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Original Article

Spermatogonial Scsα1 restrains testicular germ cell tumor formation and modulates GST expression

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Abstract: Testicular germ cell tumors (TGCTs) are developmental malignancies closely linked to aberrant germ cell differentiation, yet the underlying molecular mechanisms remain poorly defined. Here, we demonstrate that *Scsα1* is indispensable for transit-amplifying (TA) divisions of spermatogonia and the formation of elongated spermatids in *Drosophila* testes. Knockdown of *Scsα1* in spermatogonia disrupts multiple stages of spermatogenesis, impairing both spermatogonial proliferation and differentiation and ultimately leading to defective spermatid elongation. Importantly, in a *bam* heterozygous mutant background, *Scsα1* dysfunction further compromises germline differentiation, resulting in tumor-like germ cell accumulation in *Drosophila* testes. Moreover, downregulation of *Scsα1* in spermatogonia significantly altered the mRNA levels of *GstD3*, *GstD5*, *GstE6*, and *GstE1*, indicating an association between *Scsα1* dysfunction and altered glutathione S-transferase (GST) expression. Consistent with this, downregulation of *Scsα1* in spermatogonia resulted in elevated reactive oxygen species (ROS) levels. Collectively, these findings suggest a potential link between *Scsα1*, GST expression, and tumor-like germ cell accumulation in *Drosophila*, providing new insights into the molecular mechanisms underlying germline tumor development.

Keywords: *Scsα1*, spermatogonia, differentiation, *Drosophila*, testicular germ cell tumors

Introduction

Among young men, testicular germ cell tumors (TGCTs) are among the most frequently diagnosed solid malignancies, and their development is closely associated with abnormal germ cell development [1, 2]. Under normal conditions, spermatogenesis involves multiple stages, including the self-renewal and differentiation of spermatogonial stem cells and spermatogonia, meiosis of spermatocytes, and spermiogenesis [3]. During this process, germ cells must maintain a precise balance between self-renewal and differentiation to sustain sperm production and male fertility [4]. Disruption of this process can lead to abnormal accumulation of undifferentiated germ cells, spermatogenic arrest, and male infertility [5, 6], and may also contribute to the persistence of undifferentiated germ cells and their subsequent neoplastic transformation [7]. Previous studies have suggested that most TGCTs originate from germ cells with impaired development and maturation, whose precursor cells remain in an undifferentiated state for an extended period and subsequently acquire abnormal proliferative capacity [8]. Therefore, disruption of the balance between germ cell proliferation and differentiation is considered an important pathological basis for TGCT development and abnormalities in male reproductive function [2-9].

The male reproductive system of the *Drosophila* possesses a structurally well-defined and highly conserved germline stem cell microenvironment, in which germline stem cells (GSCs) and surrounding somatic cells collectively consti-

tute the apical niche of the testis, regulating germ cell fate through various signaling pathways [10-12]. The *Drosophila* testis serves as an important model system for studying tumor-like abnormal proliferation of germ cells [13]. When GSC differentiation is impaired, undifferentiated germ cells can proliferate continuously and form GSC-like cell clusters, resulting in a phenotype resembling that of testicular germ cell tumors [14]. Therefore, elucidating the key regulatory factors that influence spermatogonial differentiation is important for understanding the mechanisms underlying germ cell tumor formation and abnormalities in male fertility [13-15].

The glutathione (GSH) system serves as a crucial defense mechanism for maintaining cellular redox homeostasis. Within this system, glutathione S-transferases (GSTs) catalyze the conjugation of GSH to various electrophilic compounds and play roles in protection of oxidative species, detoxification, and cellular signaling [16-18]. A growing body of research indicates that members of the GST family are not only closely associated with male reproductive function and sperm quality [19-21], but that abnormalities in their expression and activity are also involved in tumor cell survival, proliferation, and stress adaptation [22, 23]. Therefore, GSH/GST-mediated redox regulation may serve as an important link between cellular metabolic status, germ cell fate, and tumor-like abnormal proliferation [24].

Recent studies have shown that spermatogonial differentiation is closely associated with metabolic remodeling in the *Drosophila* testis [25, 26]. Succinyl-coenzyme A synthetase α subunit 1 (*Scsα1*) encodes the α subunit of the *Drosophila* succinyl-coenzyme A synthetase, a key metabolic enzyme in the tricarboxylic acid (TCA) cycle. This enzyme is involved in the conversion of succinyl-coenzyme A to succinate and contributes to ATP/GTP production through substrate-level phosphorylation. Since the TCA cycle plays a central role in cellular metabolism and redox homeostasis [27], *Scsα1* dysfunction may not only affect cellular energy supply but also alter redox homeostasis and glutathione metabolism. However, it remains unclear whether *Scsα1* regulates spermatogonial proliferation and differentiation in *Drosophila*, or whether its metabolic function is associated with abnormal germ cell proliferation and impaired differentiation.

In this study, the Bam-Gal4 driver was used to knock down *Scsα1* in *Drosophila* spermatogonia, enabling a systematic analysis of its roles in spermatogonial proliferation, differentiation, and later stages of spermatogenesis. The expression of glutathione S-transferase (GST) family genes was further analyzed to investigate potential in redox-related regulation. We aimed to determine whether *Scsα1* dysfunction disrupts the balance between spermatogonial proliferation and differentiation and is associated with altered GST expression and abnormal accumulation of undifferentiated germ cells.

Materials and methods

Fly strains and genetic crosses

The *Drosophila* melanogaster flies used in this study were maintained on standard cornmeal medium at 25°C and approximately 60% relative humidity. The w^{1118} strain was used as the control. To investigate the role of Scsα1 in male reproductive system development, RNA interference (RNAi) targeting Scsα1 was performed using the Gal4/UAS system. UAS-Scsα1 RNAi transgenic flies (TH02217.N) were crossed with Bam-Gal4 flies to reduce Scsα1 expression in spermatogonia. Male flies of the indicated genotypes were collected for subsequent functional analyses.

Immunofluorescence staining

Testes were dissected from *Drosophila* in 1× phosphate-buffered saline (PBS; G4202-500 mL, Solarbio) and fixed in 4% paraformaldehyde for 30 min. After fixation, the testes were washed three times with PBS containing 0.3% Triton X-100 (PBST) and blocked with 5% bovine serum albumin for 30 min. The samples were then incubated with primary antibodies for 1 h at room temperature. After three washes with 0.3% PBST, the samples were incubated with the corresponding secondary antibodies for 1 h at room temperature in the dark. Following three additional washes with 0.3% PBST, nuclei were stained with Hoechst 33342 (1.0 mg/mL, C0031, Beijing Solarbio) for 5 min. The samples were subsequently mounted for microscopic examination.

