Original Article

Homeodomain protein HOMEZ is dispensable for male fertility in mice

Qiuling Yue[1#], Lina Yu[1#], Wenwen Liu[1#], Jidong Zhou[1], Xuechun Hu[2], Yixun Liu[2], Xue Zhou[1], Limin Wu[3], Bo Xu[3], Xianhong Tong[3], Xiaohua Jiang[3], Guijun Yan[1], Shun Bai[3]

[1]Center for Reproductive Medicine and Obstetrics and Gynecology, Nanjing Drum Tower Hospital, Nanjing University Medical School, Nanjing, China; [2]Department of Urology, The First Affiliated Hospital of USTC, Division of Life Sciences and Medicine, University of Science and Technology of China, Hefei, China; [3]Reproductive and Genetic Hospital, The First Affiliated Hospital of USTC, Division of Life Sciences and Medicine, University of Science and Technology of China, Hefei, China

Contributions: (I) Conception and design: S Bai, Q Yue; (II) Administrative support: S Bai, Q Yue, G Yan, X Jiang; (III) Provision of study materials: S Bai, L Yu, Q Yue; (IV) Collection and assembly of data: L Yu, W Liu, J Zhou, Q Yue; (V) Data analysis and interpretation: S Bai, L Yu, X Hu, Y Liu, X Zhou, L Wu, B Xu, X Tong, Q Yue; (VI) Manuscript writing: All authors; (VII) Final approval of manuscript: All authors.

[#]These authors contributed equally to this work.

Correspondence to: Shun Bai. Reproductive and Genetic Hospital, The First Affiliated Hospital of USTC, Division of Life Sciences and Medicine, University of Science and Technology of China, Hefei 230001, China. Email: shunbai@ustc.edu.cn; Guijun Yan. Center for Reproductive Medicine and Obstetrics and Gynecology, Nanjing Drum Tower Hospital, Nanjing University Medical School, Nanjing 210008, China. Email: yanguijun55@163.com; Xiaohua Jiang. Reproductive and Genetic Hospital, The First Affiliated Hospital of USTC, Division of Life Sciences and Medicine, University of Science and Technology of China, Hefei 230001, China. Email: biojxh@ustc.edu.cn.

Background: Homeodomain (HD) proteins contain an evolutionarily conserved helix-turn-helix (HTH) DNA-binding motif and act as transcription factors to control gene expression. A previous study showed that the HD gene *Homez* is highly enriched in adult testes. However, the role of HOMEZ in spermatogenesis and male fertility remains unknown.

Methods: Using CRISPR/Cas9 technology, *Homez* mutant mice were generated and performed histological, immunofluorescence, quantitative reverse transcription-polymerase chain reaction (qRT-PCR), Western blot and mating assays to analyze the phenotype of *Homez* mutants.

Results: Molecular phylogenetic analyses indicated that the HOMEZ is evolutionarily conserved among mammalian species. qRT-PCR and Western blot analyses showed that *Homez* is highly expressed in the testis, with a relatively increased expression trend during spermatogenesis. *Homez* mutant males were viable and showed no differences in body and testis weight compared to their wild-type. In addition, mating between *Homez* mutant males and wild-type females produced normal litter sizes. Moreover, histopathology detected complete spermatogenesis in the seminiferous tubules and mature spermatozoa in the epididymides from *Homez* knockout males. Furthermore, significantly increased transcription of three *Zbx* genes were found in *Homez* mutatnt testes compared with wild-type testes.

Conclusions: *Homez* knockout mice are fertile and are not essential for germ cell development. These findings could prevent unnecessary duplicative work by other groups.

Keywords: HOMEZ; spermatogenesis; male fertility

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Introduction

Spermatogenesis is a complex and highly morphological change process that involves spermatogonial proliferation and differentiation (mitotic divisions), meiotic divisions and spermiogenesis, including the formation of flagella, condensation of the nucleus, and expansion of mitochondria (1). The accurate regulation of gene expression by transcription factors has been identified to play an important role in mammalian spermatogenesis (2). Defects in these proteins may induce abnormal sperm parameters and male infertility.

Homeodomain (HD) proteins contain an evolutionarily conserved helix-turn-helix (HTH) DNA-binding motif and act as transcription factors to control gene expression (3). Coordinated execution of these functions is essential for diverse aspects of life during embryogenesis in most adult organisms (4). HD proteins display evolutionary conservation, and mutations in HD proteins result in developmental anomalies or arrest (3). Additionally, HD proteins play roles in tumorigenesis (5). However, the mechanism by which HD proteins mediate diverse functions remains unclear.

HOMEZ (Homedomain leucine zipper-encoding), a member of the HD protein family, contains three atypical HDs that act as DNA-binding regions, two leucine zipper-like motifs and an acidic domain (6). HOMEZ plays a critical role in *Xenopus laevis* neurogenesis as a positive regulator of Bmps and Ngnr1 and a negative regulator of Notch signaling (7), similar to the function of the ZHX (Zinc-fingers and homeoboxes) family of transcription factors that have been implicated in glioblastoma (8-10). Importantly, genome-wide analysis revealed that heterozygous variants in *Homez* are correlated with congenital heart disease (CHD) in India and China (11,12). Nevertheless, an analysis of human and murine tissues revealed highly enriched *Homez* transcript expression in adult testes (6). Therefore, HOMEZ may play a role in the reproductive process, especially in male fertility. However, whether HOMEZ affects spermatogenesis remains unclear. In the current study, we generated *Homez* knockout mice using the CRISPR/Cas9 system to evaluate the role of HOMEZ in male fertility. We present the following article in accordance with the ARRIVE reporting checklist (available at https://tau.amegroups.com/article/view/10.21037/tau-21-1169/rc).

