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Functional Analysis of Forkhead Transcription Factor Fd59a in Spermatogenesis of *Drosophila melanogaster*

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Simple Summary: Spermatogenesis, which is regulated by many different genes, is a conserved process across species to produce mature sperm for animal reproduction. Fox transcription factors can bind to DNA sequences in the promoters to regulate gene expression. FoxD subfamily members are mainly involved in metabolism and early organ development. In *Drosophila melanogaster*, FoxD subfamily member Fd59a may regulate development of nervous system and control egg-laying behavior of females. However, the functions of insect FoxD members are still largely unknown. In this study, we investigated the role of Fd59a in spermatogenesis of *Drosophila*. We found that mutation in *Fd59a* caused swelling of the apical region in the testis, resulting in only a few mature sperm in the seminal vesicle and significantly lower fertility of *Fd59a* mutant males compared to the control flies. We also found that the homeostasis of testis stem cell niche in *Fd59a* mutant and RNAi flies was disrupted, causing increased apoptosis of sperm bundles. RNA-sequencing and qRT-PCR results suggest that Fd59a can regulate expression of genes related to reproductive process and cell death. Our collective results indicated that Fd59a plays a key role in *Drosophila* spermatogenesis, which will help understand the role of FoxD members in insect spermatogenesis.

Abstract: Spermatogenesis is critical for insect reproduction and is regulated by many different genes. In this study, we found that Forkhead transcription factor Fd59a functions as a key factor in spermatogenesis of *Drosophila melanogaster*. Fd59a contains a conversed Forkhead domain, and it is clustered to the FoxD subfamily with other FoxD members from some insect and vertebrate species. Mutation in *Fd59a* caused swelling in the apical region of the testis. More importantly, only a few mature sperm were present in the seminal vesicle of *Fd59a* mutant flies compared to the control flies, and the fertility of $Fd59a^{2/2}$ mutant males was significantly lower than that of the control flies. Immunofluorescence staining showed that the homeostasis of testis stem cell niche in $Fd59a^{2/2}$ mutant and *Fd59a* RNAi flies was disrupted, and apoptosis of sperm bundles was increased. Furthermore, results from RNA-sequencing and qRT-PCR suggest that Fd59a can regulate expression of genes related to reproductive process and cell death. Taken together, our results indicated that Fd59a plays a key role in the spermatogenesis of *Drosophila*.

Keywords: spermatogenesis; forkhead; FoxD; testis; apoptosis

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1. Introduction

Forkhead box (Fox) transcription factor, which contains a highly conserved DNA binding domain of ~100 amino acids consisting of three α helices, three β folds and two ring connections, plays critical roles in organ development, innate immunity, and other processes [1]. Based on phylogenetic analysis, Fox proteins are assigned to different subclasses and named "Fox, subclass N, member X" [2]. FoxD subfamily members are mainly involved in metabolism and early organ development [3]. In mammals, FoxD1 regulates human early embryonic development and is associated with various diseases. For example, FoxD1 can promote *SLC2A1* (Solute carrier family 2 member 1) transcription and inhibit degradation of *SLC2A1* to facilitate proliferation, invasion, and metastasis of pancreatic cancer cells [4–6]. In planarian, *FoxD* gene expression was induced by wound signaling, and it was involved in head regeneration [7]. In *Drosophila melanogaster*, Fd59a/FoxD may regulate development of nervous system and control egg-laying behavior of females [8]. However, the functions of insect FoxD subfamily members are still largely unknown.

Spermatogenesis is a process to produce mature sperm for reproduction. The process of spermatogenesis is conserved from insects to vertebrates; thus, insect testis is an ideal model for studying mechanisms of spermatogenesis [9]. In *D. melanogaster*, germ stem cells (GSCs) differentiate into goniablasts under the control of stem cell niche, then goniablasts develop into spermatids through mitosis and meiosis. After nuclear elongation and individualization processes, round spermatids finally become mature sperm [10].

Spermatogenesis is regulated by various signaling pathways, such as JAK-STAT (Janus kinase-signal transducer and activator of transcription), BMP (Bone morphogenetic protein), and EGF (Epidermal growth factor) pathways [11], and by many genes [12,13]. Recent studies showed that different genes are involved in spermatogenesis of insects. For example, knockdown expression of *ribosomal protein S3* (*RpS3*) strongly disrupted spermatid elongation and individualization process in *D. melanogaster* [14]. Knockdown or mutation of *cytochrome c1-like* (*cyt-c1L*) gene in early germ cells resulted in male sterility of *D. melanogaster* [15]. Moreover, *BmHen1*, a gene in *Bombyx mori* encoding methyltransferase that modifies piRNAs, was found to regulate eupyrene sperm development [16]. These results indicate that the molecular mechanism of insect spermatogenesis is much more complicated than what we have already known about.

In our previous study, we showed that *B. mori* FoxA participated in the development of wing disc [17]. Microarray data showed that Fox genes were expressed in *B. mori* testis and *BmFoxD* was expressed at a high level [18]. In *Drosophila*, expression of *Fd59a*/*FoxD* was about 2-fold higher in the testis than in the ovary [19], suggesting that Fd59a may play a role in testis development or spermatogenesis. In this study, we showed that mutation and knockdown expression of *Fd59a* caused swelling in the apical region of testis and decreased male fertility. More importantly, loss of function in *Fd59a* disrupted the homeostasis of testis stem cell niche and induced apoptosis of sperm bundles, resulting in only a few mature sperm in the seminal vesicle. By analyzing RNA sequencing from the testis of $Fd59a^{2/2}$ mutants, we found that Fd59a may regulate expression of genes related to reproductive and metabolic processes. Our findings suggest that Fd59a plays a role in *Drosophila* spermatogenesis.

2. Materials and Methods

2.1. Fly Lines

Wild-type w^{1118} line was maintained in the laboratory [20]. *Nos-Gal4* (TB00040) and *UAS-GFP dsRNA* (BDSC9331) fly lines were obtained from Tsinghua Fly Center in Beijing, China. $Fd59a^{1}$/CyO (BDSC56819), $Fd59a^{2}$/CyO (BDSC56820) and *UAS-Fd59a* RNAi (BDSC31937) flies were obtained from the Bloomington *Drosophila* Stock Center in Indiana, USA. *Bam-Gal4* fly line was kindly provided by Professor Yufeng Wang at the School of Life Sciences, Central China Normal University, Wuhan, China.

