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The protein YWHAE (14-3-3 epsilon) in spermatozoa is essential for male fertility

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Abstract

Background: Spermatogenesis is a complex biological process highlighted by synthesis and activation of proteins that regulate meiosis and cellular differentiation occur during spermatogenesis. 14-3-3 proteins are adaptor proteins that play critical roles in kinase signaling, especially for regulation of cell cycle and apoptosis in eukaryotic cells. There are seven isoforms of the 14-3-3 family proteins encoded by seven genes (β,ε, γ, η, θ/τ, ζ and σ). 14-3-3 isoforms have been shown to have many interacting partners in several tissues including testis.

Objective: While it is known that 14-3-3 proteins are expressed in the functions of testis and spermatozoon, the role for each of the seven isoforms is not known. In this study, we investigated the roles of 14-3-3η and 14-3-3ε isoforms in spermatogenesis.

Materials and methods: To study the in vivo function of 14-3-3η and 14-3-3ε in spermatogenesis, we generated testis-specific and global knockout mice for each of 14-3-3η

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AUTHORS' CONTRIBUTIONS

AE oversaw the breeding of transgenic mice, acquired and analyzed the data, and drafted portions of the manuscript. SD oversaw and performed ATP, mitochondrial potential, and GSK3 activity assays. AI performed a variety of essential data collection and analyses. WN performed the Western blot for the phospho(Ser)14-3-3 binding motif antibody. RS performed the histological section analysis. MK performed in the data analysis. SV and DK contributed to the conception of the work, participated in the experiments, and assisted in interpreting the data and revising the manuscript. All authors read and approved the final manuscript. All authors read and approved the final manuscript.

CONFLICT OF INTERESTS

The authors declare that they have no competing interests.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

All mice used in the present experiments were housed and used at Kent State University with the approval of the Kent State University Institutional Animal Care and Use Committee following the appropriate laws, guidelines, and policies and performed in accordance with the NIH policies.

CONSENT FOR PUBLICATION

Not applicable.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

and 14-3-3ε isoforms (CKO and GKO, respectively). Computer-assisted semen analysis was used to assess sperm motility, while immunohistochemical studies were conducted to check spermatogenesis.

Results: Although both 14-3-3η and 14-3-3ε isoforms were present in mouse testis, only the expression of 14-3-3ε, but not 14-3-3η, was detected in spermatozoa. Mice lacking 14-3-3η were normal and fertile while 14-3-3ε CKO and GKO males showed infertility. Low sperm count with higher abnormal spermatozoa was seen in 14-3-3ε CKO mice. The motility of 14-3-3ε knockout spermatozoa was lower than that of the control. A reduction in the phosphorylation of both glycogen synthase kinase 3 and PP1γ2 was also seen in spermatozoa from 14-3-3ε CKO mice, suggesting a specific role of 14-3-3ε in spermatogenesis, sperm motility, and fertility.

Discussion and conclusion: This is the first demonstration that of the seven 14-3-3 isoforms, 14-3-3ε is essential for normal sperm function and male fertility.

Keywords

14-3-3; infertility; sperm motility; spermatogenesis; YWHA

1 | INTRODUCTION

The 14-3-3 (YWHA) proteins in mammals consist of seven isoforms encoded by seven independent genes: *Ywhab* (14-3-3β), *Ywhae* (14-3-3ε), *Ywhah* (14-3-3η), Ywhag (14-3-3γ), *Ywhaz* (14-3-3ζ), *Ywhaq* (14-3-3τ), and *Sfn* (14-3-3σ). Multiple isoforms usually exist in many species including plants which contain as many as 15 isoforms. The 14-3-3 proteins are highly conserved and have been shown to bind to various cellular proteins where they complement or supplement intracellular events involving protein-protein interaction or subcellular localization.[1,2] Most of the binding partners of 14-3-3 are phosphorylated proteins containing phospho-serine and phospho-threonine residues within their RSXpSXP and RX(Y/F)XPSXP amino acid sequence motifs. However, phosphorylation-dependent sites that differ significantly from these motifs have been reported[3] and some interactions of 14-3-3 do exist independent of phosphorylation. The molecular and biochemical effects of 14-3-3 binding are diverse, depending upon the nature of the interacting proteins and the signaling pathways involved. 14-3-3 proteins have been shown to regulate the localization and phosphorylation status of proteins and modulate the activity of enzymes.[4] 14-3-3 proteins also bind to over 300 proteins regulating a wide variety of cellular pathways such as transcription, translation, splicing, protein trafficking, and cell division. 14-3-3-mediated regulation of cell division is biochemically well defined. YWHA protein is known to play a key regulatory role in both mitosis and meiosis. The 14-3-3 proteins exist as homo- or heterodimers with a monomeric molecular mass of approximately 30 kDa.[5]

We have previously identified 14-3-3 proteins in oocytes and in spermatozoa.[6,7] They have been also identified in Sertoli cell adhesion and also in Leydig cells in testis. [8] In spermatozoa, 14-3-3 was first identified as a protein phosphatase (PP1γ2)-binding protein. A catalytically active form of PP1γ2 was found bound to 14-3-3 in spermatozoa. Using TAP-tag and GST-14-3-3 pull down approaches, several 14-3-3 binding proteins

were identified in testis and spermatozoon.[9] However, the functions of sperm 14-3-3 have remained elusive. In this report, we describe the phenotypes in male mice lacking 14-3-3 η or ε. The 14-3-3ε isoform is highly expressed in developing testis with its expression temporally coinciding with the onset of spermatogenesis. Data presented here show that 14-3-3ε is essential for normal spermiogenesis and sperm function whereas loss of the η isoform is dispensable. Loss of the 14-3-3ε isoform affects sperm morphogenesis and energy metabolism. The phosphorylation status and the catalytic activities of protein phosphatase PP1γ2 and glycogen synthase kinase 3 (GSK3) are altered in spermatozoa lacking 14-3-3ε. This is the first report documenting the essential requirement of one of the seven isoforms, 14-3-3ε, the loss of which cannot be compensated by the other six despite their presence and expression in testis.

2 | MATERIALS AND METHODS

2.1 | Ethics statement

All wild-type (WT), GKO, and CKO mice used in the experiments were housed in the animal facility in Kent State University. Protocols used in the experiments were approved by Kent State University Institutional Animal Care and Use Committee (IACUC) in accordance with the NIH and National Research Council's publication “Guide for Care and Use of Laboratory Animals.”

2.2 | Sperm isolation

Mice aged between 8–16 weeks were sacrificed, and the *cauda epididymis* and *vas deferens* were isolated in 1ml phosphate-buffered saline (PBS). Using a 45-mm gauge needle, the *cauda epididymis* was punctured. The spermatozoa also were squeezed out from the *vas deferens*. The epididymis was then left at 37°C for 10–15 minutes to let the spermatozoa to swim out. The spermatozoa then transferred into microcentrifuge tubes by using a large bore pipette tips. For sperm counts, 10 μL of sperm suspension was diluted 1:10 in water and 10 μL diluted spermatozoon was then transferred to a Neubauer hemocytometer for counting.

2.3 | Differential Interference Contrast (DIC) microscopy

The sperm suspension was centrifuged at 750 × *g* for 10 minutes at 4°C. The supernatant was removed and the pellet was suspended with 4% paraformaldehyde (PFA) (EM grade) in 1× PBS for 30 minutes at 4°C. The fixed spermatozoa then mounted on slides that covered with poly-L-lysine (Sigma P-8920). Spermatozoa were then observed using Olympus ix81 microscope (Olympus) using differential interference contrast system.

2.4 | Sperm and testis extracts for Western blot analysis

The sperm suspension after the counting was centrifuged at 750 × *g* for 10 minutes at 4°C. The PBS was then removed and 1% SDS was added to obtain a sperm count of 1×10^7/μL). The suspension then boiled for 5 minutes at 95°C. After boiling, the suspension was centrifuged at 16 000 × *g* for 15 minutes at room temperature. The supernatant was saved at −20°C until used for Western blot analysis.

The testis were isolated and washed with PBS. The tissue was then homogenized in homogenizing buffer (HB+) that contains Tris-HCL 20 mmol/L pH 7.0, EGTA 1 mmol/L and EDTA 1 mmol/L pH 8.0, MS-SAFE Protease and Phosphatase Inhibitor (Sigma-Aldrich # MS-SAFE). One ml of HB + buffer was used for 100 mg of tissue. The homogenate was then centrifuged at 16 000 × *g* for 15 minutes at 4°C and the supernatant stored at −20°C until use.

2.5 | Western Blot

Testis or sperm extracts were boiled for 5 minutes with Laemmli sample buffer (5×, Tris-HCL pH 6.8–7.0, Glycerol 50%, SDS 5%, Bromophenol Blue 0.05%, and DTT 250 mmol/L). The proteins were separated on either 10% or 12% SDS-PAGE and transferred to PVDF membranes. The membrane was then incubated with 5% non-fat dry milk diluted in Tris-Tween Buffer Saline (TTBS) (0.2 mol/L Tris, pH 7.4, 1.5 mol/L NaCl, 0.1% thimerosal, and 0.5% Tween 20) for 45 minutes followed by incubation with different antibodies. The following antibodies were diluted in 1% non-fat dry milk diluted in TTBS [14-3-3η: Rabbit anti-14-3-3η (Millipore Sigma AB9736, 1:1000 dilution); 14-3-3ε: anti-14-3-3ε (Santa Cruz, sc-23957, 1:1000 dilution); phospho-GSK3α/β: Rabbit monoclonal anti p-GSK3α/β (Ser21/9) antibody (Cell Signaling Technology) 1:1000 dilution; phospho-T320 PPα: Rabbit monoclonal anti p-T320 Pp1α antibody (Abcam #EP1512Y) 1:1000 dilution; PP1γ2: anti-Pp1γ2 c-terminal (YenZym, 1:5000 dilution); beta-tubulin: Rabbit monoclonal anti β-tubulin antibody (Abcam #ab52901) 1:5000 dilution; beta-actin: Mouse monoclonal anti β-actin antibody AC-15 (GeneTex# 26276) 1:2000 dilution; and Anti phospho-(Ser) 14-3-3 binding motif: Rabbit polyclonal anti p-Ser 14-3-3 binding motif antibody (Cell Signaling Technology #9601S) 1:1000 dilution]. Following primary antibody incubation, the blot was washed four times with TTBS for 5 minutes and incubated with an appropriate horseradish peroxidase-conjugated secondary antibody for 90 minutes at room temperature. The blot was then washed four times with TTBS for 10 minutes and developed with enhanced chemiluminescence (Thermo Scientific Super Signal West Pico ECL).

