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Normal spermatogenesis in *Fank1* (fibronectin type 3 and ankyrin repeat domains 1) mutant mice

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Background. The fibronectin type 3 and ankyrin repeat domains 1 gene, *Fank1*, is an ancient, evolutionarily conserved gene present in vertebrates. *Fank1*-knockdown mice have oligospermia caused by an increase in apoptotic germ cells. In this study, we investigated the in vivo function of *Fank1.* **Methods.** In this study, we generated *Fank1*-knockout mice using the CRISPR/Cas9 system. We then investigated the phenotype and in vivo function of *Fank1*. Testes and epididymis tissues were analyzed by histological and immunofluorescence staining. Apoptotic cells were analyzed in TUNEL assays. Fertility and sperm counts were also evaluated. The GTEx database were used to assess gene expression quantitative trait loci (eQTL) and mRNA expression of candidate genes and genes neighboring single nucleotide polymorphisms was analyzed by quantitative RT-PCR. **Results.** In contrast to the *Fank1*-knockdown model, no significant changes in epididymal sperm content and the number of apoptotic cells were observed in *Fank1*-/- homozygotes. In addition, a different pattern of Dusp1, Klk1b21 and Klk1b27 mRNA expression was detected in *Fank1*-knockout testis. These results reveal differences in the molecular changes between *Fank1*-knockdown mice and *Fank1* -knockout mice and provide a basic resource for population genetics studies.

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Abstract

Background. The fibronectin type 3 and ankyrin repeat domains 1 gene, *Fank1*, is an ancient, evolutionarily conserved gene present in vertebrates. *Fank1*-knockdown mice have oligospermia caused by an increase in apoptotic germ cells. In this study, we investigated the in vivo function of *Fank1*.

Methods. In this study, we generated *Fank1*-knockout mice using the CRISPR/Cas9 system. We then investigated the phenotype and in vivo function of *Fank1*. Testes and epididymis tissues were analyzed by histological and immunofluorescence staining. Apoptotic cells were analyzed in TUNEL assays. Fertility and sperm counts were also evaluated. The GTEx database were used to assess gene expression quantitative trait loci (eQTL) and mRNA expression of candidate genes and genes neighboring single nucleotide polymorphisms was analyzed by quantitative RT-PCR.

Results. In contrast to the *Fank1*-knockdown model, no significant changes in epididymal sperm content and the number of apoptotic cells were observed in *Fank1*-/- homozygotes. In addition, a different pattern of Dusp1, Klk1b21 and Klk1b27 mRNA expression was detected in *Fank1*-knockout testis. These results reveal differences in the molecular changes between *Fank1*-knockdown mice and *Fank1*-knockout mice and provide a basic resource for population genetics studies.

Key words: Fank1; male infertility; gene knockout; spermatogenesis.

Introduction

Genetic studies are widely used for identification of susceptibility loci in human disease (Johnson & O'Donnell 2009). Mouse models of gene editing are indispensable for investigations of gene function in vivo. However, the development of genetic research is restricted by the lack of progress in our understanding of gene function. Thus, large-scale knockout programs have been initiated to mutate all protein-encoding genes in the mouse (Collins et al. 2007; Skarnes et al. 2011). The CRISPR/Cas9 system has been used to target genomic loci in mammalian studies (Li et al. 2013; Mali et al. 2013; Shen et al. 2013; Wang et al. 2013), and gene knockout mice have become more commonly used in genetic studies in mice. To date, 1503 human diseases with one or more mouse models have been recorded in the Mouse Genome Informatics database (Smith et al. 2018).

The fibronectin type 3 and ankyrin repeat domains 1 gene (*Fank1*) is an ancient, evolutionarily conserved gene present in vertebrates and expressed from the meiosis phase to the haploid phase of spermatogenesis in the testis (Zheng et al. 2007). As a DNA binding protein, FANK1 recognizes the DNA sequence AAAAAG, and is implicated as a transcription factor during spermatogenesis (Dong et al. 2014). In a study of short-hairpin RNA (shRNA)-based knockdown transgenic mouse model, a reduction in sperm number and an increase in apoptotic germ cells were observed (Dong et al. 2014).

In recent years, gene editing mouse models have played an indispensable role in elucidating gene function in vivo. A number of studies have revealed phenotypic differences between

knockout (i.e., mutants) and knockdown (i.e., RNA interference) models (El-Brolosy & Stainier 2017). These phenotypic differences could be caused by gene expression compensation in mutants or off-target effects of the knockdown reagents (El-Brolosy & Stainier 2017). Both models have distinct advantages and limitations for the elucidation of gene function. However, gene knockout may be a better model of human genetic mutations.

Thus, in this study, we have generated a *Fank1*-mutant model using the CRISPR/Cas9 system to investigate the phenotype and in vivo function of *Fank1*.

Materials and Methods

Gene expression quantitative trait loci (eQTL) analysis

The publicly available RNA-seq and genotyping data of human samples from the Genotype-Tissue Expression project (GTEx, http://commonfund.nih.gov/GTEx/index) were used to assess gene expression quantitative trait loci (eQTL) for mRNA expression of candidate genes and genes neighboring single nucleotide polymorphisms (SNP). Statistical analysis was performed using GTEx (2013).

