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Tektin 3 is required for progressive sperm motility in mice

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Abstract

Tektins are evolutionarily-conserved flagellar (and ciliary) filamentous proteins present in the axoneme and peri-axonemal structures in diverse metazoan species. We have previously shown that tektin 3 (TEKT3) and tektin 4 (TEKT4) are male germ cell-enriched proteins, and that TEKT4 is essential for coordinated and progressive sperm motility in mice. Here we report that male mice null for TEKT3 produce sperm with reduced motility (47.2% motility) and forward progression, and increased flagellar structural bending defects. Male TEKT3-null mice however maintain normal fertility in two different genetic backgrounds tested, in contrast to TEKT4-null mice. Furthermore, male mice null for both TEKT3 and TEKT4 show subfertility on a mixed B6;129 genetic background, significantly different from either single knockouts, suggesting partial non-redundant roles for these two proteins in sperm physiology. Our results suggest that tektins are potential candidate genes for non-syndromic asthenozoospermia in humans.

Keywords

coiled-coil; axoneme; knockout; motility

Introduction

Sperm progressive motion and velocity are the result of highly coordinated actions of the axonemal cytoskeleton, the peri-axonemal outer dense fibers and fibrous sheath, and regulatory proteins that are involved in a repetitive cycle to produce flagellar beat and waveform. Approximately 250 proteins that form the axonemal proteome (Ostrowski et al. 2002; Pazour et al. 2005) interact to produce a remarkably fine-tuned physiological process that leads to sperm motion. Several of these proteins serve functional and regulatory roles (e.g., dyneins, radial spoke proteins, etc.) while others are constituents of the axoneme (e.g., tubulins, nexins, etc.) and maintain the structural integrity of the flagellar axoneme and accessory structures. Mutations in several of these components cause sperm motility disorders due to defect in either structural or functional integrity of the flagella (Afzelius 2004).

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Tektins are sarkosyl-insoluble coiled-coil filamentous proteins originally purified from sperm flagella of the purple sea urchin *Strongylocentrotus purpuratus* as components stably associated with 3–4 tubulin protofilaments in the wall of A and B outer doublet microtubules (Linck et al. 1985; Linck and Langevin 1982). Since then, highly conserved tektin family members have been identified in all major mammalian species studied, and are characterized by a nonapeptide carboxy-terminal signature sequence [RPNV/I/MELCRD]. In sea urchin sperm flagella, three tektins named tektin A, B and C, exist as longitudinal polymers with axial periodicities matching the tubulin lattice (Pirner and Linck 1994), which led to the 'molecular ruler' hypothesis that these proteins aid in the periodic attachment of axonemal appendages along the flagellar length. Recent cross-linking studies in sea urchin sperm have also indicated that the ~2–3nm tektin filaments consist of two isoforms of tektin A (tektin A1 and A2) that interact with tektin B to form heterodimers and other higher order multimers (Setter et al. 2006) and tektin C homodimers. The tektin filaments were also found to be associated with tubulins and were proposed to impart the outer doublets with stability against the transverse shear forces acting to pull apart the doublets in an active flagellar bend.

Whereas biochemical purification of flagellar axonemes in sea urchins identified only 3 tektins, our group and others have identified at least 5 members of this family in mammals (Cao et al. 2006; Iguchi et al. 1999; Matsuyama et al. 2005; Norrander et al. 1998; Roy et al. 2007; Roy et al. 2004). Recent proteomic studies on the peri-axonemal structures of rodent spermatozoa have detected the presence of all five tektins in the accessory structures (Cao et al. 2006). Tektin 4 (TEKT4) has been localized to the outer dense fibers by electron microscopy (Iida et al. 2006). A paralog, TEKT5, has been localized ultrastructurally to the inner surface of the mitochondrial sheath (Murayama et al. 2008). Since sperm from sea urchins and other invertebrates are devoid of accessory peri-axonemal structures, this suggests that the tektin family might have expanded in mammals to perform additional functions as components of the outer dense fibers and/or fibrous sheath. We have previously shown that deficiency of TEKT4 causes asthenozoospermia, defective flagellar beat pattern and fertility defects in male mice (Roy et al. 2007). Others have shown that mice null for the flagellar protein TEKT2 demonstrate absence of inner dynein arms in the axoneme, diminished flagellar motility and infertility (Tanaka et al. 2004). In the present study, we report the functional characterization of tektin 3 (TEKT3) in spermatozoa, flagellar motility, and male reproductive physiology in mice.

