Eef2k is not required for fertility in male mice

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Background: Eukaryotic elongation factor-2 kinase (*Eef2k*) is a protein kinase associated with the calmodulin-induced signaling pathway and an atypical alpha-kinase family member. *Eef2k*-mediated phosphorylation of eukaryotic translation elongation factor 2 (*Eef2*) can inhibit the functionality of this protein, altering protein translation. Prior work suggests *Eef2k* to be overexpressed in breast, pancreatic, brain, and lung cancers wherein it may control key processes associated with apoptosis, autophagy, and cell cycle progression. The functional importance of *Eef2k* in the testes of male mice, however, has yet to be clarified.

Methods: A CRISPR/Cas9 approach was used to generate male *Eef2k*-knockout mice, which were evaluated for phenotypic changes in epididymal or testicular tissues through histological and immunofluorescent staining assays. In addition, TUNEL staining was conducted to assess the apoptotic death of cells in the testis. Fertility, sperm counts, and sperm motility were further assessed.

Results: Male *Eef2k*-knockout mice were successfully generated, and exhibited normal fertility and development. No apparent differences were observed with respect to spermatogenesis, sperm counts, or germ cell apoptosis when comparing male *Eef2k*$^{-/-}$ and *Eef2k*$^{+/+}$ mice.

Conclusions: Male *Eef2k*-knockout mice remained fertile and were free of any evident developmental or spermatogenic abnormalities, suggesting *Eef2k* to be dispensable in the context of male fertility.

Keywords: Eukaryotic elongation factor-2 kinase (*Eef2k*); male fertility; spermatogenesis

Introduction

Infertility is estimated to impact 8–12% of couples worldwide, and is defined by a failure to conceive following unprotected frequent sexual intercourse over a one-year period. Approximately half of infertile couples are impacted by male-factor infertility (1), which can occur due to hormonal deficits (2), physical causes, sexually transmitted diseases, or environmental, lifestyle (3,4), and genetic factors (5,6). As spermatogenesis is a key process in the maintenance of appropriate male fertility, any abnormalities in the generation of spermatozoa can give rise to male-factor infertility (7). There are four primary phases of spermatogenesis: (I) the initial mitotic proliferation and spermatogonial differentiation of pre-leptotene spermatocytes; (II) the meiotic division of spermatocytes to yield spermatids; (III) spermiogenesis wherein round spermatids are converted into elongating spermatids; and (IV) spermiation, wherein elongated spermatids, as called as sperm, are released into the lumen of seminiferous tubules (8).

We have previously shown that *Tcte1*-knockout male mice are sterile as they exhibit sperm with impaired progressive motility (asthenozoospermia) (9). In our quantitative proteomic analyses, we found eukaryotic elongation factor-2 kinase (*Eef2k*) to be significantly downregulated in these *Tcte1*-knockout mice, suggesting that *Eef2k* may be an important regulator of spermatogenesis and/or sperm motility.

Eef2k is a structurally and functionally atypical kinase involved in calmodulin-mediated signaling that functions by phosphorylating and inhibiting *Eef2* to regulate protein translation and synthesis (10,11). *Eef2k* overexpression has previously been reported in breast, pancreatic, brain, and lung cancers in which it plays important roles in the regulation of apoptosis, autophagy, and cell cycle progression, making it a viable target for anticancer treatment (12-16). However, the *in vivo* functionality of *Eef2k* in the testes remains to be clarified. As such, we herein used a CRISPR/Cas9 approach to generate *Eef2k*-knockout mice, and we used these mice to explore the role of *Eef2k* in testicular development and spermatogenesis. We present the following article in accordance with the ARRIVE reporting checklist (available at http://dx.doi.org/10.21037/tau-21-18).

Methods

Eef2k-knockout mouse generation

The study was conducted in accordance with the declaration of Helsinki (as revised in 2013). Experiments were performed under a project license (No.: IACUC-2004020) granted by the Animal Ethical and Welfare Committee, in compliance with the Institutional Animal Care and Use Committee of Nanjing Medical University guidelines. To generated targeted knockout mice, Cas9 mRNA and single-guide RNAs (sgRNAs) were generated as in prior studies (7,9,17,18). Briefly, *Age*I and *Dra*I were used for the respective linearization of the Cas9 and sgRNA plasmids, which were subsequently purified using the MinElute PCR Purification Kit (Qiagen, Duesseldorf, Germany). An mMESSAGE mMACHINE T7 Ultra Kit (Ambion, TX, USA) was used to facilitate the *in vitro* transcription of Cas9 mRNA, followed by purification with the RNeasy Mini Kit (Qiagen) based on provided instructions. Similarly, sgRNAs were generated and purified with the MEGA shortscript and MEGA clear kits (Ambion), respectively. The sgRNAs used in the present study were prepared to target *Eef2k* exon 6, and were as follows: 5'-GGGGAACAACCCACATCTGAAAGAGG-3' and 5'-TGGTGGAGGTTCTTGGTGATCTGG-3'. These two constructs were then injected into murine zygotes that were obtained following the mating of WT male and super-ovulated female C57BL/6 mice. The strain is stable and easy to reproduce. The pure background C57BL/6 mice for gene knockout can ensure the high stability of genetic background and the consistency of experimental data.

