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Speriolin is a novel human and mouse sperm centrosome protein

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BACKGROUND: Oocytes in humans, mice and other mammals lack identifiable centrioles. The proximal centriole brought in by the fertilizing sperm in humans and most other mammals appears to gives rise to the centrioles at the spindle poles in the zygote, and is believed to indicate that centrioles are inherited through the paternal lineage. However, both the proximal and distal sperm centrioles degenerate in mice and other rodents. A bipolar mitotic spindle nucleates from multiple centrosome-like structures in the mouse zygote and centrioles are not seen until the blastocyst stage, suggesting that centrioles are inherited through the maternal lineage in mice. We previously identified speriolin as a spermatogenic cell-specific binding partner of Cdc20 that co-localizes with pericentrin in mouse spermatocytes and is present in the centrosome in round spermatids.

METHODS: The nature and localization of speriolin in mouse and human sperm and the fate of speriolin following fertilization in the mouse were determined using immunofluorescence microscopy, immunoelectron microscopy and western blotting.

RESULTS: Speriolin surrounds the intact proximal centriole in human sperm, but is localized at the periphery of the disordered distal centriole in mouse sperm. Human speriolin contains an internal 163-amino acid region not present in mouse that may contribute to localization differences. Speriolin is carried into the mouse oocyte during fertilization and remains associated with the decondensing sperm head in zygotes. The speriolin spot appears to undergo duplication or splitting during the first interphase and is detectable in 2-cell embryos.

CONCLUSIONS: Speriolin is a novel centrosomal protein present in the connecting piece region of mouse and human sperm that is transmitted to the mouse zygote and can be detected throughout the first mitotic division.

Key words: centriole / flagellum / fertilization / paternal inheritance / zygote

Introduction

The perpendicularly oriented pair of centrioles and the surrounding pericentriolar material (PCM) form the centrosome, an amorphous cytoplasmic region that serves as the microtubule organizing center (MTOC) in most cells. In mammals, centrioles are barrel-shaped organelles with the inner walls lined by a cylinder of nine triplets of microtubules ~0.5 μm in length and 0.2 μm in diameter (Bettencourt-Dias and Glover, 2007). The centriole pairs duplicate during S-phase with each cell cycle and along with the PCM have an essential role in organizing the mitotic spindle in M-phase and completing cytokinesis (Doxsey *et al*., 2005; Loncarek and Khodjakov, 2009). The centrioles also give rise to the basal bodies of cilia and to flagella (reviewed in Dawe *et al*., 2006; Nigg and Raff, 2009).

Formation of the mammalian sperm flagellum begins in the round spermatid when the pair of centrioles localize the side of the cell facing the seminiferous tubule lumen, with one of the pair lying against the nuclear envelope (proximal centriole) and the other (distal centriole) becoming the basal body that nucleates assembly of the nine-plus-two array of microtubules of the axoneme. A centriolar adjunct develops at the end of the proximal centriole, and nine longitudinal columns surround the distal centriole and delimit the connecting piece region of the sperm. The longitudinal columns extend from a thickened plate (capitulum) abutting the nuclear envelope into the flagellum where they are continuous with the outer dense fibers (reviewed in Eddy, 2006; Kerr *et al*., 2006). In humans (Zamboni and Stafanini, 1971; Manandhar *et al*., 2000), rhesus monkeys (Manandhar and Schatten, 2000) and most other mammals (reviewed in Manandhar *et al*., 2005), the structure of the distal centriole becomes disorganized in late spermatids, while the proximal centriole retains its structure. However, mice, rats and other rodents are different in that both the proximal and the distal sperm

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centrioles degenerate (Fawcett, 1965; Woolley and Fawcett, 1973; Manandhar *et al*., 1998).

An unusual feature of unfertilized mammalian oocytes is their lack of centrioles which are usually considered essential cell organelles. This was shown by transmission electron microscopy (TEM) for oocytes and tubal ova of the rabbit (Zamboni 1970), oocytes undergoing meiotic divisions in the mouse (Calarco *et al*., 1972; Szöllösi *et al*., 1972), human oocytes (Sathananthan *et al*., 1988), cow oocytes (Sathananthan *et al*., 1997) and sheep oocytes (Crozet *et al*., 2000). However, following fertilization, in most species, a sperm aster develops from the sperm proximal centriole. This replicates during the pronuclear stage to produce the centrioles at the poles of the mitotic spindle of the first cleavage and all subsequent cell divisions (Sathananthan *et al*., 1996, 1997; Crozet *et al*., 2000). It remains to be determined how the single sperm centriole replicates to generate two centriolar pairs.

Other studies indicated that an intact sperm centriole is associated with successful ICSI (reviewed in Schatten and Sun, 2009) and that abnormalities in the human sperm centriolar region are associated with cases of infertility (Kamal *et al*., 1999; Rawe *et al*., 2002), failure of cleavage after ICSI (Chemes, 2000; Nagy, 2000; Rawe *et al*., 2002), failures of fertilization and abnormal early embryonic cleavages (Nagy, 2000). These observations are consistent with the hypothesis of the paternal inheritance of centrioles by embryos proposed by Boveri at the turn of the twentieth century. However, parthenogenetically activated rabbit oocytes can form centrioles (Szöllösi and Ozil, 1991), and parthenogenetically activated human oocytes can form multiple asters (Terada *et al*., 2009), indicating that sperm components are not necessary for the formation of asters or centrioles in these species.

The ontogeny of centrioles in the embryos of mice and other rodents is different from that in most mammals. In addition to the lack of centrioles in oocytes and the absence of distinct centrioles in the sperm at the time of fertilization, centrioles are not present in mouse embryos until they form *de novo* in the late morula (Szöllösi *et al*., 1972) or early blastocyst stage of development (Gueth-Hallonet *et al*., 1993). It was seen by TEM that microtubules forming the meiotic spindles in oocytes emanate from several dense fibrillar aggregates comparable in appearance to MTOCs (Calarco *et al*., 1972; Szöllösi *et al*., 1972). In addition, multiple rounds of cell division can occur in mouse embryos developing parthenogenetically without the contribution of sperm components (Schatten *et al*., 1991; Schatten, 1994; Hiiragi and Solter, 2004). These and other observations have led to the belief that centrioles are maternally inherited in the mouse (reviewed in Sun and Schatten, 2007).

The PCM of centrosomes contains a variety of proteins critical for their functions. The list of proteins associated with centrosomes continues to grow from studies using indirect immunofluorescence microscopy (IIF) and immunoelectron microscopy (IEM), identifying their interactions with known centrosomal proteins, the application of proteomics and the tools of genetic analysis (reviewed in Andersen *et al*., 2003; Doxsey *et al*., 2005; Bettencourt-Dias and Glover, 2007). Although most studies focus on the basic roles of centrosomes in cell cycle regulation, some centrosomal proteins have been found to be associated with a variety of human diseases (reviewed in Badano *et al*., 2005; Nigg and Raff, 2009; Bettencourt-Dias and Glover, 2009). For example, mutations in pericentrin are associated with Seckel syndrome (Griffith *et al*., 2008) and Majewski osteodysplastic primordial dwarfism type II (Rauch *et al*., 2008). Although usually a component of the centrosome, pericentrin is not detected in spermatids or in sperm in mice (Manandhar *et al*., 1998; Goto and Eddy, 2004) and apparently does not have an essential role in the centrosomes of these cells.

