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GOLGA4, A Golgi matrix protein, is dispensable for spermatogenesis and male fertility in mice

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A B S T R A C T

During acrosome biogenesis, numerous granules formed from trans-Golgi stacks and accumulated in the concave region of the nuclear surface that is essential for acrosome formation. Several Golgi-associated proteins were involved in this process. However, the specific function of Golgi-associated proteins, especially Golgi matrix protein, during acrosome biogenesis remains elusive. In this study, we identified GOLGA4, as a Golgi matrix protein, highly expressed in mouse testes. To explore the function of GOLGA4 in spermatogenesis, we generated a *Golga4* global knockout mouse line using CRISPR/Cas9 technology and demonstrated that *Golga4* knockout males are fertile with normal morphology of testis and sperm. Furthermore, testicular histology showed no significant difference between WT and KO mice. Together, our data demonstrate that GOLGA4 is dispensable for mouse spermatogenesis and male fertility.

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1. Introduction

Mammalian spermatogenesis is a dynamic and complicated process, which can be divided into three phases: proliferation and differentiation of spermatogonia in mitosis, spermatocytes develop into haploid spermatids in meiosis, and round spermatids differentiation into spermatozoa in spermiogenesis [1,2]. The last phase, spermiogenesis, is also a complex and highly ordered biological process in which spermatids undergo acrosome biogenesis, nuclear chromatin condensation, head shaping, and flagella formation, etc. Among these, acrosome biogenesis has been considered as a critical process to facilitate sperm morphological transformation, and the Golgi-apparatus appear to play an essential role in acrosome biogenesis [3,4]. There are three phases during acrosome biogenesis: The Golgi phase, the cap phase, and the acrosome maturation phase [5]. In the Golgi phase, numerous granules formed from trans-Golgi stacks, and they accumulate in the concave region of the nuclear surface and then fuse to build a large acrosome granule. In the cap phase, the acrosome gradually expands and surrounds the nucleus. During the acrosome maturation phase, the acrosome changes its morphology and becomes mature so that they can participate in fertilization [5–7]. Although the morphogenetic changes during acrosome biogenesis have been well-documented, the precise molecular mechanism, especially the function of Golgi-associated proteins underlying this process, is poorly understood.

Golgi matrix protein genes have six members, including *Golga1*, *Golga2*, *Golga3*, *Golga4*, *Golga5*, and *Golgb1*. Previous studies have demonstrated that GOLGA3 highly expressed in pachytene spermatocytes and round spermatids, and mice with a point mutation of the *Golga3* gene exhibited abnormal spermiogenesis with defects in acrosome formation [8,9]. Interestingly, inactivation of GM130, a cis-side localized Golgi matrix protein, in mice caused the phenotype similar to human disease globozoospermia [10]. Besides, GOPC, a Golgi protein, defective male mice were also infertile accompanied by the abnormal acrosome [11,12]. GOLGA4, also known as P230/GOLGIN-245, a member of golgin subfamily A, is thought to be involved in specialized transport processes associated with maturation and/or differentiation of oligodendrocyte precursors and is required for efficient retrograde distribution of Shiga toxin from endosomes to the Golgi [13,14]. However, the specific function of GOLGA4 in spermatogenesis and male fertility remains unclear.

To investigate the role of GOLGA4 in spermatogenesis, we first

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Table 1
Primer sequences are used in this study.