Results

Tekt3 null mice are viable

To define the physiological role of TEKT3 in sperm flagellar motility and male reproduction, we generated a targeted mutation in *Tekt3* using homologous recombination (Figure 1A) in embryonic stem (ES) cells. As predicted, the targeting strategy produced a null allele as confirmed by absence of *Tekt3* transcripts in testis by Northern blot analysis (Figure 1C). Consistent with the limited expression of this gene beyond the testis, homozygous null mice were viable and had no apparent gross abnormalities. Null mice were born with the expected Mendelian frequency and approximately 1:1 ratio for both sexes.

Tekt3$^{-/-}$ males have reduced percentage of sperm with progressive motility

To investigate the role of *Tekt3* in male reproduction, we analyzed the reproductive physiology of the mutant strain in detail. Mean testis weights of wild-type (WT) and null males were not significantly different at 8–10 weeks of age (Table 1). Analysis of testis histology at 8 weeks revealed normal maturation and complete spermatogenesis in null males. Counts performed on cauda epididymal spermatozoa were also similar between WT

and *Tekt3* null males. However, the null males showed a significant reduction in the percentage of motile spermatozoa in the cauda (Table 1); whereas WT males had 87.6 ± 2.6% motile spermatozoa ($n = 5$), only 47.2 ± 3.0% of actively motile spermatozoa were present in the null males ($n = 6$) ($P < 0.0001$). Sperm without active motility were either immotile or demonstrated sluggish sideward bending that was characterized by a defective flagellar beat pattern and lack of forward progression. Motile spermatozoa had normal progressive velocity after 1 hr of incubation, and the decrease in the percentage of motile spermatozoa after incubation in M16 medium for 3 hrs was not significantly different from WT sperm (data not shown).

Sperm from *Tekt3*$^{-/-}$ males show an increase in flagellar structural defects

To investigate the cause of the reduced percent of motile sperm in null males, we compared sperm morphology between WT and *Tekt3* null males. Spermatozoa from *Tekt3* null males exhibited normal head morphology but frequently had pronounced flagellar bending at the annulus at the junction of the midpiece and the principal piece. The bending was present in 48.7 ± 4.2% of sperm from *Tekt3*-null males ($n = 7$); in contrast, WT sperm had normal head and tail morphology with only 27.0 ± 3.0% showing similar structural defects in the flagella ($P < 0.01$) (Figure 2A). Flagellar bending was typically up to 180° (Figure 2B–C), and the progressive velocity of sperm with severe flagellar bending was diminished; these spermatozoa would swim backwards, indicating a lack of propulsive force generation in the midpiece.

Fertility is maintained in *Tekt3*$^{-/-}$ males

To assess if the structural and motility defects seen in *Tekt3*-null sperm would compromise the fertility of null males, *Tekt3*$^{-/-}$ males and heterozygous littermates (*Tekt3*$^{+/-}$) on a mixed genetic background (B6;129S5-*Tekt3*^{tm1Zuk}) were first bred to wild-type (WT) females for 6-months. Mating of 4 *Tekt3*$^{+/-}$ males with WT females over a 6-month period resulted in 24 litters with an average litter size of 7.8 ± 0.5 pups per litter; surprisingly, mating of 6 *Tekt3*$^{-/-}$ males with WT females resulted in only a modest reduction in fertility with a mean litter size of 7.13 ± 0.5 pups per litter (Figure 3A) ($P > 0.05$). The number of litters sired per month by *Tekt3*-null males was 0.85 ± 0.1 compared to 0.96 ± 0.2 for WT males (Figure 3B) ($P > 0.05$). These findings suggest that on a mixed genetic background, *Tekt3*$^{-/-}$ males possess normal fertility.

We then tested the fertility of *Tekt3*$^{-/-}$ males carrying the null mutation on a 129 genetic background (129S5-*Tekt3*^{tm1Zuk}) by mating them to WT females. Similar to null males on a mixed background, 129S5-*Tekt3*$^{-/-}$ males show a modest decline in fertility (Figure 3C and 3D) that was not significantly different from WT controls. These results suggest that TEKT3 is not essential for male fertility in mice. *Tekt3* null females on both genetic backgrounds had normal fertility (data not shown), consistent with male-specific expression of *Tekt3*.