Genotyping

Edited *Eef2k* founders were identified via PCR amplification (Rapid Taq master mix, Vazyme, Nanjing, China) using the following primers: F 5'-GGAGATGGTGATCTTCATACT-3' and R 5'-GCATACTATTCTGTGGTAGGA-3', and were subcloned into the pMD19-T plasmid (TaKaRa, Wuhan, China), after which standard Sanger sequencing was performed. Those founders that exhibited *Eef2k* frameshift mutations were mated with WT C57BL/6 mice for a minimum of three generations to ensure that no off-target editing was present in these animals, yielding respective WT

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and knockout *Eef2k* alleles that were 474 bp and 399 bp in length. Sanger resequencing of *Eef2k*^{-/-} mice was conducted, with results being plotted using SnapGene (version 1.1.3). Tail biopsy-derived DNA samples were subjected to PCR amplification with appropriate primers and were separated via agarose gel electrophoresis for genotyping analyses.

Fertility analyses

Fertility testing in male mice was conducted by mating each male mouse with three WT C57BL/6 females. Females were evaluated for the presence of a vaginal plug each morning, after which birth dates and numbers of pups in each litter were assessed.

Western blotting

RIPA buffer (P0013C, Beyotime, Shanghai, China) supplemented with protease inhibitors (B14002, Bimake, TX, USA) was used to prepare protein lysates, which were subsequently separated via 10% SDS-PAGE and transferred to PVDF membranes. Blots were blocked using 5% non-fat milk in TBST for 2 h. Blots were incubated overnight at 4 ℃ with anti-EEF2K (ab45168; 1:1,000; Abcam, China) and anti-β-actin (ac026; 1:10,000; ABclonal, Wuhan, China). Blots were then washed thrice with TBST (5 min/wash), after which they were probed for 2 h with secondary antibodies (1:1,000) at room temperature (19). The High-sig ECL Western Blotting Substrate (Tanon, Shanghai, China) was used to detect protein bands.

Histological analyses

Davidson's fluid was used to fix murine epididymis or testes tissues overnight, after which the tissues were transferred into 70% ethanol. Samples were subsequently dehydrated with an ethanol gradient (80%, 90%, 100%) and were paraffin-embedded. Tissue sections (5 µm-thick) were then prepared, deparaffinized using dimethylbenzene, and testes and epididymis sections were respectively subjected to periodic acid Schiff (PAS) and hematoxylin and eosin (H&E) staining (20,21). Additionally, slides were smeared with sperm samples from the cauda epididymis, which were subsequently fixed for 30 min using 4% paraformaldehyde in PBS, after which samples were rinsed and subjected to H&E staining.

Immunofluorescence and TUNEL staining

Tissue sections and sperm samples were deparaffinized, rehydrated, and antigen retrieval was conducted in sodium citrate buffer by boiling samples for 10 min. Samples were cooled and blocked with 5% BSA in PBS for 2 h, followed by overnight probing with primary antibodies (Table S1) at 4 ℃. Samples were then washed thrice using PBST (0.05% Tween 20 in 1× PBS), probed with secondary antibodies (Table S2), and counterstained at room temperature with Hoechst 33342 (1:1,000; Invitrogen, CA, USA) for 2 h. After an additional wash, sections were mounted, and apoptotic cells were detected via the terminal deoxynucleotidyl transferase dUTP nick end-labeling (TUNEL) assay (Vazyme) based upon provided directions (22,23). An LSM800 confocal microscope (Carl Zeiss AG, Jena, Germany) was used to image and evaluate samples.

Computer-assisted sperm analysis

Sperm samples were collected by generating cross-incisions throughout the cauda epididymis and extruding the cells therein and resuspending them in human tubal fluid culture medium (In Vitro Care, MD, USA). Individual 10 µl sperm samples were subjected to a computer-assisted semen analysis (CASA; Hamilton-Thorne Research, Inc, MA, USA) of motility, progressive motility, and sperm concentrations (24).

Eepididymal sperm count

Mature sperm from homozygous and WT mice were obtained by making small incisions throughout the cauda epididymis, followed by extrusion and suspension in culture medium, after dilution, count with blood cell counting plate under microscope. The sperm on the grid line are included in the corresponding grid according to the position of the head of sperm.

Statistical analysis

Data were analyzed using Microsoft Excel and GraphPad Prism 6.0 via one-way ANOVAs or unpaired two-tailed *t*-tests. All experiments were repeated three or more times, and data were given as means ± SD. $P<0.05$ was the significance threshold for this study.

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Results

Eef2k⁻/⁻ mouse generation

To explore the functional importance of *Eef2k* in the testes of C57BL/6 mice, *Eef2k⁻/⁻* were prepared via a CRISPR/Cas9 approach (*Figure 1A*). A frameshift mutation was introduced into *Eef2k* by deleting a 76 bp portion of exon 6 of this gene, as confirmed via Sanger sequencing and PCR genotyping (*Figure 1B,C*). We were not able to detect any *Eef2k* protein in the testes of our male *Eef2k⁻/⁻* mice (*Figure 1D*), and these mice exhibited normal viability and body weight relative to their WT counterparts.