Methods

Animals

Mice were housed in the Laboratory Animal Center of the Affiliated Drum Tower Hospital of Nanjing University Medical School and kept under specific pathogen-free conditions with food and water available ad libitum. All animals were euthanized by cervical dislocation prior to the harvest of testis and epididymal samples. This study was carried out in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (Bethesda, MD, USA). Animal experiments and protocols were approved by the Institutional Animal Care and Use Committee of Nanjing Drum Tower Hospital (No. 2021AE01035).

Extraction of single-cell RNA sequencing (scRNA-seq) data

The expression of *Homez* in human single cells was obtained from scRNA-seq data on the Human Protein Atlas website (https://www.proteinatlas.org/) (13).

Homez-knockout mice generation

Using CRISPR/Cas9-mediated genome engineering, *Homez* knockout mouse in C57BL/6 background were generated by Cyagen (Cyagen Biosciences, Suzhou, China). The sgRNA sequences were as follows: 5'-GGTAAACAGCCGATCAAGCC-3' and 5'-CCCAAACTCTCTCTACACGG-3'. Two sgRNAs were co-injected with Cas9 mRNA into 100 fertilized C57BL/6 eggs. Fourteen pups were obtained and genotyped by genomic PCR. Subsequently, the genotypes were confirmed by Sanger sequencing. The percentage of founders that contained *Homez* mutations was 78% (11/14). After genotyping, serial mating of the F0 mice was performed to generate homozygous mutants.

Genotyping

Offspring were genotyped by PCR amplification (primers: *Homez*_F1: 5'-TGAGCTGTGAGAGGTGACAAAG-3'; R1: 5'-TATAAAGCCAAGACCCTGCAAAC-3'; R2: 5'-TGCCAAACTGCTGAAACCAAAC-3') followed by Sanger sequencing. All sequencing data were analyzed using SnapGene software (GSL Biotech, Chicago, IL, USA).

Fertility test

Eight-week-old sexually mature *Homez*$^{+/+}$ and *Homez*$^{-/-}$ male mice were caged with two 8-week-old *Homez*$^{+/+}$ female mice for at least 2 months. During the mating test, total number of litters and pups was recorded, and the average litter size was calculated for each genotype.

Histology analysis

For histological studies, isolated testes and epididymides from 10-week-old *Homez*$^{+/+}$ and *Homez*$^{-/-}$ mice were fixed in Bouin's solution overnight at room temperature. The tissues were dehydrated in graded ethanol (70%, 80%, 95% and 100%) and cleared in xylene, followed by paraffin embedding. Subsequently, tissue sections were cut and mounted on glass slides. After dewaxing and rehydration, sections were stained with hematoxylin and eosin (H&E) (Sigma-Aldrich, Shanghai, China). To evaluate sperm morphology, cauda epididymal sperm were isolated, fixed in 4% paraformaldehyde (PFA), spread on glass slides and followed by staining with H&E. All sections were observed using a microscope (LEICA DM2500, Germany).

Sperm parameter analysis

Epididymal sperm were obtained from 10-week-old *Homez*$^{+/+}$ and *Homez*$^{-/-}$ male mice. For the sperm count assay, sperm were suspended in 4 mL phosphate buffered saline (PBS) at 37 ℃ for 10 min, followed by fixation in 2 mL 4% PFA (in PBS). The total number of sperm was counted using a hemocytometer. For the sperm motility study, sperm were released from the cauda epididymis into 500 μL of HTF medium at 37 ℃ for 5 min, and the percentage of motile sperm was determined by a computer-aided sperm analysis (CASA) system (ML-MD06200B, China).

Quantitative RT-PCR

Total RNA was extracted from testes and sperm with TRIzol reagent (15596026, Thermo Fisher Scientific, USA). The concentration and purity of RNA were determined by absorbance at 260/280 nm. One microgram of RNA was reverse transcribed using a cDNA reverse transcription kit (RR036A, Takara, Japan). Two microliters of diluted cDNA was used for quantitative RT-PCR (qRT-PCR) by a SYBR Green dye-based assay (RR820A, Takara, Japan). Gene expression was normalized to *18S* or β-*actin*. The primers were as follows: *Homez* (mouse) forward: 5'-TGATGATGGGCAGCAGAACA-3', reverse: 5'-ATCTTCTCGTCGTGCCCATT-3'; *18S* (mouse) forward: 5'-ATGGCCGTTCTTAGTTGGTG-3', reverse: 5'-CGGACATCTAAGGGCATCAC-3'; *Zhx1* forward: 5'-GCAAGCAGACGAAAATCAACAA-3', reverse: 5'-TCTACAGGTGTAAGGATGGGAG-3'; *Zhx2* forward: 5'-ATGGCAAGCAAACGGAAATCT-3', reverse: 5'-TCCTTTGTCACATCGGACTGT-3'; *Zhx3* forward: 5'-ATGATCCCCGTTAAGACCGTG-3', reverse: 5'-CCTGAACTCACACTCTTTACAGC-3'; β-*actin* (mouse) forward: 5'-CCGTAAAGACCTCTATGCC-3', reverse: 5'-CTCAGTAACAGTCCGCCTA-3'; *Homez* (rat) forward: 5'-GCTACTTCCCCTACCCAAGC-3', reverse: 5'-TGCGTGATGTGTGAAAGACA-3'; β-*actin* (rat) forward: 5'-CACCCGCGAGTACAACCTTC-3', reverse: 5'-CCCATACCCACCATCACACC-3'.

