

Splicing factor SRSF1 is essential for homing of precursor spermatogonial stem cells in mice

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Abstract

Spermatogonial stem cells (SSCs) are essential for continuous spermatogenesis and male fertility. The underlying mechanisms of alternative splicing (AS) in mouse SSCs are still largely unclear. We demonstrated that SRSF1 is essential for gene expression and splicing in mouse SSCs. Crosslinking immunoprecipitation and sequencing (CLIP-seq) data revealed that spermatogonia-related genes (e.g., *Plzf*, *Id4*, *Setdb1*, *Stra8*, *Tial1/Tiar*, *Bcas2*, *Ddx5*, *Srsf10*, *Uhrf1*, and *Bud31*) were bound by SRSF1 in the mouse testes. Specific deletion of *Srsf1* in mouse germ cells impairs homing of precursor SSCs leading to male infertility. Whole-mount staining data showed the absence of germ cells in the testes of adult cKO mice, which indicates Sertoli cell-only syndrome (SCOS) in cKO mice. The expression of spermatogonia-related genes (*Gfra1*, *Pou5f1*, *Plzf*, *Dnd1*, *Stra8*, and *Taf4b*) was significantly reduced in the testes of conditional knockout (cKO) mice. Moreover, multiomics analysis suggests that SRSF1 may affect survival of spermatogonia by directly binding and regulating *Tial1/Tiar* expression through AS. In addition, immunoprecipitation mass spectrometry (IP-MS) and co-immunoprecipitation (Co-IP) data showed that SRSF1 interacts with RNA splicing-related proteins (SART1, RBM15, and SRSF10). Collectively, our data reveal the critical role of SRSF1 in spermatogonia survival, which may provide a framework to elucidate the molecular mechanisms of the posttranscriptional network underlying homing of precursor SSCs.

eLife assessment

In this **valuable** study, the authors characterize the role of splicing factor SRSF1 during spermatogenesis with a conditional knockout for *Srsf1* in male mouse germ cells. The requirement of SRSF1 for maturation of postnatal gonocytes into spermatogonia, and the molecular role of SRSF1 in regulating alternative splicing in juvenile testes are **convincingly** supported. The paper also provides strong evidence that the mRNA encoding *Tial*, a factor relevant for spermatogonial maintenance and male fertility, is alternatively spliced in testis and that this splicing is regulated by SRSF1. The work will be of interest to reproductive biologists and stem cell biologists.

Introduction

SCOS, also known as del Castillo syndrome or germ cell aplasia, is one of the most common causes of severe non-obstructive azoospermia (NOA) (Wang et al., 2023 [\[1\]](#)). SCOS is the presence of only Sertoli cells in the testicular tubules of the testes, with no germ cells present (Juul et al., 2014 [\[2\]](#); Wang et al., 2023 [\[1\]](#)). It is well known that abnormal self-renewal and differentiation of SSCs leads to SCOS (Kanatsu-Shinohara and Shinohara, 2013 [\[3\]](#); La and Hobbs, 2019b [\[4\]](#)). In mice, gonocytes begin homing at 0 to 3 days postpartum (dpp) and then develop into SSCs at 4 to 6 dpp for continuous self-renewal and differentiation (Lee and Shinohara, 2011 [\[5\]](#); McLean et al., 2003 [\[6\]](#); Tan and Wilkinson, 2020 [\[7\]](#)). The mechanisms regulating homing of precursor SSCs are hence crucial for forming SSC pools and establishing niches (Oatley and Brinster, 2012 [\[8\]](#)). Spermatogonia migrate to form two distinct subtypes in mice. The first subtype develops into precursor SSCs that provide an SSC population for adult spermatogenesis, whereas the second subtype transitions directly to differentiated spermatogonia that contribute to the first round of spermatogenesis but do not self-renew (Kluin and de Rooij, 1981 [\[9\]](#); Law et al., 2019 [\[10\]](#)). Therefore, homing of precursor SSCs to establish niches is essential for SSC self-renewal and differentiation.