2.6 | Immunofluorescence labeling of spermatozoa

Spermatozoon was extracted in PBS and moved into microcentrifuge tubes. The suspension was then centrifuged at 750 × *g* for 10 minutes at 4°C. The supernatant was removed and the pellet was diluted with 4% PFA in PBS for 30 minutes at 4°C for fixation. The suspension was then centrifuged at 1000 × *g* for 5 minutes and the 4% PFA was removed as a supernatant. The pellet was then washed two times with PBS and recentrifuged. After the second wash, the fixed spermatozoon was mounted on poly-L-lysine coated coverslips. The coverslips were then covered with 0.2% Triton-X for 10 minutes for permeabilization and washed two times later with PBS for 5 minutes. The coverslips were then covered with either 5% goat serum or 5% donkey serum for 45 minutes at room temperature. The cover slips were incubated overnight at 4°C with the following antibodies with the following concentration diluted in the 1% blocking buffer [pan 14-3-3: Rabbit polyclonal anti 14-3-3 (YenZym) 1:1000 dilution; 14-3-3ε: Goat anti 14-3-3ε (R&D #AF4419) 1:500 dilution]. For the negative control sample, the incubation with the primary antibodies was omitted. The samples were then washed three times with PBS for 5 minutes and incubated with the secondary antibodies (Goat anti rabbit CY3 and Donkey anti goat CY3) for 90 minutes at

room temperature. The coverslips were then washed three times with PBS for 10 minutes. Before adding the mounting media, the coverslips covered with Hoechst diluted in 1% blocking buffer for 10 minutes and washed later with PBS. The spermatozoon was then imaged and examined by confocal microscopy.

2.7 | Testis sections for histochemistry

Testis washed with PBS was incubated overnight in Bouin's fixative solution at 4°C. The tissue was then washed two times with 70% ethanol for 5 minutes. Using Shandon Citadel 2000 tissue processor (Thermos Fisher scientific), the fixed tissues were dehydrated through a graded series of increasing ethanol (70%, 80%, 90%, and 100%) and permeabilized through two changes of Citrosolve. The processed tissue was removed and embedded in Paraffin wax. The paraffin blocks were sectioned by using microtome (Leica microsystems Inc) into 10 μm sections which were transferred to a poly-L-lysine covered slide.

2.8 | Immunofluorescence labeling of testis sections

Testis sections were deparaffinized using two changes of Citrosolve for 5 minutes and rehydrated through a decreased graded series changes of ethanol (100%, 90%, 80%, and 70%). The slides were then boiled in antigen retriever solution (Sigma Citrate Buffer Antigen Retriever C9999) three times for 1 minute with resting time of 2 minutes. The slides were rest at room temperature for 30 minutes in the antigen retrieval solution. The slides then washed with deionized water for 5 minutes. The slides were then covered with 5% blocking buffer (Goat serum diluted in PBS) at room temperature for 45 minutes. The sections were incubated overnight with the mouse monoclonal anti-14-3-3ε (Santa Cruz, sc-23957, 1:500 dilution) diluted in 1% blocking buffer at 4°C. For the negative control, the incubation with the primary antibody step was omitted. The slides were then washed 3 times with PBS for 5 minutes, and the sections were incubated with the secondary antibodies (Goat anti mouse CY3) for 90 minutes at room temperature. The slides were washed four times with PBS for 10 minutes, and in the last wash, the Hoechst stain was added. The sections were mounted and the images were obtained by using FluoView 1000 Confocal microscope.

The sections were also stained with periodic acid–Schiff staining kit (Leica Biosystems) following the manufacturer's protocol. The sections were counterstained with Gill II hematoxylin for 3 minutes then rinsed with tap water for 5 minutes. The sections later were dehydrated and mounted with xylene-based mounting media.

2.9 | RNA isolation and quantitative PCR

Wild-type mice with different ages (10, 15, 20, 25 days, and 12 weeks) were used to obtain the RNA from testes. The total RNA was isolated by using TRIzol reagent (Sigma), phenol-chloroform extraction (Amersco), and isopropanol precipitation. The pellet was then washed with 75% ethanol and dissolved in DEPC-treated water. Using Nanodrop ND-1000 Spectrophotometer (Nanodrop Technologies), the RNA concentration was measured. RNA (900 ng) was used later with the QuantiTect Reverse Transcription Kit (Qiagen) for cDNA preparation.

The quantitative PCR was performed by using Rotor-Gene Q series. An average of threshold cycles were measured from triplicate reactions for SYBER green (QuantiTect SYBER green RT-PCR kit) qRT-PCR *Gapdh* was used as a housekeeping gene for an internal control. The qPCR protocol consisted of an initial denaturation at 95°C for 5 minutes followed by 35 cycles of amplification (94°C 30 seconds, 57°C for 45 seconds, and 72°C for 45 seconds). The 14-3-3ε (*Ywhae)* primer set was designed by using Primer-Blast software and to span exon-exon junctions. The size of the primer was 172 bp (forward 5'-GCCATTTTTCCTGCTCGGAC-3' and reverse 5'-CCACCATTTCGTCGTATCGC-3'). The primer set was first run on 1% agarose gel to confirm the presence of the predicted amplification products. The average of *Ct* values of each sample was analyzed by d*Ct* method.

2.10 | Transgenic mice

A mouse line with LoxP sites inserted in the *Ywhah* gene was produced for our laboratory by a commercial company (Cyagen Bioscience) using standard ES cell targeting technique. The Exon 2 of *Ywhah* gene was selected to be targeted in this line as it was described before.[10] The homozygous mice containing Loxp gene were bred with either ACTB-Cre mice (Jackson Lab) for global knockout or Stra8-iCre mice for testis-specific knockout. We also obtained a mouse line from Dr K. Toyo-oka (Drexel University) with inserted LoxP in the *Ywhae* gene. This line contains LoxP to target exons 3 and 4 of *Ywhae* gene.[11,12] This line was maintained in 129/SvEv background in floxed condition. Again, we bred this line with either ACTB-Cre (B6N.FVB-*Tmem163*$^{Tg(ACTB-cre)2Mrt}$/CjDswJ, The Jackson Laboratory) or Stra8-iCre (008208 STOCK Tg (Stra8-icre) 1Reb/J, The Jackson Laboratory) mice.

The pups from these mating were genotyped by PCR to show the disruption of the gene (Table 1).

Using mice ear punches, the DNA were isolated and used in PCR to confirm the disruption of *Ywhah* and *Ywhae* genes. For each mouse, we used three different primers. The LoxP primers were used to distinguish the WT from the mice that has floxed gene. The knockout primers were designed as an external primers that show band only in case of allele deletion. The generic Cre primers were used to detect the presence of ACTB-Cre, and the Stra8-iCre primers were used with progeny from Stra8-iCre crossbreeding.

2.11 | Fertility testing

Transgenic male mice were bred with littermate WT females over a period of 8 weeks. After mating, the following morning females were checked for a vaginal plug to determine whether copulation had occurred. The number of pups in each litter was recorded.

2.12 | In vitro fertilization

For super ovulation, 2–3 months old wild-type females were primed intraperitoneally (IP) with 5 IU of equine chorionic gonadotropin (eCG) hormone. Exactly 52 hours later, the primed females were injected IP with 5 IU of human chronic gonadotropin (hCG) hormone. Fourteen hours after the hCG injection, the females were sacrificed to obtain ovulated eggs.

Ovaries with attached oviducts with swollen ampullae were immersed in mineral oil in a dish that also contained drops of Human Tubal Fluid (HTF) media (Millipore: EmbryoMax® Human Tubal Fluid). The ampullae were disrupted and the cumulous oocytes complexes (COCs) were dragged from the mineral oil into a 285 μL drop of HTF media. Prior to this egg collection, spermatozoa from each c*auda epididymis* of 14-3-3ε conditional knockout and litter mate WT mice were extracted in 1 mL HTF media as described earlier. Following capacitation at 37°C for 1 hour in a 5% CO_2 incubator, 15 μL of the sperm suspension was added to the COCs and incubated for 4 hours at 37°C under 5% CO_2. Eggs were then washed 2 times in fresh drops of 200 μL of HTF and incubated overnight at 37°C and 5% CO_2. The following day, 2-cell stage embryos were then scored and counted.

2.13 | Sperm motility

After capacitation, sperm motility was analyzed by a computer-assisted sperm motility analyzer (CASA). At least five random fields were chosen for each sample and analyzed using the following settings: 90 frames acquired at 60 frames/s; minimum contrast of 30; minimum cell size at 4 pixels; default cell size at 13 pixels; static cell intensity of 60; low size gate of 0.17; high size gate of 2.26; low-intensity gate of 0.35; high-intensity gate of 1.84; minimum static elongation gate of 0; maximum static elongation gate of 90; minimum average path velocity (VAP) of 50 μ/s; minimum path straightness (STR) of 50%; VAP cutoff of 10 μ/s; and straight line velocity cutoff of 0 μ/s.

2.14 | Mitochondrial potential assay

The *cauda epididymis* and *vas deferens* sperm were extracted in 1 mL of HTF media and capacitated as described. After capacitation, 500 μL of sperm suspension was mixed with 100 nmol/L of cyanine dye DiIC1(5) (MitoProbe™) and incubated at 37°C and 5% CO_2 for 15 minutes. Cyanine dye DiIC1(5) is known to penetrate the living cells and primarily accumulate in the mitochondria with active membrane potential. The accumulation and the intensity of the stain are reduced when the eukaryotic cells are treated with reagents that disrupt the mitochondrial membrane potential.[13] The sperm suspensions were then analyzed by using Flow cytometry (BD Biosciences, San Jose, CA) with 633 nm excitation.

