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Tex55 encodes a conserved putative A-kinase anchoring protein dispensable for male fertility in the mouse

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Running title: *Tex55* is not essential for male sexual reproduction

Summary sentence: The conserved putative A-kinase anchoring protein TEX55 is not essential for spermatogenesis and fertility in the mouse.

Keywords: spermatogenesis, spermiogenesis, male fertility, *Tex55*, TSCPA, 4930435E12Rik, C3orf30, AKAP

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23 **Dear Editor,**

24 Hundreds of genes are involved in human male sexual reproduction [1]. We previously
25 identified conserved genes for which expression patterns indicated a role in male fertility [2].
26 This group included *Tex55* (TSCPA, C3orf30 in humans), which was proposed to possess an
27 A-kinase anchoring protein (AKAP) activity [3]. This marks *Tex55* out as potentially
28 important, because several members of this protein family are required for normal
29 spermatogenesis [4, 5]. We first confirmed that *Tex55* mRNA is detectable in adult but not
30 fetal testis (E14.5) and that the transcript is absent in fetal (E14.5) ovary samples and nearly
31 undetectable in adult ovary; *Rpl13a* was used as a control (Figure 1A). Next, we generated a
32 gene deletion model using CRISPR/Cas9 in collaboration with the Institut Clinique de la
33 Souris (www.ics-mci.fr/en/; Phenomin project; see Supplemental Methods). We obtained
34 heterozygous mice harboring a mutant *Tex55* allele lacking the first coding exon (Figure 1B).
35 To generate the *Tex55* knockout mice ($Tex55^{-/-}$) we intercrossed $Tex55^{+/-}$ males and females.
36 $Tex55^{+/+}$ mice obtained via these crosses were used as controls. We validated the gene
37 deletion phenotype using an RT-PCR assay and observed *Tex55* mRNA expression in three
38 control testes. As expected, no mRNA was detected in three homozygous mutant testes;
39 *Rpl13a* was used as a control for the presence of mRNA (Figure 1C). Immunohistochemical
40 analysis of mouse TEX55 using a newly raised antibody against the mouse protein, revealed
41 strong signals located at elongated spermatids and residual bodies in wild type testis, while
42 no signal was detected in testis from homozygous mutant mice (Figure 1D). We subsequently
43 crossed three to four months old males with mature fertile females over a period of eight
44 months and scored the litter frequency and size. We found no significant difference for both
45 parameters between control and knockout males (Supplementary materials). This result
46 suggests that *Tex55* is dispensable for male fertility in the mouse. We further analysed testes
47 from wild-type, heterozygous and homozygous mutant mice and observed no differences in

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48 testicular weight and morphology (Figure 1E, F). Likewise, histological sections of adult
49 testes revealed no alterations in tubular structure, somatic or germ cell numbers and sperm
50 morphology (Figure 1G). We also detected no differences in the expression of the Leydig cell
51 marker protein StAR in the presence or absence of TEX55 (Figure 1G) indicating that, as
52 expected, the interstitial tissue is not affected either. Given that we detect neither mRNA nor
53 protein in homozygous mutant samples, we conclude that the gene deletion approach was
54 successful. It is, in any case, conceivable that the protein plays subtle roles in spermiogenesis
55 or sperm/egg interactions that our assays were unable to detect. Another explanation is
56 genetic redundancy. Since *Efcab10* is now considered to be a *Tex55* paralog in mouse and
57 human, it could provide an explanation for our result. However, three pieces of evidence
58 argue against *Efcab10* being functionally equivalent to *Tex55*. First, single-cell RNA-
59 Sequencing (scRNA-Seq) data show that while *Efcab10* is also expressed in spermatids, it
60 peaks earlier in germline development than *Tex55* (Supplementary Figure S1). Second, the
61 level of *Tex55*/*Efcab10* sequence conservation is extremely low (10.61% target identity;
62 3.67% query identity; see www.ensembl.org (Mouse GRCm38.p6). Third, EFCAB10 lacks
63 the critical domain for AKAPs, which means that the protein likely cannot compensate for
64 *TEX55*'s postulated major role (www.uniprot.org). Alternatively, genes such as *Cabyr*,
65 *Ropn1l* and *Spa17*, which show peak expression in round and elongated spermatids and
66 possess protein kinase regulatory subunit dimerization-anchoring domains, might be
67 functionally redundant with *Tex55* (Figure S1; see also www.proteinatlas.org). Moreover,
68 scRNA-Sequencing data reveal that all three genes are expressed in late stage round
69 spermatids, whereby *Spa17* even peaks at that stage (Figure S1). The known roles of these
70 genes are also in keeping with the idea that they might compensate for *Tex55*, since *Cabyr* is
71 involved in sperm capacitation [6], *Ropn1l* is important for sperm motility and integrity [7]
72 and *Spa17* was proposed to be involved in A-kinase anchoring [8]. We note that mutants