To analyze functions of Fd59a, $Fd59a^{1}$/*CyO* males were crossed to $Fd59a^{1}$/*CyO* females to generate $Fd59a^{1/1}$ loss of function flies, while $Fd59a^{2}$/*CyO* males were crossed to $Fd59a^{2}$/*CyO* females to generate

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Fd59a$^{2/2}$ loss of function flies. To knockdown expression of *Fd59a*, *Nos-Gal4* and *Bam-Gal4* flies were crossed to *UAS-Fd59a* RNAi flies, respectively.

All flies were reared on a fresh cornmeal/yeast/brown sugar diet with p-hydroxybenzoic acid methylester as a mold inhibitor at 25°C with a photoperiod of approximately 12 L : 12 D (light : dark) [21].

2.2. Bioinformatics Analysis

Amino acid homology search was performed by BLAST at the National Center for Biotechnology Information (NCBI, https://blast.ncbi.nlm.nih.gov). Sequence alignment was done by Cluster W. Phylogenetic tree was constructed by RAxML (Random Axelerated Maximum Likelikhood) with 1000 bootstrap replications [22]. Functional protein domains were analyzed by the SMART (https://smart.embl.de/). Potential Fox binding sites in the promoter sequences of select genes were predicted using the JASPAR program (https://jaspar.genereg.net/).

2.3. RNA Isolation and Quantitative RT-PCR

To analyze the expression pattern of *Fd59a* at different developmental stages, around 50 adult flies of w^{1118} (male : female ratio is ~2 : 1) were collected and mated in cage. Then flies were transferred to a new cage 2 h later. Based on the developmental stages, embryos at 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, and 24 h after egg-laying, and the first, second, third instar larvae, early, mid and late pupae, and 1, 3 and 5-day-old adults were collected. All samples were collected into RNAex Pro reagent (Accurate Biology, Changsha, China) and kept at -80°C.

Total RNA was extracted as described previously [20]. The first-strand cDNA was synthesized from 1 μg of total RNA using HiFiScript gDNA removal cDNA Synthesis Kit (cwbiotech, Taizhou, China). Gene specific primers (**Table 1**) for the tested genes were designed based on sequences from the Flybase and synthesized by Tsingke Biotechnology (Beijing, China).

Quantitative reverse transcription – polymerase chain reaction (qRT-PCR) was performed using QuantStudio™ 6 Flex (Thermo Fisher Scientific, Waltham, USA) with ChamQ SYBR qPCR Master Mix (Vazyme, Nanjing, China) following the manufacturer's instructions. The qPCR cycling program was 95°C for 30 s, followed by 40 cycles of 95°C for 10 s, 60°C for 30 s. Relative expression level of genes was calibrated against the reference gene *Rp49* by using $2^{-\Delta\Delta Ct}$ calculation method [23].

Table 1. Primers used in this study.

Name	Forward Primer (5′–3′)	Reverse Primer (5′–3′)
Fd59a-qRT	CAGGGAAGTCAGTCGGGGGA	GTCGCCACATCGAAGGCGTA
Rp49-qRT	GCCCAAGGGTATCGACAACA	ACCTCCAGCTCGCGCACGTT
Spd-2-qRT	GTGACCCACACGACCCTCTG	GCCGAATGACCAGCCGTTTG
Fz2-qRT	TCGCGAGTCACAATTGCACC	GGACGCCACTCTACGGTGTT
Tasp1-qRT	CGGCATGCGAGTCTGTTCGG	ACACAAGGCAGCGCAAGTCTA
Debcl-qRT	ATCGACAACGGCGGATGGTT	ACGCGATCCCAAGCGAATCT
Ptp52F-qRT	TGTCCGACGATCTTTGCGCT	GGCGTAGGGGGAAAGTGGAC
Atg7-qRT	TACAACTGCTGGCCGATGAGG	GCACGGAAAGGCGAACCAAT
RnrS-qRT	GGACCGTTTGCTCGTGGAGT	GAAATCCGCGTCCAGGGTGA
CG10700-qRT	TGTGGAGGCTACGGCCAATC	TCACCACGGCTGTTTCCCAA
CG12917-qRT	CAGGGGCTTCCTTCAGTCGG	AAATAGCCAGACACGGGGGC
Nbs-qRT	ATTCCCAAAAGCCGCGCAAG	TGGGTCACCTGCCAAATGCT
Lola-qRT	CTGCTGAGATATGCGAGCCAGA	GTTCACAATGGCCTCCGCCT
Cal1-qRT	GGTGGTGGACGAGGAAACACT	TCCACAGCCTCCTTTGCCAC
Dnah3-qRT	AGAGCTGGCAAGAGCGGAAA	ACATTGCGAGACGTGGCACC
Blanks-qRT	ACGGGCCAGGAAAGAGCTTG	ACGGCTTCTTTGGCTCGACA
En-qRT	CCAACGACGAGAAGCGTCCA	CTCCGCTCGGTCAGATAGCG
Tsc1-qRT	GGTTGGCATGACTGGCTCCT	CACGTCCCGGCTGCTTGATA

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CG32817-qRT	AATCAAGTGTCTAACCCTGAACTGG	GTTGCGCCATCGAAAAGCAT
Moe-qRT	GCCTGCGAGAGGTTTGGTTCTT	TCACGTCCTGGTTCATCACCTT
Past1-qRT	ACACCCGATCACACAGCCTC	CGCCTGCACTGTGTGGCTAA
Zpg-qRT	GGGGCCTATGTGAGCGACAA	CCGCCCTCCCAAATCTTCCA

2.4. Male Fertility Test

For fertility test, 10 1-day-old virgin females and 15 3-day-old males were placed in bottles with egg collection plates as described previously [24]. Egg collection plates were changed every 24 h, and the number of embryos and larvae in the plates were counted.

2.5. Immunofluorescence Staining

Testes from 3-day-old adult flies were dissected in 10 mM phosphate-buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4) and fixed in 4% paraformaldehyde for 30 min at room temperature. Testis samples were washed in 3‰ PBT (10 mM PBS containing 0.1% Triton X-100) for 3 or more times (each for 15 min) and blocked with blocking buffer (3‰ PBT containing 5% normal goat serum) for 1 h at room temperature. Next, all samples were incubated with primary antibody prepared in dilution buffer (3‰ PBT containing 3% normal goat serum) at 4°C overnight, washed three times in 3‰ PBT, and then incubated with secondary antibody at room temperature for 3 h in darkness. Finally, samples were washed three times with 3‰ PBT, mounted using VECTASHIELD® antifade Mounting Medium containing 2 μg/ml DAPI (Vector Laboratories, Newark, USA), and observed under confocal microscopy.