Gene name	Sequence(5′-3)	Application
Golga4	F1:TGGCTTCTGTTACAGTCGGG R1:CTGAGTTCCATGGTGCGTCT R2:TTGCCTTAGTCCCATGGAGG	Genotyping
Golga1	F:ATGTTGAGCTCCTCCAGCAC R:CCGAAGGATTACATTTGACACATCA	RT-qPCR
Golga2	F:TGGGACAGGGAAAGTGGGTA R:TCCTGCCCTCTGACCTAACA	RT-qPCR
Golga3	F:TGTCCGGAGATCCATACCAAC R:AAGCAACCATGATTCCGGTTC	RT-qPCR
Golga4	F:GCAAATGGACCAGCAAGCAA R:GGGTTTTAGCGGAAGTCCCA	RT-qPCR
Golga5	F:CTCGTTGGCCACTCTGTTTTG R:TTCCTTGTCCCTGAGCGAAG	RT-qPCR
Golgb1	F:CAAAGTTGTGGGCGTGTGAG R:TCGAACCTTGCTGTCTGTCC	RT-qPCR
Gapdh	F:AGGTCGGTGTGAACGGATTTG R:GGGGTCGTTGATGGCAACA	RT-qPCR

examined the mRNA and protein levels of *Golga4* in a mouse multiple tissues. We then generated *Golga4* global knockout mouse model using CRISPR/Cas9 technology. Surprisingly, although GOLGA4 was found to express in testes predominantly, the *Golga4* knockout males were fertile with normal testicular and epididymal histology, normal sperm count, and motility. Collectively, our study herein showed that GOLGA4 is not essential for mammalian spermatogenesis, although it highly expressed in testes.

2. Materials and methods

2.1. Generation of Golga4 KO mice

Golga4 knockout mice were produced by zygote pronuclear microinjection using the CRISPR/Cas9 genome editing technique. The two pairs of single guided RNAs (sgRNAs) with the sequence sgRNA-1: TTGGTCCGGACGTCCTCCAG and sgRNA2: TGGTAATAACCGAGACGAAG were designed for targeting exon 4 and exon 11 of *Golga4* gene. After microinjection, followed by Sanger sequencing confirmation and PCR analysis, the heterozygous with 11,771-bp deletion male founder mice were allowed to inbreed to produce homozygous mice (KO). The mice were then housed in a specific pathogen-free facility under climate-controlled conditions with a 12-h light/dark cycle and were provided with water and a standard diet. All animal experiments have specifically approved by the Institutional Animal Care and Use Committee (IACUC) of Tongji Medical College, Huazhong University of Science and Technology.

2.2. RNA extraction and RT-qPCR

Trizol reagents (Invitrogen) were used for total RNA extraction and reverse transcriptional reactions contained 500 ng of purified total RNA using a PrimeScript RT reagent kit with gDNA Eraser (TaKaRa) to remove the DNA contamination. RT-qPCR was performed with SYBR green master mix (TaKaRa) on the LightCycler@96 Real-Time PCR system (Roche) according to manufactures' instructions. The relative gene expression was quantified using the comparative cycle threshold method, with the *Gapdh* expression used for normalization. All primers are listed in Table 1.

2.3. Western blot

Tissues were collected, and proteins were extracted using RIPA buffer (CWBIO, Cat# 01408). The protein lysates run on an 8% SDS-PAGE gel and transferred to PVDF membranes (Bio-Rad). Membranes were blocked in 5% non-fat milk (blocking solution) for 1 h

