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A missense mutation in the *Capza3* gene and disruption of F-actin organization in spermatids of *repro32* infertile male mice

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ABSTRACT

Males homozygous for the *repro32* ENU-induced mutation produced by the Reproductive Genomics program at The Jackson Laboratory are infertile, have low epididymal sperm concentrations, and produce sperm with abnormally shaped heads and poor motility. The purpose of the present study was to identify the mutated gene in *repro32* mice and to define the structural and functional changes causing infertility and the aberrant sperm phenotype. In *repro32*/*repro32* mice, we discovered a failure to shed excess cytoplasm and disorganization of the middle piece of the flagellum at spermiation, resulting in the outer dense fibers being wrapped around the sperm head within a bag of cytoplasm. Using a candidate-gene approach, a mutation was identified in the spermatid-specific "capping protein (actin filament) muscle Z-line, alpha 3" gene (*Capza3*). CAPZA3 protein localization was altered in spermatids concurrent with altered localization of a unique CAPZB variant isoform and disruption of the filamentous actin (F-actin) network. These observations strongly suggest the missense mutation in *Capza3* is responsible for the mutant phenotype of *repro32*/*repro32* sperm and regulation of F-actin dynamics by a spermatogenic cell-specific CAPZ heterodimer is essential for removal of the cytoplasm and maintenance of midpiece integrity during spermiation in the mouse.

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Introduction

Spermiogenesis is the process during which spermatids undergo a series of complex morphological changes that result in formation of the highly specialized spermatozoon. In the mouse, as spermatids are remodeled from round (steps 1–8), to elongating (steps 9–12), and then to condensing (steps 13–16), they form an acrosome, assemble a flagellum, and reshape and condense their nucleus (reviewed by Kerr et al., 2006). During this process, spermatids become attached to Sertoli cells by an apical ectoplasmic specialization (ES) consisting of a heterotypic adherens junction. The ES are underlain in the Sertoli cytoplasm by hexagonal arrays of F-actin bundles sandwiched between a cistern of endoplasmic reticulum and the plasma membrane. Proteins associated with the ES include F-actin, members of the laminin and integrin adhesion molecule families, and transmembrane actin-complex binding molecules, including nectins and CEACAM6 (Yan et al., 2006; Ozaki-Kuroda et al., 2002; Kurio et al., 2008).

Near the end of spermiogenesis much of the spermatid cytoplasm in shunted into a lobule that is detached during spermiation and remains behind to be phagocytosed by Sertoli cells (Russell, 1979b). A small amount of cytoplasm remains in the head and neck region and forms the cytoplasmic droplet, which moves to the midpiece–principal piece junction and usually is shed in the epididymis. Aberrant retention of excess cytoplasm by human sperm is associated with impaired sperm motility, increased reactive oxygen species (ROS) production and decreased in vitro fertilization (IVF) success, resulting in sub-fertility or infertility (reviewed by Cooper, 2005).

The mechanisms responsible for removal of spermatid cytoplasm during spermiation in mammals are unclear. However, filamentous actin (F-actin) has been shown to be involved in this process in insects (Noguchi and Miller, 2003; Sahara and Kawamura, 2004). The potential role(s) of F-actin during spermiogenesis in mammals have been inferred from morphological studies, and include involvement in shaping the acrosome (Welch and O'Rand, 1985), attachment of the acrosome to the spermatid nucleus (Russell et al., 1986), and/or removal of cytoplasm during spermiation (Russell, 1979a, 1979b). In support of the latter proposed role, intratesticular injection of cytochalasin D to depolymerize actin resulted in retention of excess cytoplasm by testicular sperm along with disruption of tubulobulbar complexes (TBCs). Intratesticular injection of taxol to disrupt microtubules did not alter removal of cytoplasm at spermiation (Russell et al., 1989).

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Unbiased approaches for identification of mutant phenotypes can be helpful in the dissection of complex processes such as spermatid cytoplasmic remodeling. The present study was undertaken to identify the mutation responsible for the aberrant sperm phenotype and male infertility in *repro32* mice. The *repro32* mutation was produced by the Reproductive Genomics program at The Jackson Laboratory (http://reproductivegenomics.jax.org/), using random N-ethyl-N-nitrosourea (ENU) mutagenesis and a breeding strategy to identify recessive mutations affecting male and/or female fertility (Handel et al., 2006; Lessard et al., 2007). Spermiogenesis in *repro32*/*repro32* mice was abnormal and sperm were present in low concentrations in the epididymis, had abnormally shaped heads, exhibited poor motility, and showed very low IVF success. We identified a missense mutation in a spermatogenic cell-specific gene that is expressed solely in spermatids and encodes a protein involved in regulating F-actin dynamics. This study provides strong evidence that F-actin has an important role in removal of cytoplasm and maintenance of middle piece organization during spermiogenesis and spermiation, and that it is the disruption of F-actin organization during these processes that results in the structurally aberrant sperm and infertility of *repro32*/*repro32* male mice.

Materials and methods

Mapping the repro32 mutation

The chromosomal localization of the *repro32* mutation was determined by genome scanning with a panel of polymorphic microsatellite markers that discriminate between C57BL/6 and C3H chromosomal regions. Genotyping was performed by PCR on DNA extracted from tail tips of G3 progeny using standard conditions (Lessard et al., 2007). PCR products were analyzed on a 3% low EEO agarose (Fisher) gels. The map position was refined by breeding additional pairs of *repro32*/+ mice to generate offspring with recombinant chromosomes. The offspring were phenotyped by fertility testing and genotyped with additional polymorphic microsatellite markers to narrow the candidate region.