Many transcription factors (e.g., FOXO1, PLZF, POU5F1, TAF4B, CHD4, BCL6B, BRACHYURY, ETV5, ID4, LHX1, POU3F1, DMRT1, NGN3, SOHLH1, SOHLH2, SOX3, and STAT3) promote SSC self-renewal and differentiation (Cafe et al., 2021 [\[11\]](#); Song and Wilkinson, 2014 [\[12\]](#)). However, the molecular mechanisms of the posttranscriptional network underlying homing of precursor SSCs are not sufficiently clear. Previous studies have identified the key RNA-binding proteins DND1 and DDX5 in SSCs with a unique and dominant role in posttranscriptional regulation (Legrand et al., 2019 [\[13\]](#); Yamaji et al., 2017 [\[14\]](#)). Surprisingly, recent studies have found that the RNA-binding proteins SRSF10, UHRF1, BUD31, and BCAS2 regulate AS in mouse spermatogonia (Liu et al., 2022 [\[15\]](#); Liu et al., 2017 [\[16\]](#); Qin et al., 2023 [\[17\]](#); Zhou et al., 2022 [\[18\]](#)). It is well known that testes are rich in AS events (Mazin et al., 2021 [\[19\]](#); Venables, 2002 [\[20\]](#)). Thus, understanding the mechanisms of AS in human reproduction can provide new insights into clinical diagnosis. However, the underlying mechanisms of how AS functions in homing of precursor SSCs are still largely unclear.

Serine/arginine-rich splicing factor 1 (SRSF1; previously SF2/ASF) is a widely studied and important splicing factor involved in cancer progression, heart development, and thymus development (Du et al., 2021 [\[21\]](#); Katsuyama et al., 2019 [\[22\]](#); Katsuyama and Moulton, 2021 [\[23\]](#); Liu et al., 2021 [\[24\]](#); Lv et al., 2021 [\[25\]](#); Qi et al., 2021 [\[26\]](#); Xu et al., 2005 [\[27\]](#)). Our previous work has shown that SRSF1 deficiency impairs primordial follicle formation during meiotic prophase I and leads to primary ovarian insufficiency (POI) (Sun et al., 2023b [\[28\]](#)). However, the underlying mechanisms by which SRSF1 regulates pre-mRNA splicing in mouse SSCs remain unknown. A mouse model with *Srsf1* conditional deletion can effectively address this uncertainty. This study showed that specific

deletion of *Srsf1* in mouse germ cells leads to NOA by impairing homing of mouse precursor SSCs. We further verified that SRSF1 directly binds and regulates *Tial1/Tiar* expression via AS, which may be critical for homing of mouse precursor SSCs.

Results

SRSF1 may have a vital role in posttranscriptional regulation in the testes

To investigate the role of SRSF1 in spermatogenesis, the dynamic localization of SRSF1 in the testis was evaluated. Fascinatingly, the results of SRSF1 and γ H2AX co-staining revealed that SRSF1 was expressed during spermatogenesis (Figure 1A and S1). RT-qPCR and Western blotting results showed that the expression of SRSF1 fluctuated during the developmental stages of the testes (Figure 1B and 1C). Concurrently, the results of SRSF1 and PLZF co-staining revealed that SRSF1 was highly expressed in the nuclei of spermatogonia (Figure 1D). To further explore the function of SRSF1 in regulating SSC self-renewal and differentiation, CLIP-seq was performed in adult mouse testes (Sun et al., 2023a). GO enrichment analyses of the SRSF1 peak-containing genes revealed that spermatogenesis-related genes were regulated by SRSF1 (Figure 2A and Table S1). In combination with previous studies, we found that spermatogonia-related genes (*Plzf*, *Id4*, *Setdb1*, *Stra8*, *Tial1/Tiar*, *Bcas2*, *Ddx5*, *Srsf10*, *Uhrf1*, and *Bud31*) were bound by SRSF1. To provide in-depth insight into the binding of spermatogonia-associated genes, the SRSF1-binding peaks of the gene transcripts were shown by using IGV (Figure 2B). The co-staining results showed localization and expression of the spermatogonia-related protein in mouse testes (Figure 2C). Together, these results suggested that SRSF1 may have a vital role in posttranscriptional regulation in the testes, particularly during spermatogonium development.