Eef2k⁻/⁻ mice exhibited normal fertility and spermatogenesis

We found that male *Eef2k⁻/⁻* mice were fertile, with no differences in litter size relative to those associated with WT control mice (*Figure 1E*). Female *Eef2k⁻/⁻* mice exhibited normal fertility too (*Figure 1E*). The intercrossing of *Eef2k⁺/⁻* mice yielded normally-sized litters with offspring corresponding to the expected Mendelian and sex ratios (data is not shown in the text). There were also no differences in testes or epididymal tissue sizes when comparing male WT and *Eef2k⁻/⁻* mice (*Figure 1F,G,H*). Normal spermatogenic cells were observed in the PAS-stained seminiferous tubules of adult male *Eef2k⁻/⁻* mice, and normal quantities of spermatid cells were similarly detected in H&E-stained samples of epididymis cauda tissue from these mice (*Figure 2A,B,C,D*). PLZF (promyelocytic leukaemia zinc finger), γ-H2ax (phosphorylated H2A histone family member X), PNA (peanut agglutinin), and Sox9 (SRY-box 9) signals were used to identify spermatogonia, spermatocytes, spermatids, and Sertoli cells respectively (25-29), and we were able to observe all four of these cell types in testis sections from WT and *Eef2k⁻/⁻* mice (*Figure 3A,B,C,D,E,F,G,H*, Table S3). TUNEL staining analyses of testicular tissue sections also revealed comparable numbers of apoptotic cells per tubule and apoptotic tubule ratios in WT and *Eef2k⁻/⁻* mice (*Figure 4A,B,C,D,E*, Table S4).

Eef2k⁻/⁻ mice exhibited normal spermatozoa

No significant morphological differences were observed in the spermatozoa of male *Eef2k⁻/⁻* mice relative to WT mice, nor were any alterations in epididymal sperm counts detected between these two groups of mice (*Figure 5A,B,C,D*). Morphological analyses also revealed the ratio of normal sperm in male *Eef2k⁻/⁻* mice to be similar to that in WT mice (*Figure 5D*). Male *Eef2k⁻/⁻* mice also exhibited no significant changes in total sperm motility or progressive motility (*Figure 5E,F*). As such, the partial deletion of *Eef2k* exon 6 did not adversely impact murine spermatogenesis or fertility.

Discussion

We herein employed a CRISPR/Cas9 approach to explore the functional importance of *Eef2k* in spermatogenesis and male fertility by generating *Eef2k*-knockout mice. Our histological, immunofluorescent, and TUNEL staining assays failed to reveal any differences in spermatogenesis between *Eef2k*-knockout mice and WT mice, suggesting that *Eef2k* is dispensable in the context of normal germ cell functionality.

We had previously shown that *Tcte1*-knockout male mice were sterile as a consequence of asthenozoospermia despite exhibiting morphologically normal sperm (9). We found *Eef2k* to be significantly downregulated in the testes of these *Tcte1*-knockout mice in quantitative proteomics analyses (9), leading us to explore the functional impact of *Eef2k* knockout on spermatogenesis. We therefore hypothesized that altered *Eef2k* expression may impact *Eef2* activity and thereby disrupt normal protein synthesis within murine sperm.

Members of the eukaryotic elongation factor protein family are important mediators of elongation during the mRNA translation (30). *Eef2* is an important member of this protein family that facilitates GTP-dependent nascent protein chain translocation from the ribosomal A-site to the P-site (31), thereby regulating protein synthesis. The *Eef2k* kinase is responsible for phosphorylating *Eef2* and thereby inhibiting its functionality, ultimately modulating translation and protein synthesis within affected cells (10,11,32).

