Disruption of the murine dynein light chain gene *Tcte3-3* results in asthenozoospermia

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Abstract

To elucidate the role of the mouse gene *Tcte3* (*Tctex2*), which encodes a putative light chain of the outer dynein arm of cilia and sperm flagella, we have inactivated this gene in mice using targeted disruption. Breeding of heterozygous males and females resulted in normal litter size; however, we were not able to detect homozygous *Tcte3*-deficient mice using standard genotype techniques. In fact, our results indicate the presence of at least three highly similar copies of the *Tcte3* gene (*Tcte3-1*, *Tcte3-2*, and *Tcte3-3*) in the murine genome. Therefore, quantitative real-time PCR was established to differentiate between mice having one or two targeted *Tcte3-3* alleles. By this approach, *Tcte3-3*^{-/-} animals were identified, which were viable and revealed no obvious malformation. Interestingly, some homozygous *Tcte3-3*-deficient male mice bred with wild-type female produced no offspring while other *Tcte3-3*-deficient males revealed decreased sperm motility but were fertile. In infertile *Tcte3-3*^{-/-} males, spermatogenesis was affected and sperm motility was reduced, too, resulting in decreased ability of *Tcte3-3*-deficient spermatozoa to move from the uterus into the oviduct. Impaired flagellar motility is not correlated with any gross defects in the axonemal structure, since outer dynein arms are detectable in sperm of *Tcte3-3*^{-/-} males. However, in infertile males, deficient *Tcte3-3* function is correlated with increased apoptosis during male germ cell development, resulting in a reduction of sperm number. Moreover, multiple malformations in developing haploid germ cells are present. Our results support a role of *Tcte3-3* in generation of sperm motility as well as in male germ cell differentiation.

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Introduction

The microtubule-based motility of cilia and flagella plays a critical role in fertility and viability in mammals. The motive force to power sperm flagella is generated by dynein motors that are attached to the nine outer-doublet microtubules of the axoneme. Apart from inner and outer axonemal dynein arms, other classes of motor proteins are also expressed in the testis. Cytoplasmic dynein motors are involved in a wide variety of fundamental cellular processes including mitotic spindle formation and orientation, vesicle transport, formation, and localization of the Golgi complex, assembly and motility of cilia and flagella or spermatid translocation (Karki & Holzbaur 1999, Miller *et al.* 1999, King 2000a,b). In general, all dynein motors are multisubunit complexes and consist of heavy, intermediate, and light chains, which differ in their molecular weight and function (Gibbons 1995, King 2000a,b). Dynein heavy chains harbor the motor domain and generate the force for

sliding of microtubules, while intermediate and light chains are involved in the assembly and regulation of the whole complex. Most of the dynein polypeptides are specific either for axonemal or cytoplasmic complexes. However, for a few light chains (TCTEX1, TCTE3, DCL1 or DNALI1), it was suggested that they could play a role in both dynein subclasses (Harrison *et al.* 1998, Bowman *et al.* 1999, DiBella *et al.* 2001, Rashid *et al.* 2006).

In mammals, an increasing number of genes encoding components of axonemal and cytoplasmic dynein complexes were identified (Tanaka *et al.* 1995, Neesen *et al.* 1997, Grissom *et al.* 2002) and the function of some of these genes was analyzed by the generation of dynein-deficient mice. These models demonstrate that the loss of the dynein function can result in embryonic lethality, disturbed body axis formation, hydrocephalus, or male infertility (Supp *et al.* 1997, Neesen *et al.* 2001, Ibanez-Tallon *et al.* 2002, Rana *et al.* 2004). In contrast, to date no mouse model for targeted inactivation of a dynein light chain is available.

Here we report the functional analysis of the dynein light chain *Tcte3-3* by targeted disruption in mice. The *Tcte3* (*Tctex2*) gene was initially identified in the testis of mice bearing the *t*-haplotype as a candidate for involvement in nonMendelian transmission (Lader *et al.* 1989, Huw *et al.* 1995). It was described as a putative sperm membrane protein (Huw *et al.* 1995) but subsequent identification of the *Chlamydomonas* homolog *LC2* revealed that this protein is a component of outer dynein arm complexes (Patel-King *et al.* 1997). Complete deletion of the *LC2/oda12* gene in *Chlamydomonas* causes loss of all outer dynein arms and a slow jerky swimming phenotype (Pazour *et al.* 1999), indicating that *LC2* is required for the outer arm assembly in *Chlamydomonas*, while another mutant lacking the 3'-end of the *LC2* gene is partially functional and allows for the assembly of outer dynein arms. In the outer dynein arm, *LC2* is associated with an intermediate chain at the base of the dynein particle. More recently it was demonstrated that the *Tcte3* expression is regulated in a tissue-specific manner. Interestingly, *TCTE3* could also be detected in cytoplasmic dynein samples derived from rat kidney, and spleen, supporting that *TCTE3* could act as a component of the cytoplasmic dynein complex in these tissues. It was proposed that the heterogeneity in the polypeptide composition that build the cytoplasmic dynein motor could reflect specific requirements for a precise temporal and spatial regulation of motor cargo-binding within different cell and tissue types (DiBella *et al.* 2001).

The human orthologous *TCTE3* gene was mapped on chromosome 6q27 (Neesen *et al.* 2002). Both the murine and human genes comprise of four exons and their encoded proteins share 87% homology. Expression studies demonstrate that human and murine *TCTE3* genes are mainly expressed during male germ cell development; however, weaker expression of *Tcte3/TCTE3* is observed in several tissues including brain, lung, and trachea (DiBella *et al.* 2001, Neesen *et al.* 2002). In addition, these analyses reveal that two transcript variants are expressed that differ by the presence of exon 3. The expression in tissues containing cilia and the putative role of *TCTE3* in the assembly and function of outer dynein arms raised the hypothesis, that *TCTE3* could be a candidate gene mutated in patients suffering from primary cilia dyskinesia (PCD). This disease is characterized by recurrent infections of the respiratory tracts and male infertility due to decreased sperm motility. A mutational analysis of 25 PCD patients revealed no disease causing mutation in the coding sequence of the human *TCTE3* gene (Neesen *et al.* 2002). However, because PCD is a genetically heterogeneous disease, *TCTE3* cannot be excluded as candidate gene. To gain further insight into the function of *Tcte3*, we have generated mice carrying a targeted disruption of the *Tcte3-3* gene by replacing the exon 2 with a neomycin resistance gene fragment.

The homozygous targeted mutation caused reduced sperm motility and resulting in some males in infertility. In infertile males, reduction or deficiency of the *Tcte3-3* gene product leads to a decreased sperm number and an increased rate of apoptosis in male germ cells. Furthermore, different abnormalities were observed in developing germ cells, supporting a basic role of *Tcte3-3* in spermatogenesis.