SRSF1 deficiency leads to SCOS

To define the specific involvement of SRSF1 in spermatogonia, we studied the physiological roles of SRSF1 in vivo using a mouse model. Considering that global SRSF1 knockout is lethal in mice (Xu et al., 2005), we used a conditional allele of *Srsf1* (*Srsf1^{Fl}*) in which exons 2, 3, and 4 of *Srsf1* are flanked by two *loxP* sites (Figure 3A). By crossing *Srsf1^{Fl}* and *Vasa-Cre* mice, we obtained *Vasa-Cre Srsf1^{Fl/del}* mice with *Srsf1* deletion in germ cells (Figure 3A and 3B). We verified the absence of the SRSF1 protein in germ cells by co-immunofluorescence analyses with SRSF1 and PLZF antibodies (Figure 3C). Subsequently, the breeding experiment indicated that cKO mice had a standard mating capacity but that the absence of *Srsf1* led to complete infertility in males (Figure 3D). Histological examination of cKO epididymides revealed that sperm could not be found in the cauda epididymis (Figure 3E). Considering the limitations of sectioning, the cauda epididymal sperm count further validated this conclusion (Figure 3F). It was clear that spermatogenesis in the testes was severely impaired. Therefore, we focused our attention on the testes. The adult cKO mice were normal in size (Figure 3G). However, the sizes of cKO mouse testes were significantly reduced (Figure 3H). Histological examination of cKO testis sections showed that no germ cells could be visualized, and only a large number of Sertoli cells were observed in the testes of cKO mice (Figure 3I). Together, these results demonstrated that SRSF1 is critical for spermatogenesis and that its absence leads to SCOS.

Loss of SRSF1 impairs spermatogonia survival

To further confirm the absence of germ cells in the testes of cKO mice, PLZF and γ H2AX co-staining was performed in adult mouse testes. These data suggested that SRSF1 deficiency impaired germ cell survival (Figure 4A). The results of VASA and TRA98 co-staining further confirmed this phenotype (Figure 4B). Considering the limitations of sectioning, we used whole-