Despite the considerable amount of knowledge about centrosomal proteins in other cell types, immunolocalization studies have identified only a few of these proteins in mammalian gametes and early embryos. The MTOCs of meiotic spindles stain for γ-tubulin in mouse oocytes (Gueth-Hallonet *et al*., 1993; Palacios *et al*., 1993; Lee *et al*., 2000), but not in human, bovine (Simerly *et al*., 1999) or pig oocytes (Manandhar *et al*., 2006). Pericentrin and centrin staining are seen at the spindle poles of mouse oocytes (Hiraoka *et al*., 1989; Carabatsos *et al*., 2000) but not of pig oocytes (Manandhar *et al*., 2006), further indicating the differences between mice and other species. In addition, NEDD1 (Ma *et al*., 2010) and proteins recognized by the human scleroderma autoantiserum 5051 (Calarco-Gillam *et al*., 1983; Hiraoka *et al*., 1989) and rabbit autoantiserum NRS-01 (Hiraoka *et al*., 1989) are localized to MTOCs of mouse oocytes, and the human autoantiserum SPJ stains the spindle poles in hamster oocytes (Hewitson *et al*., 1997).

The centrosomes in round and elongating spermatids have been found to contain γ-tubulin, often detectable by IIF as two spots and by IEM in association with the proximal and distal centrioles. This has been seen in mice (Fouquet *et al*., 1998; Manandhar *et al*., 1998), rats, hamsters, guinea pigs (Fouquet *et al*., 1998), rabbits (Fouquet *et al*., 1998; Tachibana *et al*., 2009), bulls (Simerly *et al*., 1999), rhesus monkeys (Fouquet *et al*., 1998; Manandhar and Schatten, 2000) and humans (Tachibana *et al*., 2005). The γ-tubulin is shed from late spermatids in the residual bodies in mice (Manandhar *et al*., 1998) and monkeys (Manandhar and Schatten, 2000) and is no longer detectable by IIF or IEM in sperm of most species. Although it is seen by IIF in $<5\%$ of human sperm, γ-tubulin can be detected in human and bull sperm by immunoblotting (Simerly *et al*., 1999).

Centrin has been identified by IIF in the centrosomes and by IEM associated with the proximal and distal centrioles in spermatids. This was reported for mice (Hart *et al*., 1999; Manandhar *et al*., 1999), rabbits (Tachibana *et al*., 2009), pigs (Manandhar *et al*., 2006), rhesus monkeys (Manandhar and Schatten, 2000) and humans (Simerly *et al*., 1999; Tachibana *et al*., 2005; Tachibana *et al*., 2009). While centrin is not detectable by IIF in mouse sperm (Manandhar *et al*., 1999), it is seen as two spots at the junction between the head and flagellum and in association with the tip of the flagellum separated from the sperm head in pigs (Manandhar *et al*., 2006), humans (Simerly *et al*., 1999), rhesus monkeys (Manandhar and Schatten, 2000) and rabbits (Tachibana *et al*., 2009). In addition, centrin has been detected by immunoblotting in human sperm (Simerly *et al*., 1999). The centrin in rhesus monkey spermatids was localized in two spots corresponding to the location of the centrioles. The spot associated with the proximal centriole has a substantially higher intensity than the spot associated with the distal centriolar vault, suggesting that centrin loss correlates with the loss of centriolar organization (Manandhar and Schatten, 2000).

We have identified speriolin as a novel spermatogenic cell-specific protein that co-localizes with pericentrin in the centrosome in mouse spermatocytes and continues to be present in spermatid

centrosomes in the absence of pericentrin (Goto and Eddy, 2004). Speriolin was identified as a binding partner of the Cdc20 subunit of the anaphase-promoting complex/cyclosome (APC). The APC targets proteins to the proteasome for proteolysis and contributes to enhancing and silencing the spindle checkpoint (Peters, 2002).

There is a lack of molecular data on the fate of sperm centrosomal proteins following fertilization. Although the sperm centriole usually is required for aster formation in zygotes in humans, the possible role of sperm components in centrosome regeneration in zygotes of mice is unknown. In this study, we determined the location of speriolin in mouse and human sperm and in mouse zygotes and early embryos following fertilization. While many testis-specific genes and proteins have been identified (reviewed in Matzuk and Lamb, 2002; Yan, 2009), speriolin appears to be the first spermatogenic cell-specific protein associated with the sperm centrosome that is known to be carried into the oocyte at fertilization and conserved between the mouse and human. It remains to be determined if speriolin has a role in the reassembly of functional centrosomes and spindles in the mouse zygote and is a paternal contribution to the centrosome lineage in the embryo.

Materials and Methods

Testis and sperm lysates

Lysates were prepared for western blotting as described previously (Goto and Eddy, 2004). To analyze the differential solubility of speriolin, testis homogenates were fractionated by centrifugation at 14 000*g* for 30 min, and the 0.1% NP-40-insoluble pellets were incubated for 10 min at room temperature (RT) in Ca/Mg-free phosphate-buffered saline (PBS) or PBS containing 0.5 M NaCl, 0.1 M Na_2CO_3, 1% SDS or 1% Triton X-100. All reagents used in these studies were obtained from Sigma-Aldrich (St Louis, MO, USA) unless indicated otherwise. Soluble and insoluble proteins were separated by centrifugation at 14 000*g* for 30 min, and insoluble proteins were extracted by boiling in SDS-sample buffer as described previously (Goto and Eddy, 2004). Sperm from the cauda epididymis were suspended in the same solutions without homogenization, and soluble and insoluble proteins were separated by centrifugation at 14 000*g*. Cells and tissues were from CD-1 adult mice (Charles River, Raleigh, NC, USA). All procedures involving mice were carried out according to US Public Health Service guidelines and were approved in advance by Institutional Animal Care and Use Committee at NIEHS or the University of North Carolina at Chapel Hill.

Antibodies

Mouse monoclonal antibodies to β-actin (AC-15), α-tubulin (B5-1-2) and γ-tubulin (GTU-88), as well as horseradish peroxidase (HRP)-conjugated antibody to mouse IgG and fluorescein 5-isothiocyanate (FITC)-conjugated antibody to rabbit IgG, were from Sigma-Aldrich. Alexa Fluor 546-conjugated antibody to mouse IgG was from Invitrogen Molecular Probes (Eugene, OR, USA). Rabbit polyclonal antibody to lamin B (M-20) and HRP-conjugated goat antibody to rabbit IgG were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Production and characterization of the rabbit antiserum to speriolin and its use for immunoblotting and IIF were described previously (Goto and Eddy, 2004).