2.15 | ATP assay

Spermatozoon from the *cauda epididymis* was isolated in HTF media as described above. The sperm aliquots, 30 μL each, were diluted with 270 μL of boiling Tris-EDTA buffer (0.1 mol/L Tris-HCL and 4 mmol/L EDTA; pH 7.75) as described.[14] The suspensions were then boiled for 5 minutes and frozen in a dry ice acetone mixture. Samples were then thawed and centrifuged at 15 000 × *g* for 5 minutes at 4°C, and the supernatant was diluted 1:10 using the Tris-EDTA buffer. ATP quantification was performed by using Bioluminescence Assay Kit CLS II (Roche Applied Science) with 100 μL of the diluted samples. Luminescence was measured in a Turner Biosystems 20/20 luminometer.

2.16 | GSK3 catalytic activity

Sperm suspensions were centrifuged at 700 × *g* for 10 minutes at 4°C and the pellets were resuspended in 1× RIPA buffer supplemented with 0.1% β-mercaptoethanol, 10 mmol/L

benzamidine, 1 mmol/L phenyl-methyl, sulfonyl fluoride (PMSF), 0.1 mmol/L N-tosyl-L-lysyl chloromethyl ketone (TPCK), 1 mmol/L sodium orthovanadate, 1 nmol/L calyculin A, and incubated on ice for 30 minutes and then centrifuged at 16 000 × *g* at 4°C for 20 minutes. The supernatants were collected and used for GSK3 assay. The amount of ^{32}PO4 transferred from [^{32}P] γ-adenosine triphosphate to phosphor-glycogen synthase peptide (GSK3 substrate, Millipore) was the measure of GSK3 activity. The assay buffer contained 200 mmol/L HEPES, 50 mmol/L $MgCl_2$, 8 μmol/L DTT, 5 mmol/L sodium β-glycerophosphate, 0.4 mmol/L ATP and 4 μCi of γ-P^{32} ATP. In a microcentrifuge tube, 5 μL of the assay buffer was added to 5 μL of the extract and GS2 peptide: YRRAAVPPSPSLSRHSSPHQ-pS-EDEEE (1 mg/mL). The mixture was incubated at 30°C water bath for 15 minutes. The reaction was then stopped by cooling down on ice for 10 minutes. The mixture was then applied onto a phosphocellulose cation exchanger (P18; Whatman Inc) paper cut into 1.5 cm × 1.5 cm. The paper was then washed with 0.1% (vol/vol) phosphoric acid three times for 5 minutes and then placed into scintillation vials with 2 mL of distilled water and counted with scintillation counter. Each reaction was set in triplicate. The presence of 1 mmol/L LiCl was used to inhibit GSK3 activity.[15] The lithium-sensitive activity as considered to be due to GSK3. The GSK3 activity was calculated with the following formula: Activity units/107 cells = (lithium-sensitive cpm) × (Reaction vol/spot vol)/(specific activity of P 32 ATP) × reaction time).

2.17 | Co-immunoprecipitation

Testis extracts were pre-cleaned with protein G-sepharose 4 Fast Flow beads (GE Healthcare), incubated for 2 hours at 4°C and then washed three times with HB + by centrifugation at 8000 × *g* for 2 minutes. The beads were then incubated overnight at 4°C with gentle rocking with primary antibodies [goat polyclonal 14-3-3ε (R&D #AF4419); mouse monoclonal 14-3-3ε (Santa Cruz #SC23957) and rabbit PP1γ2 (YenZym) antibody]. Serum (goat, mouse, and rabbit serum) was used as a negative control. The beads were then washed two times with homogenizing buffer. The pre-cleared extracts/lysates were added to the beads/antibodies and incubated for four hours at 4°C, and then washed 5 times with TTBS and resuspended with 2XSDS reducing sample buffer boiled for 10 minutes. The beads were centrifuged at 10 000 × *g* for 10 minutes, and the supernatants were used for Western blot analysis.

2.18 | Statistics

Statistical analyses were performed by using GraphPad Prism 6.03 (GraphPad Software Inc). The statistically significant differences in all cases between samples were considered significant for *P*-values ≤ .05.

3 | RESULTS

3.1 | 14-3-3 η and 14-3-3 ε expression in mouse testis and spermatozoon

It is known that 14-3-3 proteins are present in mammalian testis and spermatozoon,[7,9,16] but identification of each of the seven isoforms remains elusive. With the isoform specific antibodies, our Western blot analysis showed that 14-3-3ε was present in both testis and sperm lysates (Figure 1A) whereas signal for 14-3-3η was seen only in testis lysates (Figure

S2A). Next, we examined expression of 14-3-3ε mRNA in post-natal developing mouse testis using qPCR. Figure 1B shows that the expression of 14-3-3ε increased with age coinciding with the onset of spermiogenesis (20 days post-natal) and the levels were highest in adult testis. To determine the localization of the protein in testis and spermatozoon in WT mice, testes and spermatozoa were fixed and probed by immunofluorescence with mouse monoclonal antibodies against 14-3-3ε. In spermatozoa, a pan 14-3-3 antibody that detects multiple 14-3-3 isoforms was used to determine localization of the other 14-3-3 isoforms compared to 14-3-3ε. Results with the pan 14-3-3 antibody indicate that 14-3-3 proteins are primarily present in the post-acrosomal and midpiece regions of spermatozoa. In contrast, the 14-3-3ε specific antibody indicated that this isoform was primarily in the midpiece of spermatozoa (Figure 1C). In testis, 14-3-3ε was present in differentiating germ cells but apparently not in spermatogonia which are the first layer of cells within the seminiferous tubule (Figure 1D). The immunohistochemical staining of testis sections show a robust staining in spermatocytes and relatively less in spermatids toward the lumen. The qPCR data (Figure 1B) show 14-3-3ε mRNA expression increasing in day 20 post-natal developing testis reaching a maximum in adult testis, suggesting high expression in spermatids. This apparent discrepancy in protein staining and mRNA expression could be due to differential translation of the message in spermatocytes and spermatid. Nevertheless definitive identification of protein and mRNA expression should emerge from studies using isolated testicular germ cells and by in situ hybridization.

3.2 | Conditional and global knock out 14-3-3 η and ε

In order to characterize the role of 14-3-3η and ε in mouse testis, we produced mouse lines with a LoxP flanked exon 2 of *Ywhah* or a Loxp flanked exon 3 and 4 of *Ywhae* (Section 2). The floxed mice were crossbreed with Stra8-iCre mice to generate testis-specific knockout in differentiating germ cells or with ACTB-Cre mice to generate global knockouts. Stra8-Cre is driven by the Stimulated by Retinoic Acid gene 8 (Stra8) promoter and is expressed specifically during spermatogenesis.[17] On the other hand, ACTB-Cre is driven by the human beta-actin gene promoter and is expressed in all cells of the embryo by the blastocyst stage.[18]

The genotype of the pups from the knockout mice was confirmed by PCR with three different primers as shown in Figure 2A and Figure S2B. Protein expression was determined using Western blotting. While there was no expression of the 14-3-3η in the testis lysate from 14-3-3η GKO, there was an expression of the protein in the CKO testis. The expression was seen in the testis of 14-3-3η CKO mice due to the presence of 14-3-3η protein in the testicular somatic cells (Figure S2C). Similar results were obtained from the 14-3-3ε CKO and GKO where you can see some expression of the 14-3-3ε protein in the CKO compared to the WT testis. The low level of expression seen was due to the presence of 14-3-3ε protein in the testicular somatic cells (Figure 2B,C). The protein was absent in sperm lysates from the 14-3-3ε GKO and CKO mice (Figure 2B,C). Immunofluorescence microscopy analysis further confirmed ablation of the gene in testis of 14-3-3ε CKO mice; the protein was absent in the seminiferous tubules of the CKO mice (Figure 2D).

3.3 | Fertility of the 14-3-3η and 14-3-3ε knockout mice

At least three males from each line were used for fertility testing. All the males were able to mate with the WT females as evidenced by the presence of vaginal plugs. GKO and CKO for 14-3-3η male mice were fertile producing pups in litters similar to wild type and ACTB and Stra8 transgenic mice (Figure S2D). However, GKO and CKO of 14-3-3ε males were completely infertile; there were no pups produced even after 8 weeks of breeding (Figure 3A). In vitro fertilization (IVF) was performed with spermatozoa from 14-3-3ε CKO and WT litter mate mice and with eggs from WT female mice. The fertility rate (% eggs fertilized, as evidenced by advancement to the two-cell stage) of the 14-3-3ε CKO spermatozoa was 5% compared to 58% with spermatozoa from WT mice. These results indicated that both in vivo and vitro fertility were impaired in the 14-3-3ε knockout mice (Figure 3C).

3.4 | Sperm concentration, motility, and ATP levels in 14-3-3ε KO mice

Sperm numbers of the 14-3-3η GKO and CKO showed no significant difference compared to WT mice (Figure S2E). On the other hand, 14-3-3ε GKO and CKO had significantly lower sperm count than WT mice—sperm numbers were about one third of the WT (1–2 $\times 10^7$/mL in CKO and GKO compared to 3.5 in WT) (Figure 3B). Sperm motility was assessed using CASA. Total and progressive motility of 14-3-3η GKO and 14-3-3η CKO spermatozoa were comparable to motility of WT spermatozoa (Figure S3A). Total and progressive motilities of spermatozoa from 14-3-3ε GKO and CKO mice were significantly lower (Figure 4A). Velocity parameters such as average path velocity (VAP), straight line velocity (VSL), and curvilinear velocity (VCL) were also significantly low (Figure 4B).

