Deletion of Ck2β gene causes germ cell development arrest and azoospermia in male mice

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Abstract

Objectives: In humans, non-obstructive azoospermia (NOA) is a major cause of male infertility. However, the aetiology of NOA is largely unknown. Previous studies reported that protein CK2β was abundantly and broadly expressed in spermatogenic cells. Here, we investigate whether protein CK2β participates in spermatogenesis.

Materials and Methods: In this study, we separated spermatogenic cells using STA-PUT velocity sedimentation, analysed the expression pattern of protein CK2β by immunoblotting, specifically deleted Ck2β gene in early-stage spermatogenic cells by crossing Ck2βflmice with Stra8-Cre+ mice and validated the knockout efficiency by quantitative RT-PCR and immunoblotting. The phenotypes of Ck2βflf SCre+mice were studied by immunohistochemistry and immunofluorescence. The molecular mechanisms of male germ cell development arrest were elucidated by immunoblotting and TUNEL assay.

Results: Ablation of Ck2β gene triggered excessive germ cell apoptosis, germ cell development arrest, azoospermia and male infertility. Inactivation of Ck2β gene caused distinctly reduced expression of Ck2α′ gene and CK2α′ protein.

Conclusions: Ck2β is a vital gene for germ cell survival and male fertility in mice.

Liang and Wang are contributed equally to this work.

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1 | INTRODUCTION

Infertility affects about 15% of couples, and male factor contributes to approximately 50% of all infertility cases in humans. Azospermia is a major cause of male infertility, including obstructive azoospermia (OA) and non-obstructive azoospermia (NOA). NOA, caused by testicular failure, is the most severe form of male infertility. NOA accounts for about 60% of azoospermia and affects about 10% of infertile men. Clinically, NOA can be categorized into hypospermatogenesis, germ cell arrest and Sertoli cell-only syndrome. Complex genetic factors contribute to testicular failure, including autosomal chromosome abnormalities, microdeletions of the Y chromosome and single gene mutations. Mutations on dozens of genes, including SOHLH1, TAF4B, ZMYND15, MCM8, SYCE1, TEX1, TDRD7, TDRD9, MEIOB, TEX14, DNAS, MAGEB4, TEX15, FANCM, DMC1 and XRCC2 have been identified to cause NOA by pedigree analysis. However, these genes can explain only a part of male infertility, and more related genes need to be explored. Protein kinase CK2, previously known as casein kinase II, is a serine/threonine kinase in the form of tetramer with 2 catalytic subunits (α and α′) and 2 regulatory β subunits, functioning in many biological processes like cell proliferation, apoptosis and DNA damage repair. We have reported that CK2 is vital for oogenesis by participating in apoptosis and DNA damage repair process; CK2β is essential for follicle survival, depletion of which causes massive follicle atresia and eventually premature ovarian failure at young adulthood.

In mice, immunoblotting shows that CK2α, CK2α′ and CK2β are expressed in testis, and the expression of CK2β is significantly lower than CK2α and CK2α′ in spermatogenesis. Immunohistochemistry reveals that CK2α is localized at the acrosome area of spermatids; CK2α′ appears in the acrosomal and cytoplasmic region of spermatids, whereas CK2β is expressed in spermatogonia and spermatocytes. Immunofluorescence in spermatogonia reveals that CK2α is localized at the acrosome and mid-piece; CK2α′ is localized at acrosome, while CK2β displays a weak staining at the acrosome and a strong staining at the mid-piece. Ablation of the CK2α′ coding gene Ck2α′ leads to infertility of male mice, with decreased sperm count and increased spermatogonia with head abnormality. Considering the distinct localization of CK2β and CK2α′, it is quite necessary to clarify whether CK2β functions in spermatogenesis and how it functions.

Here, we specifically depleted Ck2β gene in spermatogonia by crossing Ck2β−/− mice with Stra8-Cre+ mice. We found that loss of Ck2β gene resulted in germ cell differentiation arrest and male infertility. CK2β is likely to be an essential anti-apoptotic factor participating in regulating spermatocyte survival.