Results

Expression of the murine *Tcte3* gene

Several studies have demonstrated *Tcte3* expression in male germ cells as well as in somatic tissues (Huw *et al.* 1995, DiBella *et al.* 2001). We determined the intra-testicular sites of *Tcte3* expression by northern blot experiments using RNA from different postnatal testis stages and by *in situ* hybridization. *Tcte3* expression can be detected by northern blot analysis on day 11 of postnatal testis development (Fig. 1A), which corresponds to the appearance of leptotene spermatocytes. An increased expression is observed on day 15 when pachytene spermatocytes appear. Using RT-PCR *Tcte3* transcripts were found in testicular RNA from 7-day-old mice (data not shown), indicating a weak expression of *Tcte3* in spermatogonia or somatic testicular cells. To further characterize *Tcte3* expression in male germ cells we performed *in situ* hybridization using sense and antisense *Tcte3*-ribo-probes. With the digoxigenized antisense probe, *Tcte3*-transcripts could be visualized in the cytoplasm of spermatocytes (Fig. 1B and C) and weaker staining was observed in round spermatids. In contrast to RT-PCR results no *Tcte3*-transcripts could be detected in spermatogonia or using sense *Tcte3*-ribo-probes (Fig. 1D).

To determine the localization of *TCTE3* in germ cells more precisely squeeze preparations were incubated with antibody combinations. *Tcte3*-specific antibodies were detected using a Cy3-conjugated immunoglobulin (red fluorescence signal), whereas antibodies against tubulin were marked with FITC conjugated antibodies (green fluorescence signal). Additionally, the nuclei of testicular cells were stained with DAPI (blue signal). Consistent with RNA expression analyses *TCTE3* signals were barely detected in spermatogonia. In spermatocytes, the *TCTE3* protein staining spreads over the whole cytoplasm, without any specific accumulation (Fig. 1E). A similar staining pattern was observed in dividing spermatocytes, with the exception of a more intense fluorescence signal between nuclei (Fig. 1F). In round spermatids *TCTE3* was predominantly found in the cytoplasm and overlapped with tubulin staining (Fig. 1I–P). In the region of the nucleus *TCTE3* staining was weaker, but clearly visible. In elongated spermatids *TCTE3* protein labeling was decreased, but was detectable in the cytoplasm (Fig. 1G). The microtubular manchette was clearly marked by antibodies against

Figure 1 Analyses of testicular *Tcte3* expression. (A) Northern blot analysis of testicular RNA from different postnatal developmental stages. Twenty micrograms of total testicular RNA of ten to fifteen day old mice were hybridized with a ^{32}P labeled 0.7 kb *Tcte3-3* cDNA fragment. The same blot was rehybridized with a human elongation factor II (EF-2) probe. Localization of *Tcte3*-transcripts by *in situ* hybridization. Digoxigenized labeled *Tcte3-3* antisense (B and C) riboprobe was used for hybridization of paraffin embedded testicular cross section. *Tcte3* transcripts were detected in the cytoplasm of primary spermatocytes (arrowheads) and in round spermatid stages (arrows). No staining of the cytoplasm was observed after hybridization using a sense *Tcte3-3*-riboprobe (D). Localization of the TCTE3 protein in male germ cells by indirect immunofluorescence (E–Q). TCTE3 specific antibodies were detected with Cy3-coupled antibodies (red color; E–G, K–N, and P), while FITC-coupled secondary antibodies were used to visualize microtubules (Tub), (green color; G, J, L, O, and P). Additionally, DAPI was used to stain the nucleus (blue color; E–Q). In spermatocytes the whole cytoplasm is stained (E and F). In spermatids at early elongation phase TCTE3 is concentrated in the cytoplasm while weaker staining is observed in the nucleus region (I–L). During further differentiation of spermatids intensity of TCTE3 staining decreases. Some protein can be detected around the nucleus and in the cytoplasm surrounding the microtubular manchette (G, arrows). Figure H shows round spermatids incubated with secondary antibodies only. (M–Q) Immunostaining of mouse spermatozoa with anti-TCTE3 and anti-tubulin antibodies. The TCTE3 polypeptide is detectable along the whole flagella (M, N and P). Negligible staining was observed in sperm incubated only with secondary antibodies (Q).

β -tubulin (Fig. 1G). To elucidate the distribution of TCTE3 along the flagella, mouse spermatozoa were fixed on glass slides and probed with specific anti-TCTE3 antibodies. Complete flagella were labeled (Fig. 1M–P) indicating that the TCTE3 dynein light chain is equally distributed along the flagella of mouse spermatozoa.

Generation of *Tcte3-3*-deficient mice

Tcte3-cDNA was used to screen a Sv129 mouse cosmid library and two positive clones were isolated. Positions of *Tcte3-3* exons were determined by restriction enzyme mapping, Southern blot analyses, and sequencing. Four *Tcte3-3* exons were identified (Fig. 2A) and exon 2 was selected for substitution with the neomycin resistance gene of the pPNT targeting vector (Tybulewicz *et al.* 1991). After homologous recombination, a total of 7.7 kb genomic region was deleted including the exon 2 of the *Tcte3-3* gene (Fig. 2B).

Embryonic stem (ES) cells containing the disrupted allele were selected using Southern blot analysis (Fig. 2C) and two clones (clone 1 and clone 3) were identified. Additional random integration of the targeting vector was excluded by Southern blot analysis using a neomycin gene fragment hybridization probe (Fig. 2D). Moreover, PCR amplification of a 5.1 kb fragment from ES cell DNA of clone 1 and 3 proved the correct integration of the construct (Fig. 2E). The two ES cell lines carrying the mutated allele were used to generate germ line transmitting chimeras after injection in blastocysts derived from C57BL/6 females. Chimeras were bred with C57BL/6 and 129/Sv females, respectively, to establish the *Tcte3-3*-disrupted allele on C57BL/6 $\times$ 129/Sv hybrid and on 129/Sv inbred genetic background. Genotypes of offspring were determined by Southern blot and PCR analyses (Fig. 2F). Both, female and male mice heterozygous for the *Tcte3-3*-mutated allele revealed no malformations and were fertile.

Heterozygous animals were bred to generate *Tcte3-3*-deficient mice. More than 100 offspring were genotyped at postnatal day 20 using Southern blot or PCR. Approximately 20% of analyzed offspring were genotyped as wild-type while the remaining 80% revealed a heterozygous pattern. This result indicates that either *Tcte3-3*-deficient animals are lethal or that the technique used to genotype mice is unable to discriminate between heterozygous or homozygous offspring.