The primary antibodies used were as follows: rat anti-Vasa (HUABIO; 1:1000), mouse anti-1B1 (DSHB, 1:50), mouse anti-Orb (DSHB, 1:50), mouse anti-LamC (DSHB, 1:100), mouse anti-LamDm0 (DSHB, 1:100), mouse anti-FasIII (DSHB, 1:50), mouse anti-Eya (DSHB, 1:50), rabbit anti-PH3 (#53348, CST, 1:600). Secondary antibodies conjugated to Alexa Fluor 488 (A488), Cy3, or Alexa Fluor 647 (A647) were purchased from Jackson ImmunoResearch Laboratories (USA) and used at a dilution of 1:1,000.

RNA extraction and quantitative real-time PCR (qRT-PCR)

For each biological replicate, 50 *Drosophila* testes were pooled as a single sample. Three biological replicates were prepared independently. Total RNA was extracted from *Drosophila* tissues using TRIzol reagent (15596026, Invitrogen). Approximately 100 ng of total RNA from each sample was used for reverse transcription, and first-strand cDNA was synthesized using the PrimeScript™ II First-Strand cDNA Synthesis Kit (6210A, Takara). qPCR was subsequently performed using TB Green Premix Ex Taq II (RR820, Takara) on a LightCycler® 96 Real-Time PCR System (Roche). The primer sequences were as follows: Scsalpha1-F: GGCAACTTGAAGCTGAACGG; Scsalpha1-R: AACTTGGTGCCGTACTCCAG; GstD3-F: CGATTCCCACGCTGGTAGACAATG; GstD3-R: CCGCCTGCTTCTGAATATCCTTGG; GstD5-F: CCTCTCCACCGTTTCCACTTTCG; GstD5-R: TCACCTTCTTGGCGTTGGCATAC; GstE6-F: GACGAAAGTACCCAAGGAGCGATAC; GstE6-R: ACCGATGAAACGAGACTGAAATCCG; GstE1-F: CGAGCACCTGAGCGAGGAATATG; GstE1-R: GTCCACCAAGTAGGCGGCAATG; Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)-F: GTGGTGAACGGCCAGAAGAT; GAPDH-R: GCCTTGTCAATGGTGGTGAA.

Male fertility test

Males used for fertility testing were reared under standard conditions. Each male was individually paired with three virgin w^{1118} females in a single vial and allowed to mate for 7 days. Males that produced no larvae during this period were scored as sterile.

Reactive oxygen species (ROS) assay

ROS levels in testicular tissues were determined using a tissue ROS assay kit (S0039, Beyotime, China) according to the manufacturer's instructions. Briefly, testicular tissues were homogenized in BeyoLysis™ Buffer R for Metabolic Assay at a ratio of 10 mg tissue per 100 μL buffer under cold conditions. The homogenates were centrifuged at 12,000×g for 3-5 min at 4°C, and the supernatants were collected for subsequent analysis. The tissue lysates were incubated with DCFH working solution at 37°C in the dark for 30 min. Fluorescence intensity was subsequently measured using a fluorescence microplate reader at excitation and emission wavelengths of 488 and 525 nm, respectively. ROS levels were expressed as relative fluorescence intensity.

Statistical analysis

Each experiment was performed with at least three independent biological replicates. Quantitative data are presented as the mean ± standard deviation (SD). Statistical analyses were performed using GraphPad Prism software (GraphPad, La Jolla, CA, USA). Differences between two groups were assessed using Student's t-test, whereas comparisons among multiple groups were performed using one-way analysis of variance (ANOVA) followed by Dunnett's post hoc test, where appropriate. Statistical significance is indicated as follows: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

Results

Scsα1 knockdown inhibits spermatogonial differentiation

To investigate the regulatory role of *Scsα1* in *Drosophila* testes, we used the Bam-Gal4/UAS system to reduce *Scsα1* expression in spermatogonia (Bam>*Scsα1* RNAi). First, we measured *Scsα1* expression by qRT-PCR to verify the efficiency of RNA interference. The results showed that, compared with the control group, the relative mRNA level of *Scsα1* was significantly reduced in Bam>*Scsα1* RNAi testes, indicating effective suppression of *Scsα1* expression (**Figure 1A**). Quantitative analysis of the deeply stained nuclear region at the testis apex revealed an expansion of this region, accompanied by an accumulation of early-stage germ cells was observed, suggesting impaired differentiation following *Scsα1* knockdown in spermatogonia (**Figure 1B**, **1C**). Previous studies have identified Bam as a pivotal

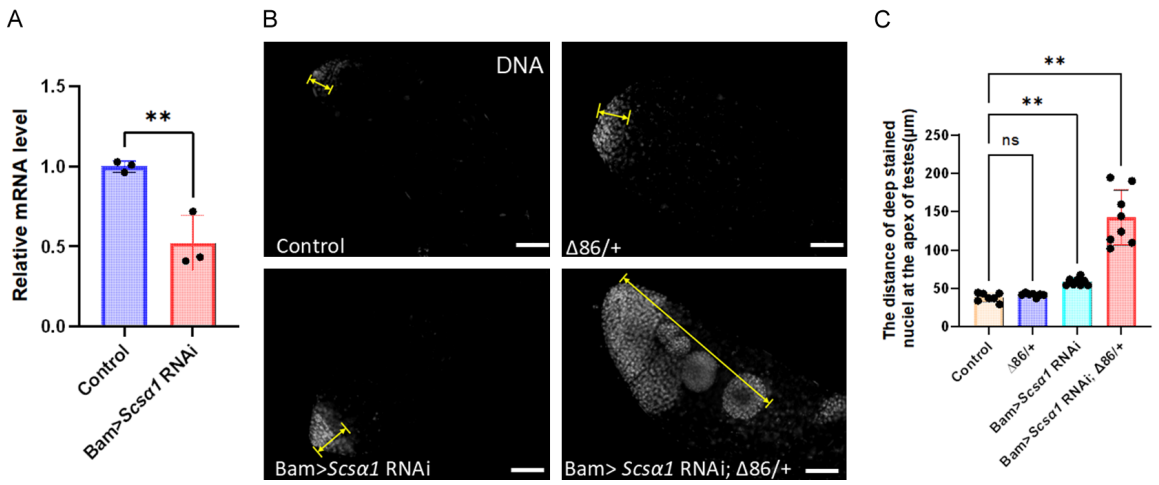


Figure 1. *Scsα1* knockdown in spermatogonia impairs germ cell differentiation in *Drosophila* testes. A. Relative mRNA levels of *Scsα1* in testes from the control (n = 3) and Bam>*Scsα1* RNAi (n = 3) groups. B. DNA staining at the testis apex in the control, Δ86/+, Bam>*Scsα1* RNAi, and Bam>*Scsα1* RNAi; Δ86/+ groups; Yellow lines indicate the extent of the densely stained nuclear region extending posteriorly from the testis apex. C. Quantification of the extent of the densely stained nuclear region in testes from the control (n = 7), Δ86/+ (n = 7), Bam>*Scsα1* RNAi (n = 8) and Bam>*Scsα1* RNAi; Δ86/+ (n = 8). ns, not significant; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$. Scale bars: 50 μm.