Generation of Fank1-knockout mice by CRISPR/Cas9

The mice were maintained and used in experiments according to the guidelines of the Institutional Animal Care and Use Committee of Nanjing Medical University (China). Cas9 mRNA and single guide RNAs (sgRNAs) were produced and purified as previously described

(Zhang et al. 2017). In brief, the Cas9 plasmid (Addgene, Watertown, MA, USA) was linearized by restriction enzyme digestion with *Age*I and then purified using a MinElute PCR Purification Kit (Qiagen, Duesseldorf, Germany). Cas9 mRNA was produced by in vitro transcription using a mMESSAGE mMACHINE T7 Ultra Kit (Ambion, Austin, TX, USA) and purified using a RNeasy Mini Kit (Qiagen, Duesseldorf, Germany) according to the manufacturer's instructions. The sgRNAs were designed on the basis of exon2 of *Fank1*. The target sequence of sgRNA was 5'-GTGGCTTCGGTTCTCCATTGAGG-3' and 5' -GTCACCTTGCCCACAACAGGAGG-3', respectively. The sgRNA plasmid was linearized with *Dra*I and then purified using a MinElute PCR Purification Kit (Qiagen, Duesseldorf, Germany). sgRNA was produced using the MEGA shortscript Kit (Ambion, Austin, TX, USA) and purified using the MEGA clear Kit (Ambion, Austin, TX, USA) according to the manufacturer's instructions. Cas9 mRNA and sgRNA were injected into mouse zygotes obtained by mating of wild-type C57BL/6 males with C57BL/6 superovulated females.

Histological analysis

Mouse testes or epididymis from at least three mice for each genotype. The tissues were fixed in modified Davidson's fluid for up to 24 h and stored in 70% ethanol. The samples were then dehydrated through a graded ethanol series and embedded in paraffin. Tissue sections (thickness 5 mm) were prepared and mounted on glass slides. After deparaffinization, slides were stained with periodic acid Schiff (PAS) for histological analysis. Apoptotic cells in testis were detected using the terminal deoxynucleotidyl transferase dUTP nick end-labeling (TUNEL) assay

(Vazyme, Nanjing, China) according to the manufacturer's instructions.

Immunofluorescence analysis

Testis sections were deparaffinized, rehydrated and boiled for 15 min in sodium citrate buffer for antigen retrieval. Sections were blocked in antibody dilution buffer (5% bovine serum albumin (BSA) in phosphate-buffered saline [PBS (137 mM NaCl; 2.7 mM KCl; 10 mM Na2HPO4 and 2 mM KH2PO4)]) for 2 h at room temperature, followed by an overnight incubation at 4°C with primary antibodies (list in Table S2). Three washes with PBST (0.05% Tween 20 in PBS) were performed prior to incubation with secondary antibody (list in Table S2) for 2 h at room temperature. Finally, sections were incubated with Hoechst 33342 (Invitrogen, Carlsbad, CA, USA) for 5 min and then mounted. Images were captured using an LSM800 confocal microscope (Carl Zeiss AG, Jena, Germany).

Fertility test

Adult males of each genotype were subjected to fertility tests. Each male was mated with three wild-type C57BL/6 females, and the vaginal plug was checked every morning. The dates of birth and number of pups in each litter were recorded.

Computer-assisted sperm analysis

Mature sperm were obtained by making small incisions throughout the cauda epididymis,

followed by extrusion and suspension in human tubal fluid (HTF) culture medium (In Vitro Care, Frederick, MD, USA). Sperm samples (10 μl) were used for computer-assisted semen analysis (CASA) (Hamilton-Thorne Research, Inc., Beverly, MA, USA). Motile sperm number, progressive sperm number and sperm concentration for the experimental and control groups were measured and analyzed.

Quantitative RT-PCR assay

Total RNA was extracted from the samples using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The concentration and purity of RNA were determined according to NanoDrop 2000C (Thermo, Waltham, MA, USA)absorbance at 260/280 nm. Total RNA (1 μg) was reverse transcribed using a HiScript II Q RT SuperMix (Vazyme, R222, Nanjing, China) according to the manufacturer's instructions. The cDNA(dilution 1:4) was then analyzed by quantitative RT-PCR in a typical reaction of 20 μl containing 250 nmol/l of forward and reverse primers, 1 μl cDNA and AceQ qPCR SYBR Green Master Mix (Vazyme, R222, Nanjing, China). The reaction was initiated by preheating at 50°C for 2 min, followed by 95°C for 5 min and 40 amplification cycles of 10 s denaturation at 95°C and 30 s annealing and extension at 60°C. Gene expression was normalized to 18 s within the log phase of the amplification curve. The primer sequences are listed in supplementary Table S2.