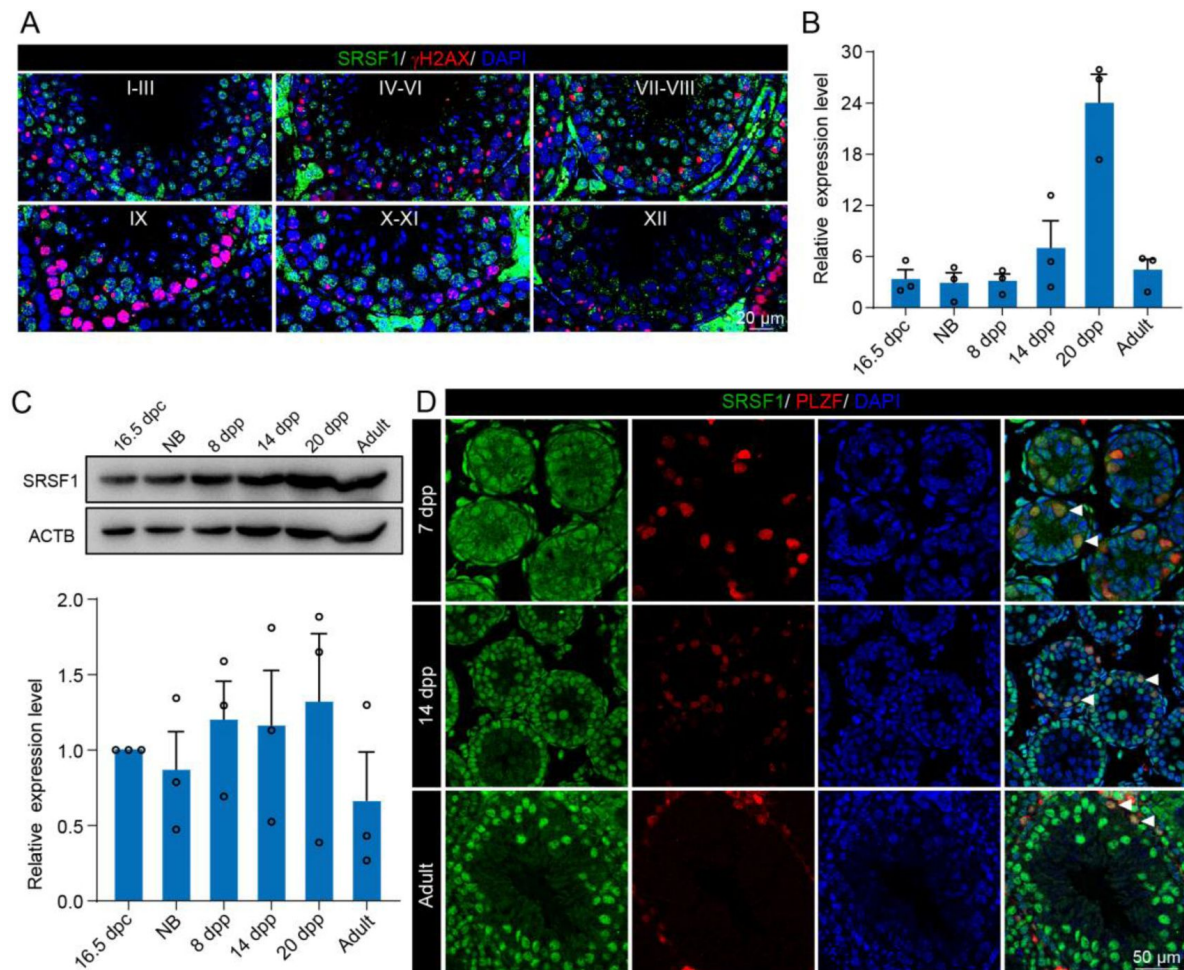


Figure 1

Dynamic localization of SRSF1 in male mouse germ cells.

(A) Dynamic localization of SRSF1 during spermatogenesis. Co-immunostaining was performed using SRSF1 and γH2AX antibodies from adult mouse testes. DNA was stained with DAPI. Scale bar, 20 μm.

(B) Expression of *Srsf1* in testes at different stages of development. The RT-qPCR data were normalized to *Gapdh*. *n*=3.

(C) Western blotting of SRSF1 expression in testes at different stages of development. ACTB served as a loading control. The value in 16.5 dpc testes were set as 1.0, and the relative values of testes in other developmental periods are indicated. *n*=3.

(D) Localization and expression of SRSF1 in spermatogonia. Co-immunostaining was performed using PLZF and SRSF1 antibodies in 7 dpp, 14 dpp, and adult mouse testes. DNA was stained with DAPI. Arrowheads, spermatogonia. Scale bar, 50 μm