regulator of germ cell differentiation, with its activity being indispensable for normal differentiation in *Drosophila* gonads [28, 29]. The heterozygous *bam* mutation (Δ86/+) further exacerbated the differentiation defects in Bam>*Scsα1* RNAi testes (**Figure 1B**, **1C**).

Scsα1 contributes to the later stage of spermatogenesis in Drosophila testes

To further analyze the effects of reduced *Scsα1* expression on the later stages of spermatogenesis in *Drosophila*, we first examined the formation of elongated spermatid clusters in the testes using DNA staining. The results showed that, compared with the control group, the number of elongated spermatid clusters was significantly reduced in the Bam>*Scsα1* RNAi group. In the Bam>*Scsα1* RNAi; Δ86/+ group, the number of elongated spermatid clusters was further significantly reduced (**Figure 2A**, **2B**). We next analyzed spermatid structures using Orb immunofluorescence staining. The results demonstrated no significant differences in the number of Orb-positive elongated spermatids among the control, Δ86/+, and Bam>*Scsα1* RNAi groups. In contrast, the Bam>*Scsα1* RNAi; Δ86/+ group exhibited a significant reduction in the number of elongated spermatids (**Figure 2C**, **2D**). These results indicate that reduced *Scsα1* expression alone is insufficient to cause a pronounced Orb-related phenotype, whereas spermiogenesis defects are significantly exacerbated when *Scsα1* knockdown is combined with the Δ86/+ background, suggesting a potential functional interaction between *Scsα1* and bam-mediated germ cell differentiation.

Scsα1 is essential for spermatogonia differentiation in Drosophila

To investigate the role of *Scsα1* in spermatogonia differentiation, we used immunofluorescence staining to analyze changes in fusome structures at the testis apex following *Scsα1* knockdown. The fusome is a specialized cellular structure connecting adjacent germ cells that undergoes dynamic changes during germ cell differentiation in *Drosophila* spermatogenesis [30]. At the early spermatogonial stage, the fusome typically appears as a punctate structure, but as germ cells differentiate, it extends to form a branched structure [31]. Therefore, changes in fusome morphology serve as an important indicator of germ cell differentiation. The results showed that, compared with the control group, the number of dot-like fusome structures increased in Bam>*Scsα1* RNAi testes (**Figure 3A**, **3B**). Furthermore, the