2 | MATERIALS AND METHODS

2.1 | Mice

To obtain Ck2β−/−:Stra8-Cre+ males, we crossed Stra8-Cre with previously reported Ck2β−/− mice, and the resulting male offspring Ck2β−/−:Stra8-Cre+ were mated with Ck2β−/− females to generate Ck2β−/−:Stra8-Cre+ male mice (C57BL/6 and 129/SvEv mixed background). Unless otherwise specified, the Ck2β−/− mouse were used as the control group. DNA extraction from mouse tail was used to genotype Ck2β−/−, Ck2β−/− and Stra8-Cre alleles, respectively. The primer pair for Ck2β−/− allele was forward: 5′-GAGGGCATAGTAGATATGAATCTG-3′ and reverse: 5′-GGATAGCAAACTCTCTGAG-3′. The primer pair for Ck2β−/− allele was forward: 5′-ATGAGTAGCTCTGAGGGTG-3′ and reverse: 5′-GGATAGCAAATCTCTGAG-3′. The primer pair for Stra8-Cre allele was forward: 5′-GTGCAAGCTGAACACAGG3′ and reverse: 5′-AGGGACACAGCATTGGAGTC-3′. All animal operations were carried out in accordance with the guidelines of the Animal Research Committee principles of the Institute of Zoology, Chinese Academy of Sciences. All mice were housed in a temperature-controlled room with a 12D:12L cycle.

2.2 | Antibodies

Antibodies used in the experiments were obtained from the following companies: rabbit monoclonal anti-CK2β antibody (1:1000) (ABGENT; AJ1128b); mouse monoclonal anti-β-ACTIN antibody (1:1000) (Easybio Technology; BE0021); mouse monoclonal anti-MVH antibody (1:200) (Abcam; ab27591); mouse monoclonal anti-CK2α antibody (1:1000) (Abcam; ab70774); and rabbit polyclonal anti-CK2α′ antibody (1:1000) (Bioworld Technology; BS6571); secondary antibodies were purchased from Zhongshan Golden Bridge Biotechnology Co, LTD.

2.3 | Isolation of mouse spermatogenic cells

Considering the sequential and synchronized occurrence of Sertoli cells, spermatagonia, spermatocytes and round spermatids in the postnatal mouse testis, isolation was performed from samples at 6, 9, and 12 weeks of age.
8 and 17 days postpartum (dpp) and adult mice, respectively.\textsuperscript{39} The isolation procedures were described previously.\textsuperscript{39,40} Briefly, testes from Ck2\(\beta^{fl/}\) and Ck2\(\beta^{fl/};SCre^{+}\) mice were dissected and decapsulated. The seminiferous tubules were minced into pieces and incubated in 8 mL phosphate-buffered saline (PBS) containing 100 \(\mu\)L 1 mg/mL collagenase (Sigma, C5138) and 100 \(\mu\)L 1 mg/mL hyaluronidase (Sigma, H3506) at 37°C for 15 minutes with gentle shaking. After centrifugation at 4°C, 200 g for 5 minutes, the cells were collected, washed with PBS, incubated in 15 mL PBS with 0.25% trypsin (Gibco, 25200-072) and 1 mg/mL DNase I (AppliChem; A3778,0050) at 37°C for 15 minutes with gentle shaking. After filtration using a 40-\(\mu\)m nylon cell strainer and centrifugation for 3 hours, the cells were separated by 2%-4% bovine serum albumin (BSA) gradient in PBS. The cell pools (10 mL/pool) were collected separately in numbered tubes and centrifuged at 4°C, 600 g for 5 minutes, and then the supernatant was removed. Adding 1 mL PBS to each odd-numbered tube, the cells were resuspended, and then, 60 \(\mu\)L suspension of each tube was added to 96-cell plate for further observation. The cellular purity and cell types were identified under phase contrast microscope based on morphological evaluation and the diameter of cells.\textsuperscript{39} Expected cell types with cellular purity (\(\geq90\%\)) were collected for immunoblotting.

2.4 | Immunoblotting

The tunica albuginea of testes was removed, the seminiferous tubules were homogenized using a homogenizer in RIPA buffer (25 mM Tris-HCI, pH 7.6, 350 mM NaCl, 1% Nonidet P-40, 1% sodium deoxycholate and 0.1% sodium dodecyl sulphate) (Solarbio Life Sciences; R0010) supplemented with protease and phosphatase inhibitor cocktail (Roche Diagnostics, 04693116001). Spermatogenic cells isolated from testes were resuspended in the above-described RIPA buffer. After transient sonication, the lysates were incubated on ice for 30 minutes and then centrifuged at 4°C, 14 000 \(\times\) g for 20 minutes. The supernatant was transferred to a new tube, and equal volume loading buffer was added. After being boiled at 95°C for 10 minutes, the protein lysates were used for immunoblotting analysis. Immunoblotting was performed as described previously.\textsuperscript{41} Briefly, the separated proteins in SDS-PAGE were electrically transferred to a polyvinylidene fluoride membrane. After incubation with primary and secondary antibodies, the membranes were scanned with Bio-Rad ChemiDoc XRS+.