Tekt3$^{-/-}$; *Tekt4*$^{-/-}$ double knockout males are subfertile on a mixed B6;129S5 genetic background

Since both tektin 3 and tektin 4 have been reported to be present in the same subcellular compartment within the peri-axonemal accessory structures (Cao et al. 2006), we then tested whether these two tektin family members play partially redundant roles in maintaining sperm motility and fertility by creating *Tekt3*$^{-/-}$; *Tekt4*$^{-/-}$ double knockouts. Double knockout mice were obtained in normal Mendelian ratios, and males and females were viable and grossly normal.

Spermatozoa from double knockout males exhibited decreased progressive motility at t = 0.5 hrs, with only 43.2 ± 5.4% having progressive motility, and was significantly different from

WT males ($P < 0.001$) (Figure 4A). At t = 0.5 hrs, the percent motility of double knockout males was not significantly different from *Tekt3*$^{-/-}$ single knockout males. However, when mated with WT females over a period of 6 months, six *Tekt3*$^{-/-}$; *Tekt4*$^{-/-}$ double knockout males produced 30 litters with a mean litter size of 5.7 ± 0.6 pups/litter that was significantly reduced from WT and single knockout males (Figure 4B). Double knockout males did not show any decrease in litters sired per month. Sperm counts and testis weights of double knockout males were not significantly different from age-matched WT controls (Table 1).

Discussion

The tektin family of proteins, first identified in sea urchin sperm flagella as 3 integral microtubule components, has expanded in mammals to at least 5 family members (Cao et al. 2006; Iguchi et al. 2002; Larsson et al. 2000; Matsuyama et al. 2005; Roy et al. 2007; Roy et al. 2004). The first tektin to be identified in mammals in an extra-axonemal location was TEKT1 that was identified in olfactory epithelial cilia and was later suggested to play a role in the nucleation of the axoneme through interaction with centrosomal proteins (Larsson et al. 2000; Norrander et al. 1998). However, in the absence of a mouse model of tektin 1 deficiency, the true function of this protein remains unknown. The *Tekt3* gene is located on mouse Chromosome 11 near the mouse *Tekt1* gene likely reflecting a gene duplication event in the recent past. Tektin 2 was identified in mice and humans as a component of the axonemal outer doublets, and male mice deficient for *Tekt2* are sterile due to sperm motility defects; significantly, both females and males also have defects in respiratory cilia as a consequence of axonemal dysfunction (Tanaka et al. 2004). A recent study indicates that single nucleotide polymorphisms in TEKT2 might have deleterious effects on sperm motility, ultrastructure and physiology in humans as well (Zuccarello et al. 2008). Our group and others have previously shown that *Tekt4* is localized to the outer dense fibers in sperm flagella in mice and rats (Iida et al. 2006; Matsuyama et al. 2005), and that loss of TEKT4 causes asthenozoospermia, inefficient energy utilization in sperm flagella, and reduced male fertility (Roy et al. 2007). Recently, TEKT5 has been localized to the inner surface of the mitochondrial sheath in rat sperm flagellar midpiece, further highlighting the extra-axonemal localization of the expanded tektin family in mammals (Murayama et al. 2008).

Here, we report the functional characterization of mouse TEKT3 in male reproductive physiology. We previously reported that mouse *Tekt3* is a male germ cell-enriched gene that is expressed predominantly in pachytene spermatocytes and round spermatids in the testis (Roy et al. 2004). Studies from other groups have identified human TEKT3, along with TEKT1 and TEKT2, also in the ciliary proteome, suggesting they might be part of the 9 + 2 axoneme (Ostrowski et al. 2002), in addition to their peri-axonemal localization in flagella.

Significantly, male mice deficient in TEKT3 produce sperm with diminished progressive motility, although sperm counts and other aspects of reproductive physiology remain unperturbed. Sperm from *Tekt3* null mice also have decreased forward progressive velocity and defective flagellar beat pattern similar to sperm from mice deficient for TEKT4; however, unlike TEKT4 null sperm, the percentage of *Tekt3* null sperm with progressive motility does not appear to drop significantly with incubation in culture medium (data not shown). We previously demonstrated that the drop in motility in TEKT4-null sperm following incubation is secondary to increased ATP consumption, presumably related to the ineffectual beat pattern in these sperm (Roy et al. 2007). Taken together, this suggests that TEKT3 might regulate sperm motility by a distinct mechanism, possibly by affecting sperm ultrastructure.