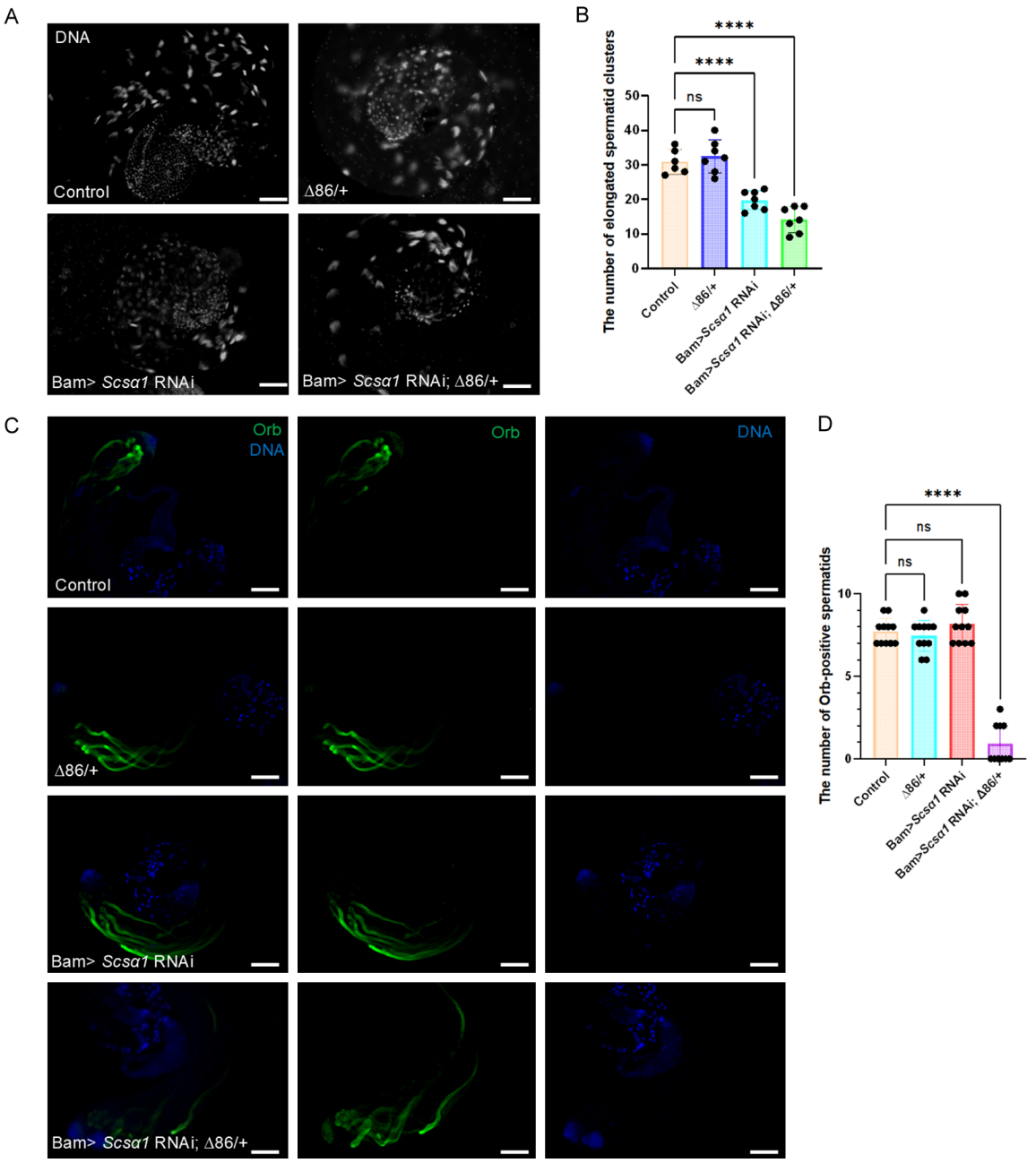


Figure 2. *Scsα1* knockdown in spermatogonia impairs elongated spermatid formation in *Drosophila* testes. (A) DNA staining at the posterior region of testes from the control, Δ86/+, Bam>*Scsα1* RNAi, and Bam>*Scsα1* RNAi; Δ86/+ groups. (B) Quantification of elongated spermatid clusters in the control (n = 6), Δ86/+ (n = 7), Bam>*Scsα1* RNAi (n = 7), and Bam>*Scsα1* RNAi; Δ86/+ (n = 7) groups. (C) Immunofluorescence staining for Orb (green) at the testis apex in the control, Δ86/+, Bam>*Scsα1* RNAi, and Bam>*Scsα1* RNAi; Δ86/+ groups. (D) Quantification of Orb-positive spermatids in the control (n = 11), Δ86/+ (n = 11), Bam>*Scsα1* RNAi (n = 11) and Bam>*Scsα1* RNAi; Δ86/+ (n = 10) groups. ns, not significant; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$. Scale bars: 50 μm for (A), and 100 μm for (C).

accumulation of punctate fusomes was further enhanced in Bam>*Scsα1* RNAi; Δ86/+ testes (**Figure 3A**, **3B**). Vasa staining further supported the finding that reduced *Scsα1* expression impaired germ cell differentiation in *Drosophila* testes (**Figure 3C**).

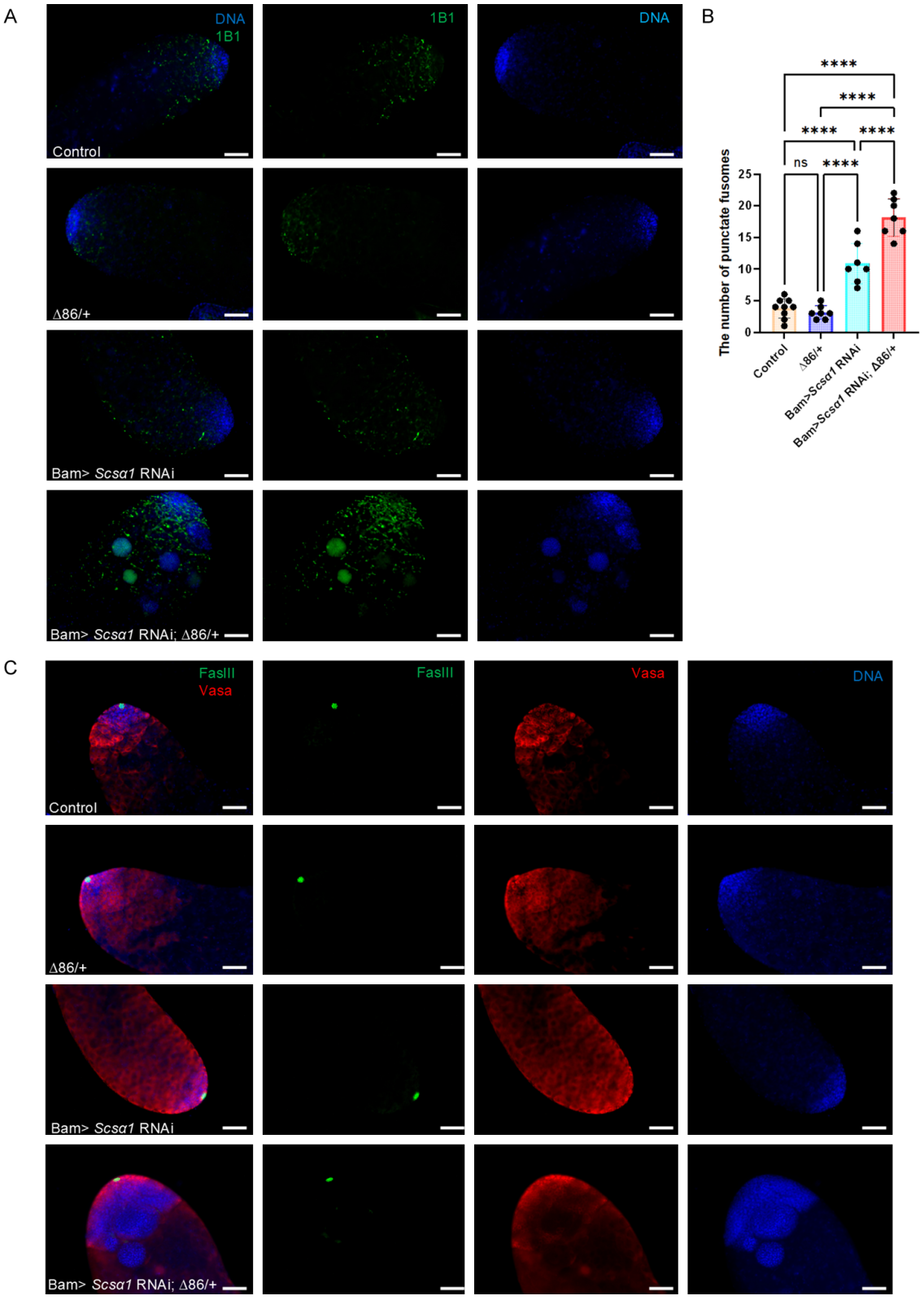


Figure 3. *Scsα1* knockdown alters fusome morphology during spermatogonial differentiation. A. Immunofluorescence staining for 1B1 (green) at the testis apex in the control, Δ86/+, Bam>*Scsα1* RNAi and Bam>*Scsα1* RNAi; Δ86/+ groups. B. Quantification of punctate fusomes at the testis apex in the control (n = 9), Δ86/+ (n = 7), Bam>*Scsα1* RNAi (n = 7), and Bam>*Scsα1* RNAi; Δ86/+ (n = 7) groups. C. Immunofluorescence staining of Vasa (red) and FasIII (green) at the testis apex in the control, Δ86/+, Bam>*Scsα1* RNAi and Bam>*Scsα1* RNAi; Δ86/+ groups. ns, not significant; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$, Scale bars: 50 μm.