Western blotting

Tissue samples were washed in PBS and lysed for 30 min in cold RIPA buffer supplemented with phosphatase inhibitors and protease inhibitors. After incubating on ice for 30 min, the lysates were centrifuged at 13,000 rpm for 15 min at 4 ℃. The concentration of protein was measured by the bicinchoninic acid (BCA) assay kit (E11201, Vazyme, China). A total of 20 μg of protein was separated on 10% standard SDS-polyacrylamide gels (SDS-PAGE). The following primary antibodies (with dilutions) were used: anti-HOMEZ (diluted 1:200 in tris buffered saline with Tween-20 (TBST), 23965-1-AP, Proteintech, China) and anti-β-actin (diluted 1:10,000 in TBST, P30002M, Abmart, China).

Isolation of germ cells

Spermatogenic cells (spermatogonia, spermatocytes, spermatids) and Sertoli cells were isolated using the STA-PUT method as previously described (14,15).

Immunofluorescence

The tissue sections were fixed in ice-cold 4% PFA, washed with PBS and blocked for 1 h in 10% goat serum in phosphate buffered saline with Tween-20 (PBS-T), followed by incubation with the following primary antibodies for 2 h at 4 ℃: anti-γH2AX (diluted 1:100 in TBST, 16-202A, Merck Millipore, USA) and anti-SOX9 (diluted 1:100 in

TBST, AB5535, Merck Millipore, USA). Nuclear DNA was stained with 4',6-diamidino-2-phenylindole (DAPI, F6057, Sigma-Aldrich, USA), and acrosomes were stained with FITC-conjugated peanut agglutinin (PNA, RL-1072, Vector Labs, USA). All stained tissues were observed using a fluorescence microscope (Leica, DM3000, Germany).

Phylogenetic analyses

Multiple alignments of amino acid sequences were downloaded from the National Center for Biotechnology Information (NCBI) database and phylogenetic trees were constructed by MEGA X software with the neighbor-joining program.

Statistical analysis

Data are presented as the mean ± standard deviation (SD). Statistical significance was assessed with two-tailed unpaired Student's *t*-test using GraphPad Prism 8.0 (GraphPad Software, CA, USA). $P<0.05$ was considered statistically significant.

Results

Homez is predominantly expressed in testes

Molecular phylogenetic analyses indicated that the HOMEZ protein is highly evolutionarily conserved among mammalian species (*Figure 1A*). To study the functional roles of *Homez* in male fertility, we first assessed the tissue- and cell-specific expression patterns of *Homez* in humans and mice. A scRNA-seq database indicated that *Homez* transcripts were enriched in round spermatids in humans (*Figure 1B*) (13). Using published RNA-seq data, we also found relative abundance of *Homez* transcripts in mouse testes (*Figure 1C*) (16). Moreover, *Homez* transcripts displayed dynamic expression patterns during spermatogenesis, with increased expression in pachytene spermatocytes and round spermatids and reduced expression in spermatozoa (*Figure 1D*) (17). Consistently, quantitative PCR (qPCR) assays of spermatogenic cells showed that *Homez* transcripts were enriched in haploid spermatids (*Figure 2A-2C*). *Homez* transcripts were also highly expressed in adult rat testes (Figure S1). Western blot analysis confirmed that in adult male mice, HOMEZ protein was detected and highly expressed in the testis, with a relatively increased expression trend during spermatogenesis (*Figure 2D,2E*). Collectively, these data demonstrate that *Homez* is a conserved and testis-enriched gene that may play a role in spermatogenesis.

Generation of Homez knockout mice

To elucidate the *in vivo* function of HOMEZ, we next generated *Homez* KO mice using CRISPR/Cas9 gene-editing technology. Two sgRNAs targeting the first intron and the second exon of *Homez* were designed, and a mutant mouse line with a *Homez* allele containing a 2,253-bp deletion was subsequently established (*Figure 3A,3B*). Heterozygous (+/–) and homozygous mutant *Homez* alleles (hereafter referred to as *Homez*$^{-/-}$) were confirmed by PCR genotyping using genomic DNA (*Figure 3C*).

Homez mutant males are fertile

The absence of HOMEZ protein in testes from adult *Homez*$^{-/-}$ mice were observed by Western blot analysis (*Figure 4A*). *Homez*$^{-/-}$ males were viable, phenotypically normal into adulthood and showed no differences in body and testis weight compared to their wild-type (WT) littermates at 10 weeks of age (*Figure 4B-4D*). *Homez*$^{-/-}$ males showed normal reproductive behavior, and mating between *Homez*-null males and WT females produced normal litter sizes (*Figure 4E*). In addition, H&E-stained testicular and epididymal sections indicated normal histological structure with complete spermatogenesis in seminiferous tubules and mature spermatozoa in *Homez*$^{-/-}$ males, respectively (*Figure 4F*).

Normal sperm parameters in Homez$^{-/-}$ *mice*

To investigate the potential role of HOMEZ in sperm maturation, we further measured sperm parameters from the epididymal cauda of *Homez*$^{-/-}$ and WT mice. The number and motility of mature spermatozoa in *Homez*$^{-/-}$ mice were similar to those of WT controls at 10 weeks of age (*Figure 5A,5B*). Moreover, *Homez*$^{-/-}$ males exhibited normal sperm morphology when compared with WT males (*Figure 5C*).