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73 lacking *Ropn1l* are fertile, mice are available for *Spa17* and engineered embryonic stem cells
74 were generated for *Cabyr* (www.mousephenotype.org), which marks them out as potentially
75 interesting candidates for double gene deletion experiments. Further mouse work involving
76 pair-wise crosses of *Tex55* mutants with mutants bearing null alleles of paralogous or
77 functionally analogous genes may reveal their redundant roles in male fertility.
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79 **Figure legend**

80

81 **Figure 1.** ***Tex55 expression and deletion***. (A) RT-PCR assay with testes and ovaries from
82 E14.5 embryos and adults. Molecular weight markers (MW). *Rpl13a* was used as a loading
83 control. (B) A schematic summarizes the wild-type (*Tex55*) and mutant ($Tex55^{DEx1}$) alleles. A
84 black line is DNA, black rectangles are coding exons. Exon numbers are shown. Single guide
85 RNA (sgRNA) positions are marked by red lines. Blue and green arrows symbolize PCR
86 primers used for genotyping. Dotted lines mark the deleted sequence. (C) RT-PCR assay;
87 lane 1 molecular weight markers (MW), lanes 2-4 wild type (+/+) and lanes 5-7 homozygous
88 mutant testes. Bands are annotated with gene names. (D) Images of an immunohistochemical
89 assay were processed using NDP.view 2 software. Wild-type (+/+) is compared to mutant (-/-
90). A black bar is 100μm. (E) Adult testes from wild-type (+/+, heterozygous (+/-) and
91 homozygous (-/-) mice are shown. (F) Testis/body weight ratio of wild type and $Tex55^{-/-}$
92 mice. (G) Images of histological sections (HE) from wild-type (+/+) and homozygous (-/-)
93 mutants are given. Images of an IHC assay for a Leydig cell marker (StAR) in wild-type and
94 homozygous mutants are shown. Black bars indicate 50 μm.

95

96 **Supplementary Figure S1.** ***Testicular expression of Tex55 and paralogous and potentially***
97 ***analogous genes***. scRNA-Seq expression data for five genes in testicular cell types are
98 shown. A color coded schematic outlines the scatter plot of data for peritubular cells (PT),
99 Sertoli cells (SE), spermatogonia (SG), spermatocytes (SC) and round spermatids at different
100 steps (rST).

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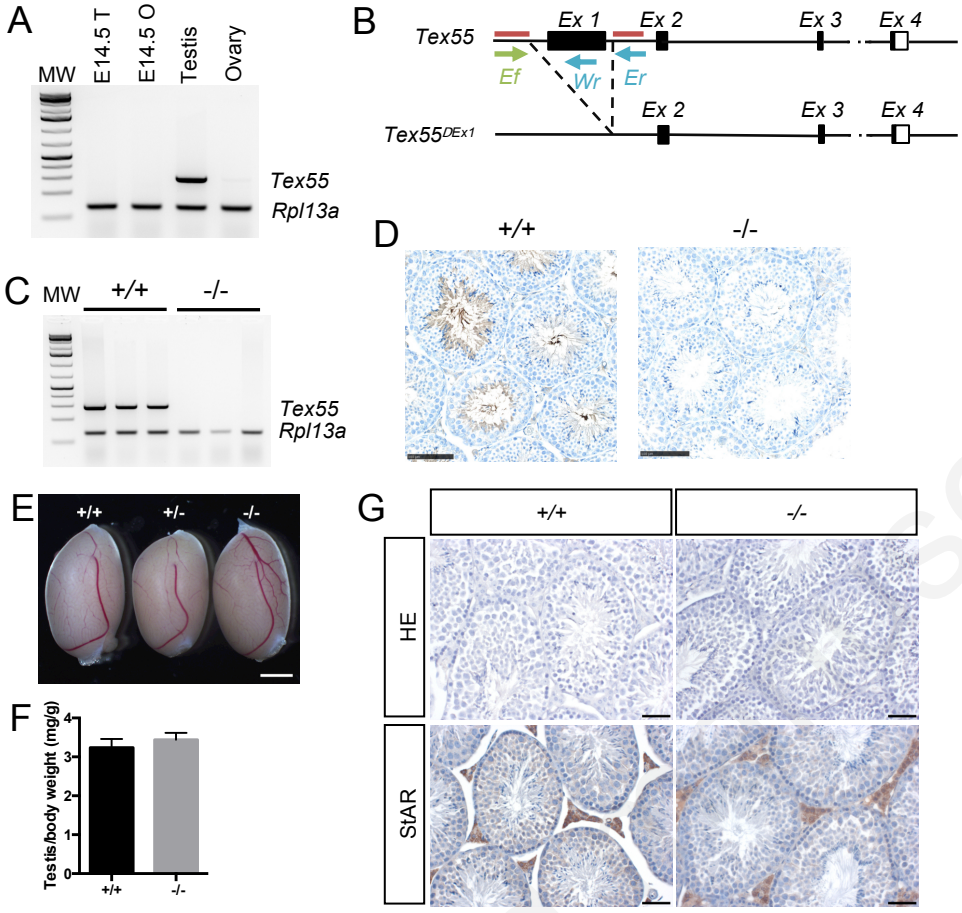