Eef2k has been extensively studied in the context of tumorigenesis and cancer treatment (33). In tumor cells, *Eef2k* plays a protective role, improving the tolerance of these highly proliferative cells to energy depletion and nutrient deprivation conditions. Because they grow rapidly, cancer cells must efficiently synthesize proteins, thereby creating significant metabolic demands and requirements for substantial amino acid availability. The ability to adapt to nutrient deprivation conditions is thus essential for these cells to live when local nutrient supplies have been
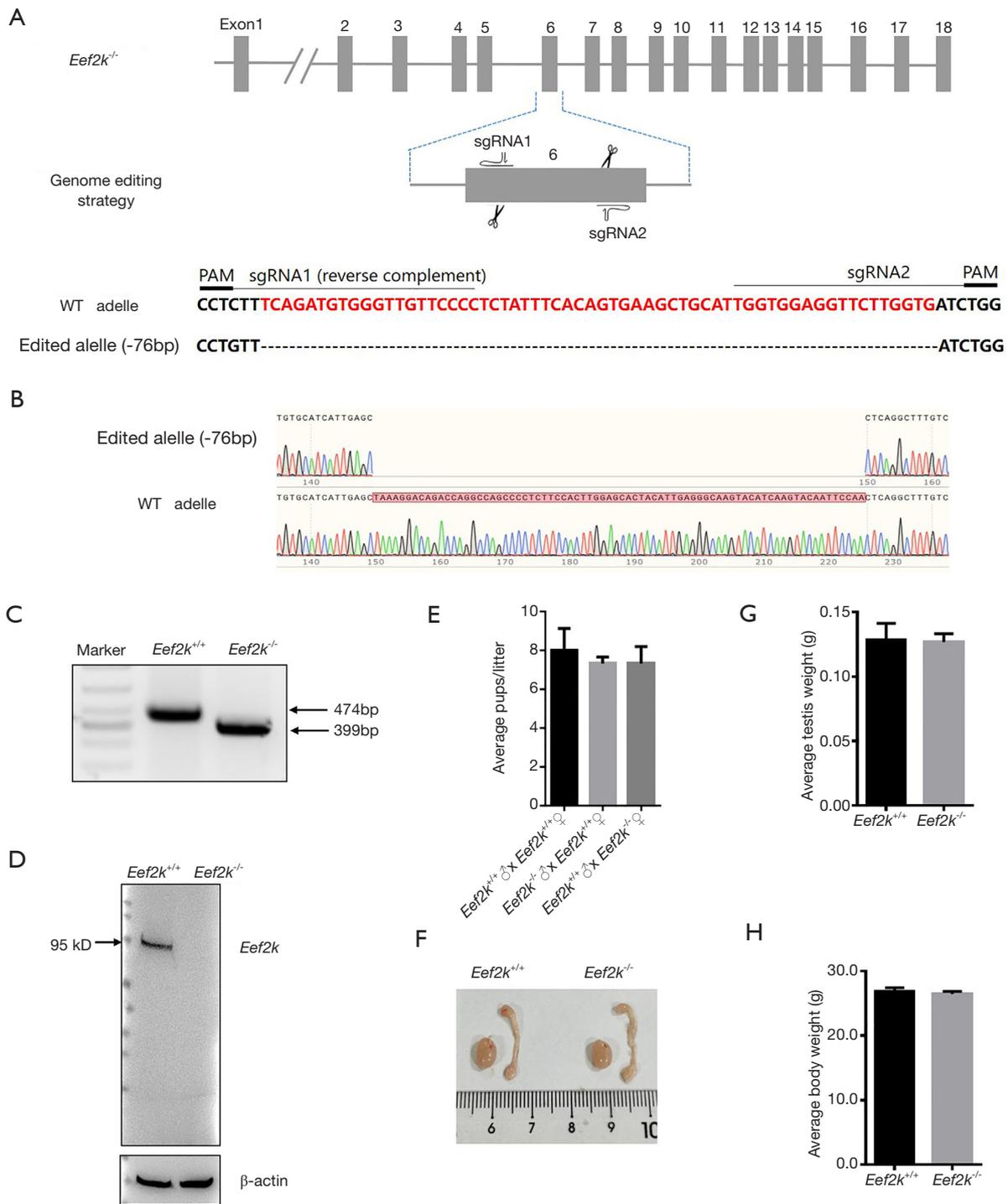
Figure 1 Generation of mutant gene and assessment of *Eef2k*^{-/-} male mouse fertility. (A) Schematic diagram of CRISPR/Cas9 targeting strategy. The sgRNAs were designed on the basis of exon 6 of *Eef2k*; (B) A 76-bp deletion from *Eef2k* exon 6 was detected in *Eef2k*^{-/-} mice by Sanger sequencing and (C) agarose gel electrophoresis analysis, detected by bio rad chemidoc XRS +; (D) *Eef2k* was not detected in *Eef2k*^{-/-} testis on western blot, detected by Tanon 5200; (E) average number of pups per litter from wild-type and *Eef2k*^{-/-} mice, n=3; (F) testis and epididymis from wild-type and *Eef2k*^{-/-} adult mice; (G,H) average testis weight and body weight ratio of 8-week-old wild-type and *Eef2k*^{-/-} mice, n=3. *Eef2k*, eukaryotic elongation factor-2 kinase.
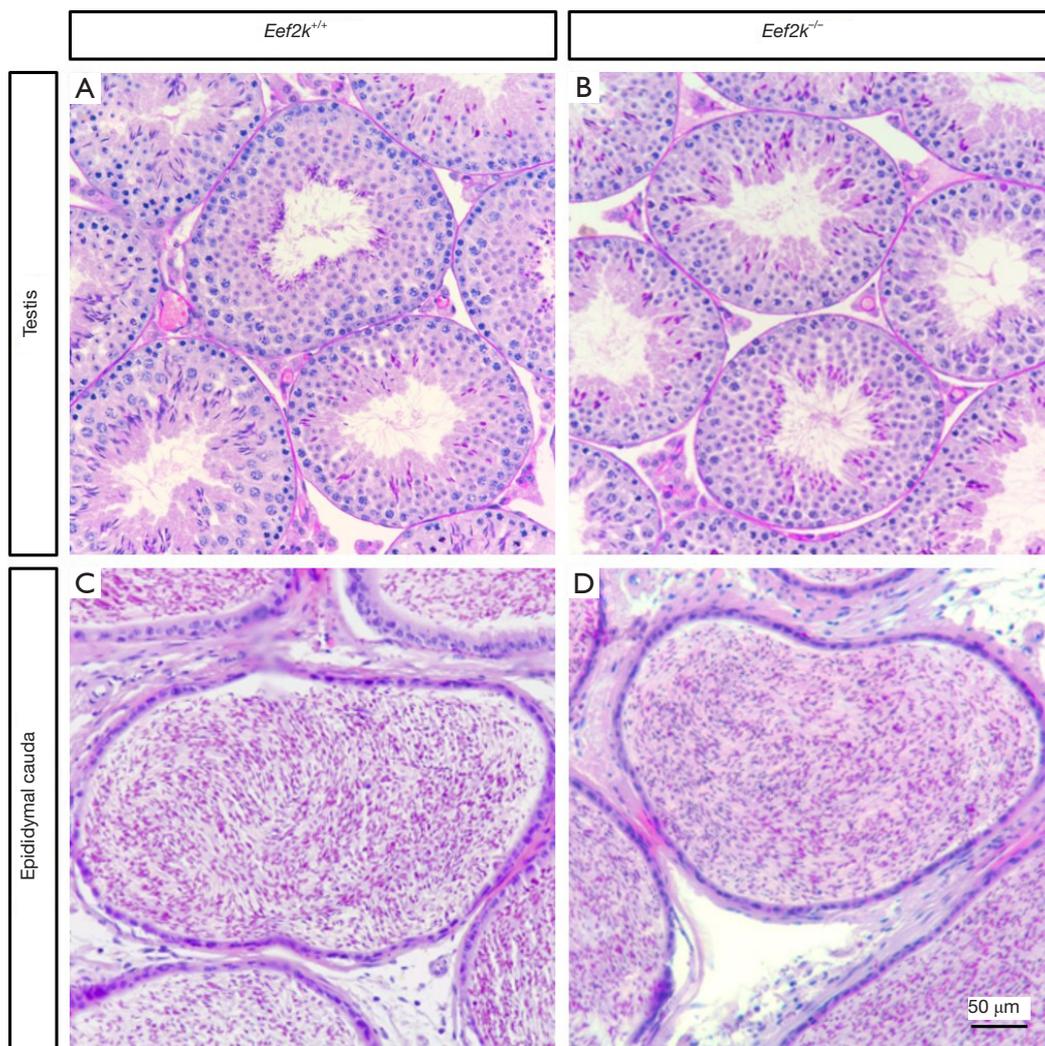
Figure 2 Histological staining showing normal-appearing spermatogenesis in $Eef2k^{-/-}$ mice. Periodic acid Schiff-stained testis sections from (A) wild-type and (B) $Eef2k^{-/-}$ mice. Hematoxylin- and eosin-stained cauda epididymis sections from (C) wild-type and (D) $Eef2k^{-/-}$ mice. $Eef2k$, eukaryotic elongation factor-2 kinase.