HOMEZ is dipensable for germ cell development

To analyze whether spermatogenesis was affected in *Homez*$^{-/-}$ mice, we performed immunostaining on germ cells at different stages. Normal γ-H2AX-positive spermatocytes

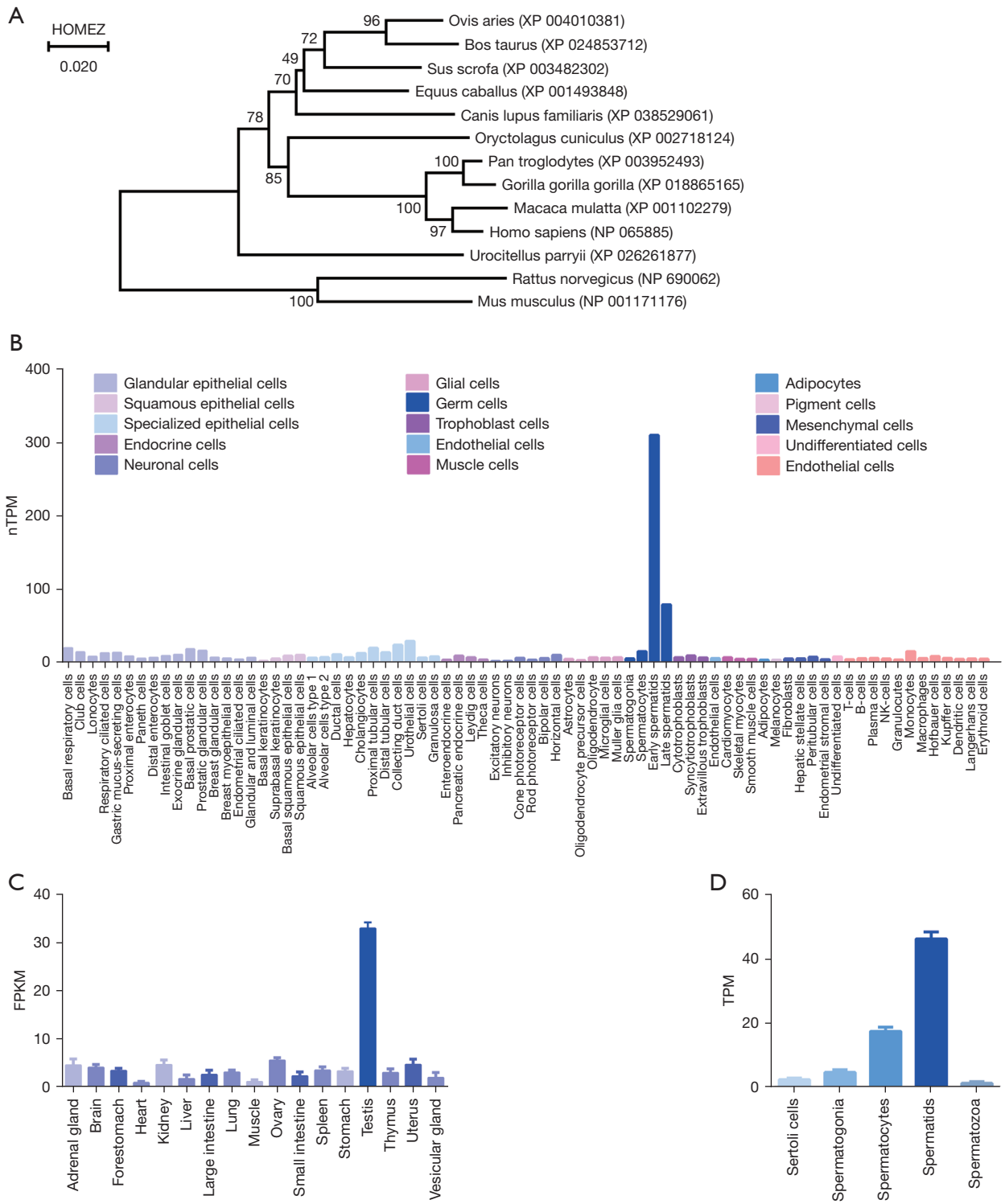

Figure 1 *Homez* is evolutionarily conserved and testis-enriched gene. (A) Phylogenetic trees of HOMEZ sequences in mammalian species. The numbers in the dendrogram are bootstrap values (%). (B) Expression pattern of *Homez* mRNA from published human single-cell RNA-seq data. (C,D) Dynamic expression levels of *Homez* mRNA in multiple mouse tissues (C) and spermatogenetic cells (D) from published RNA-seq data. *Homez*, Homedomain leucine zipper-encoding; RNA-seq, RNA-sequencing.