2.5 | Quantitative RT-PCR

Total RNA of testes from Ck2\(\beta^{fl/}\) and Ck2\(\beta^{fl/};SCre^{+}\) male mice was extracted using the RNeasy Micro Kit (Qiagen, 74004), and the first-strand cDNA was generated with cDNA synthesis kit (Invitrogen; 11754050). Glyceraldehyde-3-phosphate dehydrogenase (Gapdh) or \(\beta\)-actin was used as internal control to normalize the cDNA level of the samples. The experiment was conducted by using UltraSYBR Mixture (CoWin Biosciences; CW0957) in Roche LightCycler480 II detection system. The relative gene expression was calculated by the 2\(^{-ΔΔCt}\) method. The primers used were as follows.

\[
\begin{align*}
Ck2\alpha & \text{ forward: 5′-CTTCGGCTTAAATAGCTGGGGT-3′;}
Ck2\alpha & \text{ reverse: 5′-TCGAGAAGCACTCGGACATT-3′;}
Ck2\alpha' & \text{ forward: 5′-TCCCGAGCTGGGTGAATCAA-3′;}
Ck2\alpha' & \text{ reverse: 5′-TGTTCCACCGAGGTCTC-3′;}
\end{align*}
\]

\[
\begin{align*}
\beta\text{-Actin} & \text{ forward: 5′-GCCGTATCTCCCTCATTGC-3′;}
\beta\text{-Actin} & \text{ reverse: 5′-CAGTTGGTAACAATGCCCATGT-3′.}
\end{align*}
\]

2.6 | Tissue collection and histological analysis and immunofluorescence

Testes or caudal epididymides were dissected from Ck2\(\beta^{fl/}\) and Ck2\(\beta^{fl/};SCre^{+}\) male mice immediately after euthanasia. Testes were fixed in 4% paraformaldehyde (pH 7.4) or Bouin’s fixative overnight at 4°C. Caudal epididymides were fixed in 4% paraformaldehyde (pH 7.4) overnight at 4°C. The tissues were then dehydrated in a graded ethanol series, cleaned in xylene and embedded in paraffin. The paraffin-embedded tissues were sectioned into 5 \(\mu\)m and mounted on glass slides. After adequately drying at 48°C, the sections were deparaffinized in xylene, hydrated in a graded ethanol series and stained with haematoxylin and eosin for histological analysis.

Testes for immunofluorescent staining were fixed in 4% paraformaldehyde (pH 7.4) overnight at 4°C, dehydrated and embedded in paraffin. The paraffin-embedded tissues were sectioned into 5 \(\mu\)m and mounted on glass slides. The sections were then deparaffinized in xylene, hydrated in a graded ethanol series and stained with haematoxylin and eosin for histological analysis.

Testes for immunofluorescent staining were fixed in 4% paraformaldehyde (pH 7.4) overnight at 4°C, dehydrated and embedded in paraffin. The paraffin-embedded tissues were sectioned into 5 \(\mu\)m and mounted on glass slides. The sections were then deparaffinized in xylene, hydrated in a graded ethanol series and immersed in sodium citrate buffer (pH 6.0) and heated for 15 minutes in a microwave oven for antigen retrieval. After blocking with 5% donkey serum albumin, the sections were incubated with primary antibody at 4°C overnight and appropriate TRITC-conjugated secondary antibody. The nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI). Images were captured using a laser scanning confocal microscope (Zeiss 780 META).

2.7 | TUNEL assay

TUNEL assay was performed in accordance with the DeadEnd\textsuperscript{TM} Fluorometric TUNEL System (Promega Biosciences, G3250). Images were captured using a laser scanning confocal microscope (Zeiss 780 META).

2.8 | Breeding assay

C57BL/6J wild-type female mice with known fertility were mated with 8-week-old Ck2\(\beta^{fl/}\) and Ck2\(\beta^{fl/};SCre^{+}\) male mice. For 6 months,
the cages were monitored daily to record the number of pups and litter sizes.