exhausted. Several tumor types exhibit $Eef2k$ activation and overexpression that is associated with enhanced survival, particularly under conditions of nutrient deprivation (34-36) or acidosis (37). By disrupting translational elongation, $Eef2k$ can effectively augment nutrient deprivation resistance (12). Through a positive feedback loop associated with $Eef2k$, AMPK can reduce ERK1/2 activity and the sensitivity of tumor cells to nutrition deprivation (38). Consistent with this, $Eef2k$ overexpression was sufficient to protect RaxV12-transformed NIH3T3 cells from caloric restriction-induced cell death *in vivo* (12). $Eef2k$ can additionally promote esophageal squamous cell carcinoma (ESCC) cell proliferation, migration, and invasion, and can promote the growth of ESCC tumors in xenograft model mice (16). $Eef2k$ can also aid cell migration (14,16,39-42), potentially thereby modulating cancer progression. Spermatogonial cells are also highly proliferative in order to facilitate appropriate sperm production (43,44). As such, we hypothesized that $Eef2k$ may similarly regulate spermatogonial cell proliferation by altering the differentiation or self-renewal of these cells. However, as we observed no differences in the morphology or motility of sperm in our $Eef2k$-knockout mice, our data suggest that this protein is dispensible in
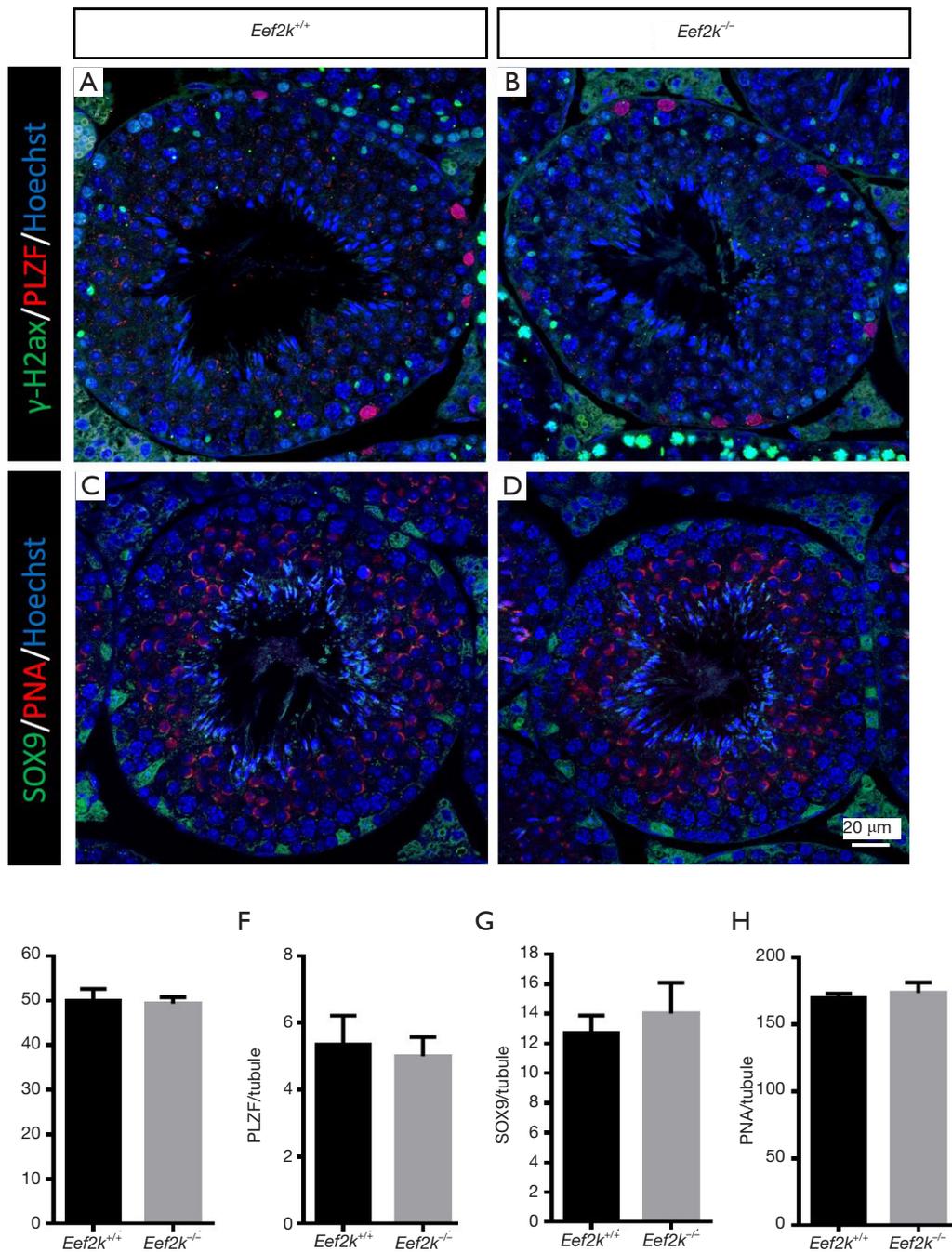
Figure 3 Immunofluorescent analysis of *Eef2k^{+/+}* and *Eef2k^{-/-}* testes. The spermatogonia (PLZF) and spermatocytes (γ-H2ax) were similar in testis sections of (A) wild-type and (B) *Eef2k^{-/-}* mice. Spermatids (PNA) and Sertoli cells (Sox9) were comparable in testis sections of (C) wild-type and (D) *Eef2k^{-/-}* mice. (E,F,G,H) Average number of cells with immune signal in stage VII tubules, n=3. *Eef2k*, eukaryotic elongation factor-2 kinase.
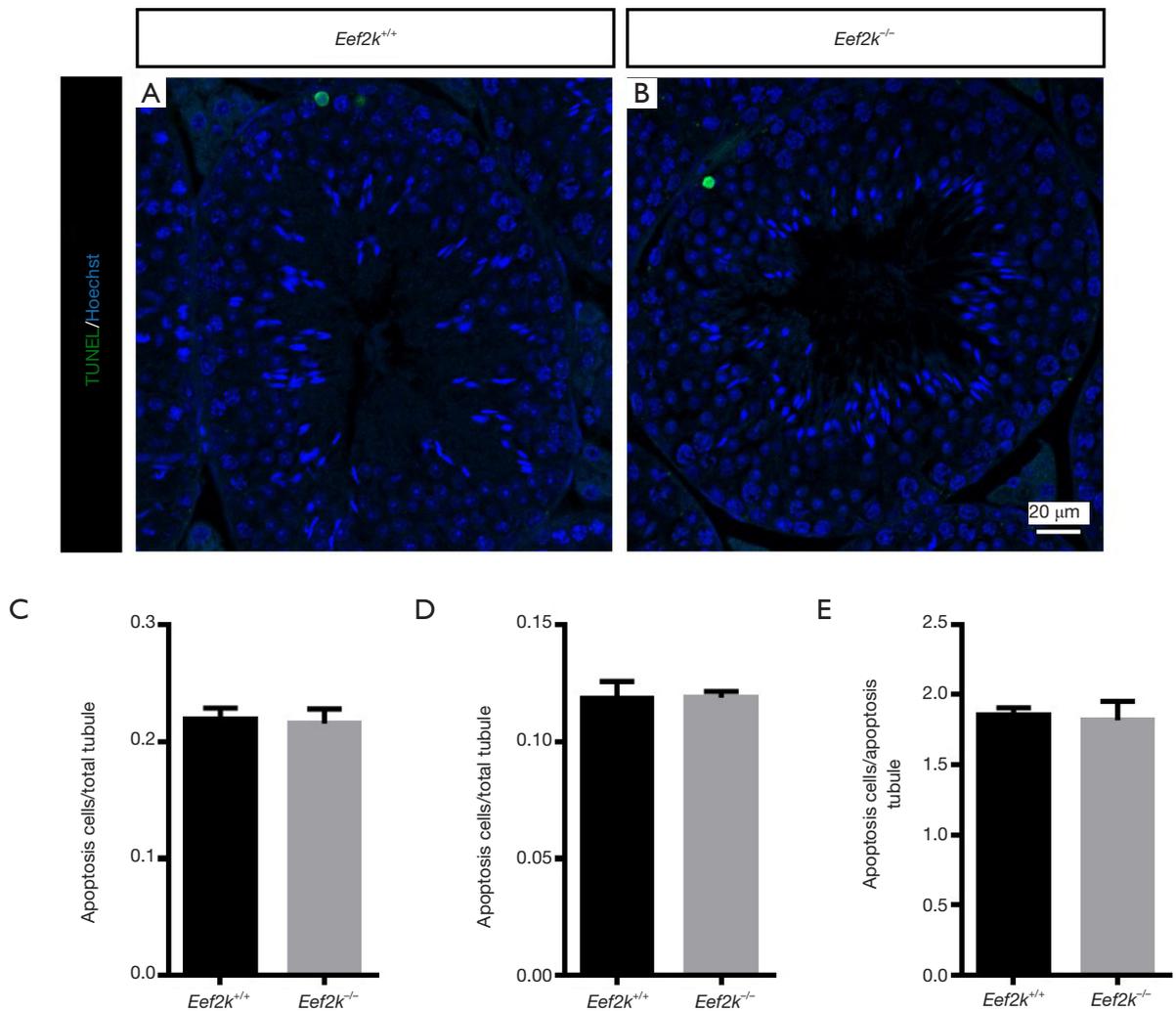
Figure 4 Analysis of apoptosis in *Eef2k*$^{+/+}$ and *Eef2k*$^{-/-}$ testes. The number of apoptotic cells was not significantly different between *Eef2k*$^{+/+}$ and *Eef2k*$^{-/-}$ mice. Testicular