Genomic organization of the *Tcte3*-locus

Huw *et al.* (1995) suggested the existence of more than one *Tcte3* gene in the murine genome. We therefore performed Southern blot analyses using different enzymes and the *Tcte3-3* cDNA as a probe (data not shown). Comparison with the restriction pattern of cosmid DNA used for the generation of the targeting

Figure 2 Targeted disruption of the mouse *Tcte3-3* gene. (A) Schematic representation of the *Tcte3* gene locus on murine chromosome 17. Positions of *Tcte3* exons are indicated by grey boxes. (B) Schematic representation of the targeting strategy. Wild-type *Tcte3-3* locus (top), targeting construct (middle) and targeted allele (bottom). Exon 2 (black box) was replaced by the neomycin resistance gene cassette. Arrows indicate direction of transcription for both the *Tcte3-3* and the neomycin resistance gene. (C) Southern blot analysis for genotyping the ES cells. Genomic DNA was restricted using KpnI restriction enzyme. An external probe (grey box, 3'-probe) gave rise to a 9.2 kb (mutant), and a 5.4 kb (wild-type) hybridization signal. (D) Hybridization with the neomycin fragment probe proved homologous recombination event in ES cell clone 1 and 3. (E) Long-range PCR was done using DNA from ES cells 1 and 3. With primers *Neo3F* and *Tc-probe-r* a 5.1 kb fragment was amplified from the targeted allele. Amplification using primer pair *Tc-geno-F* and *Tc-probe-r* resulted in a 4.8 kb fragment derived from the wild-type allele. (F) PCR genotyping of mice. Wild-type allele yields a PCR product of 900 bp using the *Tc-fin-F* and *Tc-fin-R* primers. The targeted allele is expected to generate an ~600 bp fragment using the primers *Neo3F* and *Tc-fin-R*. (G) Quantitative real-time PCR analysis of *Tcte3* mice. The average value calculated for the quantity of neomycin for each sample was plotted versus mice identification number. Putative homozygous mice have a value of ~1 indicating the presence of two neomycin copies, while heterozygous mice containing one neomycin copy have quantities of ~0.5. E, EcoRI; K, KpnI; S, Sall; N, NotI; X, XbaI; Xh, XhoI; * marks restriction sites deriving from cloning vector. Arrows (↔) indicate positions of primers used for long range PCR.

vector revealed no differences, indicating that putative copies of the *Tcte3*-locus must be very similar. Using the *Tcte3-3* cDNA, we performed a database search and identified two bacterial artificial chromosomes (BAC) clones (accession numbers AC182749 and AC173477) from gene bank, both derived from murine chromosome 17. Alignment with the *Tcte3-3* cDNA revealed three repeat units of ~31.5 kb each containing one copy of the *Tcte3* gene. The three *Tcte3* gene copies were designated as *Tcte3-1*, *Tcte3-2*, and *Tcte3-3* (Fig. 2A). We next asked whether the three genes can be

distinguished. Sequence comparison of the four exons of the three *Tcte3* genes showed that in the nontranslated sequence of exon 1 of *Tcte3-2* and *Tcte3-3*, a 7 bp sequence repeat (GCCGGAG) is missing. Moreover, in exon 3 of *Tcte3-3* at position 535 of the corresponding cDNA cytosine is replaced by guanine which results in an amino acid exchange of arginine to glycine. To examine whether all three putative *Tcte3* genes are transcribed, we searched the murine EST database for *Tcte3* EST clones. We found EST clones matching the sequence of *Tcte3-1*, *Tcte3-2*, and *Tcte3-3* indicating

that all three genes are active. Sequence analyses of the cosmid DNA fragments used to generate the targeting vector revealed complete identity with the *Tcte3-3* gene database information, however, single base substitutions were found for sequence information of *Tcte3-1* and *Tcte3-2* genomic fragments.

Because the three *Tcte3* gene copies were highly similar, a quantitative PCR was established to genotype the offspring. By this technique, we were able to differentiate between animals with one or two targeted alleles (Fig. 2G). Breeding of heterozygous offspring was performed to obtain homozygous animals lacking the *Tcte3-3* gene product. From forty-seven F2 generation animals 11 mice were genotyped by quantitative PCR as homozygous for the targeted allele while 10 mice were wild-type and 26 were heterozygous for the mutated allele.

We observed no differences in phenotypes of mice hetero- or homozygous for the *Tcte3-3* mutated allele on different genetic backgrounds or derived from different chimeric progenitors, with the exception that number of offspring was lower on 129/Sv background. Therefore, experiments were done with mice on C57BL/6 $\times$ 129/Sv hybrid background.

Tcte3-3 gene product is detectable in knock-out mice

The expression of *Tcte3-3* in homozygous *Tcte3-3*^{-/-} mice was analyzed at the protein level by western blot experiments. Whole testis protein extracts were probed with anti-TCTE3-specific antibodies. In protein extracts of wild-type, heterozygous and homozygous animals a protein band of about 22 kDa was detected (data not shown). The loading of equal amounts of protein was controlled by using anti-tubulin antibodies.

Some *Tcte3-3* homozygous mutant male mice are infertile

Tcte3-3^{-/-} males and females of the F2 generation were bred with wild-type animals to test their fertility. All *Tcte3-3* lacking females and 25 tested males were found to be fertile, however, from 10 *Tcte3-3*^{-/-} males no offspring were obtained after three month of breeding with heterozygous or wild-type females.

To elucidate the cause of infertility we analyzed testicular morphology. Testes from infertile *Tcte3-3*^{-/-} males (70.3 ± 10.1 mg) were significantly smaller compared to wild-type littermates (90.2 ± 9.3 mg; Fig. 3A). Subsequent histological analysis indicated no abnormalities in the basal part of the seminiferous tubules of *Tcte3-3*^{-/-} animals (Fig. 3C and D), where spermatogonia and Sertoli cell nuclei are located. Type A spermatogonia with large spherical nuclei were located normally, at the base of epithelium. Spermatogonia containing round nuclei with a large number

Figure 3 Histology of *Tcte3-3*^{-/-} testes. (A) shows a testis of a wild-type and a *Tcte3-3*^{-/-} male of 3 months of age. (B–H) Light microscopically analyses of testicular and epididymal semi-thin sections stained with toluidine blue. (B) In wild-type testis different stages of spermatogenesis are visible and tails of elongated spermatids reach into the lumen of tubules. (C and D) *Tcte3-3*^{-/-} seminiferous tubules show hypospermatogenesis or only focal spermatogenesis with release of premature germ cells in the lumen (arrowheads). Elongated spermatids frequently display head abnormalities (arrows). Percentage of spermatids with head deformations varies in different tubule cross sections and was estimated at ~15–20% compared to unaffected sperm. (E–G) In the epididymis, biflagellate or bend tail spermatozoa (arrowheads) and spermatozoa with head abnormalities (arrows) are also frequently found. Moreover, fewer spermatozoa are observed in cross sections compared to wild-type epididymis (H).

of heterochromatin clumps were also evident (Fig. 3C and D). In contrast, spermatocytes were significantly reduced in number (reduction was ~30% compared to wild-type littermates), while postmeiotic stages; spermatids and spermatozoa were sometimes absent in mutant testis. Histological analysis of the epididymis revealed an overall reduced number of spermatozoa in comparison

to wild-type epididymis (Fig. 3E, G and H). A considerable number of spermatozoa display head anomalies (Fig. 3E and G) and up to 40–50% of sperm showed malformation of the tail or the development of two tails (Fig. 3E and F).