Tissue collection

All animal procedures were performed in accordance with the NRC Guide for the Care and Use of Laboratory Animals and approved by the Animal Care and Use Committees of the National Institute of Environmental Health Sciences (NIEHS) and The Jackson Laboratory. In vitro fertilization (IVF) assays were performed as described previously (Eppig and O'Brien, 1996). Animals were euthanized and weighed and then one testis was removed, weighed, and immediately snap-frozen in liquid nitrogen and stored at −80 °C for later extraction of protein or RNA. The other testis was processed for histology or transmission electron microscopy as described previously (Miki et al., 2002). Sperm were collected by carefully dissecting cauda epididymides to remove blood vessels and fat, making several small cuts with iridectomy scissors and allowing the sperm to swim out into 1× PBS (Ca^{2+}/Mg^{2+}-free) at room temperature. Sperm were prepared for scanning electron microscopy (SEM) and demembranated in 1% Triton X-100 as described previously (Miki et al., 2002, 2004). Sperm counts were done using a hemocytometer.

Sequencing of candidate genes

Genomic DNA was isolated from tail tip biopsies of male *repro32*/+, *repro32*/*repro32*, and founder strain (C57BL/6J and C3H) mice using the DNeasy kit (Qiagen) according to manufacturer's instructions. Primer pairs were designed to flank the exons of each of the candidate genes and PCR was used to amplify each exon and adjacent intronic sequence (sequences are available upon request). RNA was isolated from *repro32*/*repro32* testes using the RNeasy kit (Qiagen), and 1 μg total RNA was reverse-transcribed into cDNA using oligo dT primers and MuLV reverse transcriptase (Applied Biosystems). PCR and RT-PCR products were purified using the QIAquick PCR purification kit (Qiagen) and sequenced using Big Dye Version 3.1 (ABI) on an Avant 3100 sequencer (Applied Biosystems), both according to the manufacturers' instructions. The sequences obtained were identical to those in the public database.

Reverse transcriptase PCR (RT-PCR)

RNA was isolated using Trizol reagent (Invitrogen) according to the manufacturer's instructions, and cDNA was generated from 1 μg total RNA using oligo dT primers and MuLV reverse transcriptase (Applied Biosystems). 50 ng of cDNA was amplified for 35 cycles in a reaction containing: 10 mM Gene Amp PCR buffer II, 2 mM $MgCl_2$, 1.5 mM dNTPs, 1.25 μM of each primer, and 1.25 U Taq Gold (Perkin-Elmer). Primers used for amplification of the unique *Capzb_v3* 5′ end (upper F: 5′-ACACGATGCATCCTAGCAGGC, upper R: 5′-CAGATGACAGGAGATCTTCACAC), the 3′ end common to all three known *Capzb* isoforms (lower F: 5′-TGAGTGACTGTTCCCCACAC, lower R: 5′-TGCTGCTTTCTCTTCAAGGC), and for cytoplasmic *Actb* (F: 5′-TCCGATGCCCTGAGGCTCTTTTC, R: 5′- CTTGCTGATCCACTATCTGCTGGAA) were designed to span introns to control for possible amplification of genomic DNA.

Immunoblot analysis

Protein was extracted by homogenization of wild type and *repro32*/*repro32* testes as described previously (Goto and Eddy, 2004). Twenty μg of the insoluble fraction from whole testis extracts were boiled in SDS buffer for 10 min, separated on a 10% Tris–glycine gel (Biorad), and immunoblotted using standard procedures. Antibodies to ACTB (A-5441, Sigma), CAPZA3 (GP-SH4, Progen), and CAPZB3 (GP-SH5, Progen) were used at 1:10,000, 1:1500, and 1:500, respectively. Two additional polyclonal antisera to CAPZA3 were the kind gifts of Dr. Roy Jones, Babraham Institute and Dr. Hiromitsu Tanaka, Osaka University. HRP-conjugated goat-anti-mouse (for ACTB, from Sigma) and donkey-anti-guinea pig (for CAPZA3 and CAPZB, from Jackson Immuno) secondary antibodies were each used at 1:20,000. Detection was done using the ECL Plus Detection System (Amersham).

Indirect immunofluorescence (IIF) microscopy

Spermatids were prepared for immunostaining using a procedure modified from one described previously (Vogl et al., 1986). Briefly, *repro32*/+ and *repro32*/*repro32* mice were euthanized and their testes removed, decapsulated, and placed into cold 1× PBS. The tubules were gently teased apart and then incubated in the presence or absence of 1 mg/ml (0.1%) trypsin (Sigma) for 15 min at 33 °C. The tubules were washed 3 times with 1× PBS, fixed in 4% paraformaldehyde (in 1× PBS, pH 7.2) for 10 min at room temperature, and washed 3 more times with 1× PBS. The remaining incubations were performed at room temperature in a humid chamber. Short (~1 mm) tubule segments were placed onto a Superfrost Plus positively charged slide (Fisher) and permeabilized with Automation buffer (Biomeda) for 10 min. Blocking was done for 20 min in Automation buffer containing 4.5% horse serum, and tubule segments were then incubated with primary antibody for 1 h. Following three washes in 1× Automation buffer, tubules were incubated in secondary antibody with or without fluorescent dye-conjugated phalloidin-568 (Invitrogen) diluted 1:25 (yielding approximately 265 nM final concentration, or 1 U/section) for 1 h in the dark. Three additional washes in Automation buffer were performed in the dark, and coverslips were mounted using Vectashield containing DAPI (Vector Laboratories) and sealed with