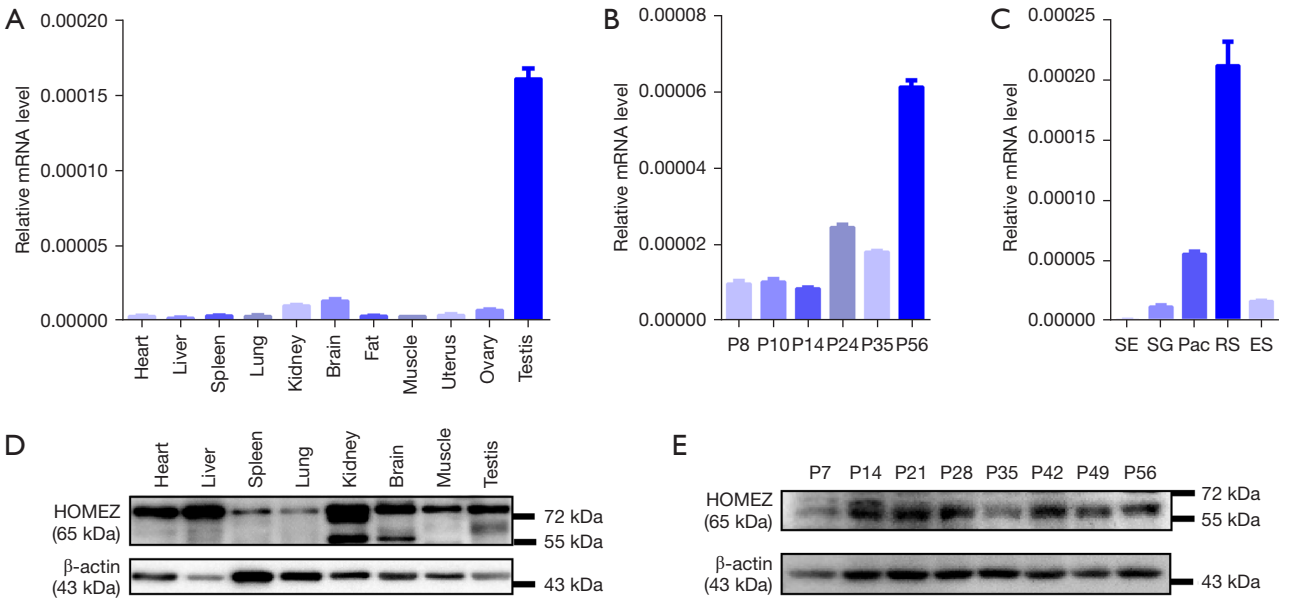

Figure 2 *Homez* is predominantly expressed in postmeiotic germ cells. (A) QRT-PCR showing the expression pattern of *Homez* mRNA in different tissues of 10-week-old mice, n=3. (B) QRT-PCR analyses of *Homez* mRNA levels in developing testes at postnatal day 8 (P8), P10, P14, P24, P35 and P56, n=3. (C) QRT-PCR showing the expression pattern of *Homez* mRNA across isolated spermatogenetic cells and Sertoli cells, n=3. (D) Western blot analyses of the expression pattern of HOMEZ protein in different tissues of 10-week-old mice. (E) Western blot showing the expression levels of HOMEZ protein in developing testes at P7, P14, P21, P28, P35, P42, P49 and P56. Gene expression was normalized to 18S. SE, Sertoli cells; SG, spermatogonia; Pac, pachytene spermatocytes; RS, round spermatids; ES, elongating spermatids; QRT-PCR, quantitative reverse transcription-polymerase chain reaction.

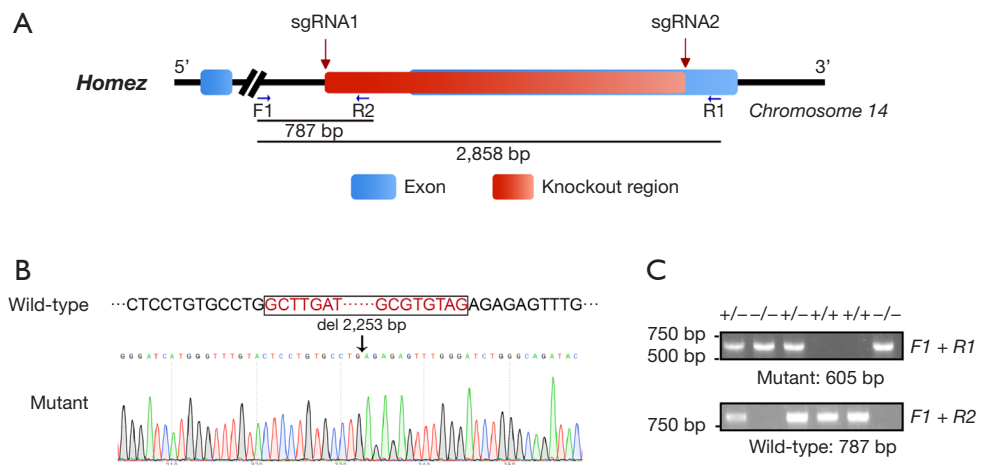

Figure 3 *Homez* knockout mice were generated by CRISPR/Cas9 technology. (A) Schematic diagram of *Homez*$^{-/-}$ mouse creation. (B) Sanger sequencing of genomic DNA showing a 2,253-bp deletion in the *Homez* gene. (C) *Homez*$^{-/-}$ mice were identified by genomic PCR. PCR, polymerase chain reaction.