Transmission electron microscopy (TEM) was used to further characterize defective cells in mutant

testis (Fig. 4). Ultrastructural analysis indicated no abnormalities in the basal portion of seminiferous tubules (spermatogonia, primary spermatocytes) and in the interstitial Leydig cells. TEM provided evidence that substantial cell death in *Tcte3*^{-/-} testis was occurring. Elongating spermatids exhibited various signs of degeneration, ranging from nuclear condensation to the formation of abnormal sperm heads resulting focally in an abrupt arrest of spermatogenesis in *Tcte3*^{-/-} males (Fig. 4A and B). In the cytoplasm of Sertoli cells and elongated spermatids large vacuoles were observed (Fig. 4C). Spermatids, which had escaped from the degeneration, in considerable numbers harbored biflagellate pattern during the elongation step (Fig. 4E and F). Some spermatids exhibited a normal morphology with elongated nucleus. Putative spermatids in more advanced stages of degeneration could not unequivocally be identified. Small clumps of heterochromatin could be distinguished in the nuclei of cells in early stages of degeneration. In the later stages of degeneration, the areas of heterochromatin became larger. Condensed chromatin and irregular pattern of organelles were seen in latest stages of germ cell degeneration (Fig. 4). Axonemal tail sections of spermatozoa analyzed by TEM demonstrated the presence of normal 9 + 2 axonemes as well as inner and outer dynein arms (Fig. 4D).

Germ cells of infertile *Tcte3*-3-deficient mice undergo apoptosis

Cellular degeneration found in *Tcte3*^{-/-} testes of infertile males suggested that spermatogenic cell apoptosis increased in *Tcte3*^{-/-} mice. Therefore,

Figure 4 Transmission electron microscopy analyses of *Tcte3*^{-/-} male germ cells. Testicular histology in *Tcte3*^{-/-} males is normal until beginning of spermatid elongation. (A) shows a normal round spermatid (rSp), while elongating spermatid start to degenerate (deSp). In (B) spermatids with normal sperm head (eSp) are located beneath degenerating elongated spermatids (deSp). In spermatids and surrounding Sertoli cells, development of vacuoles (V) becomes obvious (C). The axonemal structure is not disturbed in *Tcte3*^{-/-} sperm tails and outer dynein arms are detectable (arrows, D). In the epididymis often spermatozoa with two flagella or bend tails are observed (E, arrows indicate double tail formation; F, arrows indicate double tail formation or coiled flagella).

Figure 5 Apoptosis is increased in *Tcte3*^{-/-} testes. (A and B) show testicular sections of a *Tcte3*^{-/-} male after TUNEL reaction. TUNEL-positive staining is mostly evident in spermatogonia, however also primary spermatocytes and round spermatids are affected. Interstitial cells were not labeled. Only few cells are stained using the TUNEL assay in wild-type testis (C). (D) Quantification of the incidence of TUNEL-positive germ cells in wild-type (+/+) and *Tcte3*^{-/-} males.

Table 1 Sperm analysis in *Tcte3-3*^{+/+} and infertile *Tcte3-3*^{-/-} mice.

Number of sperm	Genotype of male	
	+/+	-/-
Epididymis × 10 ⁵	190 ± 0.4 (3)	1.6 ± 0.2 (4)
Uterus × 10 ³	5.8 ± 1.5 (3)	1.5 ± 1.2 (4)
Oviduct × 10 ¹	20 ± 2.9 (3)	0.1 ± 0.7 (4)

Number of spermatozoa extracted from *Tcte3-3*^{+/+} and *Tcte3-3*^{-/-} epididymis or from uterus and oviduct of mated females was determined. Data in sperm analysis represent the mean ± s.d. of the number of individual measurements indicated in parenthesis.

TUNEL assays were performed on *Tcte3-3*^{-/-} testicular sections (aged 3 months) in order to assess the possible cell death rate. Those regions in the *Tcte3-3*^{-/-} seminiferous tubules that contained predominantly cells in prophase I stages exhibited a high frequency (more than 50%) of cell death (Fig. 5A). At higher magnification, the majority of TUNEL-positive cells could be identified as spermatogonia and spermatocytes in most of the testicular tubules from *Tcte3-3*^{-/-} mice (Fig. 5B). In contrast, cell death was rare in the wild-type testes (Fig. 5C).

Quantification of apoptosis was carried out by counting apoptotic germ cells that were clearly stained by the TUNEL assay. A total of 25 randomly selected seminiferous tubule cross-sections were analyzed from each of two mice. The rate of germ cell apoptosis was expressed as the number of apoptotic cells per tubule. An average of 25–30 cells was found to be TUNEL positive in each of the *Tcte3-3*^{-/-} seminiferous tubules (Fig. 5D). This result shows that germ cell development in the *Tcte3-3*^{-/-} mice is impaired.

Tcte3-3-deficient spermatozoa reveal reduced motility

To investigate the causes for *Tcte3-3*^{-/-} male infertility, we determined the number of spermatozoa in the epididymis of these males as well as in the uteri/oviducts of mated wild-type females. At least three males of each genotype were analyzed. As shown in Table 1, the sperm number in the epididymis is significantly reduced in infertile *Tcte3-3*^{-/-} males in comparison to wild-type

males, indicating that production of spermatozoa is affected in the knock-out mice. Moreover, uteri and oviducts of vaginal plug positive females were extracted and examined for the presence of spermatozoa (Table 1). In the uteri of females which were bred with *Tcte3-3*^{-/-} males, a slightly reduced amount of spermatozoa was ascertained; however, nearly no spermatozoa of *Tcte3-3*^{-/-} mice were found in the oviduct (Table 1). This result suggests that the motility of *Tcte3-3*^{-/-} sperms is reduced. In contrast, fertile *Tcte3-3*^{-/-} males did not reveal reduced number of sperm in the epididymis in comparison to wild-type males.

To characterize sperm motility in more detail, we used a computer assisted sperm analyzer. Analyses were done after 1.5, 3.5 and 5.5 h after sperm extraction from the epididymis. We did not observe significant differences between spermatozoa of wild-type and heterozygous *Tcte3-3*^{+/+} mice for any of the investigated parameters (data not shown). Approximately 60–76% of spermatozoa of these mice were motile and 40–54% of the spermatozoa showed progressive motility (Table 2). In contrast, only 20–31% of the spermatozoa of *Tcte3-3*^{-/-} mice were motile and more importantly only between 11 and 18% of these spermatozoa revealed a progressive movement (Table 2).