Figure 1

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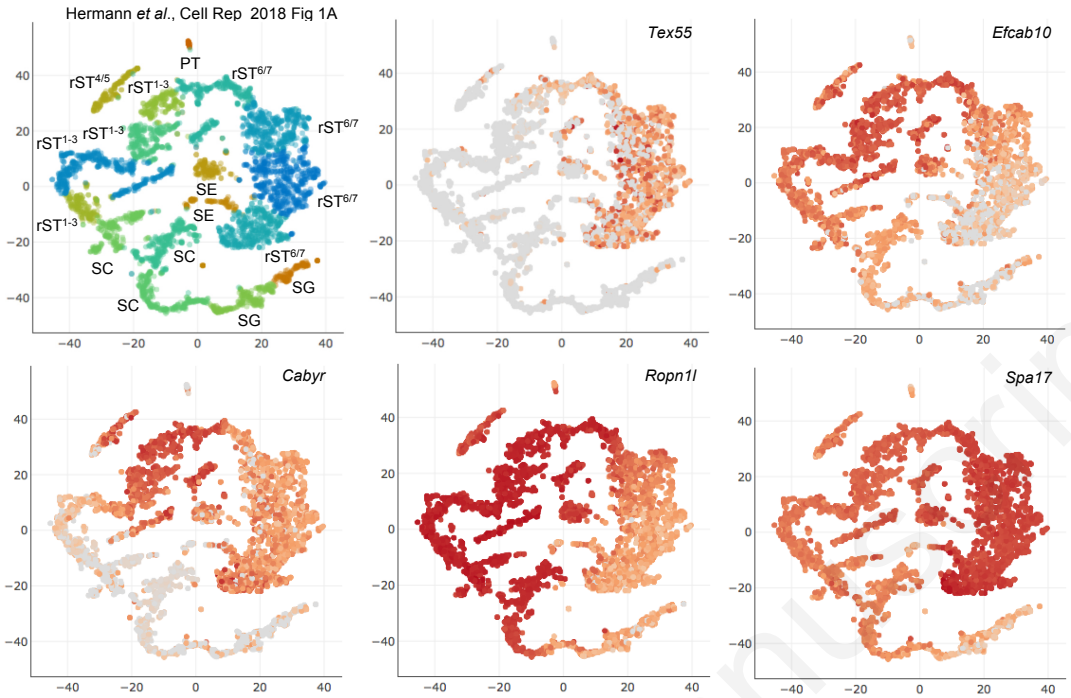

Supplemental Figure S1. scRNA-Seq expression data. A color-coded scatter plot in the top left corner shows the distribution of spermatogonia (SG), spermatocytes (SC), round spermatids at different stages (rST), Sertoli cells (SE) and peritubular cells (PT). Expression data for five genes are shown as indicated. Cells that express target genes are marked in shades of red.