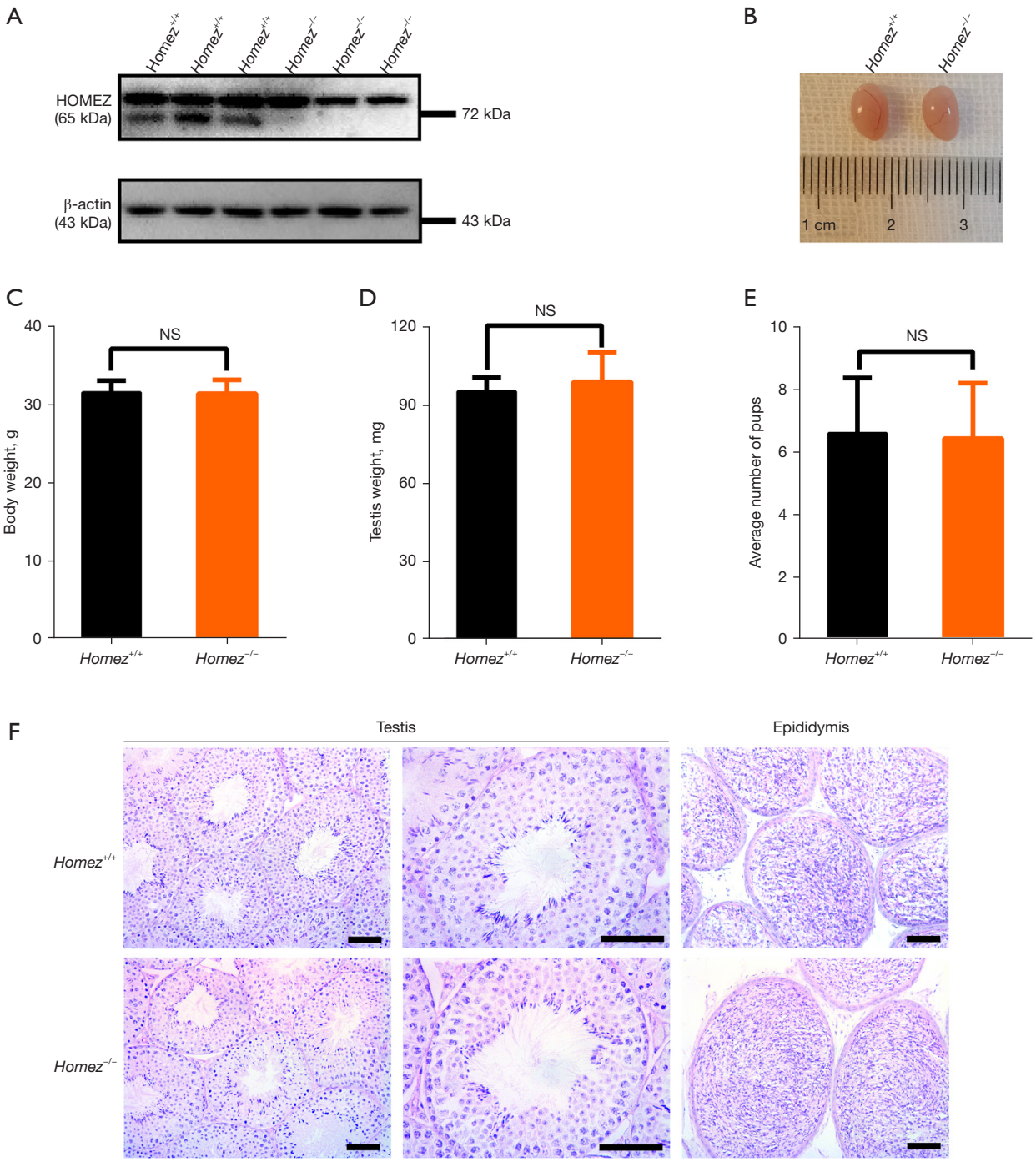

Figure 4 *Homez*$^{-/-}$ mice are fertile. (A) HOMEZ protein was not detected in adult *Homez*$^{-/-}$ testes, n=3 for each genotype. (B) Representative image of *Homez*$^{+/+}$ and *Homez*$^{-/-}$ testes from 10-week-old mice. (C) Body weight of 10-week-old *Homez*$^{+/+}$ and *Homez*$^{-/-}$ mice, n=6. (D) Testis weight of 10-week-old *Homez*$^{+/+}$ and *Homez*$^{-/-}$ mice, n=6. (E) Number of pups per litter from *Homez*$^{+/+}$ and *Homez*$^{-/-}$ males, n=12. (F) H&E staining of testes and epididymis from 10-week-old *Homez*$^{+/+}$ and *Homez*$^{-/-}$ mice. Scale bar: 50 μm. NS, no significant difference; H&E, hematoxylin-eosin.