sections from TUNEL assay of (A) *Eef2k*$^{+/+}$ and (B) *Eef2k*$^{-/-}$ mice; (C) Average TUNEL-positive apoptotic cell counts of *Eef2k*$^{+/+}$ and *Eef2k*$^{-/-}$ mice; (D) Average TUNEL positive apoptotic tubule counts of *Eef2k*$^{+/+}$ and *Eef2k*$^{-/-}$ mice, n=3. (E) Average TUNEL-positive apoptotic cell counts in each apoptotic tubule of *Eef2k*$^{+/+}$ and *Eef2k*$^{-/-}$ mice, n=3. *Eef2k*, eukaryotic elongation factor-2 kinase.
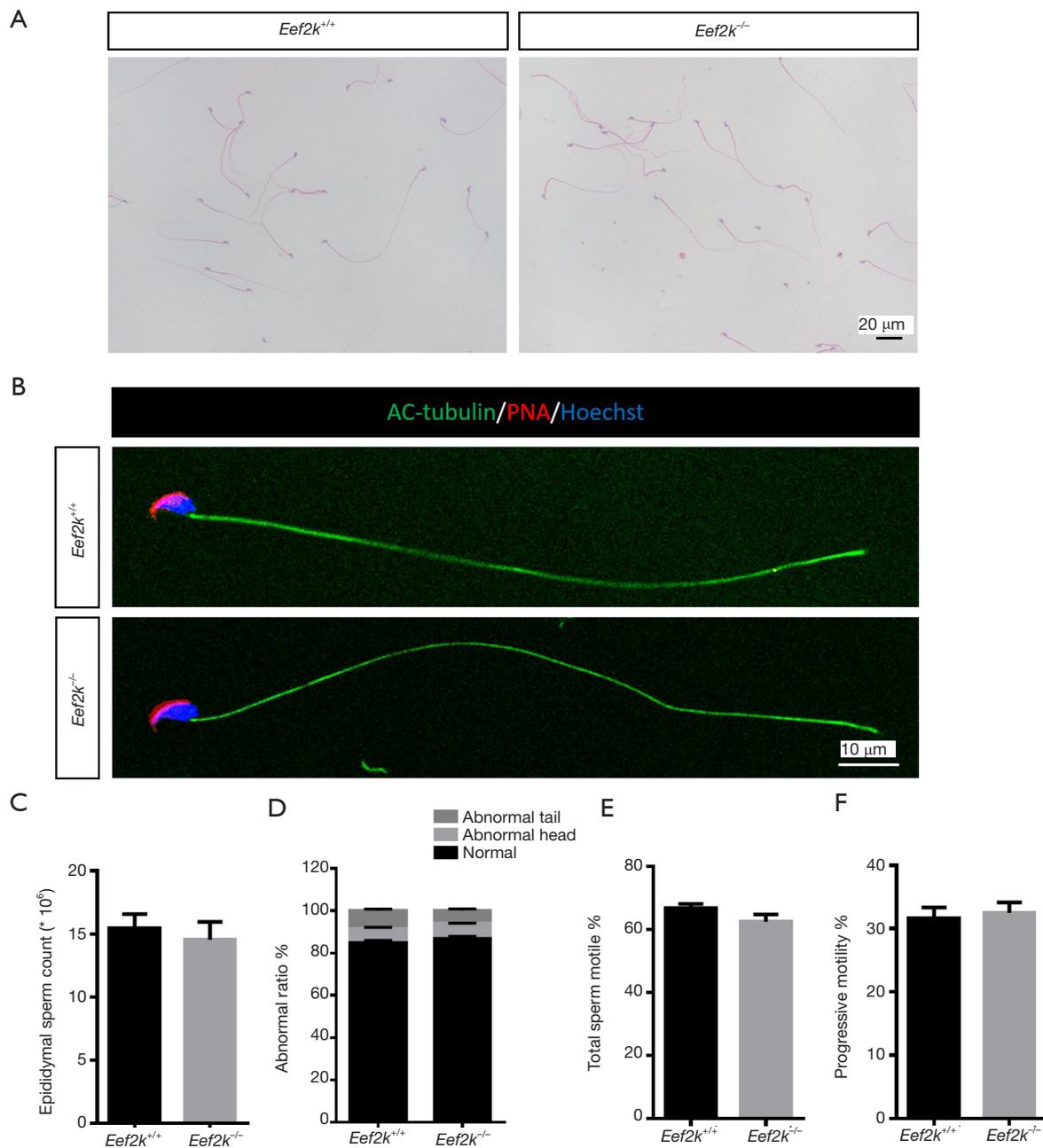
Figure 5 Analysis of spermatozoa in *Eef2k⁻/⁻* mice. (A) Hematoxylin- and eosin-stained spermatozoa from wild-type and *Eef2k⁻/⁻* mice. (B) Fluorescent detection of AC-tubulin (green) and PNA (red) from wild-type and *Eef2k⁻/⁻* spermatozoa. (C) Epididymal sperm count from wild-type and *Eef2k⁻/⁻* mice, n=3. (D) Classification of epididymal sperm morphology from wild-type and *Eef2k⁻/⁻* mice, n=3. (E) Average rate of motile sperm and (F) progressive sperm from wild-type and *Eef2k⁻/⁻* mice, n=3. *Eef2k*, eukaryotic elongation factor-2 kinase.