3.5 | Mitochondrial function and ATP level in spermatozoa from 14-3-3 knockout mice

Given the reduced motility of spermatozoa from 14-3-3ε KO mice, we examined whether general mitochondrial function and ATP production were impaired in spermatozoa from the KO mice. Mitochondrial membrane potential measured using a fluorescent probe was also considerably lower spermatozoa from 14-3-3ε KO mice spermatozoa (Figure 5A), suggesting a possible difference in oxidative phosphorylation and mitochondrial function. ATP levels in spermatozoa lacking 14-3-3ε were significantly diminished—about 50% lower compared to wild type (Figure 5B).

3.6 | Sperm morphology and spermiation defects in 14-3-3ε KO mice

Spermatozoa from both 14-3-3ε GKO and CKO were fixed and spread on slides to examine their morphology. More than 40% of the spermatozoa from both knockdowns have an abnormally bent heads and the normal spermatozoa from those knockouts were significantly fewer in number compared to the wild type (Figure 6A,B). Spermatozoa also show other defects such as vacuolated head, amorphous head, bent, and irregular midpiece. Low sperm count and low sperm motility together with morphological abnormalities of spermatozoa could be a manifestation of defects in spermiation. The histology of the testes from 14-3-3ε GKO, 14-3-3ε CKO, and WT mice was examined. Periodic acid–Schiff (PAS) stained testis sections show an abnormal localization of spermatozoa near the basement of the seminiferous instead of in the lumen as seen in testis of wild-type mice. Elongated

spermatids also appear to be disorganized and the heads look abnormal. Some seminiferous tubules show vacuoles due to germ cells sloughing (Figure 7).

3.7 | The phosphorylation status and catalytic activities of GSK3 and PP1γ2 in spermatozoa lacking 14-3-3ε

It is known that phosphorylation of the protein kinase GSK3 and the protein phosphatase PP1γ2 are increased with onset of motility in the *epididymis*. We determined the phosphorylation of these two enzymes in spermatozoa for 14-3-3ε CKO mice. Western blot analysis shows that serine phosphorylation of GSK3 and threonine phosphorylation of PP1γ2 are significantly reduced in spermatozoa lacking 14-3-3ε (Figure 8A). We also measured GSK3 activity in spermatozoa. GSK3 activity was about twofold higher in 14-3-3ε CKO compared to WT spermatozoa (Figure 8B). We had previously shown using a pan antibody that 14-3-3 is a binding partner for sperm PP1γ2.[7,9] Here, we sought to determine whether the sperm 14-3-3ε isoform binds to PP1γ2. Two different 14-3-3ε antibodies were able to immunoprecipitate PP1γ2 from sperm extracts (Figure 8C). These binding data were further confirmed with reciprocal co-immunoprecipitation of 14-3-3ε in sperm extracts with PP1γ2 antibodies (Figure 8D). Finally, we determined the presence of phosphoproteins detected with antibodies against a phsopho-(Ser) motif recognized by 14-3-3. Previous studies using this antibody showed phosphoproteins by 14-3-3 pull down from sperm extracts and also in extracts from caudal bovine epididymal spermatozoa.[7] We confirmed the presence of the 14-3-3 phospho (Ser)-binding motif containing proteins in extracts of caput and caudal epididymal spermatozoa (Figure 8E). The levels of the proteins were higher in mouse caudal compared to caput epididymal spermatozoa similar to data shown with bovine epididymal spermatozoa.[7] Western blot signal for 14-3-3 phospho(Ser)-binding motif proteins was considerably lower in spermatozoa from 14-3-3ε compared to WT mice even though less higher amounts of extracts from the KO spermatozoa were loaded (Figure 8F).

4 | DISCUSSION

The reason for the presence of seven isoforms of 14-3-3 produced from seven independent genes in higher organisms is not known. Determination of function is further complicated by the fact that some of the 14-3-3 isoforms can form heterodimers. Gene knockout has shown that some of the isoforms may have specific roles. The global knockout of 14-3-3ε is embryonic lethal, apparently due to impaired heart development and the chance of getting an adult 14-3-3ε GKO male is less than 1%. [11,19,20] Knockout of 14-3-3ζ appears to cause neurological defects.[12,21] A statement in one report on 14-3-3ζ [22] stated that males and females lacking this isoform are infertile,however, there was no supporting data or examination and discussion of the infertility phenotype. The absence of 14-3-3η in knockout mice results in deafness and degeneration of cochlear cells.[23] There was also a mention in this report that 14-3-3η knockout males were infertile but there was no supporting data or discussion. The roles of any of the isoforms, based on gene knockout studies, in male or female fertility were not known.

We focused on two isoforms to begin the process of defining the role of 14-3-3 in male reproduction. Here, we show that both 14-3-3 η and ε are present in testis, whereas spermatozoa contain only the ε isoform which is consistent with the published report of the mouse sperm proteome where they found the expression of 14-3-3 β, ε and ζ.[16] The 14-3-3ε isoform is present in the midpiece region. The exression of 14-3-3ε mRNA in post-natal developing testis showed that expression increased temporally coinciding with the expected onset of spermatogenesis and reaching the highest levels in the adult mice testis. Previous studies have also documented high levels of expression of 14-3-3ε in human brain and testis.[24]

Our results clearly show that genetic knockout of 14-3-3ε leads to male infertility. Both global knockout (GKO) and testis-specific knockout (CKO) of 14-3-3ε prevented males from producing pups when they were mated with wild-type females. Males were capable of mating, as indicated by copulation plugs in females that were shown to be otherwise fertile, but now offspring were produced. There was no other obvious phenotype observed in the males except infertility. In contrast, global or testis-specific deletion of 14-3-3η did not reduce male fertility in in vivo breeding studies, suggesting that this isoform is not required for normal testicular and sperm function in male mice. The role of any of the other five isoforms in males awaits further testing.

Infertility of males lacking 14-3-3ε may be associated with the lower sperm count in these animals, but also with many other factors. In vitro fertilization was significantly lower with spermatozoa from the 14-3-3ε CKO males (5% compared to 56% with WT spermatozoa). The data suggest that impaired sperm function in penetrating the egg is also a likely reason for infertility. Factors responsible for this might include poor motility, reduced energy production, or due to abnormally shaped spermatozoa.

The percent motility and velocity parameters of spermatozoa from 14-3-3ε knockout mice were reduced. Mitochondrial potential and ATP levels were also reduced. The exact reasons for the reduced energy production and motility in mutant spermatozoa are not known. Sperm morphology was affected with about 75% of knockout spermatozoa displaying some abnormalities including misshapen heads and bent midpiece and head neck junctions. Similar sperm abnormalities are also seen in other KO mice where spermiogenesis is affected.[25] The histological examination of 14-3-3ε KO testis suggests spermiogenesis defects such as fewer spermatozoa in seminiferous tubules, disorganized and abnormal sperm heads, sloughing cells, loss of elongating spermatids, and vacuoles where cells have sloughed. The sperm abnormalities were similar to those previously described in the germ cells of *PPP1cc* CKO mice.[26] Previous studies on 14-3-3 in testis showed that the 14-3-3θ isoform is present in Sertoli cells localized at the ectoplasmic specialization (ES), a type of adherent junction at the interface of the Sertoli cell and elongating spermatids. Localization of the 14-3-3θ isoform was also found at the blood-testis barrier (BTB). Knock down of 14-3-3θ in Sertoli cells in culture resulted in reduced levels of proteins thought to be associated with the BTB and ES junction.[27] The role of the 14-3-3 in differentiating spermatids and Sertoli cells in promoting cell contacts essential for normal spermiogenesis require additional studies.

We had previously identified 14-3-3ζ in spermatozoa and also isolated a number of its binding proteins from spermatozoon and testis. We had shown that two key signaling enzymes PP1γ2 and GSK3 are potential binding partners for 14-3-3.[7,9] Loss of 14-3-3ε may affect activity of these enzymes with further disruption of other enzymes involved in energy metabolism which are also 14-3-3 binding proteins. High protein phosphatase activity limits motility in caput epididymal spermatozoa in WT mice. Inhibiting Protein Phosphatase 1 by Calyculin A leads to motility initiation in caput spermatozoa and motility stimulation in caudal spermatozoa. Thus, PP1 activity is reduced while the cAMP level and PKA activity are increased during epididymal sperm maturation. A reduction in the PP1 activity is also associated with increased phosphorylation and decreased catalytic activity of GSK3.[28] In caudal spermatozoa from 14-3-3ε KO mice, phosphorylation of PP1 and GSK3 is low compared to the WT spermatozoa suggesting a role for 14-3-3ε in the changes in activity of the signaling during epididymal sperm maturation. Caudal spermatozoa lacking 14-3-3ε resemble WT caput spermatozoa indicating impaired epididymal sperm maturation. Reduced GSK3 phosphorylation and high GSK3 catalytic activity seen in spermatozoa lacking 14-3-3ε is also seen in spermatozoa from soluble Adenylyl Cyclase (sAC) KO mice.[28] Reduced phosphorylation of the phospho(Ser)-binding motif containing proteins in testes extracts from testis-specific knockout 14-3-3ε males is also seen with anti phospho(Ser) 14-3-3 binding motif antibodies. These antibodies largely detect proteins with a serine phosphorylation with proline at the +2 position an arginine at the −3 position. These antibodies were validated in our previous where it was shown that the antibodies reacted with proteins isolated using pull down with recombinant 14-3-3.[7] Though unlikely, we cannot rule out the possibility that the reduced signal with 14-3-3 domain antibodies could be due reduced proteins levels rather than reduced phosphorylation. An assessment of the phospho proteome of spermatozoon and testis lacking 14-3-3ε should be instructive. Overall, our observations with spermatozoa from 14-3-3 knock out mice are consistent with the suggestion that 14-3-3 binding to phosphorylated proteins is part of mechanisms involved in the regulation of the catalytic activities of the sperm signaling enzymes.