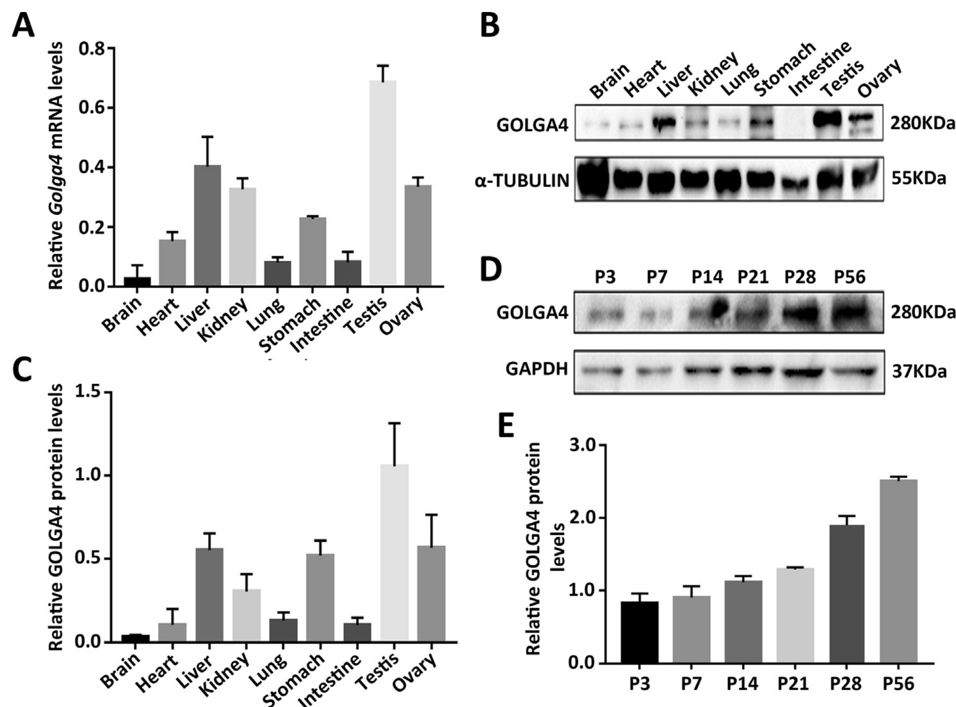

Fig. 1. The expression profile of GOLGA4 in multiple tissues and developing testes. **(A)** RT-qPCR analyses of *Golga4* mRNA expression levels in nine different organs from wild-type (WT) adult mice are shown. **(B)** Western blotting shows the GOLGA4 protein levels in nine different organs from WT adult mice. α-TUBULIN served as a loading control. **(C)** Quantification analyses of GOLGA4 protein levels in (B) are shown. Data are presented as mean ± SEM, n = 3. **(D)** Western blotting shows the GOLGA4 protein levels in mouse testes at postnatal day 3 (P3), P7, P14, P21, P28, and P56. GAPDH served as a loading control. **(E)** Quantification analyses of GOLGA4 protein levels in developing testes at P3, P7, P14, P21, P28, and P56 are shown. Data are presented as mean ± SEM, n = 3.