Results

Association of 54 SNPs with Fank1 expression in humans

Genome variants including common SNPs contribute to gene expression changes and are associated human disease. To investigate the association of the genotypes of the SNPs with *Fank1* mRNA expression, eQTL of *Fank1* and relative SNPs. The eQTL data revealed lower *Fank1* mRNA expression levels in testicular subsets with homozygous genotypes of 54 SNPs compared with that of the homozygous reference (Table S1 and Figure 1).

Fank1-/- mice are fertile and have normal spermatogenesis

To confirm the in vivo function of *Fank1*, we generated *Fank1* mutant mice using the CRISPR/Cas9 system and a 70-bp deletion of Exon 2 (Figure 2A). *Fank1*-/- mice were viable and showed normal development. Intercrossing of *Fank1*+/- mice produced offspring of normal litter size at the predicted Mendelian and sex ratios. Similar to the *Fank1*-knockdown model, *Fank1*-/- males were fertile (Figure 2B). In adult *Fank1*-/- mice, the testes and epididymis were similar in size to those of the wild-type mice (Figure 2C). However, in contrast to the *Fank1*-knockdown model, histological analysis revealed the presence of spermatogenic cells in the seminiferous tubules of adult *Fank1*-/- mice (Figure 3 and Figure 4). Furthermore, compared with the wild-type mice, there were no significant differences in the morphology of *Fank1*-/- spermatozoa found in the cauda epididymides (Figure 5A, B and C). The whole epididymal sperm content and the average numbers of motile sperm were unaffected in homozygotic male mice (Figure 5D, E and F). TUNEL analysis of testicular sections revealed that both the number of apoptotic cells per tubule and the number of tubules containing apoptotic cells were unaffected in homozygotes (Figure 6).

Expression changes in Fank1-/- testis are not consistent with those of Fank1-knockdown mice

It was reported that Dusp1, Klk1b21 and Klk1b27 were overexpressed in *Fank1*-knockdown mice and may be direct targets of Fank1 (Dong et al. 2014). However, in *Fank1*-/- testis, a reduction of Klk1b21 and Klk1b27 mRNA was detected but no increase in Dusp1 transcripts (Figure 7). These results reveal differences in the molecular changes of *Fank1*-knockdown and *Fank1*-knockout mice.

Discussion

In this study, we found that *Fank1* mRNA expression levels correlated negatively with the homozygous SNPs genotypes based on comparison with the GTEx database. This phenomenon was not detected in studies of another testicular-specific gene *Pnldc1*, which is an evolutionarily conserved gene and essential for male fertility (Zhang et al. 2017). One explanation for this result may be that Fank1 is dispensable for human reproduction. Thus, these genetic variants were retained during evolution.

The amino terminus of FANK1 contains a fibronectin type III (FNIII) domain and the carboxyl terminus includes five ankyrin repeats (ANKs), which contain binding sites for DNA, heparin and the cell surface (Skorstengaard et al. 1986). Ankyrin repeats have been found in proteins of diverse function, such as transcriptional initiators and cell-cycle regulators (Skorstengaard et al. 1986). Lack of FANK1 leads to a reduction in Klk1b21 and Klk1b27

transcripts, suggesting that FANK1 is a transcriptional factor, although transcriptional changes may also be induced as a compensatory mechanism, thus accounting for the absence of fertility changes in *Fank1*-/- males. In this study, we found no paralog of *Fank1* which may compensate for the *Fank1* mutation. Thus, we cannot explain the mechanisms underlying the phenotypic differences between the *Fank1* knockout and *Fank1* knockdown mouse models. Nevertheless, the *Fank1* knockout mouse model generated in this study provides a basic resource for studies of population genetics, and also expands our understanding of the differences in animal models established using different approaches.

Acknowledgments

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Figure 1(on next page)

The association of the genotypes of the SNPs with *Fank1* mRNA expression

eQTL analysis of *Fank1* mRNA expression level for genotypes Homo Ref, Het and Homo Alt at rs3812681, rs12770063, rs35267061 and rs61863578.