Northern blotting

The gene-encoding speriolin was provisionally named *Sprn* (Goto and Eddy, 2004) and renamed *Spatc1* (GenBank no. NM_028852) for spermatogenesis and centriole associated 1 by the Mouse Genome Informatics (MGI) program. A human multiple tissue northern blot (Clontech, Mountain View, CA, USA), containing 2 μg of poly $(A)^+$ RNA/lane, was probed with a radiolabeled *SPATC1* cDNA. The full-length 1938-bp human speriolin cDNA (*SPATC1*; GenBank no. AB092352) was isolated from a testis cDNA library using primers designed from human ESTs sequences with high identity to the mouse *Spatc1* sequence. The membrane was exposed for 12 to 35 h at −80°C using Kodak X-OMAT-AR X-ray film with an intensifying screen.

Immunofluorescence staining of sperm

Mouse sperm were isolated from the cauda epididymis of adult CD-1 mice and allowed to settle onto SuperFlost slides (Fisher Scientific, Pittsburg, PA, USA). Human semen samples, previously cryo-protected and stored in liquid nitrogen, were provided by the Andrology Laboratory, Department of Obstetrics and Genecology, University of North Carolina School of Medicine. Sperm were separated from seminal fluid by diluting 10-fold with PBS containing complete protease inhibitors (Roche Applied Science, Indianapolis, IN, USA) and washed three times in PBS containing protease inhibitors.

Sperm were permeabilized with 0.5% Triton X-100 for 2 min at 4°C and then fixed with MeOH for 20 min at −20°C and blocked with 5% normal goat serum in automation buffer (Biomeda Corporation, Foster City, CA, USA) containing 1% bovine serum albumin (BSA). Sperm were labeled for 2 h at RT in a humidified chamber with primary antibodies, followed by FITC-conjugated or Alexa-Fluor 546-conjugated secondary antibodies. Nuclei were labeled with 4′,6-diamidino-2-phenylindole (DAPI, Sigma) and slides were mounted with Vectashield (Vector Laboratories, Burlingame, CA, USA). Protein localization was determined at a magnification of ×400 using an Axioplan fluorescence microscope (Carl Zeiss, Inc., Thornwood, NJ, USA) and a SPOT digital camera (Diagnostic Instruments, Inc., Sterling Height, MI, USA). Final images were prepared using Photoshop 6 (Adobe Systems, Inc., San Jose, CA, USA).

Immunoelectron microscopy

Human sperm and CD-1 mouse cauda epididymides were fixed with 4% paraformaldehyde (PFA, Electron Microscopy Sciences, Hartfield, PA, USA) and 0.5% glutaraldehyde (Ladd Research, Williston, VT, USA) in 0.15 M phosphate buffer (pH 7.4) (Karlsson and Schultz, 1965) at 4°C for overnight, and post-fixed in 1% osmium tetroxide in the same buffer for 1 h at RT. Samples were dehydrated in ethanol and embedded in LR White resin (Ted Pella Inc., Redding, CA, USA). Sections were blocked with Aurion goat blocking solution (Electron Microscopy Sciences) for 30 min at RT, and then labeled with speriolin antiserum at 1:100 dilution in TBS buffer (50 mM Tris, 138 mM NaCl, 2.7 mM KCl, pH 8.0) containing 0.2% acetylated BSA over night at 4°C, followed by colloidal gold-conjugated antibody to rabbit IgG (15 nm gold, Ted Pella Inc.) for 1 h at RT. Finally, sections were post-stained with 4% aqueous uranyl acetate for 10 min at RT. Sections were examined in a LEO EM 910 transmission electron microscope (Carl Zeiss, Inc.) at 80 kV in the Electron Microscopy Laboratory of the Department of Laboratory Medicine and Pathology, University of North Carolina School of Medicine.

IIF staining of zygotes

Three-week-old female C57BL/6 mice were injected with 3.25 IU pregnant mare serum gonadotrophin followed 48 h later by injection of 2.2 IU human chorionic gonadotrophin (hCG). These superovulated females were mated with wild-type C57BL/6 males, and zygotes were collected from the oviducts of plug-positive females ~24 h after hCG injection. Cumulus cells were removed by incubating in 300 μg/ml

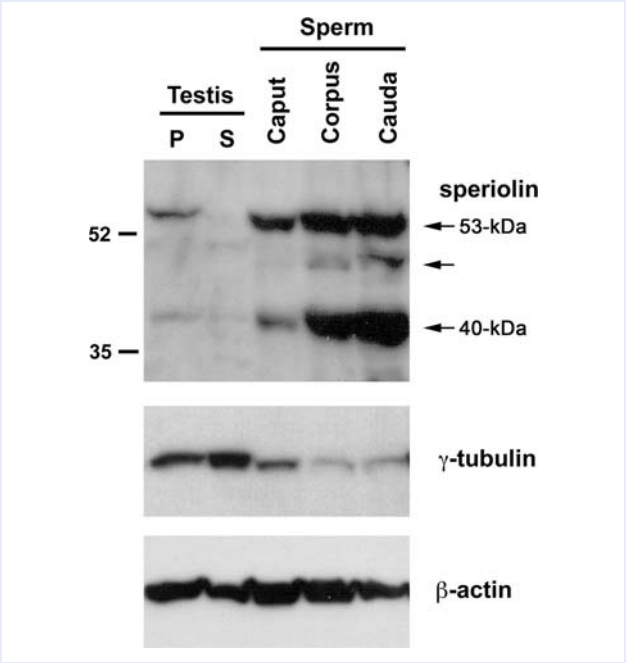

Figure 1 Speriolin is present in mouse sperm. 0.1% NP-40-insoluble (P) or -soluble (S) extracts were prepared from testis of CD-1 adult mice. Equal numbers of sperm (5×10^5) from the caput, corpus or cauda epididymis were lysed in SDS sample buffer by boiling, and proteins were separated by SDS–PAGE. Extracts were subjected to western blotting with antisera to speriolin (top panel), γ-tubulin (middle panel), or β-actin (lower panel). The γ-tubulin level decreased during sperm maturation in the epididymis. β-actin was used as a loading control for testis and sperm. The arrows indicate speriolin immunostaining.

hyaluronidase (Type IV-S; Sigma-Aldrich) at RT in M2 medium for ~5 min, and zygotes were cultured in M16 medium (Specialty Media) for 0–10 h at 37°C with 5% CO_2 in air. Zygotes were fixed with 4% PFA in PBS containing 0.1% Triton X-100 for 40 min at 37°C. Fixed zygotes were permeabilized with 0.25% Triton X-100 in PBS and then blocked with 3% BSA and 0.1% Tween 20 in PBS. They were stained with the speriolin-specific antibody followed by FITC-conjugated antibody to rabbit IgG. DAPI was used to stain DNA and zygotes were mounted with Vectashield. Speriolin localization was determined at a magnification of ×400 using an Axioplan fluorescence microscope (Carl Zeiss, Inc.), and digital images were captured with a QICAM digital camera and QCapture 2 software (QImaging, Surry, BC, Canada). Some IIF images of human sperm were obtained using a CARV confocal cell imager fitted to an Olympus BX51 microscope.