Primary antibodies used were anti-Vasa (1:50, Developmental Studies Hybridoma Bank, Iowa, USA), anti-Fasciclin III (Fas III) (1:100, Developmental Studies Hybridoma Bank, 7G10, Iowa, USA), anti-αSpectrin (1:50, Developmental Studies Hybridoma Bank, 3A9, Iowa, USA). The secondary antibodies were goat anti-rabbit 568 and goat anti-mouse 488, which were Alexa Fluor-conjugated (1:500, Invitrogen, Carlsbad, USA). Fluorescent images were captured using an FV3000 confocal microscope (Olympus, Tokyo, Japan).

2.6. TUNEL Assay

To analyze whether loss of function in Fd59a can induce cell death of sperm bundle, testes from 3-day-old adults of $Fd59a^{2/2}$ and *Fd59a* RNAi flies were dissected. The collected testes were fixed in 4% paraformaldehyde and washed with 3‰ PBT as described above.

Terminal deoxyribonucleotide transferase (TdT)-mediated dUTP nick end labeling (TUNEL) assay was performed using the one-step TUNEL apoptosis assay kit from Beyotime Biotechnology (Shanghai, China). Testis samples prepared from above were incubated with 50 μL TUNEL reaction mixture containing 5 μL of TdT enzyme and 45 μL of fluorescent labeling solution at 4°C overnight, and then washed three times in 3‰ PBT. DAPI staining was performed as described above.

2.7. RNA Sequencing

Testes from 3-day-old $Fd59a^{2/2}$ and w^{1118} flies were dissected in DEPC-treated PBS (10 mM, pH7.4). Total RNA was extracted, and RNA sequencing was performed at Majorbio Bio-Pharm Technology (Shanghai, China) using an Illumina HiSeq 2000 (Illumina, San Diego, CA, USA).

2.8. Bioinformatics Analysis of RNA-seq Data

Transcript abundances in this study were determined using Fragments Per Kilobase per Million mapped reads (FPKM) values. Differentially expressed genes (DEGs) were identified based on a $\log_2$ fold-change > 1.5 (or log2 fold-change < −1.5) and a *p*-adjust < 0.05 with three biological replicates. Gene Ontology (GO) enrichment analysis of DEGs was performed using the GOseq R package.

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2.9. Statistical Analysis

The size of apical region from *Fd59a*$^{2/2}$ and *w*^{1118} testes was quantitated by ImageJ. Three biological replicates and three technical replicates for each biological sample were performed. Statistical significance was analyzed by the student's t-test (for comparison of two groups) or one-way analysis of variance followed by a least significant difference test (for multiple comparisons among groups). Data were analyzed using GraphPad Prism version 9.0 and presented as mean ± S.E.

3. Results

3.1. Expression Profile of Fd59a in Drosophila

To understand functions of *Fd59a*, we first determined the expression profile of *Fd59a* at different developmental stages of *Drosophila* by qRT-PCR. The results showed that expression of *Fd59a* mRNA peaked twice during development from embryo to adult, with the first peak around mid-embryonic stage and then a plateau with a relatively high level from late-embryonic stage to 1st instar larval stage, and the second peak just at the metamorphosis period and maintaining at a relative high level till mid pupal stage (Figure 1A), indicating that Fd59a may be involved in *Drosophila* development.

It has been reported that loss of function in Fd59a affected egg-laying of *Drosophila* females [8]. Interestingly, microarray data from the Flybase showed that expression level of *Fd59a* was about 2-fold higher in the testis than in the ovary [19]. We performed qRT-PCR and confirmed that mRNA level of *Fd59a* was significantly higher in the testis than in the ovary of 3-fay-old *w*^{1118} flies (Figure 1B), suggesting that Fd59a may also have a function in the testis of *Drosophila*.

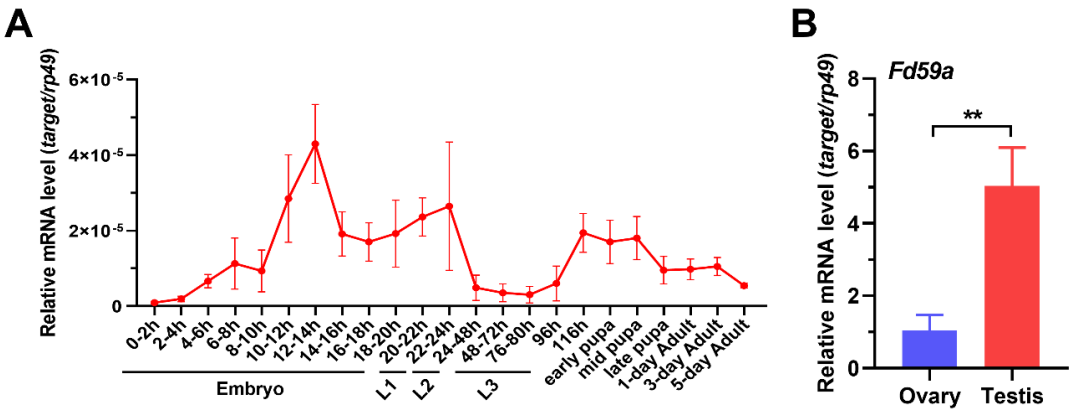

Figure 1. Expression of *Fd59a* at different developmental stages and in adult ovary and testis. (**A**) Expression of *Fd59a* at different developmental stages. *Drosophila* embryos at 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22 and 24 h after egg-laying, first (L1), second (L2), third (L3) instar larvae, early, mid and late pupae, 1, 3 and 5-day-old adult flies were collected to prepare total RNAs for analysis of transcriptional expression of *Fd59a* by qRT-PCR. (**B**) Expression of *Fd59a* in the testis and ovary of 3-day-old adult flies. Data were presented as means ± S.E., and significant difference was determined by the student's *t-test* and indicated by asterisks: ** $p < 0.01$.

3.2. Sequence and Phylogenetic Analyses of Fd59a

Fd59a gene is in the 2nd chromosome. The full-length cDNA of *Fd59a* is 1371 bp long, encoding a protein of 456 amino acid residues, with a calculated molecular weight of 49.1 kDa and pI of 5.19.

Fd59a belongs to the FoxD subfamily. To reveal the evolutionary relationship of Fd59a, Fd59a/FoxD homologous sequences from some insect and vertebrate species were blasted and downloaded from NCBI (Table 2), sequence alignment was performed, and phylogenetic tree was constructed. The results showed that all the select Fd59a/FoxD homologous proteins contain a

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conserved forkhead box domain (Figure 2A). Meanwhile, *Drosophila* Fd59a was most close to FoxD3-A of *Papilio Xuthus* and FoxD3-like of *Blattella germanica* (Figure 2B) and they contain similar functional domains (Figure 2C). These results suggest that *Drosophila* Fd59a is evolutionarily close to insect FoxD members.