Scsα1 knockdown induces spermatogonial differentiation defects

To further investigate the effects of *Scsα1* knockdown on germ cell differentiation, we examined the distribution of spermatogonia and spermatocytes in *Drosophila* testes. In the control group, spermatogonia labeled with LamDm0 were primarily confined to the apical region of the testis. In contrast, in the Bam>*Scsα1* RNAi group, this region extended further toward the posterior end of the testis. This phenotype was most pronounced in the Bam>*Scsα1* RNAi; Δ86/+ group (**Figure 4A**, **4B**). We also found that, compared with the control group, the number of spermatocytes was reduced in the Bam>*Scsα1* RNAi group, and this reduction was even more pronounced in the Bam>*Scsα1* RNAi; Δ86/+ group (**Figure 4C**). Taken together, our data suggest that reduced *Scsα1* expression inhibits the transition from spermatogonia to spermatocytes in the testis, leading to persistent accumulation of spermatogonia.

Spermatogonial Scsα1 non-autonomously regulates cyst cell homeostasis

We next investigated the maintenance of cyst cells following *Scsα1* knockdown in spermatogonia. In the control group, mature cyst cells were distributed around differentiating germ cells. However, in Bam>*Scsα1* RNAi and Bam>*Scsα1* RNAi; Δ86/+ testes, the number of mature cyst cells was significantly increased (**Figure 5A**, **5B**). These data suggest that reduced *Scsα1* expression in spermatogonia may lead to abnormal accumulation of cyst cells through non-cell-autonomous effects.

Knockdown of Scsα1 in spermatogonia induces aberrant cell proliferation

Our findings revealed that *Scsα1* knockdown in spermatogonia perturbed germ cell differentiation and was associated with persistent cell proliferation at positions distal to the FasIII-positive hub cells (**Figure 6A**). Furthermore, compared with controls, Bam>*Scsα1* RNAi and Bam>*Scsα1* RNAi; Δ86/+ testes exhibited significant increases in both the number of PH3-positive cells and the distance between PH3-positive cells and FasIII-positive hub cells (**Figure 6B**, **6C**).

Scsα1 knockdown in spermatogonia alters GST gene expression and ROS levels

Given the established role of *Scsα1* in mitochondrial metabolism, particularly its involvement in the tricarboxylic acid (TCA) cycle, we hypothesized that its loss in spermatogonia may perturb redox homeostasis through altered expression of antioxidant defense genes. To test this, we quantified mRNA levels of four GST genes-*GstD3*, *GstD5*, *GstE6* and *GstE1*-by qRT-PCR. Both Bam>*Scsα1* RNAi and Bam>*Scsα1* RNAi; Δ86/+ testes showed statistically significant reductions in the relative mRNA levels of *GstD3*, *GstD5*, and *GstE6*, along with a significant increase in the relative mRNA level of *GstE1*, compared with controls (**Figure 7A-D**). Furthermore, ROS measurements demonstrated elevated ROS levels in *Scsα1*-knockdown testes, accompanying the changes in GST gene expression (**Figure 7E**). Although male fertility was not significantly affected (**Figure 7F**), *Scsα1* knockdown caused defects in germ cell differentiation and was associated with abnormal germ cell accumulation. These findings suggest that *Scsα1* deficiency may disrupt redox homeostasis, potentially contributing to the observed germ cell differentiation defects.

Discussion

In *Drosophila*, the testicular stem cell niche provides a powerful and genetically tractable system for investigating germ cell proliferation and differentiation and has been used to study mechanisms relevant to germ cell tumorigenesis [13]. We found that reducing Scsα1 expression in spermatogonia impaired germ cell differentiation and resulted in the abnormal accumulation and persistent proliferation of early germ cells, accompanied by reduced spermatid formation. Furthermore, *Scsα1* knockdown increased the number of cyst cells. These findings suggest that *Scsα1* contributes to maintaining the balance between spermatogonial proliferation and differentiation in *Drosophila*.

Scsα1 encodes the α subunit of succinyl-CoA synthetase, a key enzyme in the tricarboxylic acid (TCA) cycle that catalyzes the conversion of succinyl-CoA to succinate, coupled with ATP/GTP production. Cell differentiation is closely associated with mitochondrial activity, and alterations in mitochondrial function and ener-