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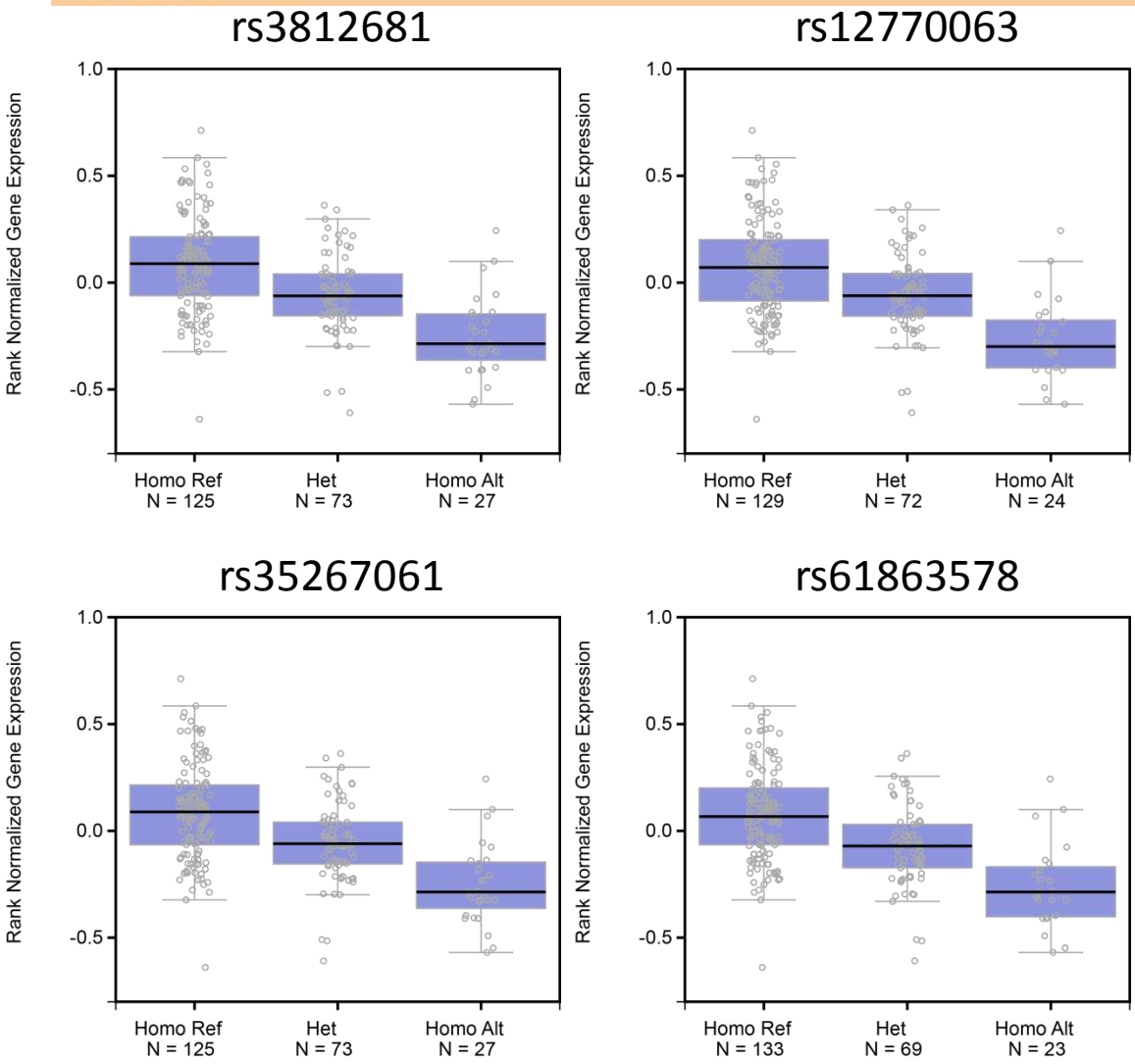

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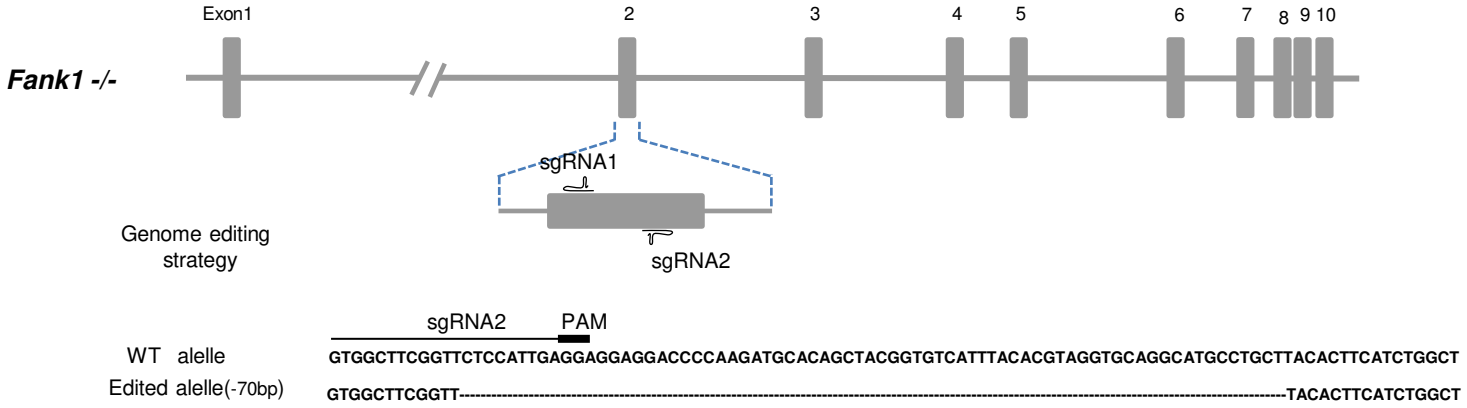
Figure 2(on next page)

Fank1-/- mice are fertile.