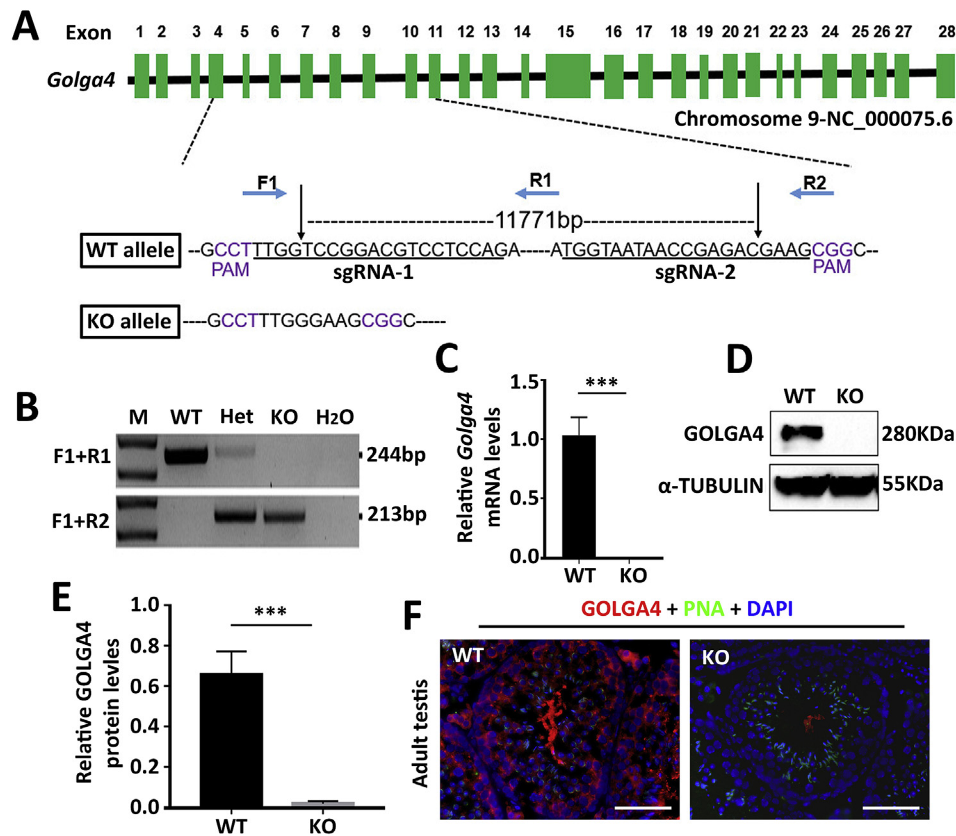

Fig. 2. Generation of *Golga4* knockout mice using CRISPR/Cas9 technique. **(A)** Schematic illustration shows the targeting strategy for generating *Golga4* knockout (KO) mice using the CRISPR/Cas9 system. Green boxes represent exons of the *Golga4* gene on mouse chromosome 9, blue arrows indicate the site of designed primers for genotyping, black underlines indicate the target regions of sgRNAs and black arrows show the cut site. The injection of the mixtures of Cas9 and sgRNA into zygotes for mutagenesis via microinjection led to 11,771-bp frame-shift deletion. **(B)** Representative genotyping gel images of *Golga4* alleles. A longer band (244bp) can be amplified from the WT allele using F1 and R1 primers, and a shorter band (213bp) can be amplified from the mutant allele using F1 and R2 primers, respectively. M, marker; Het, heterozygous; H_2O, negative control. **(C)** RT-qPCR analyses of *Golga4* mRNA levels in WT and KO adult testes are shown. Data are presented as mean ± SEM, n = 3. ***, $P < 0.001$ by student's *t*-test. **(D)** Representative Western blot image shows the GOLGA4 protein levels in WT and KO adult testes. α-TUBULIN served as a loading control. **(E)** Quantification analyses of GOLGA4 protein levels in WT and KO testes are shown. Data are presented as mean ± SEM, n = 3. ***, $P < 0.001$ by student's *t*-test. **(F)** Co-immunofluorescent staining of GOLGA4 (red) and PNA (green) in testicular sections from WT and KO mice is shown. Nuclei were stained with DAPI. Scale bar = 100 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

and incubated with primary antibodies (rabbit anti-GOLGA4 antibody, ABclone, A10216; GAPDH, Proteintech, 10494-1-AP; α-Tubulin antibody, Proteintech, 66031-1-Ig) at 4 °C for overnight. The membranes were washed with TBST three times, then incubated with a secondary antibody (HRP goat anti-mouse IgG, Abbkine, A21010-1; HRP goat anti-rabbit IgG, Abbkine, A21020-1) for 1 h. ECL (ClarityTM Western ECL Substrate, Bio-Rad) was used for chemiluminescence detection and photographed by ChemiDoc XRS + system (BIO-RAD). The band intensity was quantified by densitometry using Image J Analysis software (Research Services Branch).

2.4. Histology analysis

For histological analysis, testes and epididymides were collected and fixed in Bouin's fixative (Sigma Aldrich) for 3 h at RT. After several washes in 70% ethanol to remove excess stain, testes were embedded in paraffin. Tissues were sectioned at 5 μm thickness and stained by Periodic Acid-Schiff (PAS)-hematoxylin.

2.5. Immunofluorescence

Testes were fixed with 4% paraformaldehyde (PFA) diluted with PBS and then embedded in 50% Tissue-Tek O·C.T. compound (Sakura Finetek, 4583) in 20% sucrose on liquid nitrogen. 5 μm cryosections were cut and treated with the antigen retrieval by 0.01 mM Citrate (PH = 6.0). The sections were washed three times in PBS (10min/wash), then incubated with anti-GOLGA4 antibody (ABclone, A10216) in a humidified chamber at 4 °C for overnight. The sections were washed three times with PBS and then incubated with Alexa Fluor™ 594 Goat anti-rabbit IgG (H + L) (Invitrogen, A11032) at RT for 1 h. After washing three times with PBS (10min/wash), the sections were counterstained with DAPI and photographed with the fluorescence microscope.