Results

Speriolin is located in the connecting piece of mouse sperm

The antiserum to mouse speriolin (Goto and Eddy, 2004) detected a 53-kDa band in the pellet fraction of mouse testis extracts and major 53 and 40-kDa bands and a minor ~50-kDa band in mouse sperm extracts on immunoblots of heavily loaded gels and after long exposure. The intensity of immunostaining of the 40-kDa band increased as sperm transited the epididymis (Fig. 1). The 40 and ~50-kDa forms presumably arise by processing of the 53-kDa form, but further studies will be required for confirmation.

Speriolin was seen by IIF as a single spot in the connecting piece region at the junction between the sperm head and flagellum (Fig. 2A) and at the tip of flagella that were detached from sperm heads (Fig. 2C). The location of speriolin in the connecting piece was determined at higher resolution using IEM. In both longitudinal (Fig. 3A) and transverse sections (Fig. 3B) of mouse sperm in the

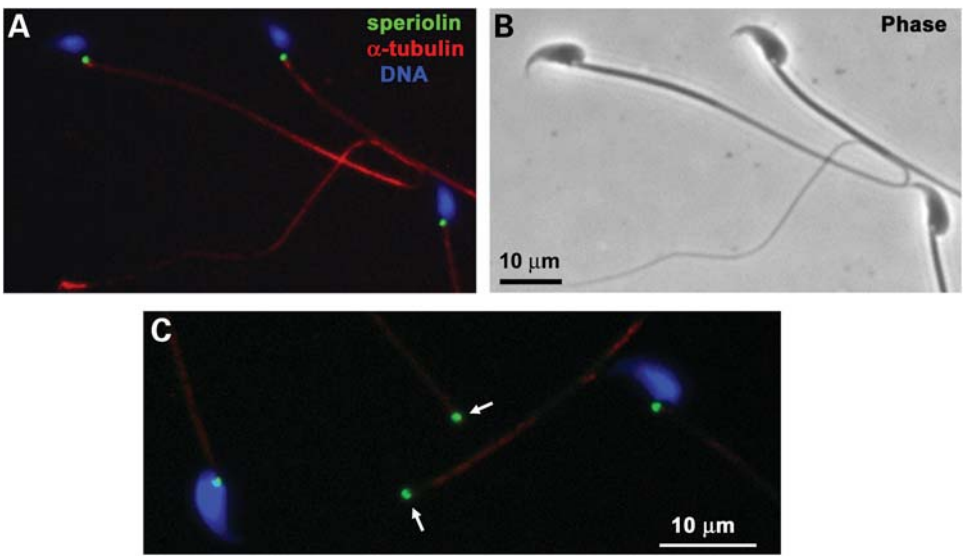

Figure 2 Speriolin is localized in the centrosomal region in mouse sperm. Sperm collected from the cauda epididymis were fixed and stained with antibodies to speriolin (green) and α-tubulin for axoneme microtubules (red), and with DAPI (blue) for DNA, and merged (**A**) or viewed by phase microscopy (**B**). Speriolin localizes to the neck region of sperm near the junction of the head and flagellum and at the proximal end of flagella (arrows) separated from the heads (**C**). DAPI, 4′,6-diamidino-2-phenylindole. Bar, 10 μm.

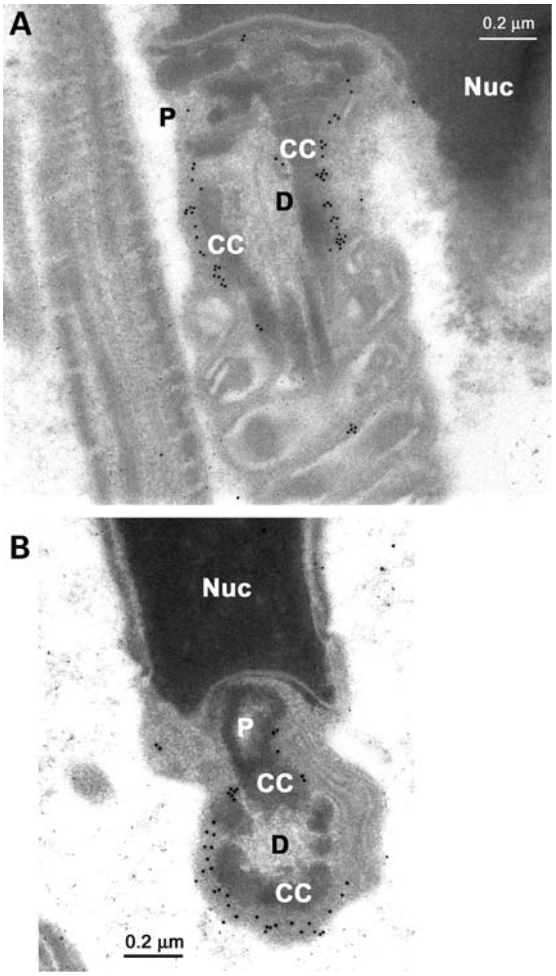

Figure 3 Electron microscopic immunolocalization of speriolin in a longitudinal section (**A**) and a transverse section (**B**) of the neck region of mature mouse epididymal sperm. Speriolin immunoreactivity was detected specifically at the periphery of the columns of the connecting piece (CC) in both sections. Proximal and distal centriolar vaults are shown. Nuc, nucleus.

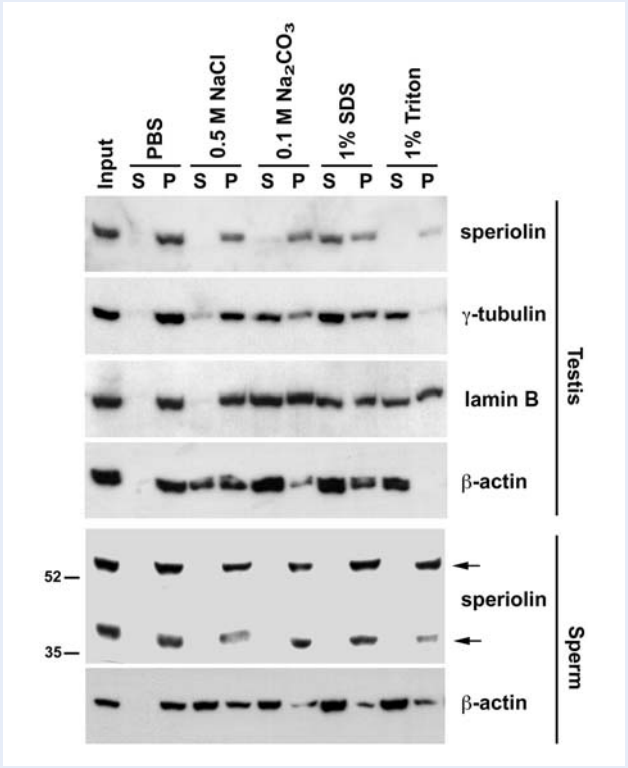

Figure 4 Differential solubilization of speriolin in testis and sperm. 0.1% NP-40-insoluble fractions (Input) were prepared from testis or cauda epididymal sperm as indicated. The insoluble fractions were suspended in the indicated solutions and then separated by 14 000*g* centrifugation into supernatant (S) and pellet (P) fractions. The extracts were analyzed by western blotting using antibodies to speriolin, γ-tubulin, lamin B or β-actin as indicated. Antibody to lamin B was used as a loading control for the 1% Triton insoluble fraction.

cauda epididymis, immunogold particles were distributed mainly at the periphery of the segmented columns surrounding the distal centriolar vault of the connecting piece (Fawcett, 1965, 1970) where the distal centriole was detectable until late in spermiogenesis.