The low ATP level and impaired mitochondrial function could be the reasons behind low motility in the 14-3-3ε KO spermatozoa. There is evidence that 14-3-3ε plays a large role in mitochondrial function. It has been known that induction of the steroidogenesis in Leydig cells triggers the translocation of 14-3-3ε from the cytosol into the outer membrane of mitochondria.[29] The 14-3-3ε is also named as mitochondrial import stimulation factor (MSF) when it forms a heterodimer with 14-3-3ζ or γ. At the outer mitochondrial membrane, Voltage Dependent Anion Channel (VDAC) competes with the Steroidogenic Acute Regulatory protein (STAR) to bind to 14-3-3ε. The VDAC channel helps in transferring the fatty acid ions, reactive oxygen species, calcium, cholesterol, and metabolites like ATP and ADP through the membrane. It also appears to mediate apoptosis.[24,29] VDAC has three isoforms in mammals originating from VDAC genes 1, 2, and 3. Gene knockout of VDAC 1, 2, or 3 in a mouse cell line cause a reduction in respiration. VDAC 1 KO mice appears to be unaffected by the missing gene while VDAC 2 knockout mice were never generated due to the anti-apoptotic function. Surprisingly, VDAC 3 knockout mice show an infertility phenotype in mouse male. VDAC 3 is localized in the acrosomal and midpiece regions of mouse spermatozoa.[30] Future work should focus on

changes that occur in sperm mitochondria in the absence of 14-3-3ε. The exact reason for infertility phenotype in mice lacking 14-3-3ε is still not known. Infertility could be due multiple factors related to the function of 14-3-3ε in testis and spermatozoa. Determination of the precise biochemical mechanisms underlying the role of 14-3-3ε in sperm function needs further investigation.

Our earlier studies with 14-3-3ζ identified a number of binding proteins. The determination of the binding partners for 14-3-3ε should now be done, given its importance in normal sperm function. Previously, we showed that 14-3-3ζ may form a heterodimer with 14-3-3ε in testis.[9] If this were the case, we predict knock out 14-3-3ζ could have the same phenotype as the 14-3-3ε knockout. Finally, it is feasible to envision possibilities of using disruption of 14-3-3 binding protein partners as a novel approach to male contraception.

In conclusion, our study has shown for the first time that out of the seven 14-3-3 isoforms one isoform (14-3-3ε) is essential for spermiogenesis and normal sperm function. The absence of 14-3-3ε alters male mouse fertility by causing oligospermia, asthenospermia, and teratospermia. The deletion of 14-3-3ε alone in male germ cells causes defects, indicating that other isoforms of 14-3-3 cannot compensate for loss of the epsilon isoform in testis. The absence of 14-3-3ε alters spermiogenesis as well as altering other key proteins known to regulate sperm motility.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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DATA AVAILABILITY STATEMENT

All data sets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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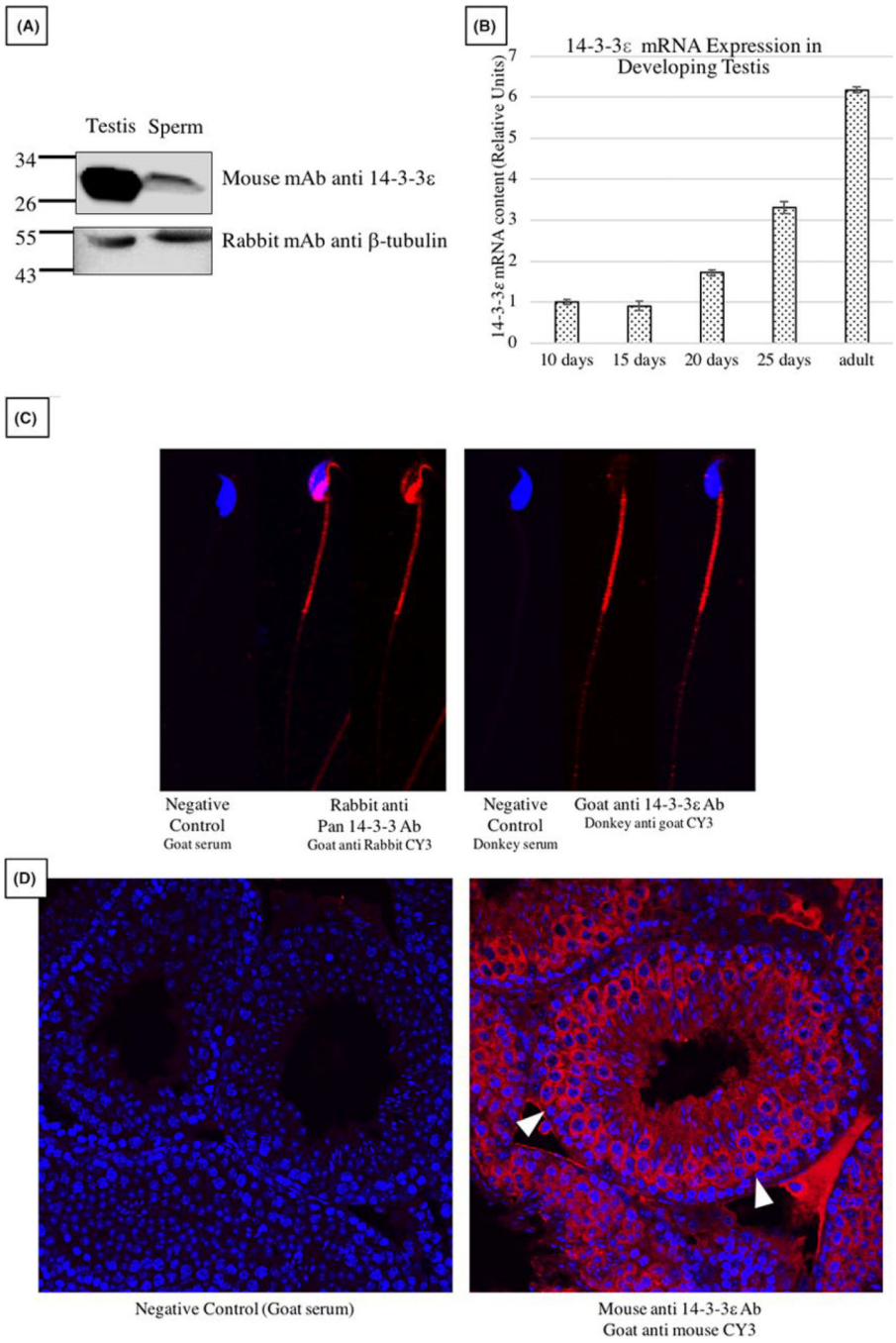

FIGURE 1.

A, Western blot analysis of testis and sperm lysates from WT mice shows detection of 14-3-3ε in both testis and sperm lysates. The blot later was probed with β-tubulin to show equal sample loading. B, Testes were collected from pups at the post-natal day shown. Messenger RNA was isolated by using TRIzol reagent. Expression of 14-3-3ε mRNA was quantified with qPCR using specific primers for 14-3-3ε and GAPDH primers as a housekeeping gene. The expression of 14-3-3ε increases with age and the highest expression was found in mature adults. These data are representative of a triplicate experiment, and

error bars represent standard of error (SE). C, Spermatozoon was collected from WT mice, fixed with 4% PFA for 45 minutes, and stained with either polyclonal goat anti-14-3-3ε or polyclonal rabbit anti-Pan 14-3-3 as a primary. The slice then stained with either anti-goat antibody CY3 or anti-rabbit antibody CY3 counterstained with Hoechst. Cells were imaged using confocal microscopy. Pan 14-3-3 is localized in post-acrosomal regions and midpiece while the 14-3-3ε is only localized in the midpiece of mouse spermatozoa. D, Testis from WT mice were fixed in Bouin's fixative solution overnight, processed, and sliced into 10 μm sections. The slice was incubated with mouse monoclonal anti-14-3-3ε antibody followed by secondary anti-mouse antibody CY3 and counterstained with Hoechst. 14-3-3ε shows expression in differentiating germ cells but not in the spermatogonia (white arrows) which locates in the first layer of the seminiferous tubules