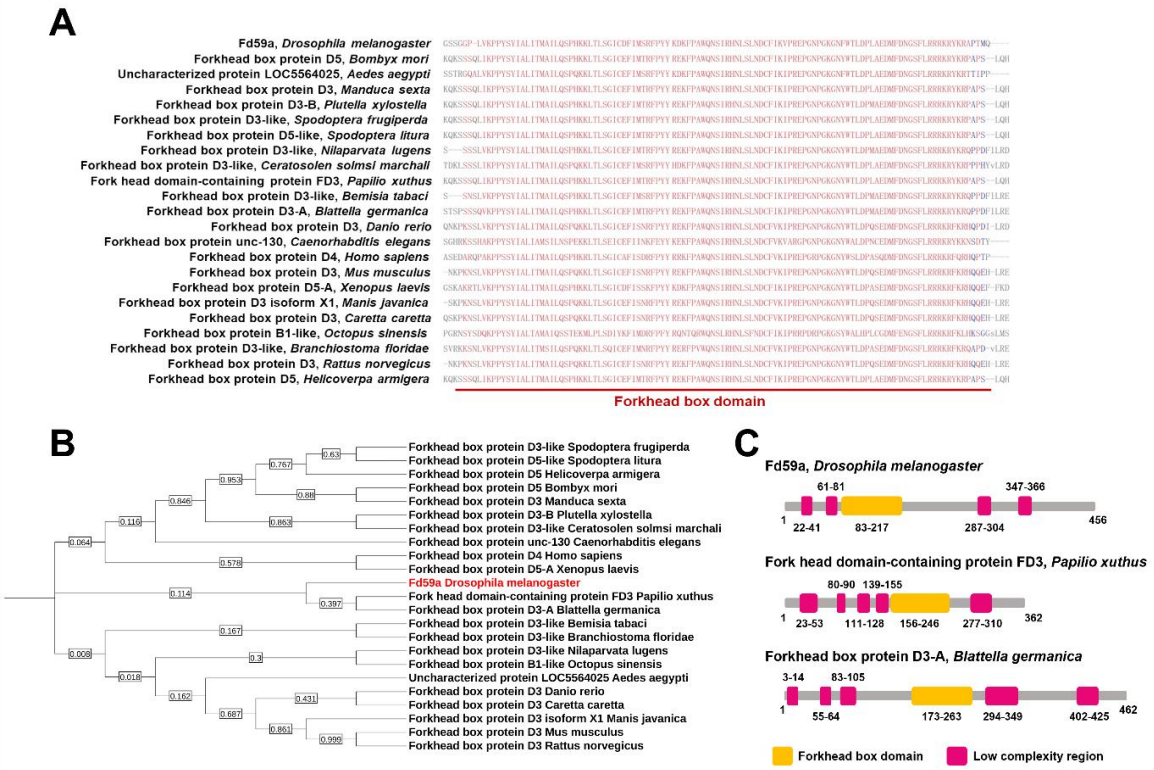

Figure 2. Sequence analysis of *D. melanogaster* Fd59a. Sequence alignment (**A**), Maximum Likelihood phylogenetic tree (**B**) and functional domains (**C**) of Fd59a with its homologous proteins from some insect and vertebrate species. For detailed information of the sequences, see **Table 2**.

Table 2. Fd59a homologous protein sequences used in the phylogenetic tree.

Proteins	Species	Accession Number
Forkhead box protein D5	*Bombyx mori*	XP_004922516.1
uncharacterized protein LOC5564025	*Aedes aegypti*	XP_001648348.3
Forkhead box protein D3	*Manduca sexta*	XP_030032680.1
Forkhead box protein D3-B	*Plutella xylostella*	XP_037961710.1
Forkhead box protein D3-like	*Spodoptera frugiperda*	XP_035435321.1
Forkhead box protein D5-like	*Spodoptera litura*	XP_022815528.1
Forkhead box protein D3-like	*Nilaparvata lugens*	XP_039277739.1
Forkhead box protein D3-like	*Ceratosolen solmsi marchali*	XP_011504225.1
Fork head domain-containing protein FD3	*Papilio xuthus*	KPJ03207.1
Forkhead box protein D3-like	*Bemisia tabac*	XP_018914635.1
Forkhead box protein D3-A	*Blattella germanica*	PSN41724.1
Forkhead box protein D5	*Helicoverpa armigera*	XP_021187147.2
Forkhead box protein D3	*Danio rerio*	NP_571365.2
Forkhead box protein unc-130	*Caenorhabditis elegans*	NP_496411.1
Forkhead box protein D4	*Homo sapiens*	NP_997188.2
Forkhead box protein D3	*Mus musculus*	NP_034555.3
Forkhead box protein D5-A	*Xenopus laevis*	NP_001081998.1
Forkhead box protein D3 isoform X1	*Manis javanica*	XP_036880296.1
Forkhead box protein D3	*Caretta caretta*	XP_048718258.1

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Forkhead box protein B1-like	*Octopus sinensis*	XP_029653697.1
Forkhead box protein D3-like	*Branchiostoma floridae*	XP_035698942.1
Forkhead box protein D3	*Rattus norvegicus*	NP_542952.1

3.3. Loss of Function in Fd59a Affects Testis Development and Male Fertility

To determine the role of *Fd59a* in testis development in *D. melanogaster*, testes from $Fd59a^{1/1}$ and $Fd59a^{2/2}$ loss of function mutant flies as well as w^{1118} flies were dissected. $Fd59a^{1}$ and $Fd59a^{2}$ were two mutant types of *Fd59a*, with $Fd59a^{1}$ as a hypomorphic mutation and $Fd59a^{2}$ as a null allele [8]. We found that the apical region of testes from the $Fd59a^{1/1}$ and $Fd59a^{2/2}$ mutant flies was swelled (Figure 3A,B), with only a few mature sperm in the seminal vesicle of $Fd59a^{2/2}$ mutant testis compared to full of mature sperm in the w^{1118} flies (Figure 3A). As the phenotype of $Fa59a^{2/2}$ flies was more significant than that of $Fa59a^{1/1}$ flies, we carried out our following studies in the $Fa59a^{2/2}$ mutant flies. When $Fd59a^{2/2}$ males were crossed with w^{1118} females, the hatching rate of F1 flies was significantly decreased compared to the control (Figure 3C). Together, these results suggest that Fd59a plays a critical role in the development of testis and/or spermatogenesis of *D. melanogaster*.