(A) Schematic diagram of CRISPR/Cas9 targeting strategy; (B) Average pups per litter of wild-type and *Fank1*-/- mice; (C) Testis and epididymis from wild-type and *Fank1*-/- adult mice; (C) Average testis weight/body weight. g

A



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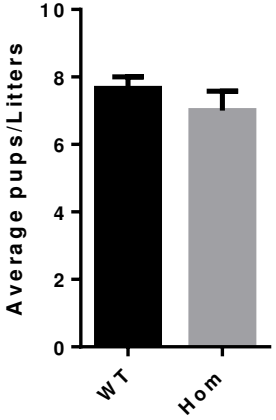

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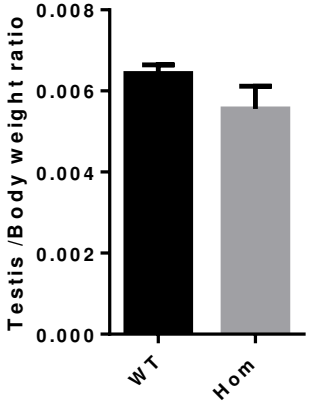

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Figure 3(on next page)

Spermatogenesis appears normal in *Fank1*-/- mice.

(A) Sections of periodic acid Schiff-stained testis from wild-type and *Fank1*-/- mice; (B) Sections of hematoxylin and eosin-stained cauda epididymis from wild-type and *Fank1*-/- mice.

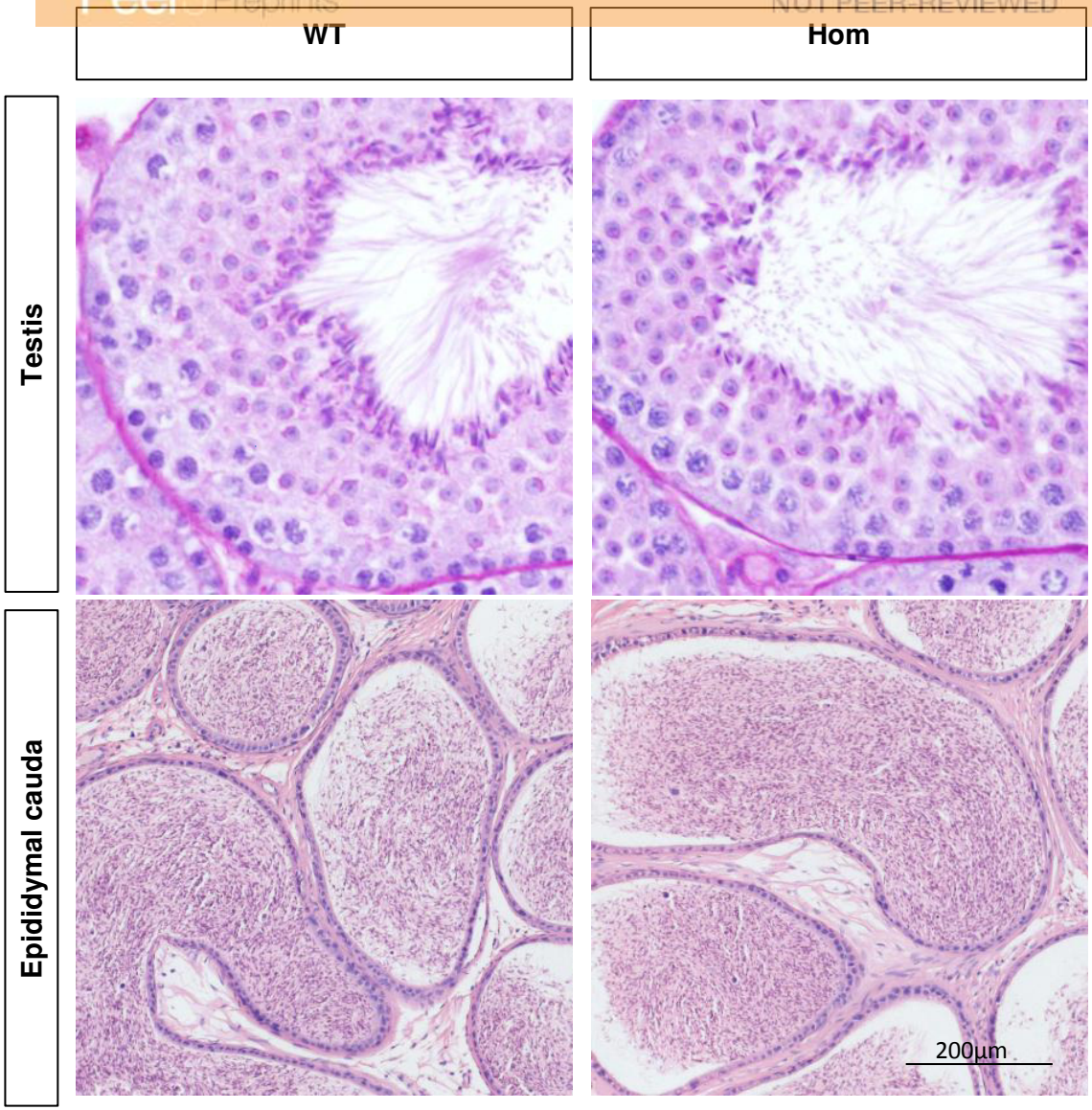

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Figure 4(on next page)

Spermatogenic markers appear normal in *Fank1*-/- mice.