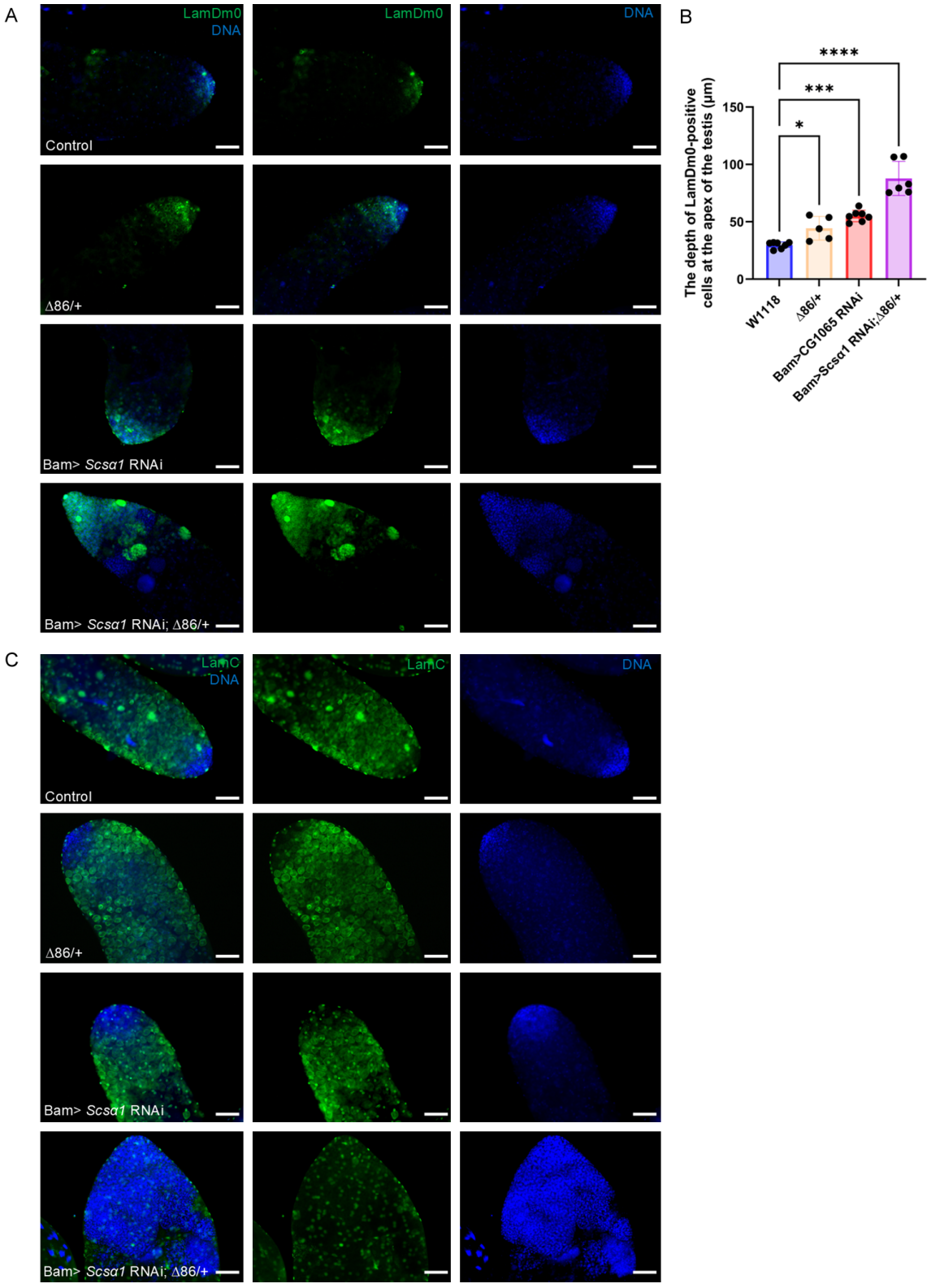


Figure 4. *Scsα1* knockdown alters the distribution of spermatogonia and spermatocytes in *Drosophila* testes. A. Immunofluorescence staining of LamDm0 (green) at the testis apex in the control, Δ86/+, Bam>*Scsα1* RNAi and Bam>*Scsα1* RNAi; Δ86/+ groups. B. Quantification of the LamDm0-positive region from the testis apex in the control (n = 7), Δ86/+ (n = 5), Bam>*Scsα1* RNAi (n = 7) and Bam>*Scsα1* RNAi; Δ86/+ (n = 6) groups. C. Immunofluorescence staining for LamC (green) at the testis apex in the control, Δ86/+, Bam>*Scsα1* RNAi and Bam>*Scsα1* RNAi; Δ86/+ groups. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$. Scale bars: 50 μm.

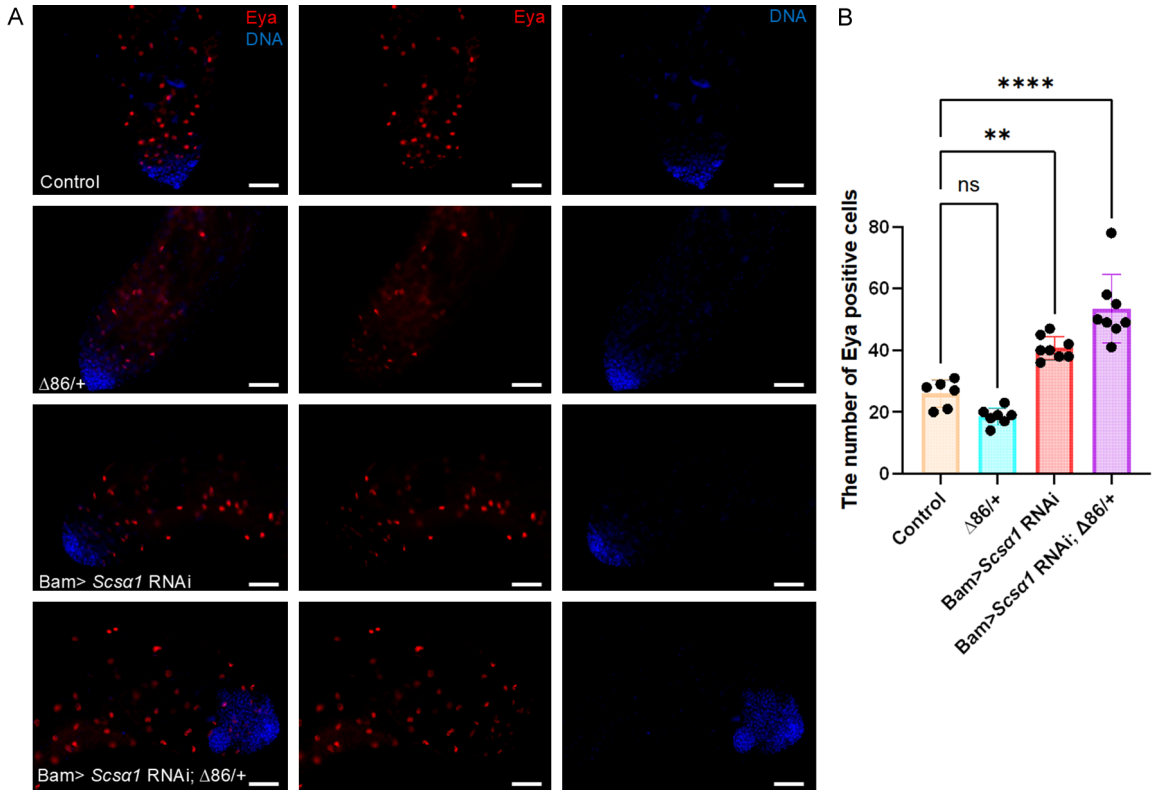


Figure 5. *Scsα1* knockdown in spermatogonia increases the number of Eya-positive cyst cells in *Drosophila* testes. A. Immunofluorescence staining for Eya (red) at the testis apex in the control, Δ86/+, Bam>*Scsα1* RNAi and Bam>*Scsα1* RNAi; Δ86/+ groups. B. Quantification of Eya-positive cells in the control (n = 6), Δ86/+ (n = 7), Bam>*Scsα1* RNAi (n = 8), and Bam>*Scsα1* RNAi; Δ86/+ (n = 8) groups. ns, not significant; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$. Scale bars: 50 μm.

gy metabolism may accompany changes in cellular differentiation states [32]. In this study, reduced *Scsα1* expression was associated with an increase in punctate fusomes and significant increases in the number of PH3-positive cells and their distribution beyond the apical region, consistent with impaired spermatogonia differentiation and persistent mitotic activity. Notably, reduced *bam* gene dosage exacerbated the germ cell differentiation defects and abnormal proliferation associated with *Scsα1* knockdown. Previous studies have shown that Bam is a key factor promoting spermatogonial exit from mitotic proliferation and initiation of differentiation, whereas reduced *bam* gene dosage can cause some spermatogonia to undergo additional transit-amplifying divisions, thereby aggravating differentiation defects [33, 34]. *Scsα1* was knocked down in spermatogonia, a cell population in which Bam is expressed. *Scsα1* knockdown alone resulted in relatively mild defects in germ cell differentiation; however, these phenotypes were markedly enhanced in the Bam heterozygous background. This suggests that *Scsα1* deficiency may increase the susceptibility of spermatogonia to differentiation defects when *bam* gene dosage is reduced.