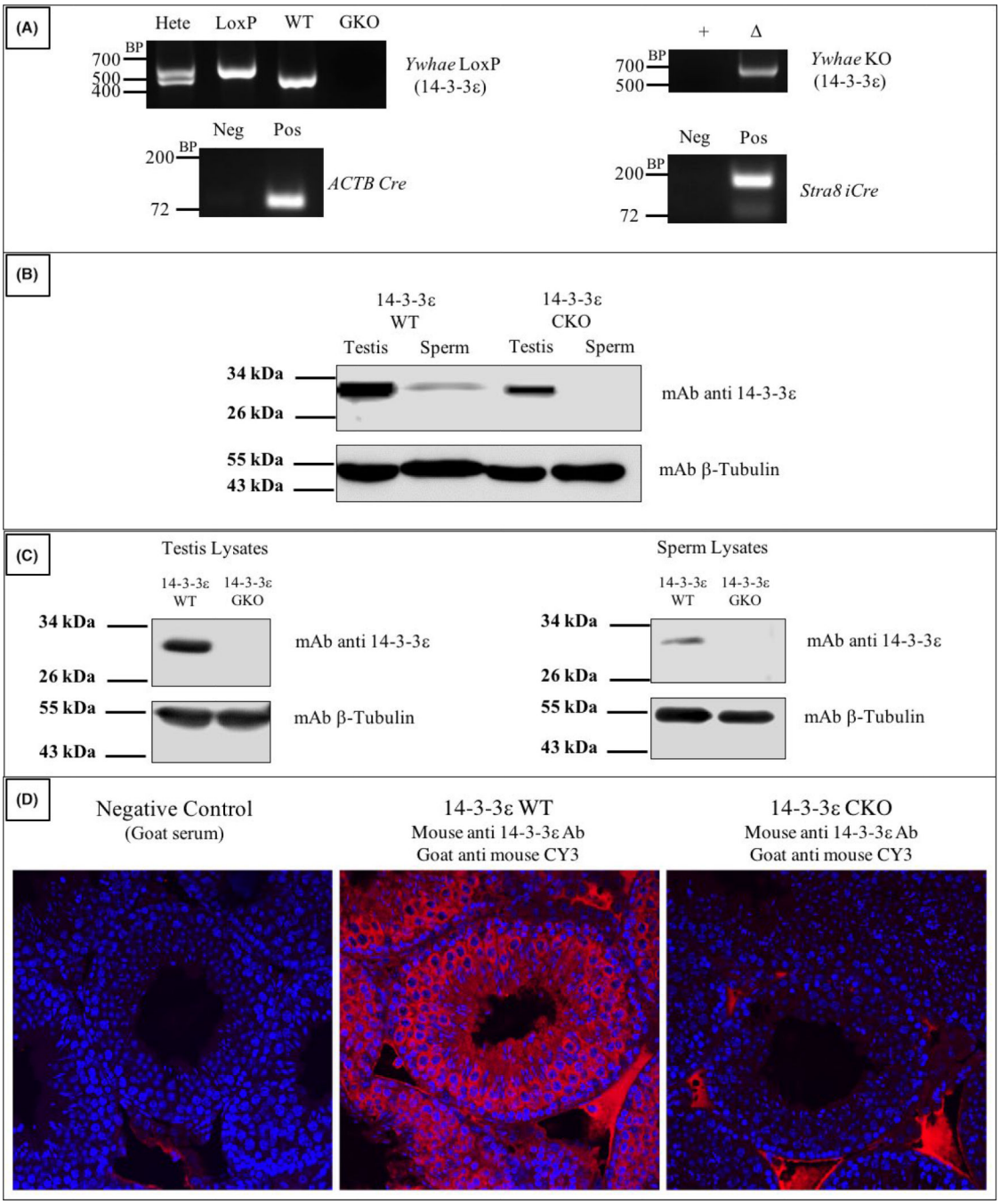

FIGURE 2.

A, Whole animal genotyping identifies those males with global and testis-specific gene deletion. 14-3-3ε LoxP primers indicate the presence of LoxP on the 14-3-3 genes, if the upper band is present. The lower band indicates WT and absence of a band indicates a possible gene deletion (-/-). The 14-3-3ε KO primers indicate a deletion (Δ) of one or both alleles if a band is present. The presence of a LoxP band using the 14-3-3ε LoxP primers, a band in the KO primers and a band in the Stra8 Cre indicates a germ cell-specific knockout (-/LoxP) since the second LoxP will be deleted later in the testis. On the other hand, the

presence of both LoxP and WT bands with no Cre (LoxP/WT) Cre negative indicates a full functional gene and a WT phenotype in this mouse. (B and C) Western blot analysis for both testis and sperm lysates of 14-3-3ε GKO and CKO were used to confirm the absence of the protein. Testis and sperm lysates from 14-3-3ε GKO show no expression for the 14-3-3ε protein. The lower expression in the testis of the 14-3-3ε CKO is due to the presence of the protein in the somatic cells. The blot was re-probed with a β-tubulin antibody to confirm the equal protein loading. D, The testis slices from both WT and 14 3 3ε CKO were incubated with primary antibody against 14-3-3ε (mouse mAb 14-3-3ε 8c3 from Santa Cruz) followed by rabbit anti mouse CY3 (Red) and counter stained with Hoechst (Blue, nuclei). Multiple sections were imaged using confocal microscopy. The protein 14-3-3ε is not detected in the seminiferous tubules of 14-3-3ε CKO animals in testes sections that were processed and imaged in parallel with sections from WT mice

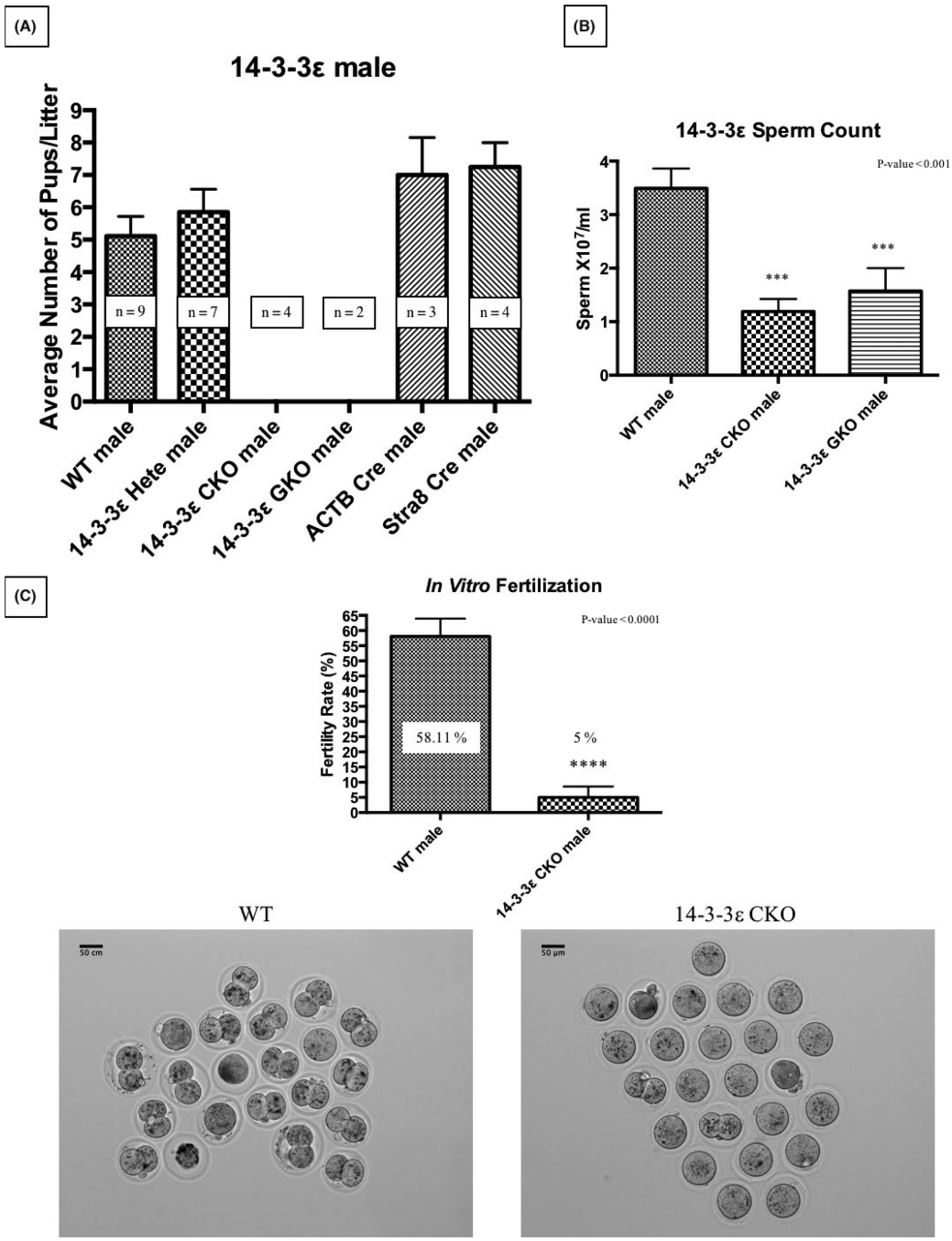

FIGURE 3.

A, The fertility of the mice of 14-3-3ε GKO and CKO was tested by in vivo breeding of transgenic males with WT females. The average number of pups born was recorded for at least 3 breeding pairs for each category. Males with the 14-3-3ε deletion produced no pups during the 8 weeks. (n) represents the number of breeding pairs. B, Spermatozoa from WT and 14-3-3ε KOs were extracted in HTF media. The sperm suspension was then diluted 1:10, and the sperm count was measured by Neubauer hemocytometer. At least three mice were used for the analysis. Prism software was used to perform an ANOVA test. 14-3-3ε

GKO and CKO spermatozoa were significantly lower than the WT sperm count (*P*-value = .0005). Error bars in all graphs represent SE. C, In vitro fertilization of spermatozoa from 14-3-3ε CKO knockout males. For each of 3 different 14-3-3ε CKO males and 3 WT males, eggs from two WT females were collected and inseminated. The percent of fertilized eggs (indicated by development to the two-cell stage) in cells fertilized by spermatozoa from 14-3-3ε CKO mice was significantly lower than for eggs fertilized by WT spermatozoa (*P*-value < .0001). Three representative examples of fertilization and percent of two-cell development of wild-type males are shown. The images shown at the bottom of the figure are representative of fertilized eggs inseminated by spermatozoa from wild-type males and spermatozoa from 14-3-3ε CKO males