this context. This analysis revealed that the loss of *Eef2k* expression did not adversely impact sperm motility, morphology, or progressive motility in these mice.

Overall, our data indicate that *Eef2k* does not play a key role in murine spermatogenesis, and they provide a basis for future analyses of the mechanistic basis for spermatogenesis in these mice.

Conclusions

EEF2K is a protein that is expressed at low levels in asthenospermatic human sperm and *Tcte1*-knockout mouse sperm. However, the knockout of this protein did not adversely impact fertility, spermatogenesis, or development in male C57BL/6 mice. As such, these results indicate that *Eef2k* is dispensable in the context of male fertility. Due to the limited energy and funds, there is no further research in proteomes of the germ cells.

Acknowledgments

Footnote

Reporting Checklist: The authors have completed the ARRIVE reporting checklist. Available at http://dx.doi.org/10.21037/tau-21-18

Data Sharing Statement: available at http://dx.doi.org/10.21037/tau-21-18

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at http://dx.doi.org/10.21037/tau-21-18). The authors have no conflicts of interest to declare.

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. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). Experiments were performed under a project license (No.: IACUC-2004020) granted by the Animal Ethical and Welfare Committee, in compliance with the Institutional Animal Care and Use Committee of Nanjing Medical University guidelines.

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Table S1 List of antibodies

Antibodies	SOURCE	IDENTIFIER
Mouse monoclonal anti-Acetylated Tubulin	Sigma, St.Louis, MO, USA	Cat#T7451
Rabbit polyclonal anti-SOX9	Millipore, Billerica, MA, USA	Cat#AB5535
Rabbit polyclonal anti-gamma H2A.X (phospho S139)	Abcam, Cambridge, MA, USA	Cat#ab2893
Goat polyclonal anti-PLZF	RnD Systems, Minneapolis, MN,USA	Cat#AF2944

SOX9, SRY (sex determining region Y)-box 9; PLZF, Promyelocytic leukemia zinc finger.

Table S2 List of antibodies

Antibodies	SOURCE	IDENTIFIER
Donkey anti-Mouse IgG, Alexa Fluor 488	Invitrogen, Carlsbad, CA, USA	Cat#A-21202
Donkey anti-Rabbit IgG, Alexa Fluor 488	Invitrogen, Carlsbad, CA, USA	Cat#A-21206
Cy3-AffiniPure Donkey Anti-Goat	Jackson, West Grove, PA,USA	Cat#705-165-147

IgG, Immunoglobulin G; PA, Pennsylvania; USA, united states of American.

Table S3 Statistics of cells with fluorescent signal in tubules

	γ-H2AX/per tubule	PLZF/per tubule	SOX9/per tubule	PNA/per tubule
$Eef2k^{+/+}$-1	51	4	11	176
$Eef2k^{+/+}$-2	45	7	12	165
$Eef2k^{+/+}$-3	54	5	15	169
$Eef2k^{-/-}$-1	47	4	17	158
$Eef2k^{-/-}$-2	52	6	15	184
$Eef2k^{-/-}$-3	49	5	10	179

PLZF, Promyelocytic leukemia zinc finger; SOX9, SRY (sex determining region Y)-box 9; PNA, peanut agglutinin.

Table S4 TUNEL-positive apoptotic cells and tubules count

	Total tubules	Apoptosis cells	Apoptosis tubules	Apoptosis cells/ total tubules	Apoptosis tubules/ total tubules	Apoptosis cells/ apoptosis tubules
$Eef2k^{+/+}$-1	235	56	31	0.24	0.13	1.81
$Eef2k^{+/+}$-2	233	49	25	0.21	0.11	1.96
$Eef2k^{+/+}$-3	249	52	29	0.21	0.12	1.79
$Eef2k^{-/-}$-1	253	59	30	0.23	0.12	1.97
$Eef2k^{-/-}$-2	280	62	32	0.22	0.11	1.94
$Eef2k^{-/-}$-3	235	45	29	0.19	0.12	1.55

TUNEL, TdT-mediated dUTP Nick-End Labeling.