Notably, *Scsα1* knockdown significantly downregulated *GstD3*, *GstD5*, and *GstE6*, while upregulating *GstE1*. Previous studies have reported that knockdown of the mitochondrial energy metabolism gene *Ant2* alters the expression of multiple GST family members, including *GstD3* and *GstD5*, accompanied by impaired spermatogonial differentiation, increased apoptosis, and a lack of mature sperm, ultimately leading to male infertility [35]. Glutathione transferases catalyze the conjugation of glutathione to a variety of substrates and play important roles in cellular detoxification and protection against oxidative stress [36]. Thus, the dysregulation of GST family genes following *Scsα1* knockdown suggests a potential link between altered mitochondrial metabolism, impaired

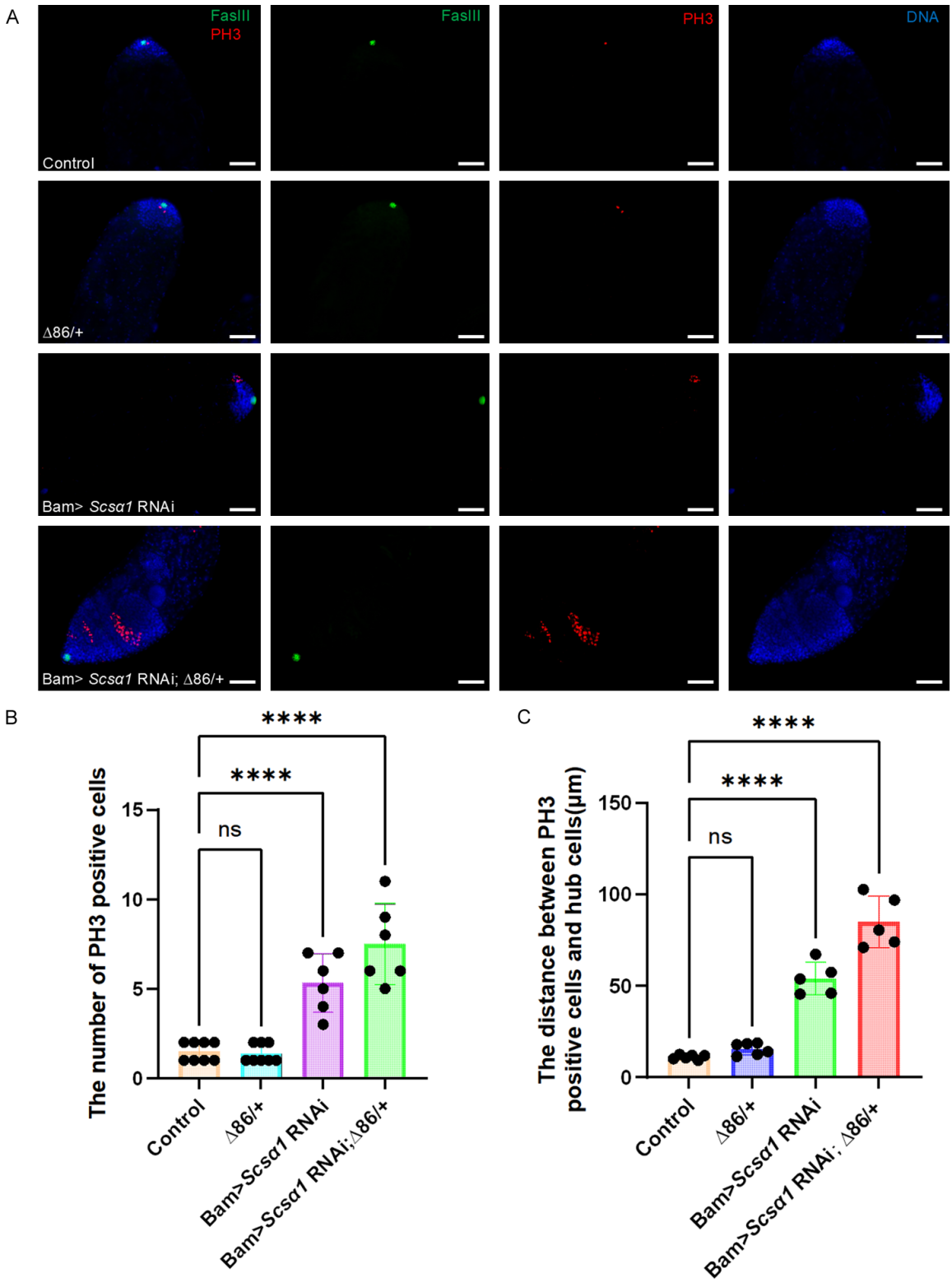


Figure 6. *Scsα1* knockdown in spermatogonia increases mitotic activity in *Drosophila* testes. A. Immunofluorescence staining for PH3 (red) and FasIII (green) at the apex in the control, Δ86/+, Bam>*Scsα1* RNAi and Bam>*Scsα1* RNAi; Δ86/+ groups. B. Quantification of PH3-positive cells in the control (n = 8), Δ86/+ (n = 8), Bam>*Scsα1* RNAi (n = 6), and Bam>*Scsα1* RNAi; Δ86/+ (n = 6) testes. C. Quantification of the distance between PH3-positive cells and hub cells in the control (n = 6), Δ86/+ (n = 6), Bam>*Scsα1* RNAi (n = 5), and Bam>*Scsα1* RNAi; Δ86/+ (n = 5) groups. ns, not significant; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$. Scale bars: 50 μm.