Certain functional parameters were evaluated in more detail (Fig. 6). Curvilinear velocity represents the total distance passed through by a sperm in a time unit (Fig. 6A and B). Most spermatozoa of wild-type males showed velocities of >200 µm/s, whereas the speed of *Tcte3-3*^{-/-} sperm from infertile and fertile males was decreased to ~100–150 µm/s. Furthermore, a reduction in the average path velocity (Fig. 6C and D) and in the straight line velocity (Fig. 6E and F) of *Tcte3-3*^{-/-} spermatozoa was observed, which have only 50–60% of the velocities estimated for spermatozoa of wild-type animals. The straight line velocity represents the straight line progressive movement of a sperm between the beginning and the end of the measurement divided by the time elapsed. In contrast, no significant alterations were observed for beat frequency (Fig. 6I) or the linearity (Fig. 6J) of motile *Tcte3-3*^{-/-} spermatozoa.

Interestingly, the loss of *Tcte3-3* function is correlated with a slight reduction in the amplitude of the sperm tail

Table 2 Analysis of the sperm motility of *Tcte3-3*^{+/+} and *Tcte3-3*^{-/-} mice.

Genotype of male	Incubation time (h)	Total number of measured spermatozoa (%)	Number of motile spermatozoa (%)	Number of spermatozoa with progressive movement (%)
+/+	1.5	2895 (100)	2196 (76)	1575 (54)
	3.5	3069 (100)	2066 (67)	1356 (44)
	5.5	3681 (100)	2245 (61)	1520 (41)
-/-	1.5	4259 (100)	1326 (31)	748 (18)
	3.5	3974 (100)	1054 (26)	582 (14)
	5.5	4204 (100)	833 (20)	453 (11)

Motility of spermatozoa from *Tcte3-3*^{+/+} and *Tcte3-3*^{-/-} males was determined after 1.5, 3.5, and 5.5 h using a computer-assisted semen analysis system.

Figure 6 Results of the computer assisted analysis of sperm motility. Different parameters, (A–F) sperm velocities (μm/s), (G and H) lateral amplitude of the sperm tail beat (μm), (I) beat frequency of the sperm flagella (Hz) and (J) forward movement (%) of wild-type spermatozoa (+/+) and *Tcte3-3*^{-/-} sperm (-/-) were measured after 1.5 and 3.5 h incubation time. *Tcte3*-deficient spermatozoa showed significantly reduced swim velocities and a decreased lateral amplitude in comparison to spermatozoa of wild-type and heterozygous mice ($P < 0.001$ by Mann–Whitney *U* test). No significant difference is observed in the linearity of sperm movement. The beat frequency of *Tcte3-3*^{-/-} sperm is slightly increased. For all parameters the medians (25–75%) and percentiles (5–95%) are shown.

beat (Fig. 6G and H). While in wild-type animals the lateral amplitude of most of spermatozoa flagella was more than 10 μm, whereas the amplitude in the majority of *Tcte3-3*-deficient spermatozoa was decreased to < 8 μm.

Discussion

Over the past years, an increasing number of axonemal and cytoplasmic dynein genes were identified in different species, but particularly in mammals, specific functions of most of these polypeptides are still unknown. To our knowledge, the present study is the first report of the targeted disruption of a dynein arm light chain gene in mouse which can result in male infertility. *Tcte3* genes are mainly expressed in testis and somatic tissues containing ciliated epithelia, implying a role in ciliary and flagellar motility (Rappold *et al.* 1987, DiBella *et al.* 2001, Neesen *et al.* 2002). Moreover, *Tcte3* expression was also demonstrated in liver and spleen and it was suggested that *Tcte3* expression in these tissues was due to its possible role as a component of the cytoplasmic dynein complex. In this regard *Tcte3* could be involved in distinct cargo-binding activities (DiBella *et al.* 2001). The different expression patterns of individual dynein genes might indicate that mammalian dynein genes have adapted specific features for ciliary and/or flagellar beating and potentially other cellular processes in both embryonic development as well as in the adult organism. Our results support this hypothesis because loss of *Tcte3-3* function in some males on the one hand resulted in decreased sperm motility supporting its function as an axonemal dynein, on the other hand increased apoptosis of male germ cells and multiple malformations within the differentiating spermatozoa are observed, indicating an important role of *Tcte3-3* in spermatogenesis.

It is surprising that the inactivation of the *Tcte3-3* gene copy has such phenotypic effects in some mice, considering that three putative *Tcte3* gene repeat units were found on murine chromosome 17. Data derived from the EST libraries suggests that all three copies are transcribed, although the expression level of each individual gene is unknown. Alignment of sequence information available for *Tcte3-1* and *Tcte3-2* genomic fragments with our *Tcte3* cosmid DNA used for the generation of the targeting vector revealed single nucleotide substitutions, whereas complete identity with the *Tcte3-3* genomic fragments were found. Therefore, it is most probable that we have inactivated the *Tcte3-3* gene by homologous recombination. Interestingly, the TCTE3-3 amino acid sequence differs in position 125 from the sequences of TCTE3-1 and TCTE3-2. An arginine is replaced by glycine. It is unclear whether this exchange is of any functional relevance for the TCTE3-3 function.

Our western blot analyses revealed no detectable differences in the amount of TCTE3 protein between testicular protein extracts from wild-type, heterozygous or homozygous *Tcte3*-3-deficient mice. A recent report indicating that *Tcte3* expression is regulated by sense–antisense transcript pairs (Chan *et al.* 2006). The authors stated that different species of *Tcte3* antisense transcripts exist and that some were not polyadenylated and capped. The *Tcte3* antisense amplicons revealed 100% complementarity either to exons 3 and 4 or only to exon 4. *Tcte3* antisense transcripts were only detected in testis, while *Tcte3* sense transcripts were also observed in other adult tissues. This could explain that the phenotype in *Tcte3*-3^{-/-} males seems to be restricted to male reproductive organs. The authors suggested that *Tcte3* antisense transcripts serve as a noncoding regulatory RNA and are involved in the vigorous control of gene expression necessary for spermatogenesis (Chan *et al.* 2006). The homozygous inactivation of the *Tcte3*-3 gene copy by homologous recombination might disturb the balance between *Tcte3* sense and antisense transcripts towards degradation of *Tcte3* transcripts in male germ cells. In this content it should be mentioned that results of situ hybridization experiments using a sense *Tcte3* probe argues against a strong expression of *Tcte3* antisense transcripts within the murine testis. However, we cannot exclude that experimental conditions prevent efficient binding of the 76 bp of the sense probe covering exon 4 to *Tcte3* exon 4-specific antisense transcripts. Moreover, the sensitivity of *in situ* hybridization could be insufficient to detect low amounts of antisense transcripts, because *Tcte3* transcripts were also not visualized in spermatogonia although RT-PCR results indicated low expression of *Tcte3* in these cells.