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Supplementary Methods

Ethics Statement

Studies involving animals, including housing and care, method of euthanasia and experimental protocols were conducted in accordance with French legislation for Laboratory Animal Care, issued from the transposition of the European Directive 2010/63/UE. This Directive stipulates in article 1, alinea 2 that euthanizing laboratory animals for the sole purpose of investigating their tissues does not require prior authorization by an ethics committee. The animal facility was licensed by the French Ministry of Agriculture (C35-238-19). Animals were sacrificed using CO2, according to a method in annex IV of the directive. All experiments were supervised by Soazik P. Jamin who is licensed for animal experimentation by the French Ministry of Agriculture (92–299).

Tex55 mouse gene expression

Single-cell RNA-Sequencing (scRNA-Seq) data from mouse testicular cells were visualized using the ReproGenomics Viewer (http://rgv.genouest.org, [1]), [2].

RT-PCR assays

Adult and E14.5 ovaries and testes were dissected out from C57BL6 mice. The genomic DNA from the yolk sac was used to confirm the sex by PCR (see below). Total RNA was extracted using RNeasy Plus Mini kit (Qiagen, Courtaboeuf, France) for adult tissues or Arcturus Pico pure (ThermoFisher Scientific, Illkirch, France) for fetal gonads following the manufacturer's guidelines. Complementary DNAs (cDNAs) were synthesized with iScript RT Supermix (Biorad, Marnes-La-Coquette France) according to the manufacturer's instructions. Polymerase chain reaction (PCR) was set up with the four primers (*Tex55* and *Rpl13a*) and the Taq DNA polymerase (Qiagen). The amplification conditions were 5 minutes at 95°C, followed by 30 cycles of 94°C for 1 minute, 58°C for 1 minute, and 72°C for 1 minute, with a final 10 minute extension at 72°C. The primers we used are listed in Table 1.

Table 1. Oligonucleotide sequences of PCR primers used for RT-PCR

Name	DNA sequence
m-Tex55-714-s	5'-GCTCCCATCCAAAGTTTCAA-3'
m-Tex55-1010-a	5'-TCCACATAGCGAAGTGCAAC-3'
m-Rpl13a-L1	5'-CATGAGGTCGGGTGGAAGTA-3'
m-Rpl13a-R1	5'-GCCTGTTTCCGTAACCTCAA-3'

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Production of Tex55 heterozygous and homozygous mutant mice

The *Tex55* mutant mouse line was established at the Institut Clinique de la Souris - PHENOMIN (http://www.phenomin.fr) by a CRISPR/Cas9 approach [3]. The first coding exon of *Tex55* (www.ensembl.org; ENSMUSE00000354274) encoding the N-terminal 297 amino acids was deleted using an approach based on two pairs of sgRNAs. sgRNA identification numbers refer to the MIT specificity score (http://crispor.tefor.net/crispor.py; Table 2). 161 fertilized oocytes from C57BL/6N mice were microinjected with a mixture of four sgRNAs (12 ng/µl each) and spCas9 mRNA (25 g/µl). Among 16 pups (putative founders) analyzed by PCR six showed clear deletions of exon 1. Germline transmission of the mutated allele was achieved and a mouse line (Pus5945-13-DEL) was established, whereby all mice bore the same exon 1 deletion that was confirmed by junction sequencing. Finally, *Tex55*$^{+/-}$ mice were intercrossed to generate homozygous mutants. *Tex55*$^{+/+}$ mice were used as controls.

Table 2. sgRNA sequences

Name	**Sequence**
5'-sgRNA gR71	GGCAGGTTCAGGCAGCGTGG
5'-sgRNA gR85	AATCCAGATGGCCCACCTAT
3'- sgRNAs gR79	GGTCAAAGGGATGTGCCGGG
3'- sgRNAs gR84	GGTGGCATGTGTTAGTTATA

Mouse genotyping

Mouse genotyping was performed using DNA extracted from tail tips or yolk sac and a standard PCR assay. The primer sets 493Rik-Ef/493Rik-Er/493Rik-Wr were used to discriminate *Tex55*$^{+/+}$, *Tex55*$^{+/-}$ and *Tex55*$^{-/-}$ genotypes and the primer sets mZFY-1A/mZFY-1B were used to identify male and female fetuses. In post-natal mice, the genotyping was repeated twice at weaning and at the time of collection. Oligonucleotide primer sequences are shown in Table 3.