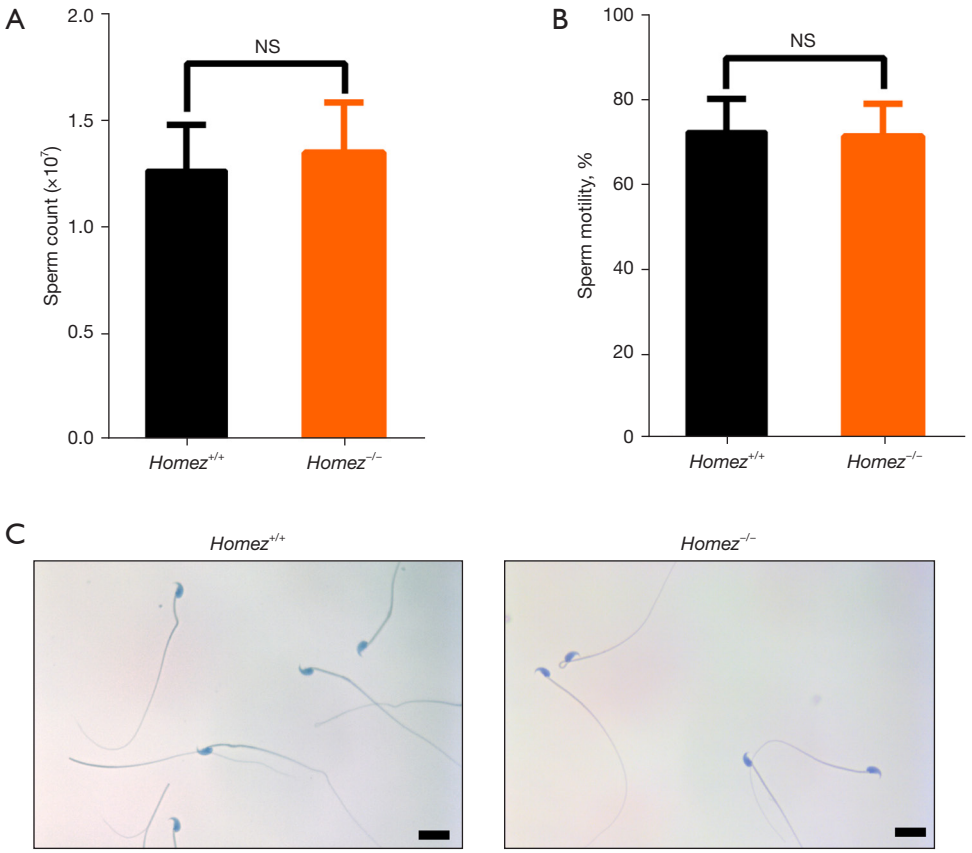

Figure 5 *Homez*$^{-/-}$ mice show normal sperm parameters. (A,B) Sperm count and motility from 10-week-old *Homez*$^{+/+}$ and *Homez*$^{-/-}$ mice, n=6. (C) H&E staining shows sperm morphology from 10-week-old *Homez*$^{+/+}$ and *Homez*$^{-/-}$ mice, n=6. Scale bar: 20 μm. NS, no significant difference; H&E, hematoxylin-eosin.

and PNA-positive spermatids were found in seminiferous tubules from *Homez*$^{-/-}$ testes (*Figure 6A,6B*). Further analysis showed similar numbers of SOX9-positive Sertoli cells in *Homez*$^{-/-}$ and WT males (*Figure 6C,6D*). Collectively, these data show that *Homez* deficiency does not affect spermatogenesis.

Functional redundancy between HD genes

Since the *Homez* and *Zhx* family is a subset within the HD family (6), we next explore the mRNA expression of *Zhx* family genes in WT and *Homez*$^{-/-}$ males. *Zhx1*, *Zhx2* and *Zhx3* expression levels were upregulated in the testes of *Homez*$^{-/-}$ testes compared with those in the testes of WT mice (*Figure* 7).

Discussion

In the current study, we highlight that *Homez* is a highly conserved and testis-enriched gene, especially in postmeiotic cells. Using CRISPR/Cas9 technology, we explored whether the homeobox gene *Homez* plays a role in fertility among mice. Together with the histological and immunofluorescence results, our findings revealed normal fertility in *Homez* knockout mice, with no difference in spermatogenesis compared to that of wild-type males.

Several HD proteins have been implicated in fertility. The HD gene *Otx2* is essential for the hypothalamic-pituitary-gonadal (HPG) axis, and male Otx2 heterozygous mice exhibit a progressive loss of fertility (18,19). *Vax1* plays a role in neuronal fate determination, and heterozygous