Between 30–50% of sperm produced by *Tekt*3 null males have structural defects typically associated with abnormal bending of the flagella between 90° and 180°. Additionally there

was thinning of the midpiece, and these sperm often would swim backwards with respect to the head orientation, suggesting a defect in force generation in the midpiece. This is reminiscent of the structural defects seen in *Sept4*-null mice (Ihara et al. 2005; Kissel et al. 2005) which have sperm with absence of the annulus, an enigmatic ultrastructural entity of unknown composition and function localized to the midpiece-principal piece junction (Fawcett 1970). In light of our findings, it would be interesting to study how annulus formation is affected in *Tekt3*-null mice.

The presence of both TEKT3 and TEKT4 in flagellar accessory structures led us to explore the phenotype of double knockout mice lacking both these proteins. Consistent with our previous report that TEKT4 null sperm exhibit a gradual decrease in progressive motility and excessive consumption of ATP after incubation > 1.5 hrs (Roy et al. 2007), shortly after extraction from the cauda (t = 0.5 hrs), no significant drop in sperm percent motility was seen in double knockout males compared to single *Tekt*3 null males. The fertility of *Tekt3*$^{-/-}$; *Tekt4*$^{-/-}$ males was found to be significantly reduced with respect to single *Tekt3*$^{-/-}$ males or WT males, suggesting a possible additive effect of the two mutations. The time-dependent decrease in progressive motility in *Tekt4* null sperm and the structural defects in *Tekt3* null sperm might additively reduce the fertility potential of these sperm, although evidence for this is lacking. Nevertheless, the double knockout males provide an interesting model to study the phenotype in a sensitized genetic background. The fact that the double knockout males can sire offspring suggests that other tektin family members, likely TEKT1, TEKT2 and/or TEKT5 may serve partially redundant roles with TEKT3. This possibility needs to be formally verified in future experiments.

In conclusion, TEKT3 appears to play a non-redundant and important role in maintaining progressive motility of sperm. Male mice lacking TEKT3 can still sire offspring, suggesting that the mechanism for hyperactivation is intact in these sperm and that the flagellar bending defects may not be critical in preventing the generation of hyperactivated motility during fertilization. Further studies are needed to clarify the mechanism of the structural defects seen in *Tekt3* null sperm and the functional relation of this protein with other members of the tektin family.

Materials and Methods

Cloning of the *Tekt3* gene

The *Tekt3* gene consists of 8 exons spanning 33.3 kb on mouse chromosome 11, and the human *TEKT3* gene resides in a syntenic region of human chromosome 17p12. A 452-bp *Tekt3* fragment from exon 2 was used to probe a 129S6/SvEv mouse genomic lambda Fix II phage library (Stratagene, La Jolla, CA) to clone the gene. Briefly, the library was plated onto NZCY plates at a density of approximately 500,000 plaques, transferred onto HyBond-N filters (Amersham Biosciences, Piscataway, NJ), and hybridized with [^{32}P]-dCTP random-primed probe in Church buffer at 65°C. Filters were washed and exposed overnight at −80°C. Two overlapping lambda FIX II recombinant clones (T3-1 and T3-3) extending from ~1.5 and 2.3 kb upstream of exon 2 to intron 4 of *Tekt3* were obtained after a secondary screen and were subcloned into the *Not* I site of pBluescript SK (+) vector (Stratagene, La Jolla, CA) for ease of manipulation.

Generation of *Tekt3* knockout mice

A 2,057 bp *Asp718-Hind*III fragment containing part of exon 2 and intron 2 was cloned into pGEM-7zf; the *Asp718* site was subsequently cleaved, filled-in with Klenow polymerase, and religated to create a *Sna*BI site. Flanking *Xba*I and *Sac*I sites were then used to subclone the 3′ arm into the p*Pgk1-HPRT1* vector (Matzuk et al. 1992). A 6.3 kb *Asp718-Asp718* 5′

arm containing parts of intron 1 and exon 2 was subcloned into the vector (Figure 1A) to create the targeting construct that after homologous recombination would insert into and delete 218 bp of exon 2 including the initiation ATG codon, replacing it with the *Pgk1-HPRT* cassette. We predicted that this construct would create a null allele for *Tekt3*.