Sperm speriolin resists solubilization

Speriolin was previously found to be insoluble in 0.1% NP40 containing 1 mM dithiothreitol (Goto and Eddy, 2004). To examine the solubility of speriolin further, pellets from testis or sperm homogenates were extracted with 0.5 M NaCl or with 0.1 M Na_2CO_3 alone or in solutions containing 1% SDS or 1% Triton X-100. Speriolin was detected in the 1% SDS supernatant fraction of testis extracts, but found only in the pellet fractions of testis and sperm extracted with the other solutions (Fig. 4). In addition, speriolin from sperm was solubilized only in SDS–PAGE sample buffer containing 5% 2-mercaptoethanol, suggesting that it becomes disulfide bond cross-linked to form multimers or complexes with other proteins while sperm undergo epididymal maturation. Since speriolin has an N-terminal leucine-zipper (LZ) domain, ODF2 was evaluated as a possible binding partner. ODF2 is a major component of the sperm outer dense fibers (Schalles *et al.*, 1998) and has two LZ motifs in the C-terminal region. This C-terminal region also is present in cenexin, encoded by a splice variant of *Odf2* and a ubiquitously distributed centrosome scaffold component (Nakagawa *et al.*, 2001; Hüber and Hoyer-Fender, 2007; Hüber *et al.*, 2008). However, when FLAG-tagged speriolin was co-expressed with EYFP-tagged ODF2 in COS7 cells and immunoprecipitated using a monoclonal antibody to FLAG, ODF2 did not co-immunoprecipitate with speriolin (data not shown).

The human gene for speriolin

A full-length 1938-bp human speriolin cDNA (*SPATC1*; GenBank accession no. AB092352) was isolated from a testis cDNA library using primers designed from human EST sequences with high identity to the mouse *Spatc1* sequence. It encodes a 591-aa protein with a calculated mass of 62.4-kDa and pI of 8.23. The N-terminal region (aa 1–60) has 93% identity with the mouse speriolin LZ motif-containing region, and the C-terminal region (aa 444–591) has 90% identity with the mouse speriolin Cdc20-binding region. However, human speriolin

contains a 163-amino acid (aa) region (aa 237-399) that is not present in the mouse protein (Fig. 5A). The human *SPATC1* gene is located on chromosome 8q24.3 and contains five exons, as does the mouse *Spatc1* gene (Fig. 5B). When *SPATC1* expression was analyzed by northern blot analysis using the full-length cDNA as a probe, transcripts were detected only in testis (Fig. 5C). In addition, the antiserum to mouse speriolin recognized a protein of ~60-kDa in human sperm (Fig. 5D). To confirm that the antiserum to mouse speriolin recognizes human speriolin, FLAG-tagged human speriolin was expressed in COS7 cells, and the antiserum was shown to recognize the recombinant protein (Fig. 5D, FLAG-h-speriolin), as did a monoclonal antibody to FLAG (data not shown). The recombinant protein migrated as two bands of ~78- and 103-kDa (Fig. 5D). It remains to be determined why the recombinant protein produced in COS7 cells appears as two bands and migrates differently than speriolin extracted from human sperm.

Speriolin is located in the connecting piece of human sperm

Human sperm were triple-labeled with antiserum to speriolin, a monoclonal antibody to α-tubulin to identify the axoneme, and with DAPI to stain the nucleus. Speriolin was seen by IIF as a single spot at the base of the flagellum in the connecting piece region of ~90% of the sperm (Fig. 6A). However, speriolin was not detected in some sperm with abnormally shaped heads or short flagella (Fig. 6A). Two distinct spots occasionally were observed in the neck region of human sperm (Fig. 6B). Seeing either one or two centrin spots in human (Simerly *et al.*, 1999), mouse (Manandhar *et al.*, 1999) and pig sperm (Manandhar *et al.*, 2006) was suggested to be due to the orientation of the sperm and may explain the variation seen here. Immunostaining of human sperm with antibodies to Cep57 and TSKS also detected one or two distinct spots in the neck region (Xu *et al.*, 2008).

The subcellular location of speriolin in human sperm was examined by IEM using the antiserum to mouse speriolin. In contrast to mouse sperm (Fig. 3), immunogold particles were located mainly at the periphery of the proximal centriole in human sperm (Fig. 7) and not at the periphery of the segmented columns of the connecting piece surrounding the distal centriolar vault. A diagram of the subcellular localization of speriolin in mouse and human sperm is shown in Fig. 8.

Speriolin is transmitted to the zygote

The fate of mouse sperm speriolin following fertilization was determined by immunostaining with speriolin antiserum in combination

