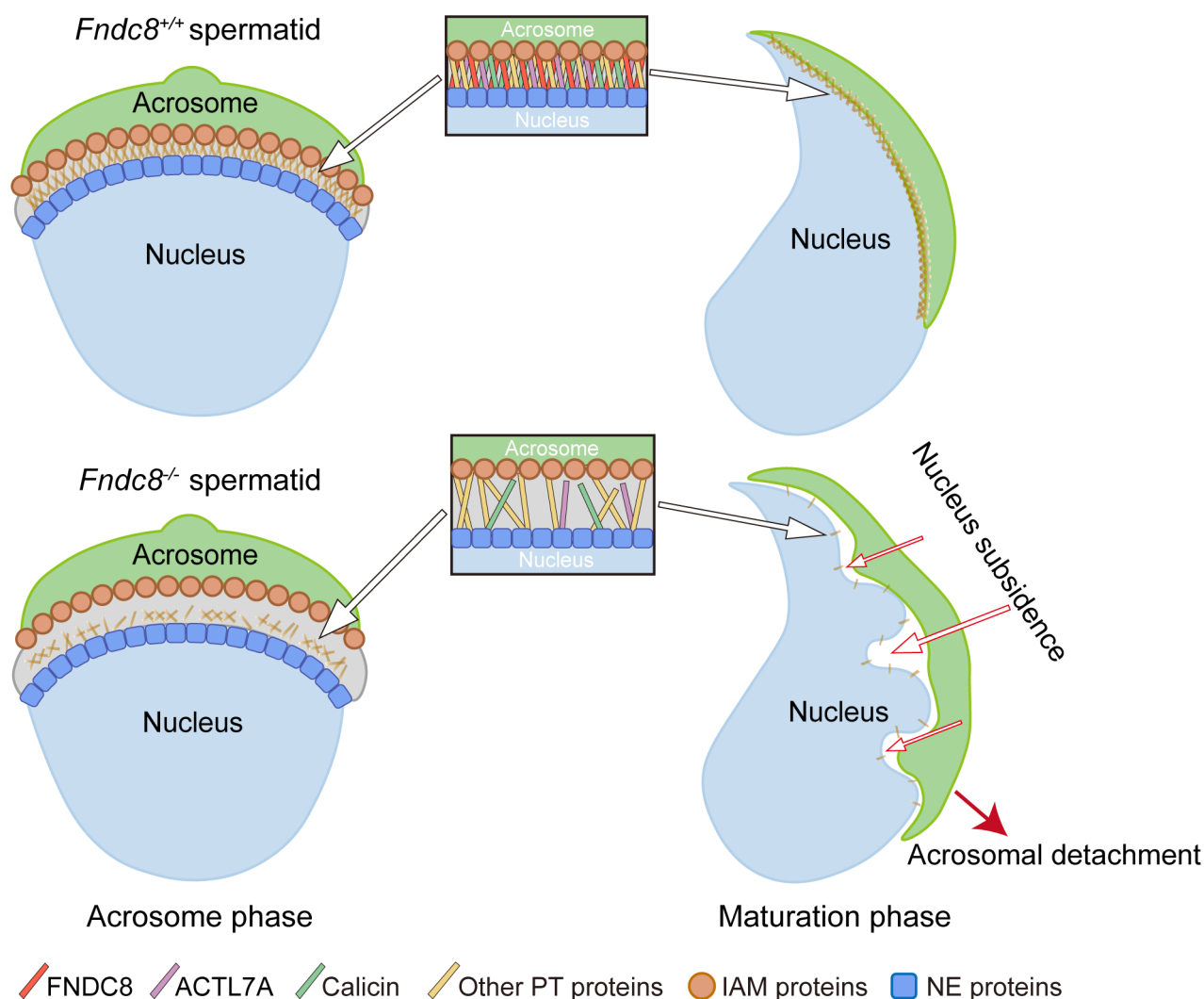


Figure 7 Proposed model for the role of FNDC8 in sperm head shaping

Schematic illustrating abnormal sperm head shaping pattern caused by disruption of the perinuclear proteins CCIN and ACTL7A in *Fndc8* knockout spermatozoa.

nuclear compaction and acrosome anchoring to the nuclear membrane.