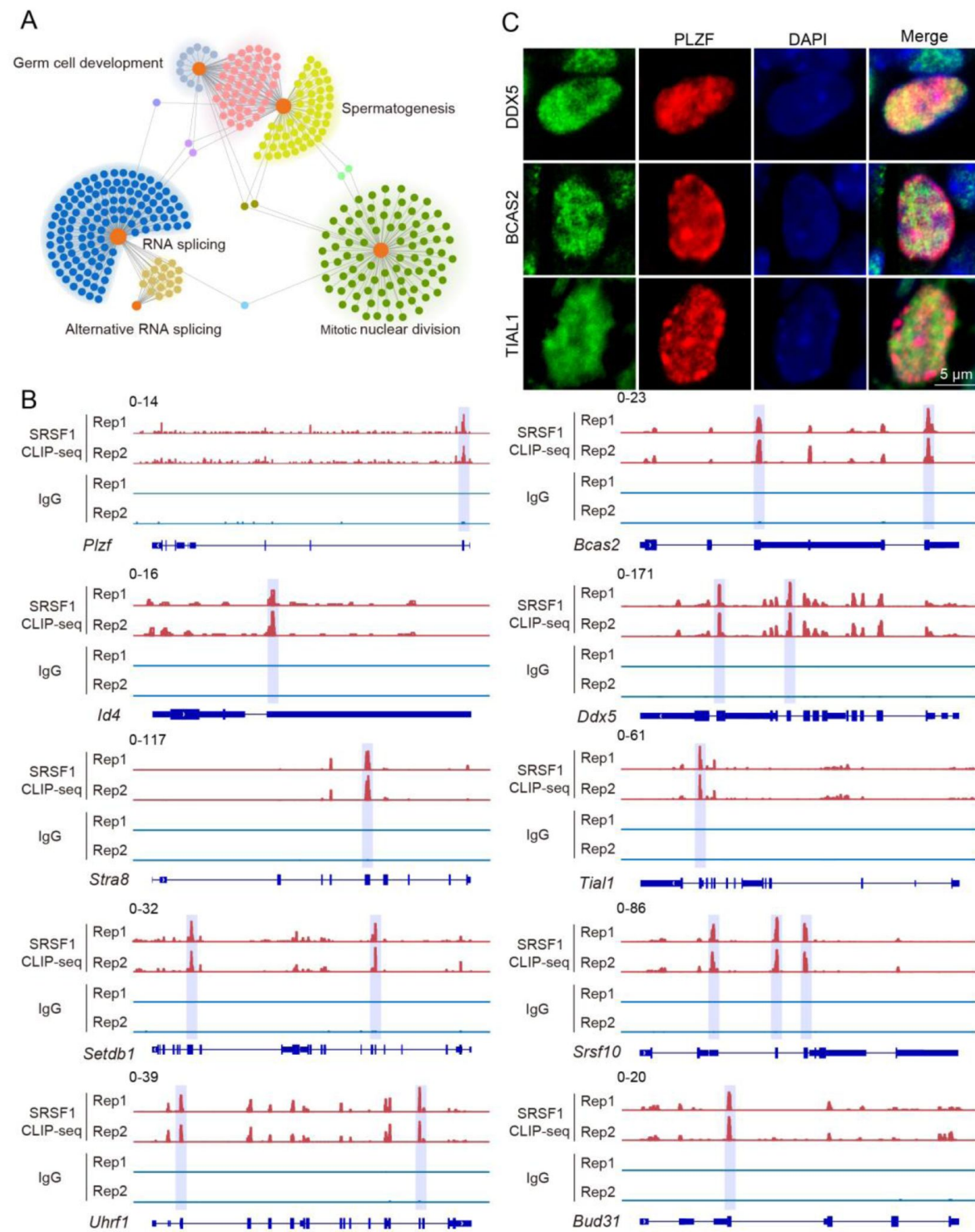


Figure 2

SRSF1-binding genes have an essential role in spermatogonia.

(A) Network showing GO enrichment analyses of SRSF1-binding genes.

(B) Representative genome browser views of spermatogonia-related gene transcripts bound by SRSF1. Higher peaks are marked by a lavender area.

(C) Localization and expression of the spermatogonia-related protein in mouse testes. Scale bar, 5 μ m.