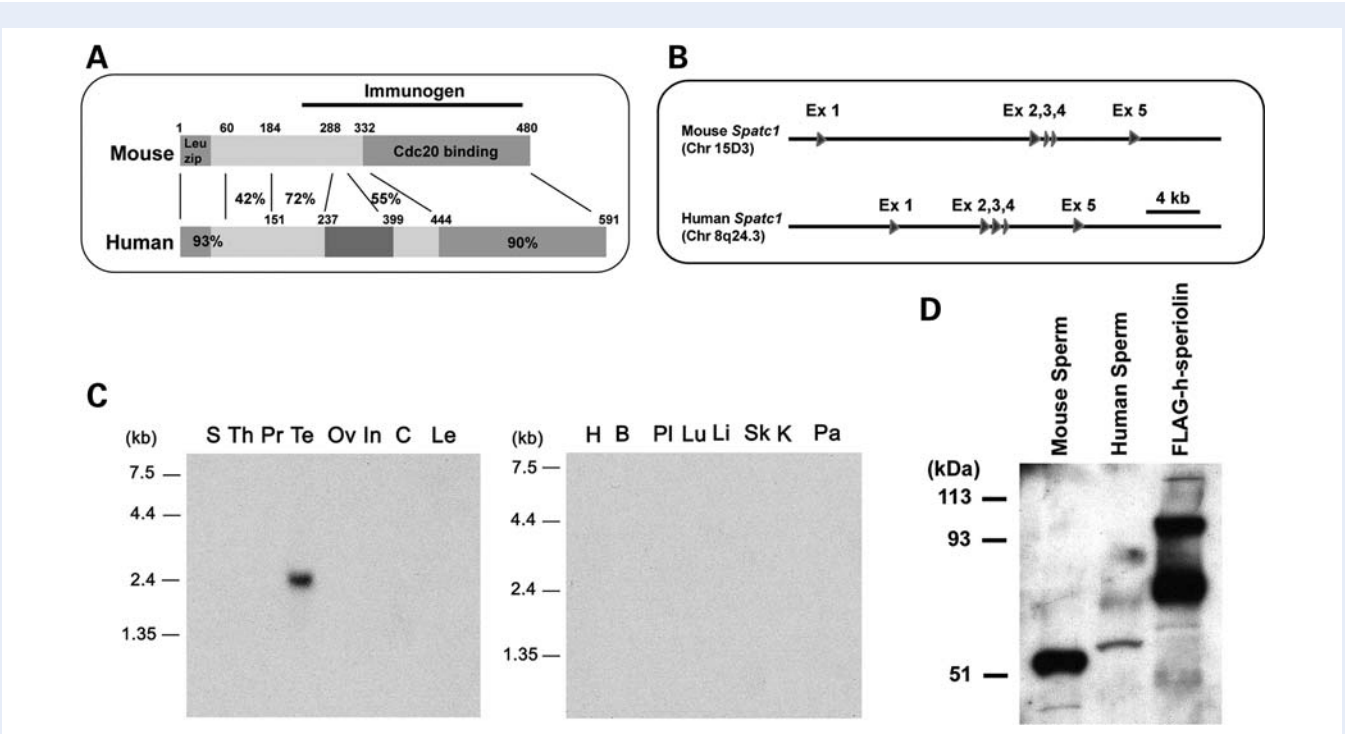

Figure 5 Speriolin is evolutionally conserved in mammals. (**A**) Diagram of the conserved domains of speriolin. The N- and C-terminal regions of human speriolin are 93 and 90% identical with mouse speriolin, respectively, while the central region shows only 35% identity. The minimum region required for binding to Cdc20 and the region used for raising antisera to mouse speriolin (immunogen) are indicated. (**B**) Genomic structure of mouse and human *Spatc1* genes. Mouse and human *Spatc1* genes are encoded by five exons and show structural similarity. (**C**) Northern blot analysis of human mRNAs. Human tissue mRNA blots were probed with human speriolin cDNA. The human speriolin mRNA was detected only in testis. S, spleen; Th, thymus; Pr, prostate; Te, testis; Ov, ovary; In, small intestine; C, colon; Le, peripheral blood leukocyte; H, heart; B, brain; Pl, placenta; Lu, lung; Li, liver; Sk, skeletal muscle; K, kidney; Pa, pancreas. (**D**) Expression of speriolin in human sperm. Lysates were prepared from mouse and human sperm and from COS7-expressing FLAG-tagged human speriolin (FLAG-h-speriolin) as positive control. Proteins were separated by SDS–PAGE followed by western blotting with an antiserum generated against mouse speriolin.

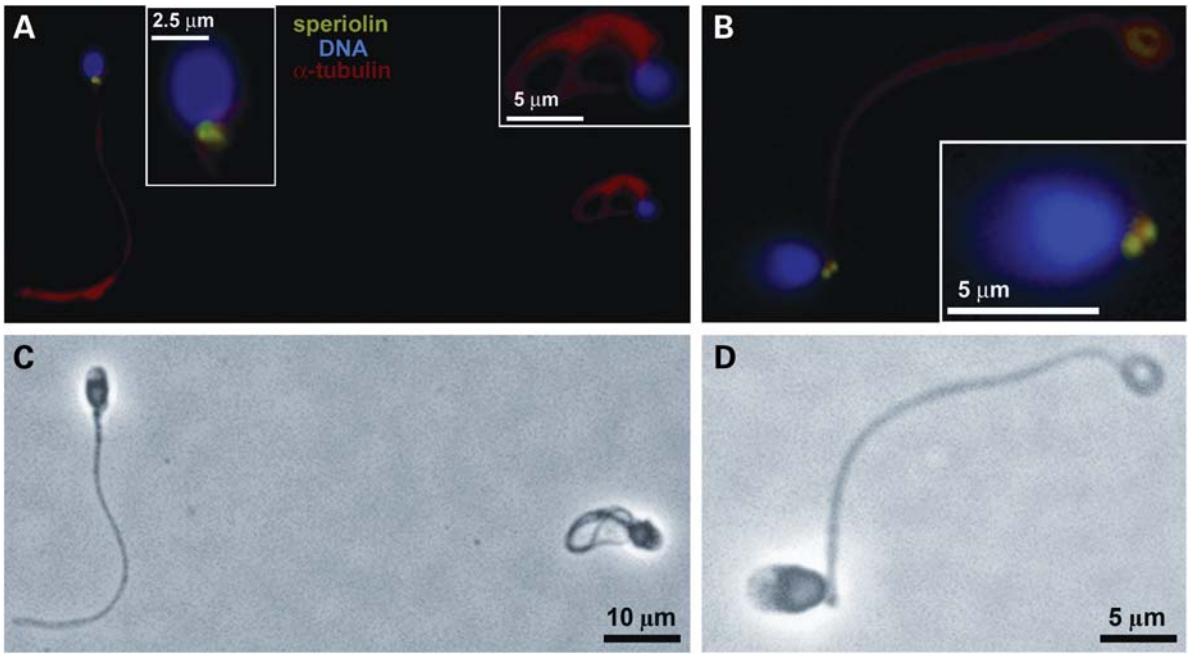

Figure 6 Speriolin is localized at the centrosomal region in human sperm. Ejaculated human sperm from normal, healthy donors were fixed and stained (**A** and **B**) with antibodies specific to speriolin (green) and α-tubulin (red), and with DAPI (blue), or (**C** and **D**) viewed by phase microscopy. Speriolin localizes to the neck region of sperm (A; higher magnification in left inset) similar to its localization in mouse sperm. The localization of α-tubulin is disrupted in abnormal sperm and no speriolin signal is seen (A; higher magnification in right inset). Some sperm showed no signal and others (B, higher magnification in inset) showed two spots in the neck region.

with DAPI to label nuclei. Speriolin usually was detected in recently fertilized oocytes as a single spot at the end of a sperm flagellum, immediately adjacent to a decondensing sperm head (Fig. 9A). A decapitated flagellum was often observed in the cytoplasm during interphase and prophase, and occasionally during metaphase and in 2-cell embryos. Speriolin disassociated from the sperm flagellum and appeared to undergo duplication or to split to form two spots at the periphery of the paternal pronucleus (Fig. 9B) or occasionally the maternal pronucleus during interphase. The spots remained detectable during prophase (Fig. 9C) and in 2-cell embryos (Fig. 9D). In the more than 70 zygotes obtained from 8 plug-positive females, speriolin was detected clearly in all zygotes in interphase and prophase, in 3 of 6 zygotes in metaphase, and in 2 of 7 2-cell embryos. Unfertilized oocytes were not labeled by antiserum to speriolin (data not shown).