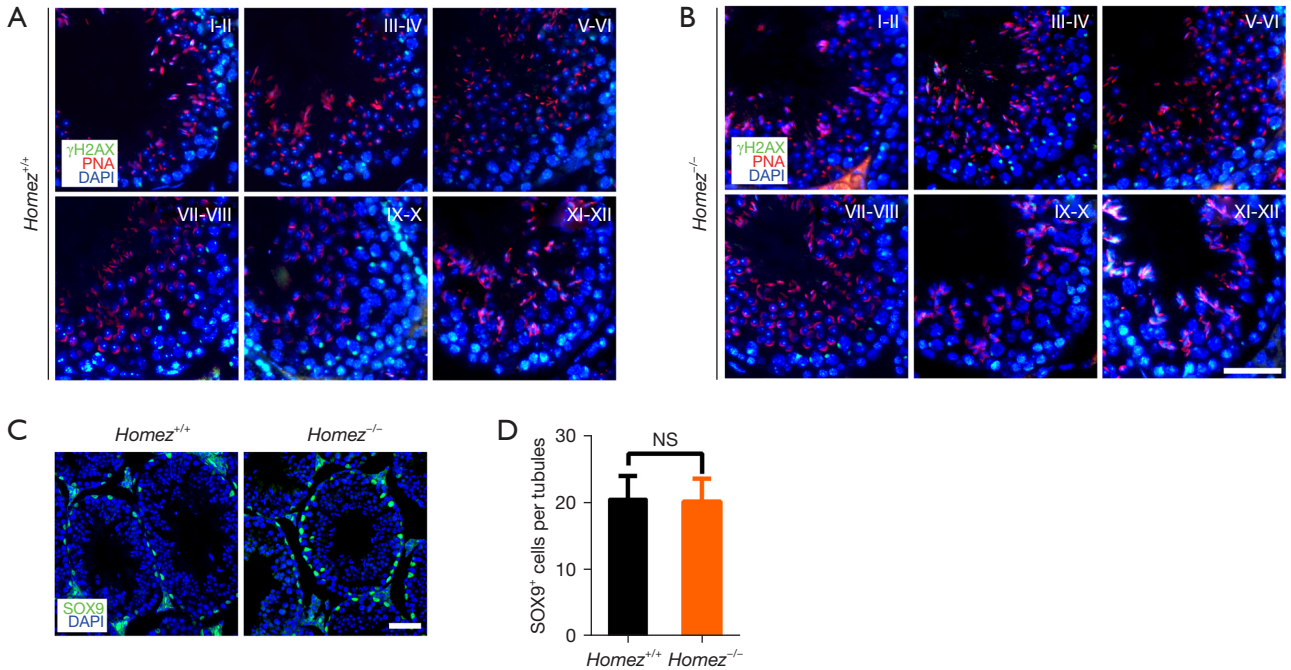

Figure 6 Normal spermatogenesis in *Homez*$^{-/-}$ males. (A,B) Immunodetection of γH2AX and PNA in testis sections from 10-week-old *Homez*$^{+/+}$ and *Homez*$^{-/-}$ mice. (C) Immunostaining of the Sertoli cell marker SOX9 in testis sections from 10-week-old *Homez*$^{+/+}$ and *Homez*$^{-/-}$ mice. (D) Quantification of SOX9-positive cells in seminiferous tubules from 10-week-old *Homez*$^{+/+}$ and *Homez*$^{-/-}$ mice. A total of 40 tubules per genotype were analyzed. Scale bar: 50 μm. NS, no significant difference; γH2AX, phosphorylated histone H2AX; PNA, peanut agglutinin; SOX9, SRY-Box Transcription Factor 9.

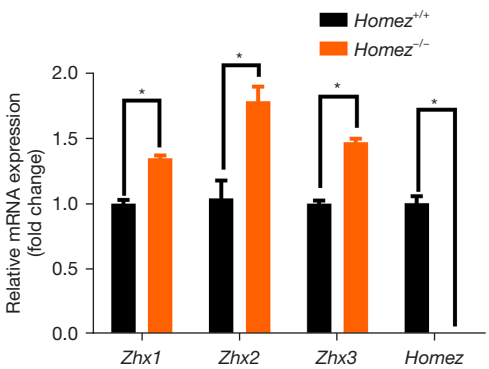

Figure 7 Functional compensation between *Homez* and *Zhx*. QRT-PCR showing the mRNA expression levels of *Homez* and *Zhx* genes in testes from 10-week-old *Homez*$^{+/+}$ and *Homez*$^{-/-}$ mice, n=6. Gene expression was normalized to β-actin. *, P<0.05. *Zhx*, Zinc-fingers and homeoboxes; QRT-PCR, quantitative reverse transcription-polymerase chain reaction.

mutation in *Vax1* alters GnRH expression, causing male subfertility in mice (20). In addition, conditional knockout of RHOX10, an HD-harboring homeobox protein, causes spermatogenic defects and male infertility (21). Another homeobox gene, *Meis1*, is essential for Sertoli cell-mediated regulation of male fertility (22). Nevertheless, the function of HD proteins in fertility, especially those proteins mainly expressed in germ cells, remains largely unknown.

Since a number of HD proteins mediate various physiological processes, including male reproduction, the high expression of *Homez* transcripts in germ cells indicate that they may be involved in spermatogenesis. However, we found that different stages of spermatogenic cells were present in *Homez*$^{-/-}$ testes. It is worth noting that many testis-enriched and evolutionarily conserved genes are not essential for male fertility in mice. This may be due to functional redundancy in these genes. Functional

redundancy among HD family proteins has been found in vertebrates (23,24). Genomic and phylogenetic analyses indicate that the ZHX family and HOMEZ are a subset within the superfamily of HD proteins, of which the three HDs from ZHX3 and HOMEZ share high sequence identity (HD1, 39%; HD2, 48%; HD3, 28%) (6), indicating that HOMEZ may have redundant functions with the ZHX family in male reproduction. Interestingly, our study also revealed significantly increased transcription of three *Zhx* genes in *Homez*$^{-/-}$ testes compared with WT testes. Among the three ZHX genes, *Zhx3* transcripts were highly enriched in the testis, especially in haploid germ cells, which implies potential functional redundancy in male fertility between ZHX3 and HOMEZ. Although we found that HOMEZ is not essential for fertility using a cohousing assay under normal laboratory mating conditions in this study, it may be required for normal fertility during stress conditions.

In conclusion, our findings demonstrate that HOMEZ does not affect fertility in male mice, even though *Homez* is highly expressed in postmeiotic cells. One possible explanation for the normal fertility observed in HOMEZ knockout mice may be the overlapping characteristics between HOMEZ and ZHX proteins.

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Footnote

Reporting Checklist: The authors have completed the ARRIVE reporting checklist. Available at https://tau.amegroups.com/article/view/10.21037/tau-21-1169/rc

Data Sharing Statement: Available at https://tau.amegroups.com/article/view/10.21037/tau-21-1169/dss

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://tau.amegroups.com/article/view/10.21037/tau-21-1169/coif). The authors declare no conflicts of interest.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. This study was carried out in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (Bethesda, MD, USA). Animal experiments were approved by the Institutional Animal Care and Use Committee of Nanjing Drum Tower Hospital (No. 2021AE01035).

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Supplementary

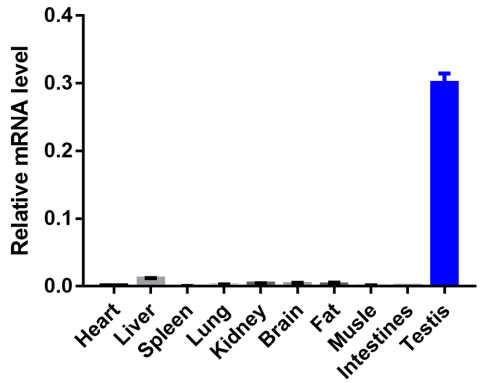

Figure S1 *Homez* is enriched in rat testes. Tissues from 10-week-old Sprague-Dawley (SD) male rats were used for qRT-PCR, n=3. Gene expression was normalized to *β-actin*. qRT-PCR, quantitative reverse transcription-polymerase chain reaction.

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