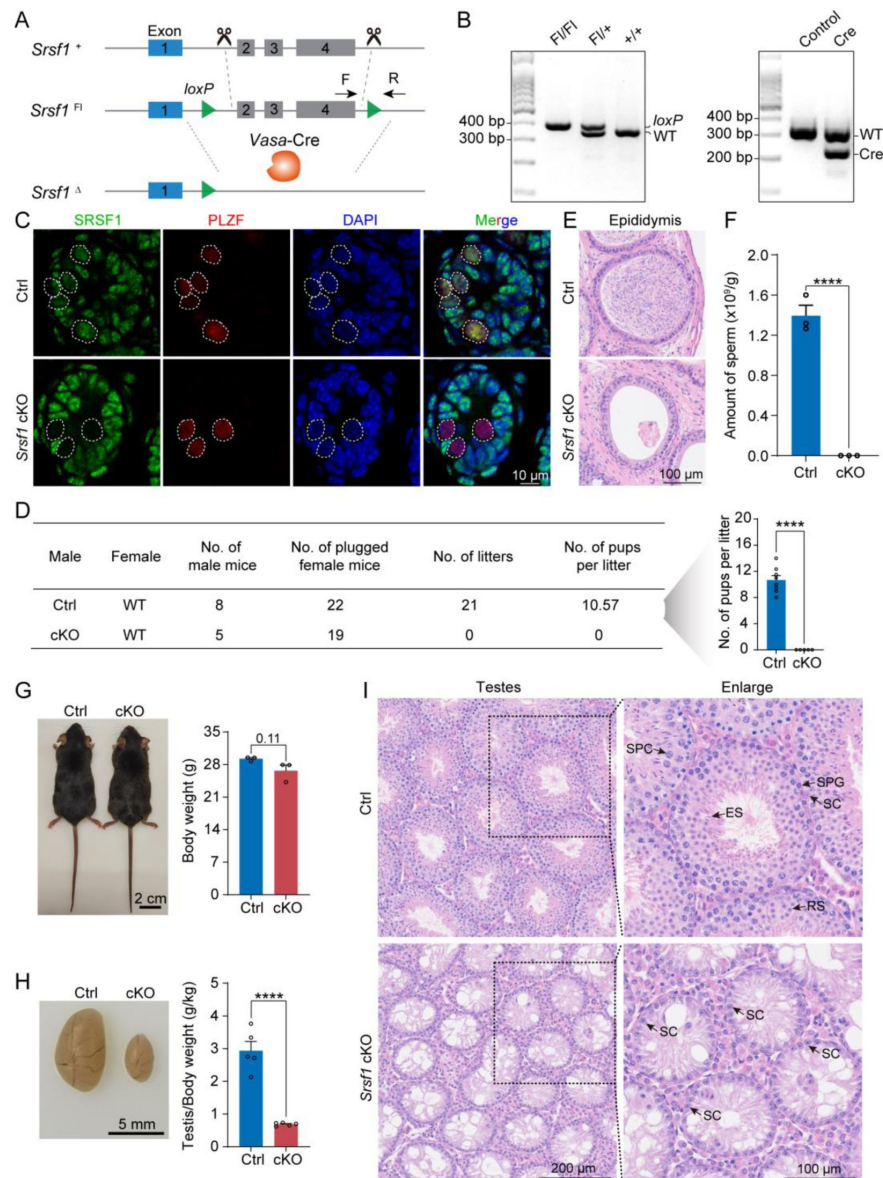


Figure 3

SRSF1 plays critical roles in spermatogenesis and male fertility.

(A) *Vasa-Cre* mice were crossed with *Srsf1*^{Fl/Fl} mice to generate *Srsf1* cKO mice. Cre-mediated deletion removed exons 2, 3, and 4 of *Srsf1* and generated a null protein allele.

(B) Genotyping PCR was performed using *Vasa-Cre* and *Srsf1* primers.

(C) Co-immunostaining of SRSF1 and PLZF in 7 dpp Ctrl and cKO testes. DNA was stained with DAPI. Scale bar, 10 μm.

(D) Fertility test results showed a male infertility phenotype in the cKO mice (*n*=5) compared to the Ctrl mice (*n*=8). The number of pups per litter was determined in the cKO (*n*=5) and Ctrl (*n*=8) mice.

(E) Haematoxylin-eosin-stained epididymis sections from adult Ctrl and cKO mice were obtained. Scale bar, 100 μm.

(F) Cauda epididymal sperm counting was performed. *n*=3.

(G) Normal body weight in cKO mice. The body sizes and weights of adult Ctrl and cKO mice are shown as the mean ± SEM. *n*=3.

(H) Testis atrophy in adult cKO mice. Testis sizes and weights of adult Ctrl and cKO mice are shown as the mean ± SEM. *n*=5.

(I) Haematoxylin-eosin-stained testis sections from adult Ctrl and cKO mice were obtained. Scale bar, left panel: 200 μm, right panel: 100 μm. SC, Sertoli cell; SPG, Spermatogonium; SPC, spermatocyte; RS, round spermatid; ES, elongated spermatid.