The spermatogonia (PLZF), spermatocytes (γ-H2AX), spermatids (PNA) and Sertoli cells (Sox9) are comparable in testis sections from both wild-type and *Fank1*-/- mice.

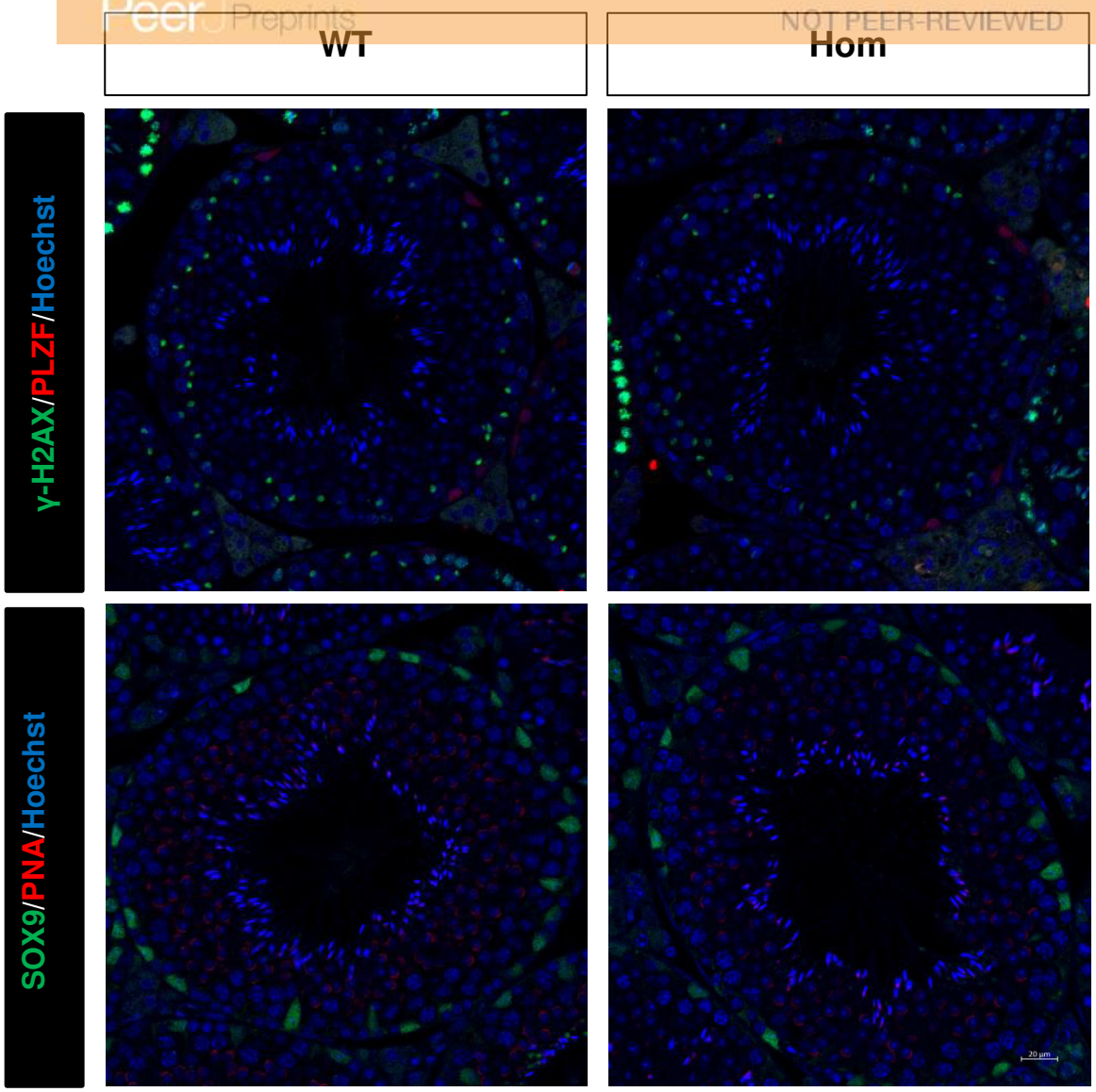

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Figure 5(on next page)

Spermatozoa appear normal in *Fank1*-/- mice.

(A) Hematoxylin and eosin-stained spermatozoa from wild-type and *Fank1*-/- mice; (B) Fluorescence detection of AC-tubulin, PNA from wild-type and *Fank1*-/- spermatozoa; (C) Cauda epididymal sperm contents from wild-type and *Fank1*-/- mice; (D) Average rate of motile sperm and (E) progressive sperm from wild-type and *Fank1*-/- mice; (F) Abnormal epididymal sperm count from wild-type and *Fank1*-/- mice.