2.9 | Statistical analysis

Paired two-tailed Student’s t test was used for statistical analysis. The data were considered statistically significant when \( P < .05 \) (*), .01 (**) or .001 (***)).

3 | RESULTS

3.1 | Deletion of Ck2β by Stra8-Cre results in male infertility

To study the potential role of protein CK2β during spermatogenesis, we first isolated the spermatogonia, spermatocytes, round spermatids and Sertoli cells from mouse testes to detect the expression of CK2β. Immunoblotting analysis showed that CK2β was expressed from spermatogonia to round spermatids and the highest expression level was detected in spermatocytes, while no expression was detected in Sertoli cells (Figure 1A and 1B). These results suggest that CK2β may participate in spermatogenesis.

To explore the potential function of protein CK2β during spermatogenesis, we mated Ck2β mice, in which exons I–II were targeted, with Stra8-Cre mice to generate Ck2βfl/Δ;Stra8-Cre mice (referred to as Ck2βfl/Δ;SCre)(Figure S1). In Stra8-Cre mice, Cre recombinase was specifically expressed in early-stage spermatogonia from 3 days after birth onward and peaked in preleptotene spermatocytes at 7 dpp. Quantitative RT-PCR and immunoblotting analysis confirmed that the expression of CK2β in testes from Ck2βfl/Δ;SCre mice was efficiently deleted at both mRNA and protein levels (Figure 2A, 2B and Figure S2).

| Breeding assay of Ck2βfl/Δ; SCre+ males in 6 mo Group | Number | Litters/male (mean ± SEM) | Pups/litter (mean ± SEM) |
|---|---|---|---|
| Ck2βfl/Δ        | 6        | 7.67 ± 0.75 | 8.11 ± 1.45 |
| Ck2βfl/Δ;SCre+  | 6        | 0           | 0           |
To determine the effects of Ck2β depletion on male fertility, we carried out a breeding assay by mating Ck2βfl/Δ or Ck2βfl/Δ;SCre+ male mice with C57BL/6J wild-type females of tested fertility for 6 months. Continuous breeding observation indicated that Ck2βfl/Δ;SCre+ males were completely infertile (Table 1).

3.2 | Ck2β depletion causes testicular atrophy and azoospermia

To determine the causes of infertility in Ck2βfl/Δ;SCre+ males, we first examined whether the infertility was due to testicular dysfunction and the consequential functional azoospermia. Ck2βfl/Δ;SCre+ males were observed to develop normally, without defects until at least 12 months of age. However, the testes of adult Ck2βfl/Δ;SCre+ males exhibited a marked reduction in size (Figure 3A). Statistical analysis revealed that the testis weight ratio of the testes of Ck2βfl/Δ continued to increase from day 12 to month 2 after birth and slightly decreased from month 2 to month 12, with a mean testis weight ratio of 0.2493%, 0.3088%, 0.4736%, 0.7709%, 0.6978% and 0.6345% corresponding to days 12, 17, 24, months 2, 8 and 12 after birth, respectively (Figure 3B). By comparison, the testis weight ratio of Ck2βfl/Δ;SCre+ males showed small changes from day 12 to month 12 after birth, with a mean testis weight ratio of 0.1942%, 0.2454%, 0.2859%, 0.3012%, 0.2550% and 0.2194% corresponding to days 12, 17, 24, months 2, 8 and 12 after birth, respectively (Figure 3B).

Further histological analysis showed that no mature spermatozoa were present in the epididymal lumens of Ck2βfl/Δ;SCre+ males (Figure 4). These data demonstrate that Ck2β
deletion results in testicular atrophy and azoospermia in mice, and these phenotypes do not change with increasing age after adulthood.

3.3 | Ck2β knockout results in spermatogenesis arrest in male mice

To clarify the cause of testicular atrophy and azoospermia in Ck2βfl/Δ;SCre+ males, we next examined the first wave of spermatogenesis at 12 dpp, 15 dpp and 17 dpp corresponding to leptotene, zygotene and pachytene spermatocytes, respectively.