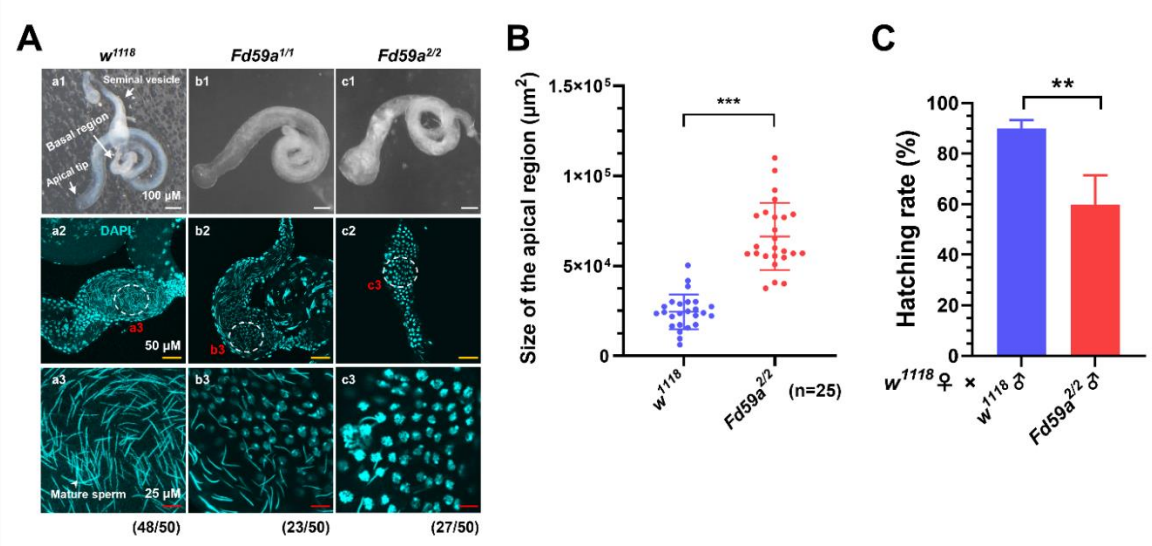

Figure 3. Mutation in *Fd59a* affects testis development and reduces the number of mature sperm in the seminal vesicle. (**A**) Overall appearance of testes and seminal vesicles from w^{1118}, $Fd59a^{1/1}$ and $Fd59a^{2/2}$ flies, nuclei were stained with DAPI. Scale bar in a1, b1, c1 is 100 μm, in a2, b2, c2 is 50 μm, and in a3, b3, c3 is 25 μm. The arrowhead in a3 represents mature sperm. Numbers below the images indicate the pairs of testes with similar phenotypes in the images. (**B**) Quantitative measurements of the apical region of testis from $Fd59a^{2/2}$ and w^{1118} flies. (**C**) Male fertility test of w^{1118} and $Fd59a^{2/2}$ flies. Data were presented as means ± S.E., and significant difference was determined by the student's *t-test* and indicated by asterisks: ** $p < 0.01$, *** $p < 0.001$.

3.4. Loss of Function in Fd59a Affects Spermatogenesis

In the apical tip of testis, hub cells are surrounded with GSCs and CySCs to form the stem cell niche, and proliferation and differentiation of testis stem cells are controlled by the niche. Disruption of niche homeostasis can lead to swelling of testis and defect in spermatogenesis [25]. To clarify the role of Fd59a in spermatogenesis, Fas Ⅲ and Vasa antibodies were used to label the hub cells and germ cells, respectively, and αSpectrin antibody was used to label spectrosomes and fusomes for early germ cell development. The results showed that distribution of GSCs and CySCs was scattered in the $Fd59a^{2/2}$ testis compared to the control testis (Figure 4A, a1-a4, b1-b4, a6, b6), and a strong pattern of fusome and spectrosome formation was displayed in the control testis, while only a few fusomes and spectrosomes were observed in the $Fd59a^{2/2}$ mutant testis (Figure 4A, a3, a5, b3, b5).

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These results suggest that Fd59a may play a role in maintaining the homeostasis of testis stem cell niche.

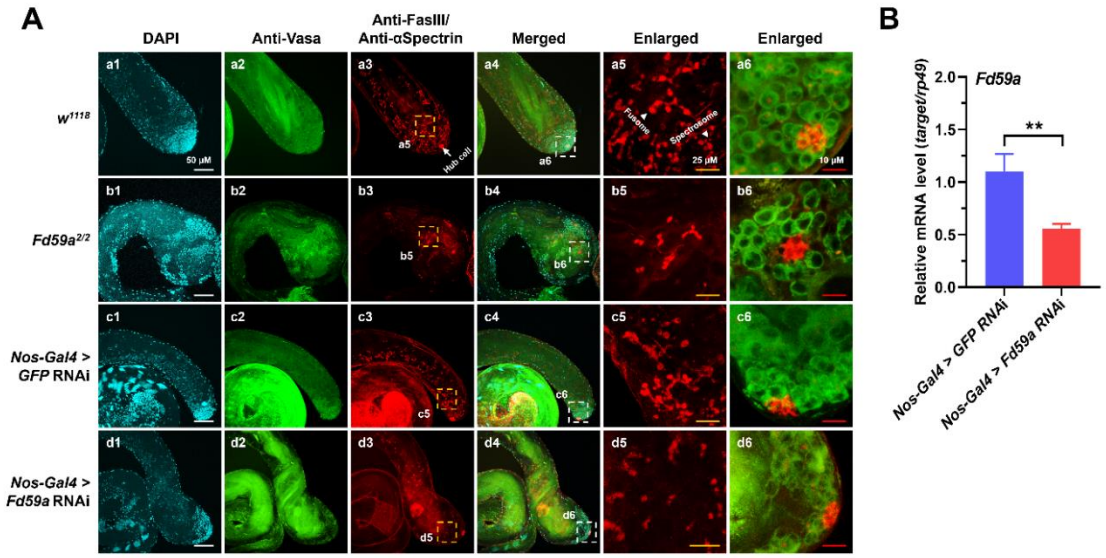

Figure 4. Loss of function of Fd59a in the testis disrupts the homeostasis of testis stem cell niche. (**A**) Immunostaining of testis. Testes from the *w*^{1118}, *Fd59a*$^{2/2}$, *Nos-Gal4>GFP* RNAi and *Nos-Gal4>Fd59a* RNAi flies were labeled with anti-Vasa (green), anti-FasIII (red) and anti-αSpectrin (red) antibodies, and nuclei were stained with DAPI (blue). (a1-a4, b1-b4, c1-c4, d1-d4) The apical region of testis, (a5, b5, c5, d5) enlarged part of the apical region showing fusomes and spetrosomes, (a6, b6, c6, d6) enlarged part of the apical tip showing the stem cell niche. In the testis of *Fd59a*$^{2/2}$ and *Nos-Gal4>Fd59a* RNAi flies, the distribution of germ cells labeled with anti-Vasa (green) antibody was disrupted, and only a few fusomes and spectrosomes labeled with anti-αSpectrin (red) antibody were observed. Scale bar in a1-a4, b1-b4, c1-c4 and d1-d4 is 50 μm, in a5, b5, c5 and d5 is 25 μm, and in a6, b6, c6 and d6 is 10 μm. The arrow in a3 represents hub cell, and arrowheads in a5 represent fusomes and spectrosomes. (**B**) Expression of *Fd59a* in the testis of *Nos-Gal4* RNAi flies. Data were presented as means ± S.E., and significant difference was determined by the student's *t-test* and indicated by asterisks: ** $p < 0.01$.