Speriolin is evolutionarily conserved

Since the identification of speriolin and cloning of the mouse *Spatc1* gene (Goto and Eddy, 2004), a putative splice variant and a paralog in the mouse genome and orthologs in 31 other species have been identified (Ensembl ENSMUSP00000073809; Ensembl release 56). Most orthologs are in eutherian mammals, but sequences encoding proteins with significant identity to speriolin are present in the genomes of a marsupial (opossum; ESNODP00000013049), monotreme (platypus; ENSOANP00000023834), lizard (ENSACAP00000 05223), fish (fugu and pufferfish; ENSTRUP00000037960 and ENSTNIP00000021588) and urochordate (tunicate; ENSCSAVP0000 0010140) genomes. These orthologs encode C-terminal regions with high identity to the Cdc20-binding region in mouse and human speriolin. In addition, the orthologs in a majority of mammals encode an N-terminal sequence with significant identity to the region containing the LZ motif in mouse speriolin. Previous studies demonstrated that deletion of the LZ motif of EYFP-tagged speriolin disrupted centrosomal targeting in cultured cells (Goto and Eddy, 2004). The conservation of the N- and C-terminal regions in human and mouse speriolin suggests that the 163-aa acid region in the middle portion of human speriolin could account for the differences in speriolin localization in human and mouse sperm.

Discussion

Speriolin has been shown to be a novel spermatogenic cell-specific protein that co-localizes with the centrosomal protein pericentrin in mouse spermatocytes, is present in the centrosome of round spermatids, co-immunoprecipitates from testis homogenates with γ-tubulin and is localized at the base of the flagellum in condensing spermatids (Goto and Eddy, 2004), strongly suggesting that it is a spermatogenic cell-specific centrosomal protein. A significant difference between the sperm from mice and humans is that both centrioles degenerate in mouse sperm (Manandhar *et al.*, 1998), while the distal centriole degenerates and the proximal centriole retains its structural organization in human sperm (Schatten, 1994; Palermo *et al.*, 1997; Manandhar *et al.*, 2000). In the present study, we identified another difference: that speriolin was located at the periphery of the

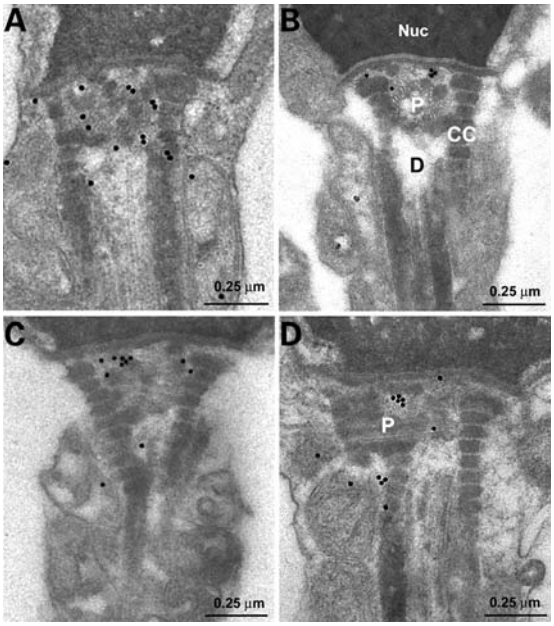

Figure 7 Localization by IEM of speriolin in longitudinal sections of ejaculated human sperm. The nine triplets of microtubules of the proximal centriole (P) are seen, in cross-sections from a frontal view (**A**, **B** and **C**), to be surrounded by speriolin. The cylinder of nine triplets of microtubules of the proximal centriole (P in panel **D**) is seen in cross-section from a lateral view (D). The distal centriole vault (D), longitudinal columns in the connecting piece (CC) and the nucleus (Nuc) are indicated in panel B. IEM, immunoelectron microscopy. Bar, 0.25 μm.

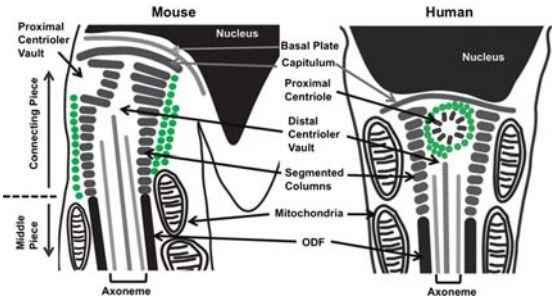

Figure 8 Schematic representation of the subcellular localizations of speriolin (green) in mouse (left panel) and human (right panel) sperm. Mouse sperm and human sperm are drawn from lateral and frontal views, respectively.

disorganized distal centriole in mouse sperm and was associated with the intact proximal centriole in human sperm (Fig. 8).

Other centrosomal constituents have been identified in sperm of some mammalian species, although the fate of these proteins after fertilization has not been determined. In studies using IIF, γ-tubulin was not detected in mouse (Manandhar *et al*., 1998), bull (Simerly *et al*., 1999) or human sperm (Fouquet *et al*., 1998; Simerly *et al*., 1999; Tachibana *et al*., 2005). However, immunoblotting detected γ-tubulin in bull and human sperm (Simerly *et al*., 1999) and, in this study, in mouse sperm. This suggests that γ-tubulin is present either at levels too low to be detected by IIF or is lost during processing for IIF. While centrin was not detected by IIF in elongated spermatids or sperm of mice (Manandhar *et al*., 1999; Goto and Eddy, 2004), it was seen by IIF in sperm from pigs (Manandhar *et al*., 2006), bulls (Simerly *et al*., 1999), rhesus monkeys (Manandhar and Schatten, 2000), rabbits and humans (Tachibana *et al*., 2005, 2009). However, the fate of sperm centrin following fertilization has not been reported, and speriolin appears to be the first sperm centrosomal protein shown to be detectable by IIF in early mouse embryos.

Although mouse sperm lack a distinct centriole and human sperm contain one centriole, *Xenopus* and *Drosophila* sperm have two centrioles (reviewed in Manandhar *et al*., 2005). Thus, the rule of centrosomal lineage in the zygote is not conserved evolutionarily. While it is clear that the sperm is required for aster formation in oocytes in most sexually reproducing species (Barroso *et al*., 2009), the sperm-specific key molecules contributing to the centrosomal regeneration in fertilized oocytes remain unknown.

In addition to studies in mouse and human, a few studies in other mammals have examined the structure of sperm centrioles and their contribution to the oocyte at fertilization. By TEM, it was seen that the proximal centriole is retained in domestic cat, tiger (Schmel and Graham, 1989), little brown bat (Fawcett and Ito, 1965), rabbit (Zamboni and Stafanini, 1971), Russian hamster (Fawcett, 1975), sheep (Crozet *et al*., 2000), long-fingered bat (Bernard and Hodgson, 1988) and bovine (Sathananthan *et al*., 1997) sperm. In addition, the sperm centriole was seen in sheep (Crozet *et al*., 2000) and bovine (Sathananthan *et al*., 1997) zygotes. Although sequences are available for only some of these species, it is interesting that cat (Ensembl ENSDCAP00000010716) and bovine (Ensembl ENSBTAP00000037249) speriolin contain a region highly similar to the 163-aa sequence present in human speriolin and absent in mouse speriolin. In contrast, rat speriolin (Ensembl ENSRNOP00000041285) lacks this region and like the mouse lacks a distinct proximal centriole in sperm (Woolley and Fawcett, 1973).