After insertion of the *MC1-tk* (thymidine kinase) cassette, the construct was electroporated into HPRT-deficient AB2.2 mouse ES cells derived from 129S7/SvEvBrd-*Hprt*^{bm2} (129S7) strain, and ES cell clones were selected in M15 medium containing HAT (hypoxanthine, aminopterin and thymidine) and FIAU [1-(2′-deoxy-2′-fluoro-B-D-arabinofuranosyl)-5′-iodouracil] as described previously (Matzuk et al. 1992). Proper targeting was verified by Southern blot analysis (Figure 1B) using *Pvu*II-digested and *BamH*I-digested DNA and 5′ internal or 3′ external probes, respectively. Four correctly targeted ES cell clones, out of 186 clones screened (2.15% targeting efficiency), that carried the *Tekt3*^{tm1Zuk} mutation (herein called *Tekt3*$^{-}$) were expanded, and these mutant clones were injected into recipient C57BL/6J blastocysts to obtain several high-percentage chimeric males (estimated from coat color mosaicism) from two different ES cell clones (201A12 and 201F11). Chimeric males were bred to females of both the C57BL/6J (B6) and 129S5/SvEvBrd (129S5) strains to obtain mice heterozygous for the *Tekt3* targeted mutant allele (*Tekt3*^{tm1Zuk}; herein called *Tekt3*$^{-}$). Male and female F1 heterozygotes were intercrossed to produce *Tekt3* homozygous null mutant (*Tekt3*$^{-/-}$) male and female F2 progeny (Figure 1B–C).

Northern blot analysis

Northern blot analysis was performed on total testis RNA extracted from mice of different genotypes as indicated (Fig. 1C). Briefly, 15 μg of total RNA was electrophoresed and transferred onto nylon membranes. A cDNA fragment that encompasses exons 4–7 was labeled with [^{32}P]-dATP using the Strip-EZ kit (Ambion, Austin, TX) and used as a probe. The membrane was hybridized in UltraHyb buffer (Ambion), washed, and subjected to autoradiography. A mouse glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) cDNA probe was used as a loading control.

Fertility analysis

Mutant mice and control littermates were mated to wild-type females beginning at 42 days of age. One male and one female were housed in a single cage and maintained for the entire duration. The number of litters and pups per litter born over a 6-month period were recorded to estimate the mean litter size and the average litters born per month. All animal experiments were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee of Baylor College of Medicine.

Sperm motility analysis

Caudal spermatozoa from adult (6-wk old) mice were prepared in pre-warmed M16 medium. Briefly, cauda epididymides were minced in pre-warmed M16 medium (Sigma-Aldrich, St. Louis, MO), and sperm were allowed to swim out by incubation at 37°C under 5% CO_2 in air for 30 min. Sperm were collected by centrifugation at 650xg for 10 min, and adjusted to a concentration of 10^6/ml. For analysis of percent motility, 20 μl of an aliquot of sperm diluted to 10^6/ml was spotted onto a glass slide and covered with a 22×22mm cover slip. After allowing 2 min for the sperm to settle down, a total number of 100 sperm (both motile and immotile) were scored manually using differential interference contrast (DIC) optics and a 20X objective. The procedure was repeated twice for each sample and averaged.

Analysis of sperm morphology

For analysis of sperm morphology, aliquots of caudal spermatozoa were prepared as above in pre-warmed M16 medium and diluted to 10^5/mL. A drop of paraformaldehyde was added to immobilize the sperm, 3 μl-aliquots were transferred into 12 μl MicroCell fixed-depth chambers (Conception Technologies, San Diego, CA) for visualization, and imaging was performed with differential interference contrast optics at 40X magnification. At least 100 sperm were counted for each genotype, and both head and tail morphology were scored.