Mammalian FoxD1 is related to apoptosis, as knockdown expression of *FoxD1* facilitated apoptosis in HNSCC (head and neck squamous cell carcinoma) cells [26]. To determine whether loss of function in Fd59a can induce apoptosis in the testis, TUNEL assay was performed. TUNEL positive signals were detected in sperm bundles of the *Fd59a*$^{2/2}$ mutant flies but not in the *w*^{1118} flies (Figure 5A, a1-a4, b1-b4), suggesting that loss of function in Fd59a induced apoptosis of spermatid.

To further confirm the role of Fd59a in testis, expression of *Fd59a* in GSCs was knocked down by *Nos-Gal4* (Figure 4B), and similar phenotypes, such as swelling in the apical region of testis, fewer mature sperm in the seminal vesicle and apoptosis of sperm bundles, were observed in the *Nos-Gal4>Fd59a* RNAi flies (Figure 4A, c1-c6, d1-d6, and Figure 5A, c1-c4, d1-d4). Meanwhile, knockdown expression of *Fd59a* in the 4–16 stage of spermatogonia by *Bam-Gal4* (Figure 5C) also induced apoptosis of sperm bundles (Figure 5A, e1-e4, f1-f4). Moreover, only a few mature sperms were observed in the seminal vesicles of the *Nos-Gal4>Fd59a* RNAi and *Bam-Gal4>Fd59a* RNAi flies, while the control flies were filled with mature sperm (Figure 5B). These combined results suggest that loss of function of Fd59a in the testis resulted in disruption of the stem cell niche during spermatogenesis and increase in apoptosis of sperm bundles, finally leading to fewer mature sperm in the seminal vesicle.

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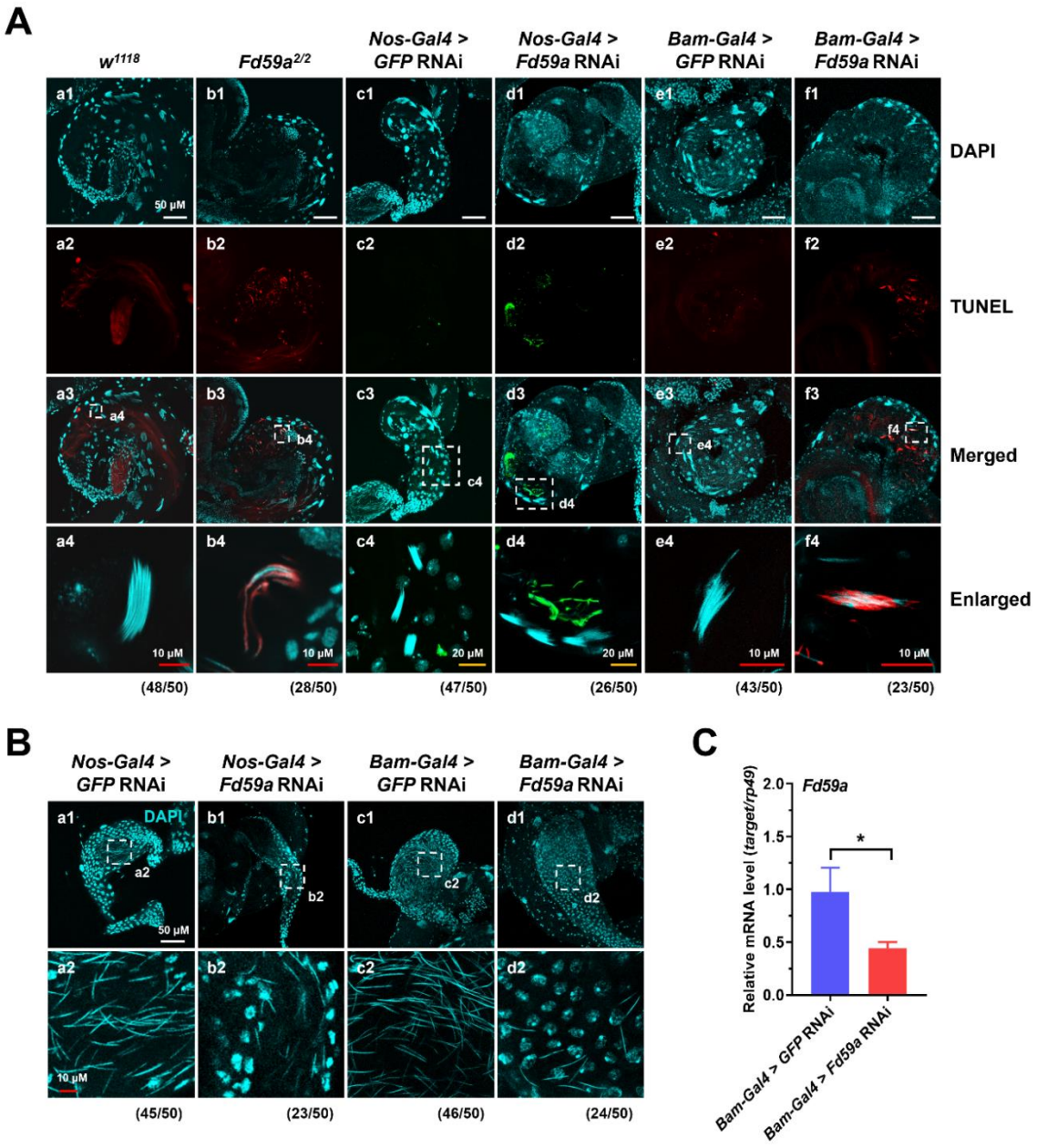