As shown in Figure 5A, the testes of Ck2βfl/Δ males were populated by spermatocytes of various stages, while the seminiferous tubules of Ck2βfl/Δ;SCre+ mice contained only few spermatocytes. Statistical analysis revealed that the number of germ cells of testes from Ck2βfl/Δ males continued to rise from 12 dpp to 17 dpp, but these numbers were almost unchanged in Ck2βfl/Δ;SCre+ males (Figure 5B), suggesting that Ck2β knockout affected the prophase of meiosis I at the first wave of spermatogenesis in mice.
To determine whether this effect would continue to adulthood, we examined the seminiferous tubules of both Ck2βfl/Δ and Ck2βfl/Δ;SCre+ males at 2 months of age. Histological analysis showed that the seminiferous tubules of Ck2βfl/Δ males were filled with spermatogonia, spermatocytes, round or elongated spermatids (Figure 6A); however, there are only a few germ cells and no round or elongated spermatids in the seminiferous tubules of the Ck2βfl/Δ;SCre+ males (Figure 6A). To confirm these observations, we performed immunofluorescence of the germ cell marker MVH on testicular sections. The results showed that the number of MVH-positive germ cells was dramatically reduced in Ck2βfl/Δ;SCre+ tubules compared with those in Ck2βfl/Δ tubules (Figure 6B). The diameter of the seminiferous tubules of Ck2βfl/Δ;SCre+ males dropped to half of that in Ck2βfl/Δ males (Figure 6C).

To precisely explore the stage affected by Ck2β knockout during spermatogenesis, we analysed the numbers of spermatogonia, leptotene, zygotene, pachytene and diplotene spermatocytes in Ck2βfl/Δ and Ck2βfl/Δ;SCre+ males at 2 months of age. Germ cell types were identified based on the size, the positioning in seminiferous tubules, the status of chromatin (the amount and distribution of heterochromatin, chromatin threads thickness and looseness) of cells. The results demonstrated that the numbers of zygotene, pachytene and diplotene spermatocytes were significantly reduced, but that of spermatogonia and leptotene spermatocytes were unchanged in Ck2βfl/Δ;SCre+ males, compared with Ck2βfl/Δ males. These findings suggest that Ck2β knockout may not affect spermatogonial proliferation, differentiation and entrance into meiosis in mice, but severely impairs spermatocyte survival and causes spermatogenesis arrest (Figure 6D).

3.4 | Deletion of Ck2β triggers germ cell apoptosis

To further examine the causes of decreased numbers of spermatocytes, detection of apoptosis in testes was carried out by the TUNEL assay. The results showed that more than half of the tubules had...
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TUNEL-positive germ cells in Ck2βfl/Δ;SCre+ males compared with less than 20% of that in Ck2βfl/Δ males (Figure 7A and 7B). Besides, the number of TUNEL-positive germ cells was also obviously increased in Ck2βfl/Δ;SCre+ males (Figure 7A and 7C). The above data indicated that deletion of Ck2β triggered germ cell apoptosis, and consequently caused reduction in the number of spermatocytes.

3.5 | Inactivation of Ck2β causes distinctly reduced expression of Ck2α′ but not Ck2α

To clarify the forms of CK2 functions in spermatogenesis, immunoblotting was carried out to assess protein levels of CK2α, CK2α′ and CK2β in testes of Ck2βfl/Δ and Ck2βfl/Δ;SCre+ mice. Compared with Ck2βfl/Δ mice, the level of CK2β protein, as expected, was significantly reduced in testis extracts from Ck2βfl/Δ;SCre+ mice (Figure 8A and Figure S3). Meanwhile, the level of CK2α′ protein was also significantly down-regulated in testes of Ck2βfl/Δ and Ck2βfl/Δ;SCre+ mice (Figure 8A and Figure S3). By comparison, the levels of CK2α protein showed no variation in testes of Ck2βfl/Δ and Ck2βfl/Δ;SCre+ mice (Figure 8A and Figure S3). The results suggest that CK2 presumably functions in the forms of α′2β2 in spermatogenesis and the reduced expression of CK2α′ protein in testes of Ck2βfl/Δ;SCre+ is assumably due to degradation resulting from decreased stability of CK2α′ protein without CK2β.

To test the hypothesis, the expression of CK2α, CK2α′ and CK2β in the mRNA level was detected in testes by RT-PCR. Results found that...
the mRNA expression of both Ck2α’ and Ck2β in testes of Ck2βΔΔ was significantly higher than that in Ck2βΔΔ;SCre’ (Figure 8B and Figure S4), while the mRNA expression of Ck2α in testes of Ck2βΔΔ was remarkably lower than it in testes of Ck2βΔΔ;SCre’ (Figure 8B and Figure S4).