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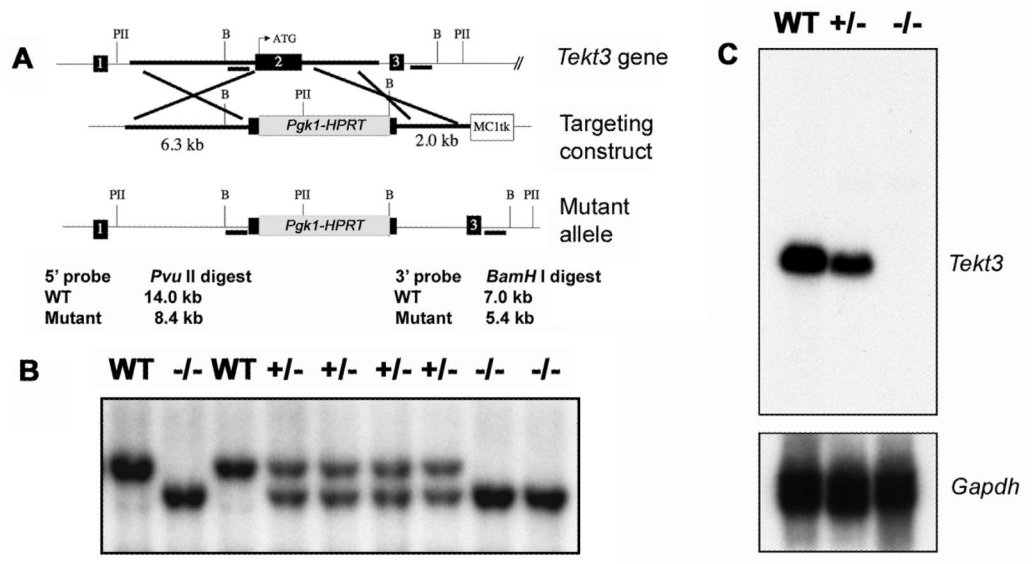

Figure 1. Targeted disruption and generation of *Tekt3*$^{-/-}$ mice

(A) *Tekt3* genomic locus and targeting vector for generation of a *Tekt3*-null allele. The *Pgk1-HPRT* cassette was used to delete 218 bp in exon 2 (containing the start codon) and homologous recombination was achieved in AB2.2 ES cells. 5′ and 3′ probes were used to distinguish wild-type and mutant alleles. (PII, *Pvu* II; BI, *BamH* I). (B) Southern blot analysis of *BamH* I-digested tail DNA from a litter of pups using the 3′ external probe. The probe detects a 7.0-kb wild-type (WT) allele and a 5.4-kb mutant allele. (C) Northern blot hybridization of total testis RNA from WT, *Tekt3* heterozygous (+/−), and *Tekt3* homozygous null (−/−) mice.

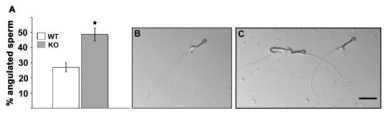

Figure 2. Structural defects in *Tekt3* null flagella

(A) Higher proportion of caudal spermatozoa from *Tekt3* null males exhibit flagellar bending at the annulus (48.7 ± 4.2%) compared to control littermates (27.0 ± 3.0%); *n*=7. (B) and (C) The flagellar bending in null spermatozoa (arrows) is typically 180° at the junction of the midpiece and principal piece. Values represented are mean ± SEM. (* $P < 0.01$; T-test).