Orthologous genes to murine *Tcte3* have been reported in several species including human (TCTE3), the green algae *Chlamydomonas* (LC2) and the rainbow trout (LC2; Patel-King *et al.* 1997, Inaba *et al.* 1999, Neesen *et al.* 2002). Loss of the complete LC2 gene in *Chlamydomonas* cause structural defects in the axoneme (Pazour *et al.* 1999). Owing to a defect in protein complex assembly all outer dynein arms are missing and mutants have a decreased flagellar beat frequency. Interestingly, another mutant with a deletion in the 3'-end of the LC2 gene is able to produce low amounts of complete LC2 protein which is sufficient for the assembly of a variable number of outer dynein arms. This mutant also revealed reduced swim velocities (Pazour *et al.* 1999). In mice the inactivation of one *Tcte3* gene copy could have a similar effect. However, *Tcte3* expression seems to be sufficient for the assembly of outer dynein arms, because defects in the axonemal structure were not detected by electron microscopy. Another putative explanation is that in mouse the TCTE3 polypeptide is not necessary for the assembly of outer dynein arms. Nevertheless, some *Tcte3*-3^{-/-} males are infertile due to altered sperm motility. The majority of

Tcte3-3-deficient spermatozoa are unable to migrate into the female oviduct. Thus, loss of *Tcte3*-3 function can lead to a higher percentage of completely immotile spermatozoa as well as reduction of both velocity and progressive motility of the remaining motile spermatozoa, which results in asthenozoospermia. In this context it is important to note that TCTE3 seems to play a central role in the activation of sperm motility. In rainbow trout, LC2 is phosphorylated in a cAMP-dependent manner when sperm motility is activated (Inaba *et al.* 1999). A putative sequence motif for phosphorylation by cAMP-dependent protein kinase is localized in the N-terminal region of salmon and trout LC2 polypeptides, but is also found in human and murine TCTE3 proteins (Inaba *et al.* 1999, Neesen *et al.* 2002). It is tempting to speculate that in *Tcte3*-3^{-/-} spermatozoa-reduced TCTE3 polypeptide amounts result in an ineffective activation by the cAMP-dependent phosphorylation which causes less progressive movement of sperm. In this context, it should be mentioned that TCTEX4 which is encoded by an alternative splice variant of *Tcte3* was found to interact with the protein kinase CSNK2B subunit. Casein kinase 2 (CSNK2B) is involved in phosphorylation of a variety of nuclear and cytosolic proteins and could play an important role in cell proliferation and transformation (Bai *et al.* 2003).

Reduced sperm motility has also been implicated in human male infertility and moreover, asthenozoospermia is considered to be a major cause of impaired male fertility. In several studies, patients with unexplained asthenozoospermia were analyzed for ultrastructural sperm defects (Wilton *et al.* 1992, Courtade *et al.* 1998) and in 20–30% of these patients no abnormalities of their sperms were observed. The phenotype of *Tcte3*-3 knockout mice strengthens the assumption that loss of the *Tcte3* dynein light chain function could cause asthenozoospermia in humans.

Electron microscopical investigations demonstrate degeneration of germ cells mainly occurring in elongating spermatids. It is not known, how the loss of *Tcte3*-3 function results in degeneration of male germ cells and an increased apoptotic rate. However, several recent reports gave evidence for a correlation between *Tcte3* expression and apoptosis. In *Ovo1* deficient mice, germ cell degeneration and defective sperm production is observed. *Ovo1* encodes a member of the *ovo* family of zinc-finger transcription factors. Microarray analysis indicated that in *Ovo1*^{-/-} testis ~200 genes are differentially expressed. One of the genes that are down regulated is *Tcte3*. Interestingly, down regulation of *Tcte3* expression already occurs even if apoptosis is minimal (Li *et al.* 2005). Further support for the correlation between *Tcte3* and apoptosis comes from studies using ethylene glycol monomethyl ether (EGME). EGME induces cell death via an apoptotic process (Ku *et al.* 1995). Spermatocytes in early and late pachytene stages seem to be more sensitive to the

apoptotic stimulus of EGME (Chapin *et al.* 1984). *Tcte3* was found to be downregulated early after EGME-induced testicular lesion (Wang & Chapin 2000). How *Tcte3* is involved in the apoptotic process of male germ cells is not understood. However, *Tcte3* could directly induce meiotic gene activation, because *Tcte3* can bind to the IME2 promoter which is involved in the expression of meiotic genes in yeast (Malcov *et al.* 2004).

A recent study suggested an interaction between TCTE3 and CREB3L4 (ATCE1) by yeast two hybrid assay (Stelzer & Don 2002). *Creb3l4* is a novel mouse cyclic adenosine monophosphate-responsive element-binding protein-like gene that is expressed in postmeiotic male germ cells. It has significant sequence homology to a subfamily of *CREB* genes with the LZIP peptide as a prototype. CREB plays a crucial role during spermatogenesis and activation of CREB leads to expression of many genes necessary for germ cell differentiation, whereas expression of an unphosphorylated dominant negative form of CREB in Sertoli cells induces apoptosis of spermatocytes (Scobey *et al.* 2001). CREB3L4 showed specific binding to the nuclear factor- κ B-binding element and it was suggested that CREB3L4 could function as an activator of late haploid genes. The physiological correlation between *Creb3l4* and *Tcte3* is unknown; however, it is tempting to speculate that loss of *Tcte3-3* affects *Creb3l4* function. This could result in impaired activation of late haploid genes and could lead to spermatid degeneration. Interestingly, *Creb3l4*-deficient mice are fertile (Adham *et al.* 2005). However, males reveal reduced spermatogenesis, caused by an increased apoptosis of meiotic and postmeiotic germ cells similar to the disturbances in germ cell differentiation observed in *Tcte3-3*-deficient mice.

At present, we can only speculate why some *Tcte3-3*-deficient males are infertile while others are fully fertile. Because *Tcte3-3*^{-/-} males older than one year are still fertile it is unlikely that infertility is an age-related problem. More likely, modifying genes exist that due to the mixed background could influence fertility in *Tcte3-3*-deficient males.

Taken together, we have generated mice lacking a functional *Tcte3-3* gene supporting a vital role of *Tcte3* for male germ cell survival and proper development.