Table 3. Oligonucleotide sequences of PCR primers used for mouse genotyping

Name	**DNA sequence**
493Rik-Ef	5'-CTGTTCTCACTGGATCTTCCTGTTG-3'
493Rik-Er	5'-GATCCTTCCTGAGTGGCCTGAG-3'
493Rik-Wr	5'-GCCAATGGACGAGCAAGTATGA-3'
mZFY-1A	5'-CCTATTGCATGGACTGCAGCTTATG-3'
mZFY-1B	5'-GACTAGACATGTCTTAACATCTGTCC-3'

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Production of TEX55 polyclonal antibodies

The rabbit polyclonal antibodies against mouse TEX55 were produced by ProteoGenix (Schiltigheim, France). Two rabbits were immunized with two peptides including the peptide TC-16 (TDTNDYRETGQRLQPC) that was conjugated to keyhole limpet hemocyanin (KLH) as a carrier molecule. The rabbits were boosted for 70 days and the antibodies were titrated by ELISA (Table 4) and affinity purified against the antigen according to standard protocols. The purified antibody was stored at -80°C in PBS, 0.02% sodium azide, pH 7.4.

Table 4. ELISA titration for TEX55 antibodies

Dilution	**Blank**	**Negative**	**1:1000**	**1:4000**	**1:16000**	**1:32000**	**1:64000**
Rabbit 1	0.057	0.055	1.925	1.496	1.025	0.685	0.364
Rabbit 2	0.061	0.052	1.968	1.514	1.174	0.752	0.418

Testicular morphology

Testes from one year old males were dissected, weighed and observed under a stereo microscope Zeiss SteREO Discovery.V8 connected to an AxioCam IIc3 camera (Zeiss, France).

Immunohistochemical analysis of TEX55 protein expression and localisation

Immunohistochemistry experiments were performed on sections of eight week old mouse testes that were fixed in 4% paraformaldehyde and embedded in paraffin. Paraffin-embedded tissue was cut at 4 µm, mounted on positively charged slides and dried at 58°C for 60 minutes. TEX55 immunohistochemistry on mouse samples was performed by the Biosit H2P2 platform on the Discovery Automated IHC Stainer using the Ventana DABMap detection kit (Ventana Medical Systems, Tucson, USA). Following deparaffination with Ventana EZ Prep solution at 75 °C for 8 minutes, antigen retrieval was performed using Tris-based buffer solution CC1 (Ventana Medical Systems) at 95°C to 100°C for 36 minutes. Endogen peroxidase was blocked with Inhibitor-D 3% H2O2 (Ventana Medical Systems) for 8 minutes at 37°C. After rinsing with reaction buffer (Ventana Medical Systems), slides were incubated at 37°C for 60 minutes with TC-16 antibody (Proteogenix, 1:1000). After rinsing, signal enhancement was performed using the DABMap kit (Ventana Medical Systems) and the slides were incubated with the secondary antibody (biotinylated horse anti-rabbit) for 32 minutes. Slides were then counterstained for 16 minutes with hematoxylin and four minutes with bluing reagent before they were rinsed. After removal from the instrument, slides were manually dehydrated and a cover slip was placed on them. The slides were scanned and images were processed using NDP.view 2 software (gimage correction 1.5, brightness 110% and contrast 115%; Hamamatsu, Japan).

StAR immunochemistry on mouse testes was performed as follows. After antigen retrieval in citrate buffer, the endogenous peroxidase was inactivated with H_2O_2. The slides were then blocked in 10% BSA for one hour and the primary antibody (StAR, CST 8449, 1:200, Cell Signaling Technology) was incubated overnight at 4°C. After rinsing with PBS, the biotinylated secondary antibody (goat anti-rabbit, Dako, 1:500) was incubated for two hours at room temperature. The Vectastain ABC complex (Eurobio, Les Ulis, France) was used to amplify the signal and the reaction was revealed with DAB Quanto (Fisher Scientific) for one

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minute. Histological sections were examined using an AxioImager microscope equipped with an AxioCam ICc1 camera and ZEN v.2.3 Blue edition (Zeiss, France).

Assay for male fertility

The control and knockout males were bred with wild-type females for a period of six months and the total number of pups was recorded.

Table 5. Mating assay of *Tex55* mutant versus wild type mice.

Genotype	No of males	No of pups	No of litters	average litter size	standard deviation	mating period
+/+	2	96	14	7.14	3.55	32 weeks
-/-	4	180	29	6.21	2.88	32 weeks

Statistical analysis

Prism 6 (GraphPad Software, San Diego, USA) was used to run the statistical tests and results showing a p-value <0.05 were considered to be statistically significant. A two-tailed student t-test was performed and the graphs display the mean standard deviation.

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