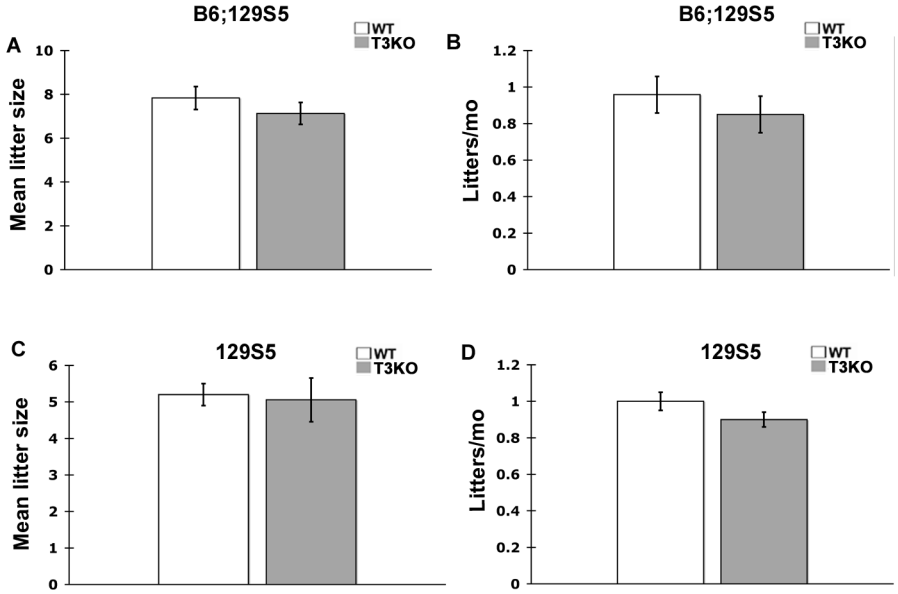

Figure 3. Fertility is maintained in *Tekt3* null males

(A) *Tekt3* null males on a mixed genetic background can sire offspring with normal litter size (7.13 ± 0.5 pups/litter); *n*=6. (B) Null males sire 0.85 ± 0.1 litters per month compared to 0.96 ± 0.2 litters per month for WT males. On an inbred 129S5 background, both litter size (5.05 ± 0.6 pups/litter) (C) and litters sired per month (0.9 ± 0.04) (D) are comparable to WT littermate controls; *n*=5. ($P > 0.05$; T-test).

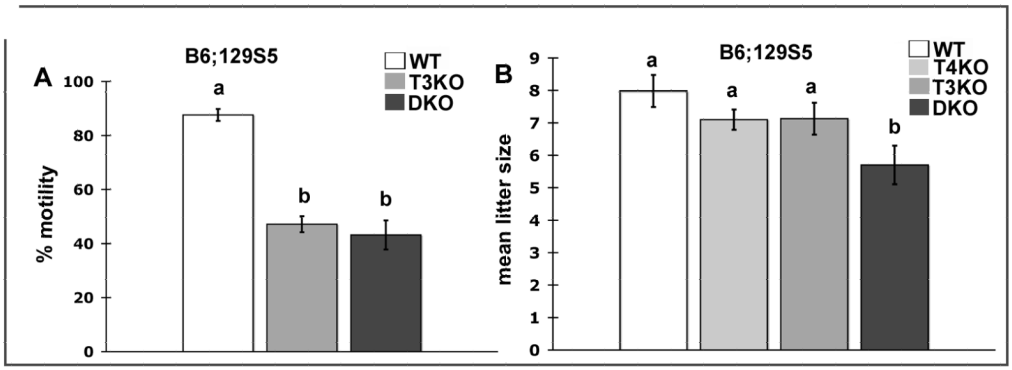

Figure 4. Reproductive physiology and fertility of *Tekt3*$^{-/-}$ *Tekt4*$^{-/-}$ double knockout males

(A) At t = 0.5 hrs, percent motility of caudal sperm from *Tekt3*$^{-/-}$; *Tekt4*$^{-/-}$ double knockout males is reduced to 43.2 ± 5.4, similar to *Tekt3*$^{-/-}$ males which have a mean percent motility of 47.2 ± 3.0% but significantly different from WT males (87.6 ± 2.6%). (B) *Tekt3*$^{-/-}$; *Tekt4*$^{-/-}$ males (DKO) are subfertile with a modest reduction in mean litter size (5.7 ± 0.6 pups/litter) that is significantly different from WT (7.98 ± 0.3 pups/litter) and *Tekt3*$^{-/-}$ males (T3KO) (7.13 ± 0.5 pups/litter) and *Tekt4*$^{-/-}$ (T4KO) (7.1 ± 0.3); *n*=6. Values are expressed as mean ± SEM. Test groups differing in the letters above the bars (a and b) have a statistically significant difference ($P < 0.01$; one-way ANOVA and Tukey-Kramer HSD test). WT, wild type; SKO, *Tekt3*$^{-/-}$; DKO, *Tekt3*$^{-/-}$; *Tekt4*$^{-/-}$.

Table 1

Reproductive physiology of *Tekt3*$^{-/-}$ mice

Genotype	Age	Testes weight, mg	Sperm count, × 10^7/ml	% motile sperm
WT	8–10 weeks	100.6 ± 1.6(*n*=3)	3.1 ± 0.5(*n*=3)	87.6 ± 2.6(*n*=5)
B6; 129S5-*Tekt3*$^{-/-}$	8–10 weeks	99.5 ± 1.2(*n*=6)	2.7 ± 0.2(*n*=3)	47.2 ± 3.0 *(*n*=6)
B6; 129S5-DKO	8–10 weeks	93.65 ± 1.14(*n*=5)	2.61 ± 0.1(*n*=5)	43.2 ± 5.4 *(*n*=5)

Values are mean ± SEM. Sperm counts and motility percentage were calculated by combining sperm from both cauda epididymides of mice at the ages indicated. WT, wild-type; DKO, *Tekt3*$^{-/-}$, *Tekt4*$^{-/-}$;

*$P < 0.0001$.