Materials and Methods

Construction of the *Tcte3-3* gene disruption vector

Tcte3-3 cDNA was isolated by RT-PCR experiment from murine testicular RNA using primers *Tc3-5* 5'-GCC CTG TGC TTC GCG GCA TCT GAG C-3' and *Tc3-3* 5'-GCT GGT CCT GAG CTA TTC ACA ATA G-3'. The resulting PCR product was cloned into pGEM-T easy vector (Promega) and subsequently sequenced.

For isolation of a *Tcte3-3* genomic clone a J1 129/Sv mouse genomic library was screened using *Tcte3* cDNA as a probe.

A 5 kb *SpeI/KpnI* fragment and a 3.8 kb *XbaI* fragment were isolated from the cosmid DNA and cloned into the pBlueScript vector (Stratagene, La Jolla, CA, USA) using the same restriction sites. The 5 kb fragment was restricted using *XbaI* and *EcoRI* restriction enzymes. The resulting 3.5 kb fragment was then ligated into *XbaI/EcoRI* restricted DNA of the pPNT vector (Fig. 2B). The 3.8 kb fragment was restricted by *NotI* and *XhoI* enzymes and was ligated into the *NotI/XhoI* sites of the pPNT vector (Fig. 2B). The resulting targeting vector was linearized with *NotI* and used for transfection of ES cells.

A 1.4 kb fragment was isolated from the 5 kb *SpeI/KpnI* fragment by cutting with *EcoRI* restriction enzyme and was used as a 3'-external probe for screening the DNA of recombinant R1 ES cell clones.

Generation of *Tcte3-3* chimeric mice

The ES cell line R1 was cultured as previously described (Wurst & Joyner 1993). Confluent plates were washed in PBS buffer and trypsinized. The cells were suspended in PBS buffer at 2×10^7 cells/ml. One milliliter of aliquots were mixed with 50 μ g linearized targeting vector and electroporated at 250 V and 500 μ F. Cells were cultured in nonselective medium in the presence of G418-resistant embryonic mouse fibroblasts. After 36 h the medium was changed containing G418 at 350 μ g/ml and ganciclovir at 2 μ M. Ten days later resistant clones were picked and transferred into 24-well trays.

Two *Tcte3-3* targeted 129/Sv ES clones (designated as clone 1 and clone 3) were cultured and injected into 3.5 dpc blastocysts derived from C57BL/6J mice, which were then implanted into pseudo pregnant CD1 mice to generate chimeric mice. Nine chimeras were obtained by two independent injections of targeted ES clones. Chimeric offspring were bred with C57BL/6 and 129/SvJ female mice to generate F1 animals on respective genetic background (C57BL/6J $\times$ 129/Sv and in 129/Sv). Heterozygous offspring were bred to obtain homozygous mice. All animal experiments were reviewed and approved by the Institutional Animal Care and Use Committee of the University of Goettingen, Germany.

DNA and PCR analyses

Genomic DNA was extracted from ES cells and mouse tails and digested with *KpnI*. After electrophoresis the DNA was transferred onto Hybond N membranes (Amersham) and hybridized with a ³²P-labeled 1.4 kb – 3'-external probe (Fig. 2B). The targeted *KpnI* fragment has a size of 9.2 kb while the corresponding wild-type allele has a size of 5.4 kb. Absence of additional random integration of the targeting vector was checked by hybridization using a neomycin phosphotransferase II probe.

In order to confirm the successful targeting of the *Tcte3-3* locus, a long-range PCR experiment was carried out using DNA from putative heterozygous ES cells and the TaKaRa LA Taq PCR kit (Cambrex, Verviers, Belgium). The first PCR covered 5.1 kb of the genomic sequence using primer set *Neo3F* and *Tc-probe-r* (5'-GTA CTG TGC TCC AGA CCA TGA TAA G-3') to amplify the targeted allele fragment. The *Tc-probe-r* primer was located

outside the 5 kb *SpeI/KpnI* fragment (Fig. 2B). In a second PCR the primer pair *Tc-geno-F* (5'-ATG TCT GTT CTA ATC ATG CCT TTT T-3') and *Tc-probe-r* was used to amplify the wild-type *Tcte3-3* locus fragment of 4.8 kb size (Fig. 2E).

For PCR analysis 1 µg genomic DNA and 10 pmol of each primer *Tc-fin-F* 5'-CAC AGA TCT TAA GAG AAA GAC TGA GAG AGT C-3', *Tc-fin-R* 5'-GGT AGA GAG GTT CAG AGT ATG CTA CCT T-3' and *Neo3F* 5'-CCT TCT ATC GGC TTC TTG ACG AG-3' were used. Cycling conditions were 1 min at 94 °C, 1 min at 55 °C and 2 min at 72 °C. After 35 cycles the products were separated in a 1% (w/v) agarose gel. The wild-type allele (primer combination *Tc-fin-F-Tc-fin-R*) has a size of 900 bp while the mutated allele (primer combination *Tc-fin-R-Neo3F*) has a size of ~600 bp (Fig. 2F).

Genotyping by quantitative real-time PCR

Owing to the putative presence of highly similar copies of the *Tcte3* gene in the mouse genome, genotyping of *Tcte3-3* mice was based on quantitative PCR amplification of the neomycin gene (*neo*) using specific primers *Neo-447f* 5'-C TTG TCG ATC AGG ATG ATC TGG-3' and *Neo-597r* 5'-G GCC ATT TTC CAC CAT GAT ATT-3'. Serial dilutions of sample and standard DNA's were made with buffer AE (Qiagen). DNA of *pelota* heterozygous mice (Adham *et al.* 2003) containing one neomycin copy at concentrations of 20, 10, 5, 2.5, and 1.25 ng/µl was used as standard while each sample DNA (*Tcte3-3* mice) was used at a concentration of 10 ng/µl. Primers and probes were designed accordingly to generate amplicons <150 bp in length, enhancing the efficiency of PCR amplification.

Real-time quantitative PCR was performed using double stranded DNA binding dye Syber Green PCR Master Mix (Applied Biosystems, Darmstadt, Germany) in an ABI GeneAmp 7000 Sequence Detection System. Each reaction was run in triplicate and melting curves were constructed by using the Dissociation Curves Software (Applied Biosystems) to ensure that only a single product was amplified. Quantitative real-time PCR of DNA specimens and standards were conducted in a total volume of 10 µl with 5 µl of 1×TaqMan Master Mix, 2.5 µl of each forward and reverse primer in a final concentration of 1 µM and 2.5 µl DNA. Thermal cycler parameters were 2 min at 50 °C, 10 min at 95 °C and 40 cycles involving denaturation at 95 °C for 15 s and annealing/extension at 58 °C for 1 min. Standard curves of the threshold cycle number versus the log number of gene copies were generated and were used to extrapolate the number of neomycin copies. Quantitative real-time PCR results were reported as the number of copies for *Pelota*/neomycin and the mean were calculated.