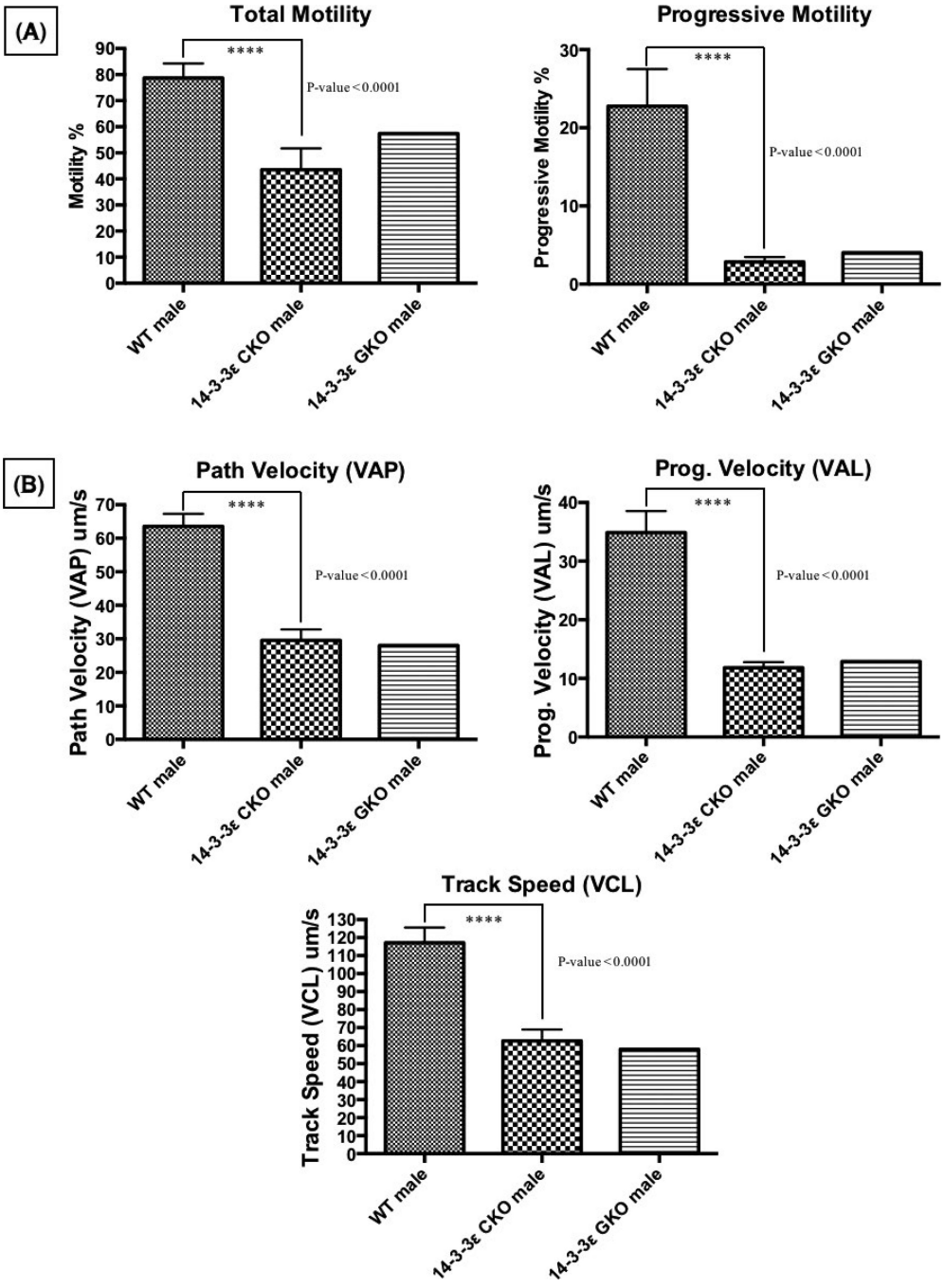

FIGURE 4.

(A and B) Spermatozoa from WT and 14-3-3ε CKO and GKO were extracted in HTF media and incubated in 37°C with 5% CO_2 for 45 minutes for capacitation. Using CASA, the sperm total and progressive motility were measured for each categories. Prism software was used to analyze the significance difference between each group. At least five different fields from each mouse were measured and averaged. The chance of getting an adult 14-3-3ε GKO male is <1% due to the embryonic lethality (11). The motility and the speed parameters spermatozoa were measured from only one 14-3-3ε GKO mouse. However, the motilities

and the speed parameters measurements for the 14-3-3ε WT and CKO were averaged from at least three mice for each category. The *t*-test shows significantly lower total and progressive motilities for 14-3-3ε CKO compared to the WT with *P*-value < .0001. C, The speed parameters (VAP, VSL, and VCL) for 14-3-3ε WT and CKO were measured. 14-3-3ε CKO show a significantly lower speed parameters when it compared to WT with *P*-value < .0001. Error bars in all graphs represent SE

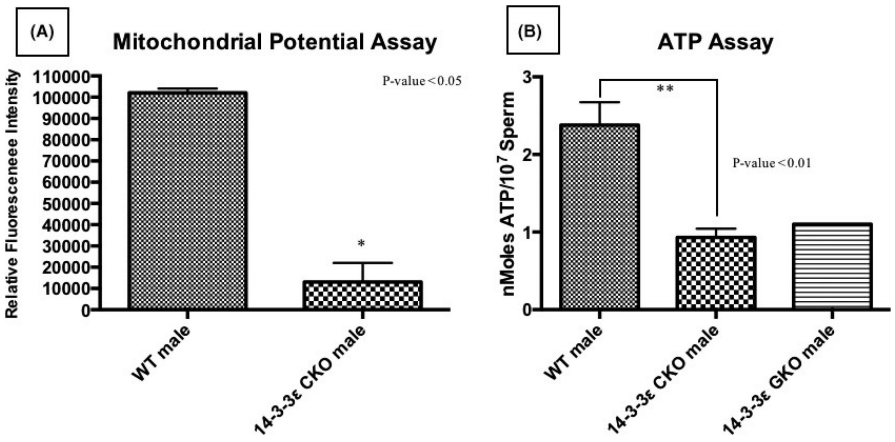

FIGURE 5.

A, Spermatozoa from WT and 14-3-3ε CKO male were extracted in HTF media. The cell suspensions were incubated with the membrane potential sensitive dye MitoProbe™DiIC1(5); Thermo Fisher, M34151. The intensities were measured by using flow cytometry. The results suggest that mitochondrial membrane potential is significantly reduced in the absence of 14-3-3ε (*P*-value = .0104). B, Wild-type and knockout spermatozoa were collected, and ATP levels were determined by a luciferase assay as described in Section 2. ATP levels in the 14-3-3ε CKO spermatozoa were significantly lower as opposed to that of WT spermatozoa. Values are means ± SEM (n = 4); *P*-value = .0038. The ATP levels for 14-3-3ε GKO represents one experiment

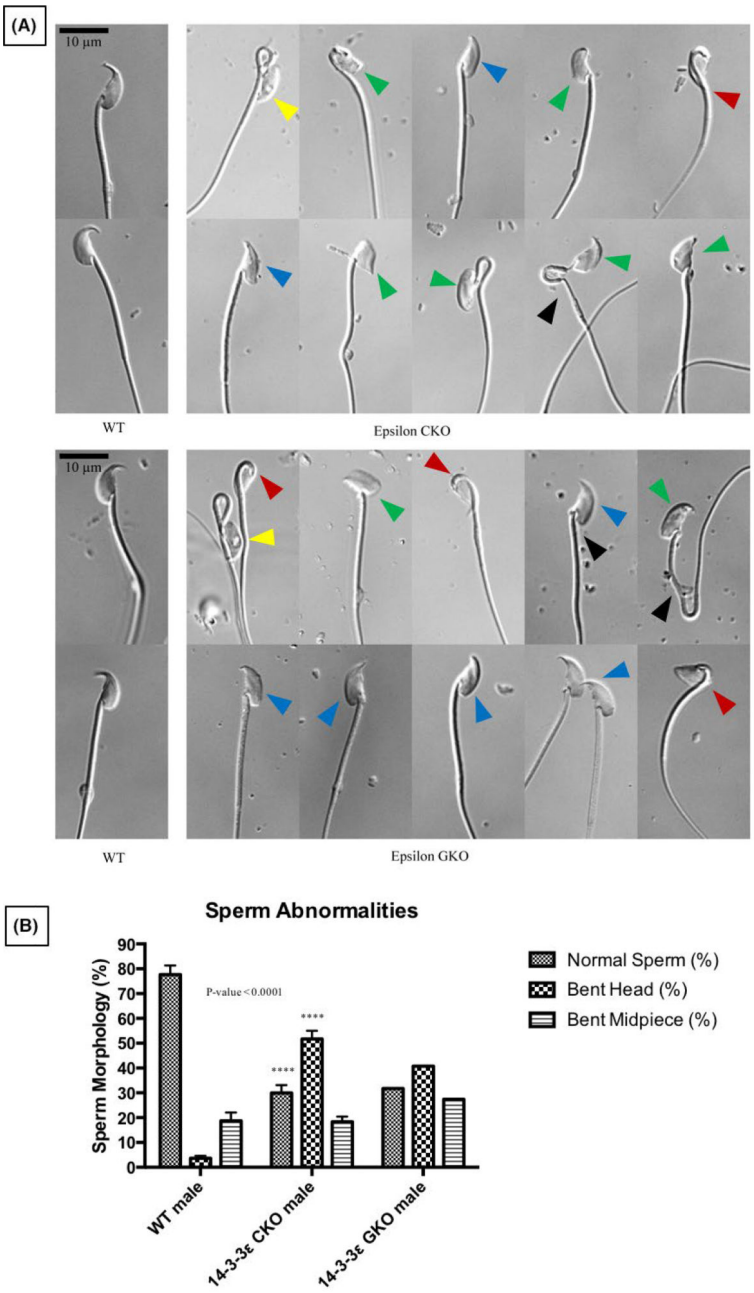

FIGURE 6.