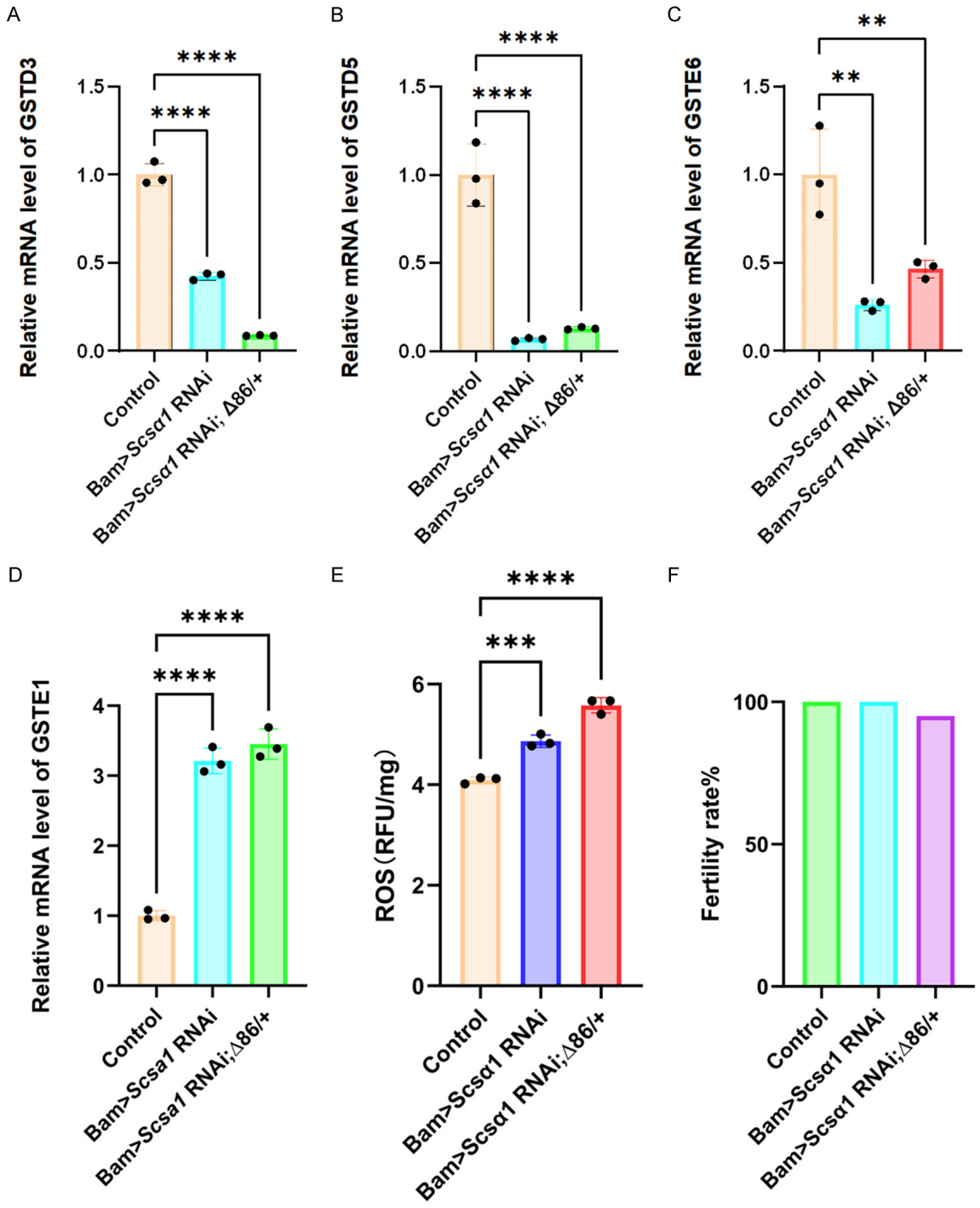


Figure 7. *Scsα1* knockdown in spermatogonia alteres GST gene expression and ROS levels in *Drosophila* testes. (A-D) Relative mRNA levels of *GSTD3* (A), *GSTD5* (B), *GSTE6* (C) and *GSTE1* (D) in testes from the control (n = 3), Bam>*Scsα1* RNAi (n = 3), and Bam>*Scsα1* RNAi; Δ86/+ (n = 3) testes. (E) ROS levels in testes from the control (n = 3), Bam>*Scsα1* RNAi (n = 3), and Bam>*Scsα1* RNAi; Δ86/+ (n = 3) testes. (F) Male fertility rates in the control (n = 20), Bam>*Scsα1* RNAi (n = 20), and Bam>*Scsα1* RNAi; Δ86/+ (n = 20) testes. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

antioxidant defense, and defective germ cell differentiation. Furthermore, the changes in GST gene expression were accompanied by elevated ROS levels in *Scsα1*-knockdown tes-

tes, suggesting a potential disturbance of cellular redox homeostasis. However, whether GST dysregulation contributes directly to *Scsα1*-associated differentiation defects or occurs as a consequence of impaired germ cell differentiation remains to be determined.

To date, *Scsα1* has not been directly implicated in TGCT pathogenesis. In *Drosophila* testes, disruption of germ cell differentiation combined with persistent proliferative capacity can result in tumor-like accumulation of undifferentiated germ cells [37]. This model has been used to study germ cell fate abnormalities relevant to TGCT biology [13]. In the present study, *Scsα1* knockdown caused impaired spermatogonial differentiation, accumulation of undifferentiated germ cells, and an increase in PH3-positive cells, exhibiting features consistent with abnormal germ cell proliferation. Previous studies have indicated that changes in tumor cell metabolism can occur alongside remodeling of the local microenvironment [38]. The increase in Eya-positive cyst cells observed in this study further suggests that *Scsα1* knockdown may affect not only germ cell homeostasis but also the surrounding somatic cell population.

It should be noted that the present study was conducted in *Drosophila*, and the relevance of these findings to human cancer biology and clinical TGCT remains uncertain. Our findings suggest that Scsα1 contributes to spermatogonial differentiation and that its reduced expression may promote tumor-like germ cell accumulation under certain genetic conditions. Further studies are needed to determine whether *Scsα1* or its human orthologs play similar roles in germ cell differentiation and contribute to TGCT development. An additional limitation is the use of a single RNAi line to reduce *Scsα1* expression. Although the observed phenotypes support a potential role for Scsα1 in germ cell differentiation, off-target effects cannot be completely excluded. Future studies using independent RNAi lines and genetic rescue experiments will be important to validate the specificity of these findings. Moreover, the present study has not established a causal role for GST dysregulation in the elevated ROS levels or germ cell differentiation defects associated with *Scsα1* knockdown. Our data demonstrate changes in GST gene expression accompanied by increased ROS levels, but the underlying molecular relationship remains unclear. Further mechanistic studies are therefore needed to determine whether GST-mediated redox regulation contributes to the observed phenotypes.

Collectively, these findings support a role for *Scsα1* in maintaining the balance between germ cell proliferation and differentiation and suggest that its reduced function may favor the accumulation of abnormal, incompletely differentiated germ cells. These observations provide a basis for further investigation of the relationship between mitochondrial metabolism, germ cell differentiation, and tumor-like proliferation, although the underlying molecular mechanisms remain to be established.

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Disclosure of conflict of interest

None.

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