4 | DISCUSSION

NOA is problematic for males as it results in infertility. Accumulating studies have shown that gene mutations contribute to NOA; however, the molecular mechanisms underlying the disorder are still poorly understood. Immunoblotting revealed that protein Ck2β was expressed in spermatogonia, spermatocytes and round spermatids, and no expression was seen in Sertoli cells. These findings are consistent with previous reports that Ck2β protein is abundantly and broadly expressed in early spermatogenesis as shown by in situ hybridization and immunohistochemistry in mice.34,35 However, our results are inconsistent with researches on rats,43 which indicates that Ck2β protein is located in both germ cells and Sertoli cells. This discrepancy may be caused by variations between species.

A study reports that Ck2α’/− mice die in mid-embryogenesis with severe developmental defects in the neural tube and heart,44 but there is no specific study of its role in gametogenesis. The fertility of Ck2α’/− females is not affected, while Ck2α’/− males are infertile.34 Male mice lacking Ck2α’ displayed oligoospermatogenesis and globoospermia; germ cells display extensive degenerative changes characterized by nuclear abnormalities at the stages from spermatogonia to early spermatids, including the first spermatogenesis wave.34,36 Zygote-specific knockout of Ck2β results in embryonic lethality after implantation with decreased cell proliferation but no signs of apoptosis.38 We have reported that oocyte-specific knockout of Ck2β given rise to ovarian follicle atresia and POF, which are related to down-regulated PI3K/AKT signalling and failed DNA damage response signalling.33

In this study, using Stra8 promoter-driven Cre recombinase, Ck2β gene is effectively deleted in male mouse germ cells from the early stage of spermatogonia, which facilitated to explore the roles of Ck2β in spermatogenesis. The weak expression of protein Ck2β present in the testes of Ck2βΔΔ;SCre’ mice probably comes from Leydig cells or other somatic cells in testes. In consistence with the result of Ck2α’ knockout,33 Ck2β mutant male mice are also infertile. Ck2β deletion severely blocks the first wave of spermatogenesis. The number of spermatogonia and leptotene spermatocytes has no change in adult mice, while the number of zygote, pachytena and diplotene spermatocytes is significantly lower in Ck2β mutant mice than control mice. It seems that Ck2β protein functions at the spermatocyte stage but not spermatogonia stage or the onset of meiotic prophase. Furthermore, the increased number of apoptotic spermatocytes implies that Ck2β protein plays a critical role in spermatocyte survival. However, it is unknown whether Ck2β protein functions in spermiogenesis because the blockage of spermatogenesis by Ck2β deletion occurs at the spermatocyte stage. Considering studies in somatic cells29–32 and oocytes,33 it is likely that CK2β protein functions via apoptosis or DNA damage signalling pathways in mouse spermatogenesis.

In this study, we find that deletion of protein Ck2β in testes results in significant reduction in protein Ck2β. In contrast, the level of protein Ck2α shows no obvious difference in Ck2β mutant testes and control testes. Considering previous reports that Ck2α’ protein is indispensable to spermatogenesis,34 CK2 presumably functions in spermatogenesis in the form of α’β2, and the degradation of Ck2α’ protein is caused by decreased stability of Ck2α’ protein without Ck2β. However, down-regulation of Ck2α’ at the mRNA level is observed in Ck2β mutant, indicating that Ck2β knockout disrupts Ck2α’ transcription and consequently disturbs the function of Ck2α’ protein per se or as a catalytic subunit of CK2 holoenzyme. In addition, it is confusing that Ck2α has no changes in the protein level while the mRNA expression is obviously elevated. From above results, the mutual regulation of the three subunits is complicated.

In summary, we identify Ck2β as a vital gene for germ cell survival and male fertility in mice, but whether it is a candidate gene for NOA in humans needs further clarification.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

QXL, ZBW, FL, QYS and WPQ designed the experiments; QXL, FL, LWL and JYQ performed experiments and analysed data; QXL wrote the manuscript; QXL, QYS, OFC, BB and HS revised manuscript. All authors read and approved the final revised manuscript.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION
Additional supporting information may be found online in the Supporting Information section.

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