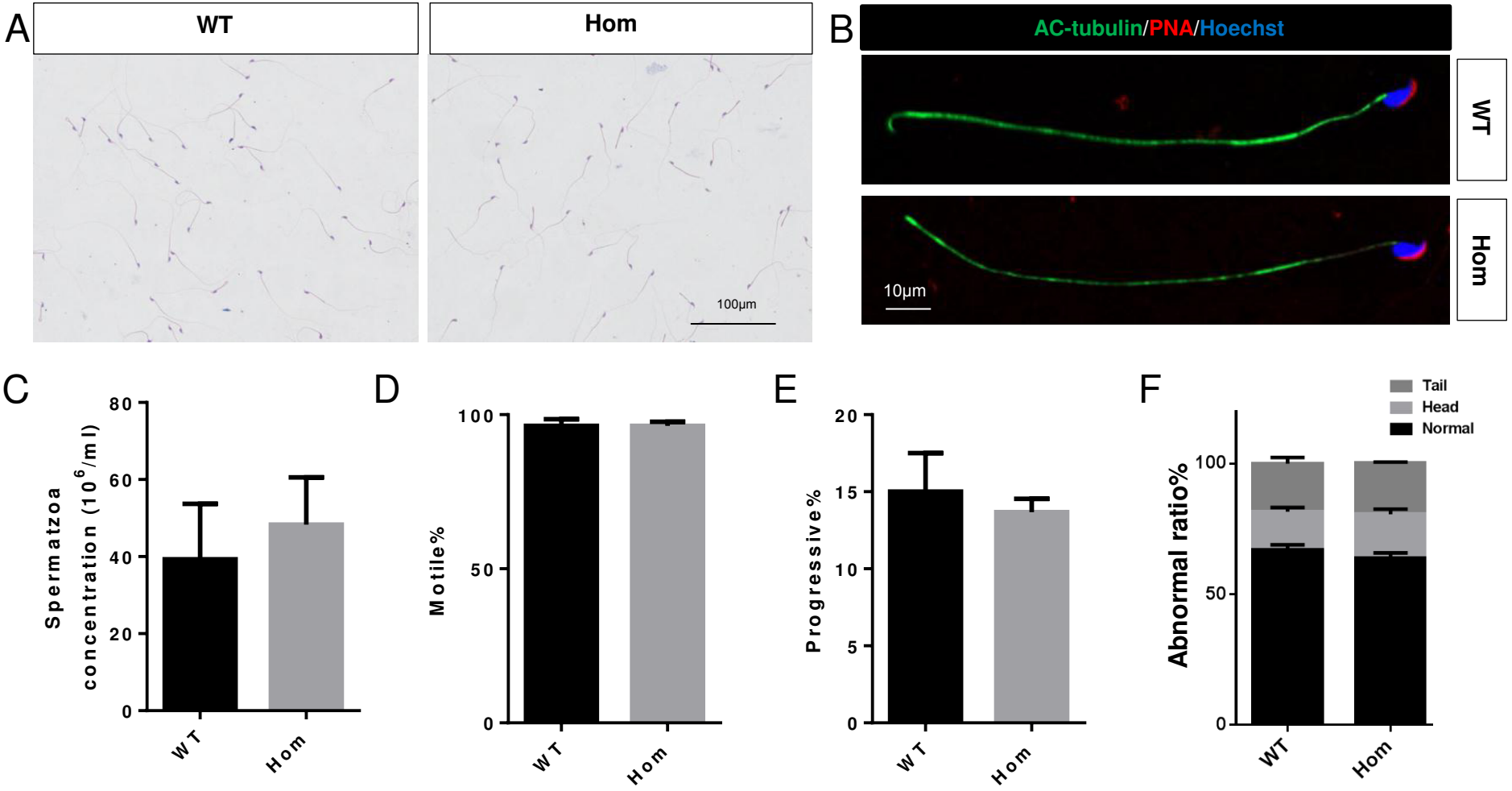

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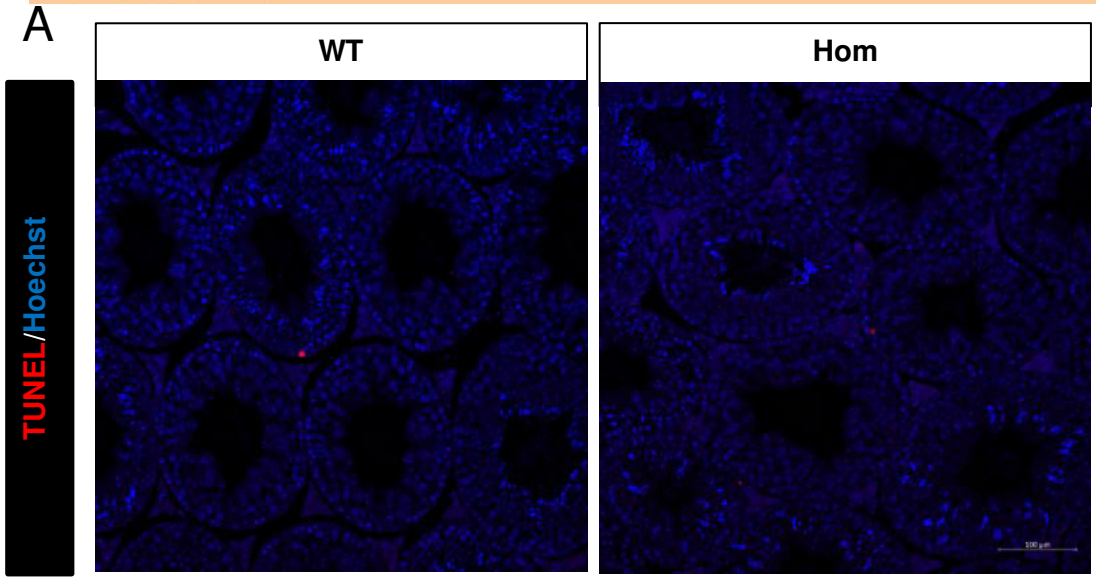
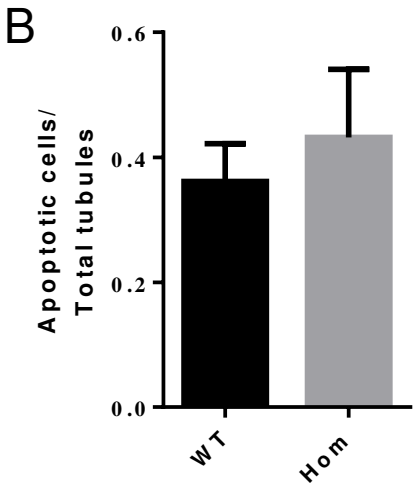
Figure 6(on next page)

Apoptotic cells are not increased in *Fank1*-/- testes.

(A) TUNEL assay of wild-type and *Fank1*-/- testes; (B) Average apoptotic cells per seminiferous tubule; (C) Average apoptotic cells per seminiferous tubules.