In situ hybridization, northern blot analysis and RT-PCR experiments

Detection of *Tcte3-1*, *Tcte3-2*, and *Tcte3-3* transcripts on paraffin sections was performed using digoxigenized riboprobes as previously described (Neesen *et al.* 1994). For *in situ* hybridization a 339 bp *Tcte3* cDNA probe was used covering part of exon 2, exon 3, and 76 bp of exon 4 of the *Tcte3* gene.

For northern blot experiments total RNA was isolated from adult mouse tissues and from different testicular postnatal stages using the RNA-NOW reagent (ICN, Frankfurt, Germany) according to the manufacturer's instructions. Approximately 20 µg total RNA of each tissue was loaded on a 1.2% (w/v) agarose-formaldehyde gel, electrophoretically separated and transferred onto Hybond C membrane (Amersham). Filters were hybridized with a ³²P-labeled 0.7 kb *Tcte3-3* cDNA fragment. Integrity and equal amounts of loaded RNA were tested by rehybridizing the blots using a ³²P-labeled 1.6 kb *BamHI/BglII* fragment of the human elongation factor 2 cDNA or a β-actin probe. Hybridization was carried out at 65 °C using Rapid-hyb hybridization solution (Amersham), containing 300 µl denatured salmon sperm DNA (10 µg/µl). Filters were washed 15 min in 2×SSC (0.3 M NaCl, 0.15 M Tris), 0.2% (w/v) SDS at 65 °C and for 10 min in 0.2×SSC, 0.2% (w/v) SDS at 65 °C.

For RT-PCR experiments the OneStep RT-PCR kit (Qiagen) was used. The cDNA synthesis was performed with *Tcte3*-specific primers *Tc3-5* and *Tc3-3* and 5 µg total testicular RNA. The resulting 690 bp PCR fragments were separated on a 1% (w/v) agarose gel. As controls for the integrity of the RNA, fragments of the murine glyceraldehyde-3-phosphate dehydrogenase RNA using primers *Gapdh-f* (5'-CAT CAC CAT CTT CCA GGA GC-3') and *Gapdh-r* (5'-ATG ACC TTG CCC ACA GCC TT-3') were amplified.

Generation of *Tcte3*-specific antibodies

The *Tcte3-3* cDNA was isolated from pGEM-T plasmid DNA by *EcoRI* digestion and was ligated into *EcoRI* restricted pET 41b vector DNA (Novagen, Darmstadt, Germany). TCTE3-GST fusion protein was purified according to the manufacturer's instructions. Approximately 100 µg/ml purified fusion protein was mixed with an equal volume of Freund's adjuvant (complete or incomplete) and injected three times subcutaneously into female New Zealand rabbits. Enrichment of specific TCTE3 antibodies was performed by eluting immunoglobulin bound to filter fixed fusion protein as described (Rashid *et al.* 2006). Moreover, we used polyclonal goat anti-TCTE3 antibodies (Santa Cruz Biotechnology, Heidelberg, Germany).

Fertility test and sperm motility analyses

To examine the fertility of *Tcte3-3*-deficient mice, 30 mature males were mated, each with two CD1 females for at least 12 weeks. Females were checked for the presence of vaginal plugs.

Testes and epididymides of wild-type, heterozygous and mutant homozygous mice were dissected in M2 media (Sigma-Aldrich Chemie). Spermatozoa were allowed to swim out of the epididymis for 20 min at 37 °C. A drop of the sperm solution was transferred to an incubation chamber which was set at a temperature of 37 °C. Sperm movement was quantified using the computer-assisted semen analysis system (CEROS version 10, Hamilton Thorne Research, Beverly, MA, USA). At least 2500 motile sperms each of five *Tcte3-3*^{+/+}, five *Tcte3-3*^{+/-}, and seven *Tcte3-3*^{-/-} mice were analyzed.

Statistical analysis was performed using the Statistica software program (StatSoft Inc., Tulsa, OK, USA). Data of fertility and sperm analysis were compared using the Mann–Whitney *U* test and Student's *t*-test.

Apoptotic assay

Slides containing testicular paraffin thin sections were processed for TUNEL assay to assess the number of cells undergoing apoptosis using an ApopTag *in situ* detection kit (Chemicon International, Inc., Temecula, CA, USA). Slides were pre-treated with H₂O₂ and incubated with the reaction mixture containing TdT and digoxigenin-conjugated dUTP for 1 h at 37 °C. Labeled DNA was visualized with a peroxidase-conjugated antidigoxigenin antibody and 3,3'-diaminobenzidine as chromagen. The percentage of cell death was determined by the number of cells exhibiting brown nuclear staining per section and results were compared to wild-type littermate.

Electron microscopy and immunohistochemical analyses

Testicular and epididymal tissues were cut into small pieces and fixed by immersion in 3.5% (v/v) glutaraldehyde and 1% (w/v) paraformaldehyde in 0.1 M cacodylate buffer (ph 7.4). Specimens were washed in 0.1 M cacodylate buffer containing 0.1 M sucrose and postfixed in 1% (w/v) osmium tetroxide. Semithin sections were stained with toluidine blue and photographed using a Zeiss light microscope. Ultrathin sections were contrasted with 1% (w/v) uranyl acetate in cacodylate buffer and examined by electron microscopy.

For squeeze preparations, testes were isolated and placed into RPMI medium (Sigma–Aldrich Chemie). The testis was dissected and a small piece of a seminiferous tubule was transferred onto a slide. The tissue was covered by a coverslip, squeezed with little pressure and frozen in liquid nitrogen. Thereafter, the coverslip was removed. The slide was incubated for 3 min in 90% (v/v) ethanol and then air-dried. Freshly prepared spermatozoa were washed three times with PBS and a drop of the sperm solution was fixed on a glass slide by air-drying. Cells were treated with PBS containing 1% (v/v) Triton X-100 for 5 min. After a blocking step for 1 h in PBS containing 5% (v/v) normal goat serum, 3% (w/v) BSA and 1 × roti-block-solution (Roth, Karlsruhe, Germany), cells were covered with 20 µl purified anti-TCTE3 antibody solution (diluted 1:5 with PBS) and incubated overnight in a humidified chamber at 4 °C. Additionally, anti-tubulin MABs were added in a final dilution of 1:50. After four washes with PBT, slides were incubated with secondary antibodies (goat-anti-rabbit-Cy3 and goat-anti-mouse-FITC) in a final concentration of 1:50 in PBS for one hour at room temperature. Finally, slides were washed four times in PBT, covered with a drop of vector shield solution containing DAPI stain and examined using a B×60 microscope (Olympus, Hamburg, Germany) with fluorescence equipment and 'Analysis' software program (Soft Imaging System, Muenster, Germany).

Declaration of interest

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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