Figure 5. Loss of function of Fd59a in the testis induces apoptosis of sperm bundles. (**A**) Detection of apoptotic cells in the testis. Testes from the *w*^{1118}, *Fd59a*$^{2/2}$, *Nos-Gal4>GFP* RNAi, *Nos-Gal4>Fd59a* RNAi, *Bam-Gal4>GFP* RNAi and *Bam-Gal4>Fd59a* RNAi flies were stained with TUNEL assay for apoptotic cells, and nuclei were stained with DAPI (blue). (a1-a3, b1-b3, c1-c3, d1-d3, e1-e3, f1-f3) The basal region of testis, (a4, b4, c4, d4, e4, f4) enlarged part of the basal region showing the sperm bundles. In the testis of *Fd59a*$^{2/2}$, *Nos-Gal4>Fd59a* RNAi and *Bam-Gal4>Fd59a* RNAi flies, many TUNEL signals were detected in the sperm bundles, and only a few TUNEL signals were detected in the basal region of *w*^{1118}, *Nos-Gal4>GFP* RNAi and *Bam-Gal4>GFP* RNAi flies, but not in the sperm bundles. Scale bar in a1-a3, b1-b3, c1-c3, d1-d3, e1-e3 and f1-f3 is 50 μm, in a4, b4, e4 and f4 is 10 μm, and in c4, d4 is 20 μm. (**B**) DAPI staining of seminal vesicle. Seminal vesicles from *Nos-Gal4>GFP* RNAi, *Nos-Gal4>Fd59a* RNAi, *Bam-Gal4>GFP* RNAi and *Bam-Gal4>Fd59a* RNAi flies were stained with DAPI. Scale bar in a1, b1, c1, d1 is 50 μm, in a2, b2, c2, d2 is 10 μm. Numbers below the images indicate the pairs of testes with similar phenotypes in the images. (**C**) Expression of *Fd59a* in the testis of *Bam-Gal4* RNAi flies. Data were presented as means ± S.E., and significant difference was determined by the student's *t-test* and indicated by asterisks: * $p < 0.05$.

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3.5. Fd59a Regulates Gene Expression in the Testis

To better understand how Fd59a plays a role in regulating testis development and spermatogenesis, we carried out RNA-sequencing (RNA-seq) with RNAs isolated from the testes of w^{1118} (control) and $Fd59a^{2/2}$ males. Totally 1863 differential expressed genes (DEGs) with at least 1.5-fold change (p-adjust < 0.05) were identified by RNA-seq, with 854 genes upregulated and 1009 genes downregulated in the testis of $Fd59a^{2/2}$ flies (Figure 6A). This result indicated that Fd59a serves as a transcription factor in the testis.

Gene Ontology (GO) analysis showed that 120 differentially expressed genes (DEGs) are involved in the reproductive process and 475 DEGs participate in the metabolic process (Figure 6B). Among the reproduction related DEGs, many genes have been shown to play a role in gonad development and spermatogenesis, such as *Fz2* and *Zpg* [27,28]. There are also 57 DEGs which are involved in cell death, including *Rnrs* and *Ptp52F* [29,30].

To confirm RNA-seq data, totally 20 DEGs associated with reproductive process and cell death were selected for qRT-PCR analysis (Table 3). The results showed that expression profiles of all the select DEGs in the testis detected by qRT-PCR were consistent with those of the RNA-seq data (Figure 6C). Then, 2000 bp promoter sequences upstream the transcriptional start sites of these select genes were downloaded, and the potential Fox binding sites were predicted using JASPAR database (the relative profile score threshold was set to 85%). Except for *CG32817* promoter, several Fox binding sites were predicted in the promoter of each select gene, with more than 10 potential Fox binding sites in the promoters of *Spd-2*, *Cal1*, *Blanks*, *Ptp52F*, *Lola* and *Debcl* genes (Table 3). This result further supported that Fd59a is an upstream regulator of these genes. Taken together, our results suggest that Fd59a serves as a transcription factor to regulate expression of genes involved in reproduction, cell growth and death in the testis.