2.6. Sperm counting

The cauda epididymis was dissected from adult mice. Sperm was squeezed out from the cauda epididymis and incubated in HTF medium for 30 min at 37 °C under 5% CO_2. Sperm counting analyses performed, as described previously [15].

3. Results

3.1. GOLGA4 is preferentially expressed in mouse testes

To explore the expression profile of *Golga4*, we examined the mRNA and protein levels in multiple tissues from adult mice by RT-

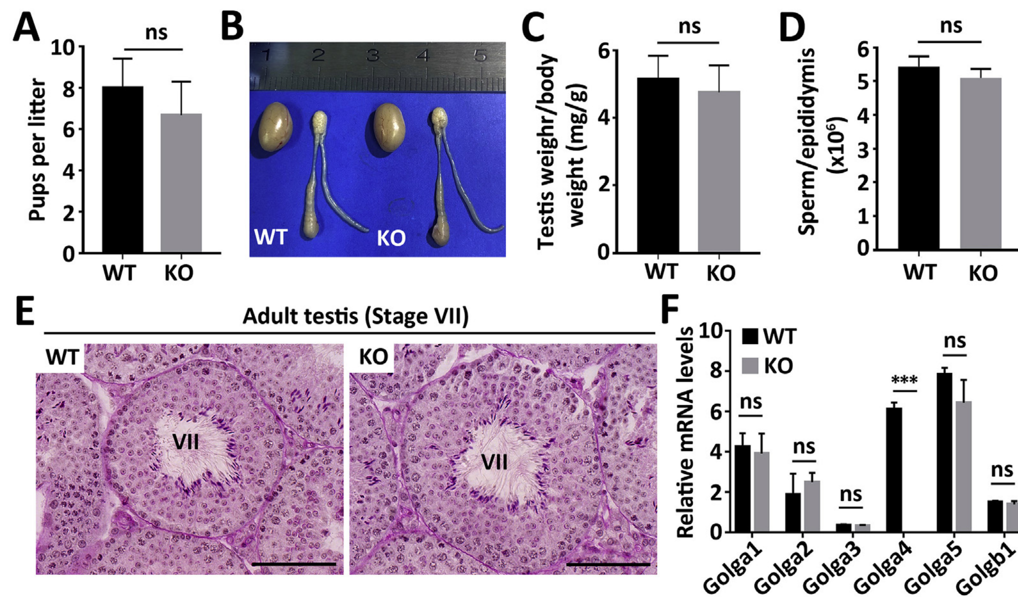

Fig. 3. GOLGA4 deficiency in mice does not cause defective spermatogenesis. (A) The histogram shows the number of pups per litter derived from WT males and KO males. Data are presented as mean ± SEM, n = 10. ns, not significant. **(B)** The gross morphology of the testis from WT and KO mice is shown. **(C)** The histogram shows the ratio of testis weight to body weight from WT and KO adult mice. Data are presented as mean ± SEM, n = 3. ns, not significant. **(D)** The histogram shows the number of sperm retrieved from one cauda epididymis of WT and KO mice. Data are presented as mean ± SEM, n = 3. ns, not significant. **(E)** Periodic acid-Schiff (PAS) staining of stage VII testicular seminiferous tubules from WT and KO mice. Scale bar = 100 μm. **(F)** RT-qPCR analyses of mRNA levels of six Golgi matrix protein family genes in WT and KO testes are shown. Data are presented as mean ± SEM, n = 3. ns, not significant. ***, $P < 0.001$ by student's *t*-test.

qPCR and Western blot, respectively. Our results showed that GOLGA4 is highly expressed in testis, followed by liver, stomach, and ovary (Fig. 1A–C). Furthermore, we also detected the mRNA and protein levels in postnatal developing testes at postnatal day 3 (P3), P7, P14, P21, P28, and P56. The data showed that GOLGA4 is continually expressed from P3 until P56 and displays the highest level at P56 (Fig. 1D–E).