A, Both WT and 14-3-3ε CKO spermatozoa were extracted in HTF media and then fixed in 4% PFA. The spermatozoa were imaged using DIC optics. Abnormal morphology indicated by arrows as following: vacuolated head (yellow), amorphous head (green), bent head (red), irregular midpiece (black), and hookless sperm head (blue). B, Abnormalities counts were made of spermatozoa apparent from WT (n = 4), 14-3-3ε CKO (n = 4), and 14-3-3ε GKO (n = 1). The abnormal spermatozoa with bent head are significantly higher in the 14-3-3ε CKO spermatozoa (*P*-value < .0001)

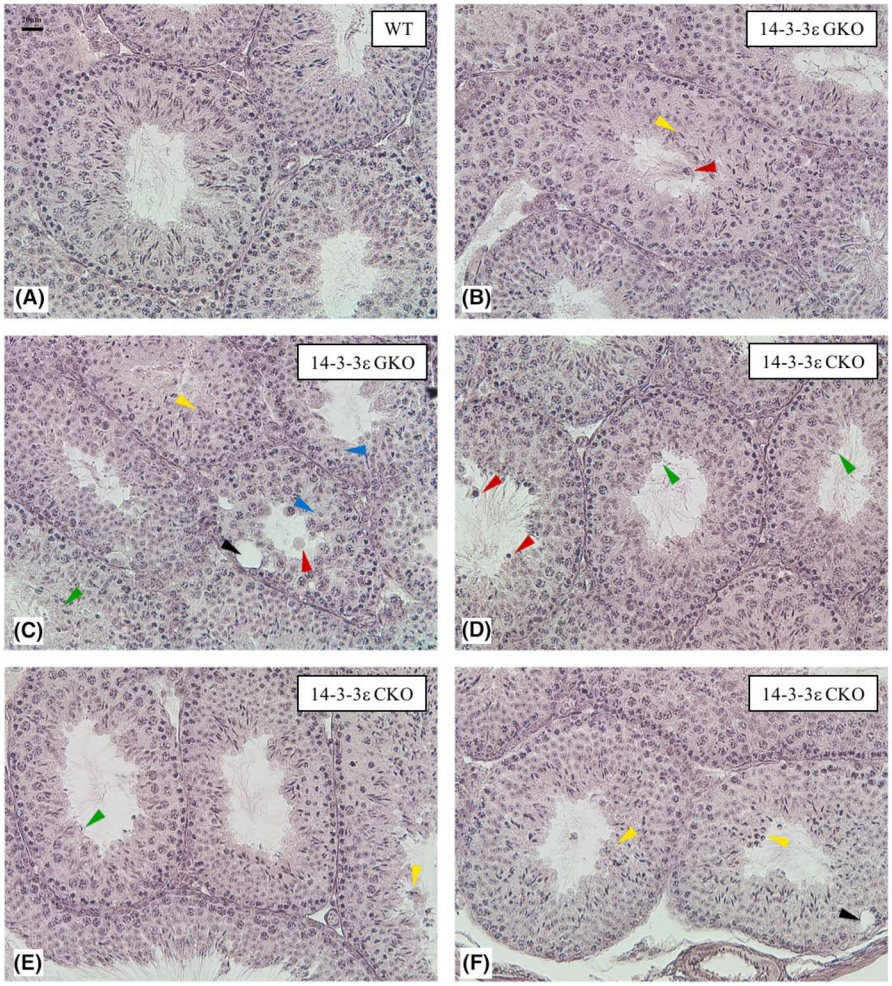

FIGURE 7.

Testis from WT (A) and 14-3-3ε GKO (B and C) and 14-3-3ε CKO (D-F) mice were fixed in Bouin's solution overnight. The tissue was then dehydrated, embedded in paraffin wax and 10 μm thick sections were prepared. Tissues slice were rehydrated and stained with periodic acid–Schiff (PAS) stain. The testes from 14-3-3ε GKO and CKO mice have fewer spermatozoa in the lumen of seminiferous tubules. Some spermatozoa appear to be localized near the basal lamina suggesting abnormal spermiogenesis and poor transport to the lumen. Abnormalities in the testes of the knockout mice are indicated by colored arrows as following: Disorganized and abnormal sperm heads (Yellow); Sloughing cells (Red); Loss of elongating spermatids (Blue); Failure of spermiation (Green); Vacuoles where cells have sloughed (Black)

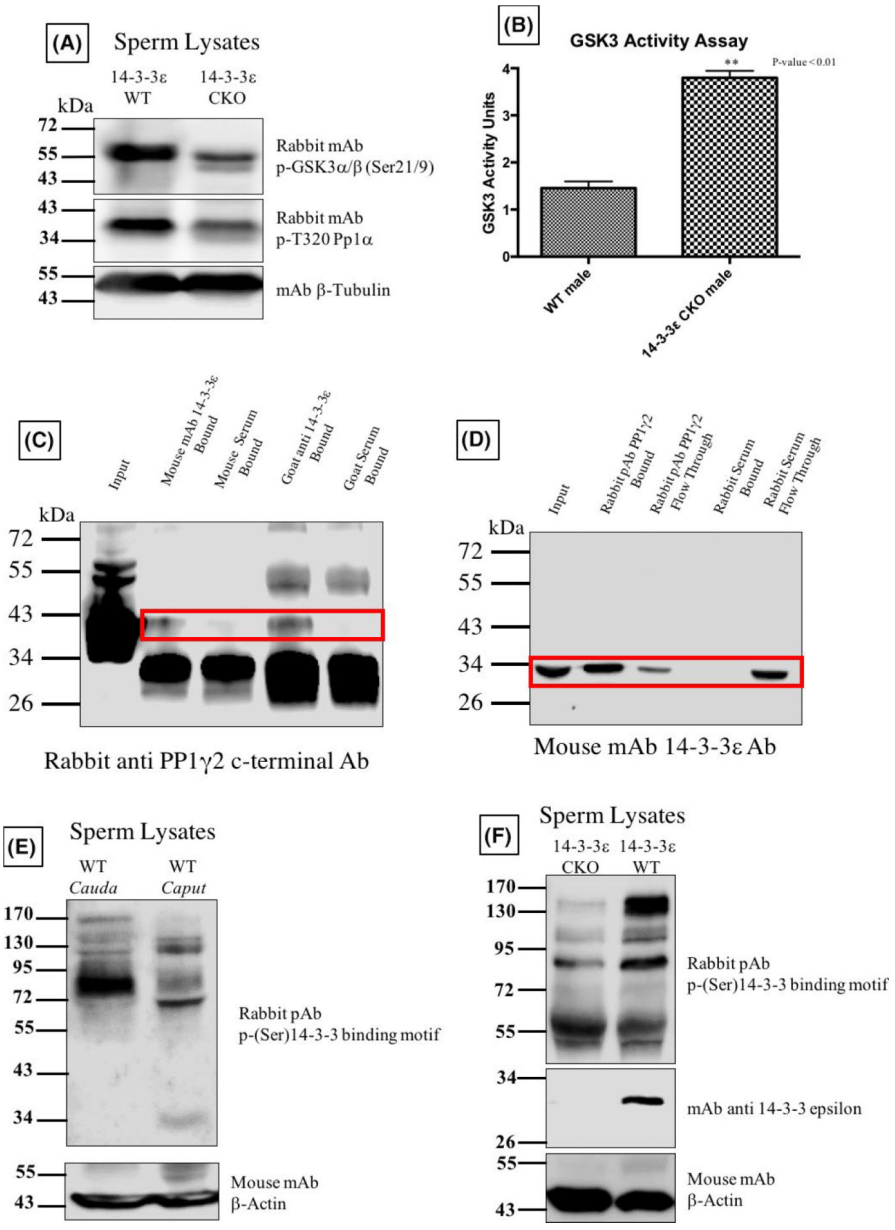

FIGURE 8.

A, Western blot analysis suggests that expression or post-translational modification of proteins known or likely to be involved in sperm function may be altered in the absence of 14-3-3ε. The 14-3-3ε CKO spermatozoa show a decrease in the phosphorylation of serine and tyrosine for GSK3α/β. The threonine phosphorylation of PP1 also reduced. The blot later was probed with β-tubulin to show equal sample loading. B, Catalytic activity of GSK3 was measured using GS2 peptide as a substrate as described in Materials and Methods. Unit activity is defined as nmoles of 32PO42- incorporated/min/107 spermatozoa. Catalytic activity of GSK3 is significantly higher in 14-3-3ε conditional KO compared to wild-type spermatozoa. Values are means ± SEM (n = 6); *P*-value = .0077. C, Co-immunoprecipitation experiment indicates and interaction of 14-3-3ε with PP1γ2 as PP1γ2 protein is pulled down by two different antibodies for 14-3-3ε (Mouse monoclonal and Goat polyclonal

antibodies). D, A reciprocal co-immunoprecipitation experiment shows the presence of 14-3-3ε protein when it is pulled down with the PP1γ2 c-terminal antibody. (E and F) Western blot analysis of sperm extract probe with anti phospho(Ser)14-3-3 binding motif antibody. E, Sperm number adjusted to (1×10^7 spermatozoon/μL). Mouse caput and caudal sperm extract were made as described after isolation in TBS. F, WT vs KO sperm extract were made from spermatozoa suspended in HTF for one hour to promote capacitation during the IVF. Lower panel for E and F show the blot developed with β-actin to show equal loading. The middle panel of blot F shows the presence of 14-3-3ε in WT but not in the CKO

TABLE 1

Primers used to detect LoxP and *Ywha* genes

PCR primers	Primers sequence	Size (bp)
Ywhah LoxP (14-3-3η)	Forward: 5′- TAATTGTGAGCCACCCGAAATGA -3′ Reverse: 5′- GCCAACGACCAATGCCAATTATAG -3′	WT:226 Floxed:292
Ywhah KO (14-3-3η)	Forward: 5′- CCTGATCTAGGATAGCTAGGGCTACATAG -3′ Reverse: 5′-AGTATACCTTTTGGAGACAGGATCTATTATAGCC -3′	Deletion gives band at: 390
Ywhae LoxP (14-3-3ε)	Forward: 5′- GCATGTGTTTGTCTGTCAGAGGAC -3′ Reverse: 5′- AGGTACCAAAACAGTAAGCCATCTCCCTA -3′	WT:450 Floxed:536
Ywhae KO (14-3-3ε)	Forward: 5′- TTCTTTTGTAGAAATTGGGGAAGGTCATGG -3′ Reverse: 5′- AGGTACCAAAACAGTAAGCCATCTCCCTA -3′	Deletion gives band at: 664
Generic Cre	Forward: 5′- GCG GTC TGG CAG TAA AAA CTA TC -3′ Reverse: 5′- GTG AAA CAG CAT TGC TGT CAC TT -3′	Cre Positive: 100
Stra8-iCre	Forward: 5′- GTGCAAGCTGAACAACAGGA-3′ Reverse: 5′- AGGGACACAGCATTGGAGTC -3′	Cre Positive: 179

Note: Wild-type males that served as control animals (litter mates) were genotyped as one of the following (homozygous WT, homozygous LoxP with no Cre and LoxP/WT with no Cre) while the heterozygous genotyped as follow (LoxP/WT stra8-iCre positive, WT/-, LoxP/- with no Cre). The global knockout males were genotypes as a homozygous for the KO (-/-), which should show no band with the LoxP primers, while the conditional knockout males were genotyped as LoxP/- with Stra8-iCre positive.