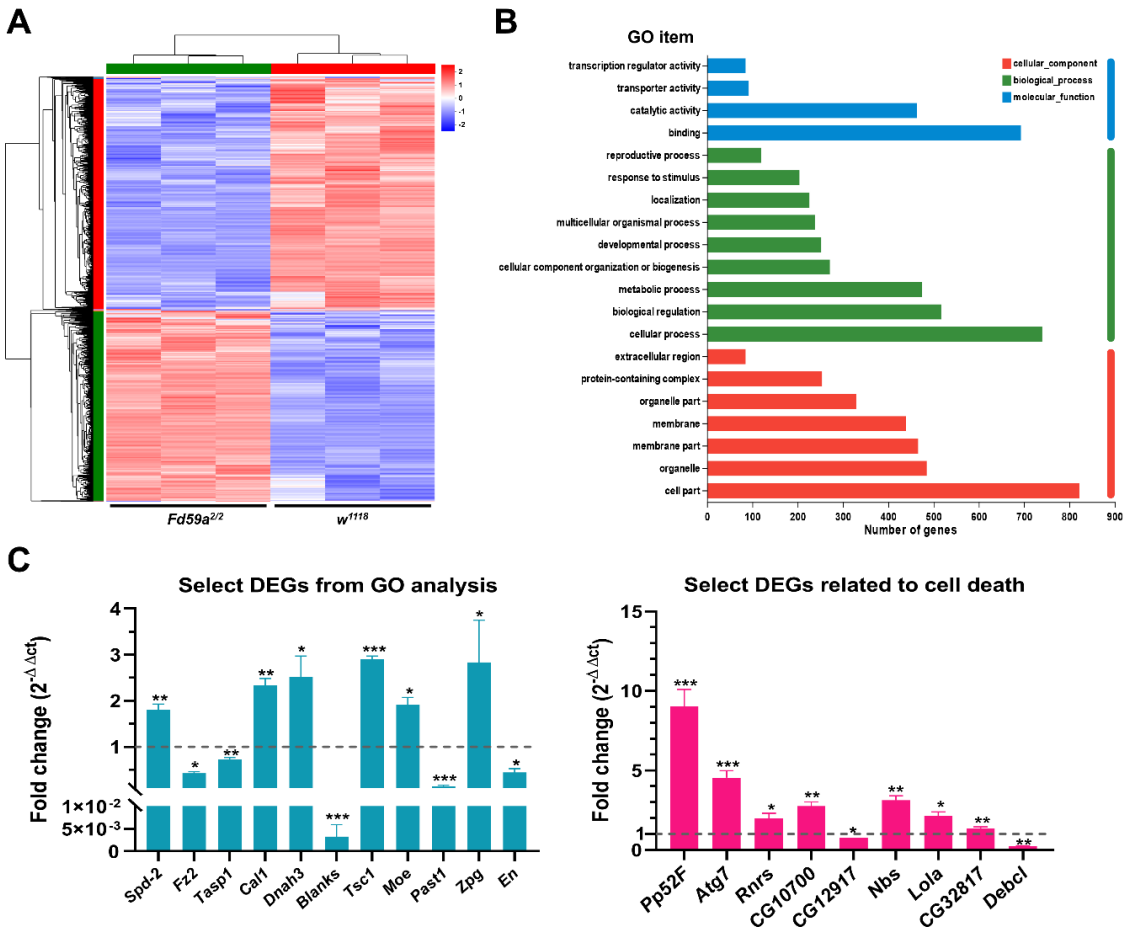

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Figure 6. RNA-seq analysis of RNAs from the testes of *Fd59a*$^{2/2}$ and *w*^{1118} flies. (**A**) Heatmap of differentially expressed genes (DEGs) in the testis of *Fd59a*$^{2/2}$ flies relative to *w*^{1118} flies. (**B**) GO analysis of DEGs between *Fd59a*$^{2/2}$ and *w*^{1118} fly testes. (**C**) qRT-PCR validation of select DEGs from RNA sequencing data. Data were presented as means ± S.E., and significant difference was determined by the student's *t-test* and indicated by asterisks: * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

Table 3. Differentially expressed genes selected for qRT-PCR validation and the number of predicted Fox binding sites in the 2 kb promoter regions.

Gene Symbol	Log_2 Fold Difference	Relative Expression	Biological Functions	Forkhead Binding Sites
GO analysis related genes				
Spd-2	1.08	Up-regulated	Involved in sperm aster formation	13
Fz2	-1.66	Down-regulated	Germ-line stem cell niche homeostasis	6
Tasp1	-1.35	Down-regulated	Involved in spermatogenesis	7
Cal1	0.61	Up-regulated	Female meiosis chromosome segregation	10
Dnah3	0.70	Up-regulated	Involved in sperm competition	6
Blanks	-0.72	Down-regulated	Involved in sperm individualization	11
Tsc1	0.76	Up-regulated	Negative regulation of developmental growth	4
Moe	0.67	Up-regulated	Oocyte anterior/posterior axis specification	10
Past1	-2.62	Down-regulated	Involved in sperm individualization	3
Zpg	1.33	Up-regulated	Male germ-line stem cell population maintenance	5
En	-1.40	Down-regulated	Involved in gonad development	8
Cell death related genes				
Ptp52F	3.44	Up-regulated	Involved in larval midgut cell programmed cell death	15
Atg7	1.60	Up-regulated	Involved in autophagy	2
RnrS	0.68	Up-regulated	Involved in activation of cysteine-type Endopeptidase activity involved in apoptotic process	9
CG10700	0.822	Up-regulated	Involved in execution phase of apoptosis	9
CG12917	-0.62	Down-regulated	Involved in apoptotic DNA fragmentation	4
Nbs	0.78	Up-regulated	Involved in intrinsic apoptotic signaling pathway in response to DNA damage	2
Lola	0.74	Up-regulated	Involved in nurse cell apoptotic process	14
CG32817	0.78	Up-regulated	Involved in extrinsic apoptotic signaling pathway	0
Debcl	-1.92	Down-regulated	Programmed cell death involved in cell development	11

4. Discussion

Drosophila testis is an ideal system to study spermatogenesis. In this study, we found that FoxD transcription factor Fd59a contributes to spermatogenesis of *Drosophila*. So far, little is known about the functions of *Drosophila* Fd59a/FoxD and other insect FoxD members. It was reported that Fd59a was expressed in octopaminergic neurons and regulated egg-laying behavior of females [8]. *Drosophila* CHES-1-like/FoxN inhibited germline stem cell differentiation by upregulating *Dpp* expression and ectopic expression of *CHES-1-like* reduced male fertility significantly [31]. In *B. mori*, Fox family genes were expressed in the testis, with *BmFoxL2-2* and *BmFoxD* at a higher level than other BmFox genes [18]. However, the function of BmFoxD in the testis is still unknown.

In this study, we showed that spermatogenesis was disrupted, and apoptosis of sperm bundles was induced in *Fd59a* mutant and RNAi flies. Spermatogenesis is a complex process regulated by multiple signaling pathways and many different genes. Over-activation of the JAK-STAT signaling pathway led to overgrowth of testis and disrupted structure of testis stem cell niche in *Drosophila* [25,32]. Over-activation of the JNK or loss of the Notch signaling caused cell death in the testis of *Drosophila* [33,34]. In mammals, deletion of *Stat3* in Foxd1 cell lineage protected mice from kidney fibrosis [35]. Hypoxia-inducible factors (HIFs) regulated genes related to oxygen homeostasis, and lack of Hif-p4h-2 (HIF prolyl-4-hydroxylases) in FoxD1 lineage led to dysregulation of genes involved

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in the Notch signaling pathway [36]. These results suggest that there is a genetic interaction between mammalian FoxD subfamily members and the JAK-STAT and Notch pathways. However, mRNA levels of genes related to the above three signaling pathways did not change significantly in the *Fd59a*$^{2/2}$ testis (RNA-seq data), indicating that Fd59a is not involved in the JAK-STAT, JNK or Notch signaling pathway. Therefore, insect FoxD members may function differently from mammalian FoxD subfamily members.

Results from RNA-seq and qRT-PCR showed that many genes related to reproduction and cell death were differentially expressed in the testis of *Fd59a*$^{2/2}$ flies. Among the reproduction related DEGs, *Fz2* and *Zpg* have been reported to regulate germ stem cell development in *Drosophila* testis [27,28], while *Blanks* functioned in sperm individualization [37]. Among cell death related DEGs, *Ptp52F* enhanced autophagy and apoptosis in *Drosophila* midgut [30]. In addition, serval potential Fox binding sites were predicted in the promoters of select DEGs. Thus, Fd59a acts as a transcription factor to regulate expression of genes involved in spermatogenesis and in maintaining survival of sperm cells.

It was reported that octopamine was essential for increasing GSCs in mating *Drosophila* females [38], and β-adrenergic-like octopamine receptor (OctβR) was strongly expressed in adult testis [39]. In *Fd59a*$^{2/2}$ adult testis, *Octβ2R* expression was down-regulated, thus, it is possible that Fd59a regulates spermatogenesis partly through regulating expression of *Octβ2R*, and *Fd59a* may be a key factor linking the nervous system to male reproduction system.

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