3.2. Generation of Golga4 knockout mice

To determine the physiological role of *Golga4* in spermatogenesis, we next generate *golga4* knockout mice using CRISPR/cas9 technology. Two sgRNAs were designed to target exon4 and exon11, respectively (Fig. 2A), and specific primers were designed for genotyping. After an indel mutation containing an 11,771-bp deletion was confirmed by direct sequencing analysis, specific primers for wild-type (WT) or homozygous mutant (KO) allele was designed and used for genotyping (Fig. 2B). We intercrossed the mouse line (11,771-bp deletion) to obtain subsequent generations and confirmed the absence of both mRNA and protein of *Golga4* in KO testes (Fig. 2C–F). In addition, observation of the KO mice revealed no apparent development abnormalities or differences in sexual behavior.

3.3. GOLGA4 is dispensable for spermatogenesis

To identify the role of GOLGA4 in spermatogenesis and male fertility, we carried out the fertility test, by which mating sexually mature wild type (WT) or KO males with fertility proved females for six months. The average number of offspring per litter and the number of litters shows no discernible change (Fig. 3A), indicating that KO males are completely fertile. Subsequently, we analyzed the testis size and weight, the sperm count derived from caudal epididymis in WT and KO mice. The results showed there are no significant differences between WT and KO mice (Fig. 3B–D). Consistent with these results, histological analyses of testis revealed no germ cell development and spermiogenesis defects in KO mouse seminiferous tubules (Fig. 3E). These data suggest that *Golga4* is dispensable for spermatogenesis in mice. In addition, to ask whether other Golgi matrix protein genes may compensate with the GOLGA4 deficiency in mice, we further examine the mRNA level of *Golga1*, *Golga2*, *Golga3*, *Golga5* and *Golgb1* in WT and KO mouse testes. Interestingly, we did not observe significant changes in other Golgi matrix protein genes in KO testes compared with that of WT testes (Fig. 3F).

4. Discussion

The acrosome is a unique organelle formed by the fusion of Golgi-derived vesicles, which is controlled by several genes, including *Hrb*, *Gopc*, *Pick1,* and *Spaca1*, and is associated with globozoospermia [12,16–18]. Some Golgi-associated proteins, such as GOLGA3 and GOPC, have been proved to be necessary for mouse fertility [8,9,11,12]. In this study, we aimed to explore the role of Golgi-associated protein, GOLGA4, in spermatogenesis. Despite the high expression of GOLGA4 in testis, the deletion of *Golga4* showed no impact on male fertility. A possible explanation for the dispensability of GOLGA4 in male fertility is functional redundancy because several previous studies have demonstrated the gene redundancy is the foremost possible reason to cause no overt phenotypes in mouse loss-function study [19–22]. Although the mRNA level of other paralog members of *Golga4* showed no significant changes in KO testes in this study, such as *Golga1*, *Golga2*, *Golga3*, *Golga5,* and *Golgb1*, we hypothesized that there are other unknown transcriptional factors may compensate for GOLGA4, which need to identify further. Notably, the *Golga4* KO mice are generated and analyzed under laboratory comfortable conditions in which provided an ideal environment. Thus, we cannot exclude the possibility that *Golga4* KO mice may display abnormal spermatogenesis under external stimulus conditions.

In summary, in this study, we demonstrated that the GOLGA4 deficiency in mice does not cause any apparent infertility

phenotype. To the best of our knowledge, this is the first study reporting that *Golga4* is dispensable for male fertility in mice by *in vivo* functional analyses. Our data will help researchers in the field of reproductive genetics for prioritizing the targeted genes and devising prediction tools for fertility and avoiding research duplication.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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