The evolutionary conservation of speriolin suggests that it has an essential biological role common to diverse species. Our previous northern analyses indicated that speriolin is testis-specific and that the protein is expressed in meiotic and post-meiotic germ cells (Goto and Eddy, 2004). In addition, UniGene data indicate that nearly all ESTs that map to the mouse *Spatc1* (Mm.159156), human *SPATC1* (Hs.97726) and dog *SPATC1* (Cfa.6997) genes are from the testis. Previous studies showed that the N-terminal region (aa 1–76) contains an LZ domain which apparently has a key role in targeting mouse speriolin to the centrosome, that a central region (aa 161–284) interacts directly or indirectly with γ-tubulin and that the C-terminal region (aa 283–480) is required for Cdc20 binding (Goto and Eddy, 2004). These regions are conserved in the orthologs in mammalian and some non-mammalian species, suggesting that testis-specificity, centrosomal targeting, γ-tubulin binding and interaction with Cdc20 are conserved properties of speriolin. In addition, EYFP-tagged mouse speriolin (EYFP-speriolin) localized at the centrosome in BALB/3T3 cells (Goto and Eddy, 2004) and co-localized with FLAG-tagged human speriolin in BALB/3T3 cells (data not shown), providing further evidence that mouse and human speriolin contain centrosome-targeting sequences.

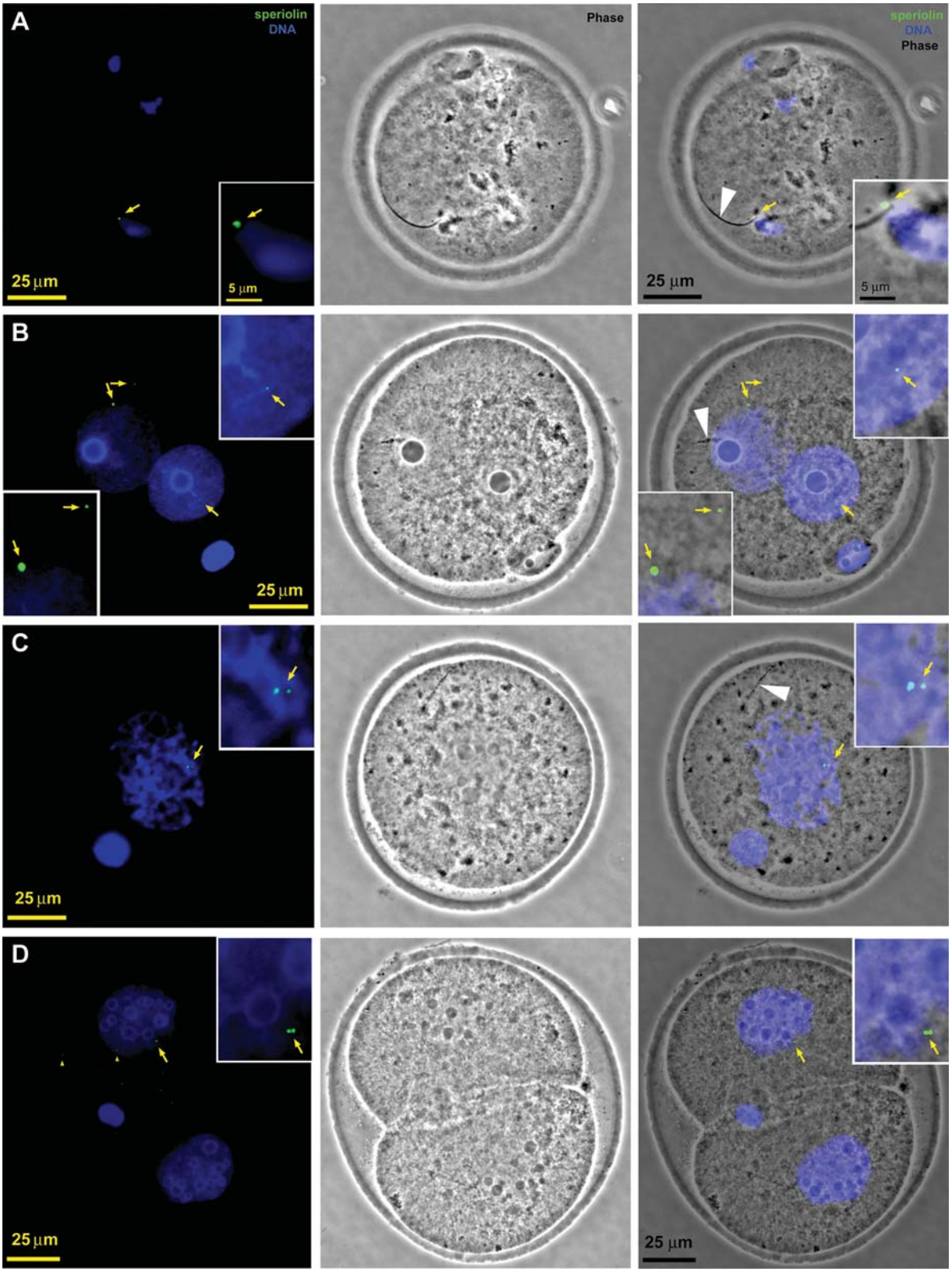

Figure 9 Speriolin is present in mouse zygotes. Fixed zygotes were stained with an antibody specific to speriolin (green) and with DAPI (blue) for DNA. Speriolin (arrow) was first seen (**A**) in zygotes in the neck region of the sperm flagella (white arrowhead). Although most of the sperm flagellum (white arrowhead) was gone by interphase (**B**) and prophase (**C**), speriolin remained detectable adjacent to the chromatin. Speriolin was occasionally detected in 2-cell embryos (**D**). Bar, 25 μm. The insets in panels are magnified views of speriolin staining identified with arrow. Bar, 5 μm.

We found that speriolin is brought into the mouse oocyte at fertilization, duplicates or is split during the first interphase, and remains detectable throughout the first meiotic division and in some 2-cell embryos. Although it is not known if speriolin is carried into the human oocyte at fertilization, it is intriguing that speriolin is associated with the human sperm centriole believed to nucleate the assembly of the centrosome and spindle apparatus in the zygote. The integrity of the sperm centrosome is considered critically important for successful fertilization and embryo development in humans (reviewed by Schatten and Sun, 2009). In our studies, speriolin was not detected in ~10% of human IVF donor sperm and those usually had abnormal morphology. The association between abnormalities in sperm centrioles and defects in sperm motility in infertile men (Chemes, 2000; Nagy, 2000; Rawe *et al*., 2002; Schatten and Sun, 2009) suggests that speriolin might be a useful IIF marker for sperm centrosome abnormalities causing infertility.

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