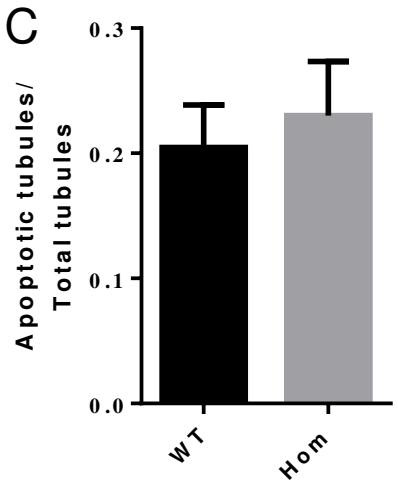


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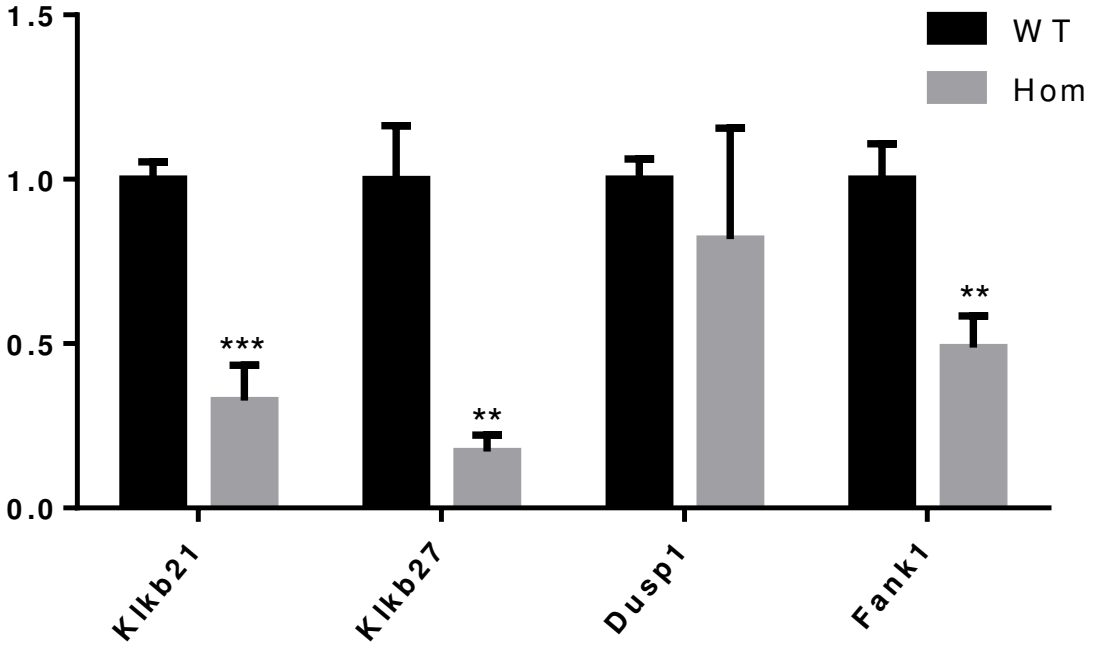
Figure 7 (on next page)

Expression changes in Fank1-/- testis.

Quantitative RT-PCR analysis of *Dusp1*, *Klk1b21*, *Klk1b27* and *Fank1* in testis.



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