The acroplaxome is located between the inner acrosomal and nuclear membranes, and serves as a critical structural bridge during head morphogenesis. Previous studies have reported that SPACA1, an inner acrosomal membrane protein, anchors the acrosome to the acroplaxome via interaction with ACTL7A (Chen et al., 2016), and the disruption of either SPACA1 (Fujihara et al., 2012) or ACTL7A (Xin et al., 2020) leads to the detachment of the developing acrosome from nucleus membrane. In the present study, FNDC8 was found to interact with ACTL7A, and its loss led to a marked reduction in ACTL7A protein abundance and mislocalization within the acroplaxome. These alterations coincided with acrosomal displacement during the acrosome phase of spermiogenesis, indicating that destabilization of ACTL7A may underlie the detachment phenotype observed in *Fndc8*^{-/-} spermatozoa. Taken together, these findings suggest that FNDC8 collaborates with CCIN and ACTL7A to maintain PT integrity, secure acrosome-nucleus attachment, and facilitate proper shaping and protection of the sperm head during spermiogenesis.

Fndc8 knockout resulted in significantly impaired sperm motility, likely reflecting underlying defects in flagellar

structure. A similar phenotype has been reported in *Ccin* knockout mice (Zhang et al., 2022b). Thus, in addition to their essential roles in maintaining PT integrity, FNDC8 and CCIN may also participate in sperm flagellum biogenesis. Given the close spatial association between the PT and manchette during spermiogenesis, these proteins may also be involved in intramanchette transport either directly or indirectly, with their gene knockout also potentially affecting flagellum biogenesis. In addition, ACTL7A—previously implicated in chromatin stabilization during spermatogenesis (Ferrer et al., 2023)—has been shown to localize to the PT. While *Actl7a* knockout mice display largely intact nuclear morphology, their sperm exhibit disorganized PT and acrosomal abnormalities, partially recapitulating the phenotypes observed in *Fndc8* knockout mice. As FNDC8 remains confined to the PT without detectable nuclear localization, its depletion likely disrupts the sperm nuclear surface rather than histone replacement in the sperm head.

Teratozoospermia is a prevalent etiology of male infertility (Curi et al., 2003). Although recent studies using mouse models have elucidated pathogenic mechanisms for specific forms (Jiao et al., 2021), the molecular basis of many cases remains unresolved. While direct clinical evidence linking FNDC8 dysfunction to teratozoospermia is currently

unavailable, comparative analyses indicate that FNDC8 is evolutionarily conserved from reptiles to mammals, with high sequence similarity between murine and humans orthologs. This conservation suggests that mutations in human *FNDC8* may contribute to male infertility in a subset of teratozoospermia cases. Given that FNDC8 deficiency results in the reduced expression and aberrant localization of CCIN and ACTL7A—both essential for PT integrity—FNDC8 may play a crucial role in maintaining proper PT structure and function. These findings identify FNDC8 as a potential molecular determinant of human teratozoospermia and provide mechanistic insight into its etiology.

SUPPLEMENTARY DATA

Supplementary data to this article can be found online.

COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHORS' CONTRIBUTIONS

Conceptualization: W.L., J.Y.L., C.H.L., B.B.W., and H.B.W. Methodology: J.Y.L., C.H.L., B.B.W., L.Y.W., Y.J.M., H.B.W., and Y.L.Z. Investigation: J.Y.L., C.H.L., and H.B.W. Visualization: J.Y.L., C.H.L., B.B.W., and L.Y.W. Supervision: W.L. Writing—original draft: J.Y.L. Writing—review and editing: J.Y.L., B.B.W., L.Y.W., and W.L. All authors read and approved the final version of the manuscript.

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