Unpaired Student's *t* test determined significance; exact *P* value *P* ≥ 0.05, *****P* < 0.0001. The points and error bars represent the mean ± SEM.

mount immunostaining to perform a comprehensive analysis and found that germ cells were indeed absent in the testes of adult cKO mice (**Figure 4C**). To dynamically analyse the loss of germ cells, we collected testes from 5 dpp, 7 dpp, and 14 dpp mice. Morphological results showed that the testes of 7 dpp and 14 dpp cKO mice were much smaller than those of Ctrl mice (**Figure 5A**). To determine the presence of germ cells in cKO testes, VASA staining was performed in 5 dpp, 7 dpp, and 14 dpp Ctrl and cKO testes. The results showed that germ cells were still present in cKO mice but were significantly reduced in 7 dpp and 14 dpp cKO testes (**Figure 5B**). The germ cell count per tube showed a significant reduction in the number of 7 dpp and 14 dpp cKO testes, especially 14 dpp cKO testes (**Figure 5B**). In addition, TUNEL results showed that apoptosis significantly increased in cKO testes (**Figure 5C**). These data suggested that the absence of SRSF1 causes apoptosis in a large number of spermatogonia that are unable to survive.

Loss of SRSF1 impairs homing of precursor SSCs

To further investigate the reason for the failure of spermatogonia to survive, we observed homing of precursor SSCs in the testes of mice at 5 dpp. Interestingly, the results of VASA and SOX9 co-staining showed that partial germ cells could not complete homing in 5 dpp cKO testes (**Figure 6A** and **6B**). In mice, starting at 3 dpp, cytoplasmic FOXO1 in some gonocytes begins to enter the nucleus (Goertz *et al.*, 2011). These cells further develop into prospermatogonia, which are expected to develop into spermatogonial stem cells (Goertz *et al.*, 2011). Thus, immunohistochemical staining for FOXO1 was performed on 5 dpp mouse testis sections (**Figure 6C**). Further, germ cell statistics of FOXO1 expression in the nucleus showed a reduced number of prospermatogonia in cKO mice (**Figure 6D**). And germ cells in which FOXO1 is expressed in the nucleus similarly undergo abnormal homing (**Figure 6E**). Thus, all the above data indicated that SRSF1 has an essential role in the homing of precursor SSCs.

SRSF1 is essential for gene expression in spermatogonia

To investigate the molecular mechanisms of SRSF1 in spermatogonia, we isolated mRNA from 5 dpp cKO and Ctrl mouse testes and performed RNA-seq (**Figure S2**). RNA-seq and RT-qPCR results showed a significant reduction in the expression of *Srsf1* in 5 dpp cKO mouse testes (**Figure 7A**). Western blotting results showed that SRSF1 expression was significantly reduced in the testes of cKO mice at 5 dpp (**Figure 7B**). Hence, for Ctrl and cKO samples, the confidence level of the RNA-seq data was high. The volcano map and cluster heatmap showed 715 downregulated and 258 upregulated genes identified by RNA-seq data in 5 dpp cKO mouse testes (**Figure 7C**, 7D and Table S2). These gene GO enrichment analyses indicated abnormal germ cell development and cell cycle arrest in 5 dpp cKO mouse testes (**Figure 7E**). Surprisingly, the heatmap showed that spermatogonia associated gene (*Gfra1*, *Pou5f1*, *Plzf*, *Nanos3*, *Dnd1*, *Stra8*, and *Taf4b*) expression was significantly reduced in the testes of cKO mice at 5 dpp (**Figure 7F**). Simultaneously, visual analysis using IGV showed that the peak of SSC-related genes was significantly decreased (**Figure 7G**). Next, we validated the abnormal expression of spermatogonia associated genes (downregulated: *Gfra1*, *Pou5f1*, *Plzf*, *Dnd1*, *Stra8*, and *Taf4b*; stabilized: *Nanos3*) by RT-qPCR (**Figure 7H**). Together, these data indicated that germ cell-specific deletion of *Srsf1* impairs the expression of spermatogonia associated genes.

SRSF1 directly binds and regulates the expression and AS of *Tial1/Tiar*

Multimomics analyses were carried out in a subsequent study to identify the molecular mechanisms by which SRSF1 regulates spermatogonia survival. Considering that the CLIP-seq data were obtained from adult mouse testis, the set of genes bound by CLIP-seq was restricted to those expressed only in the 5 dpp mouse testis RNA-seq data. we found that 3543 of the 4824 genes bound by SRSF1 were expressed in testes at 5 dpp. Venn diagram data revealed that nine out of

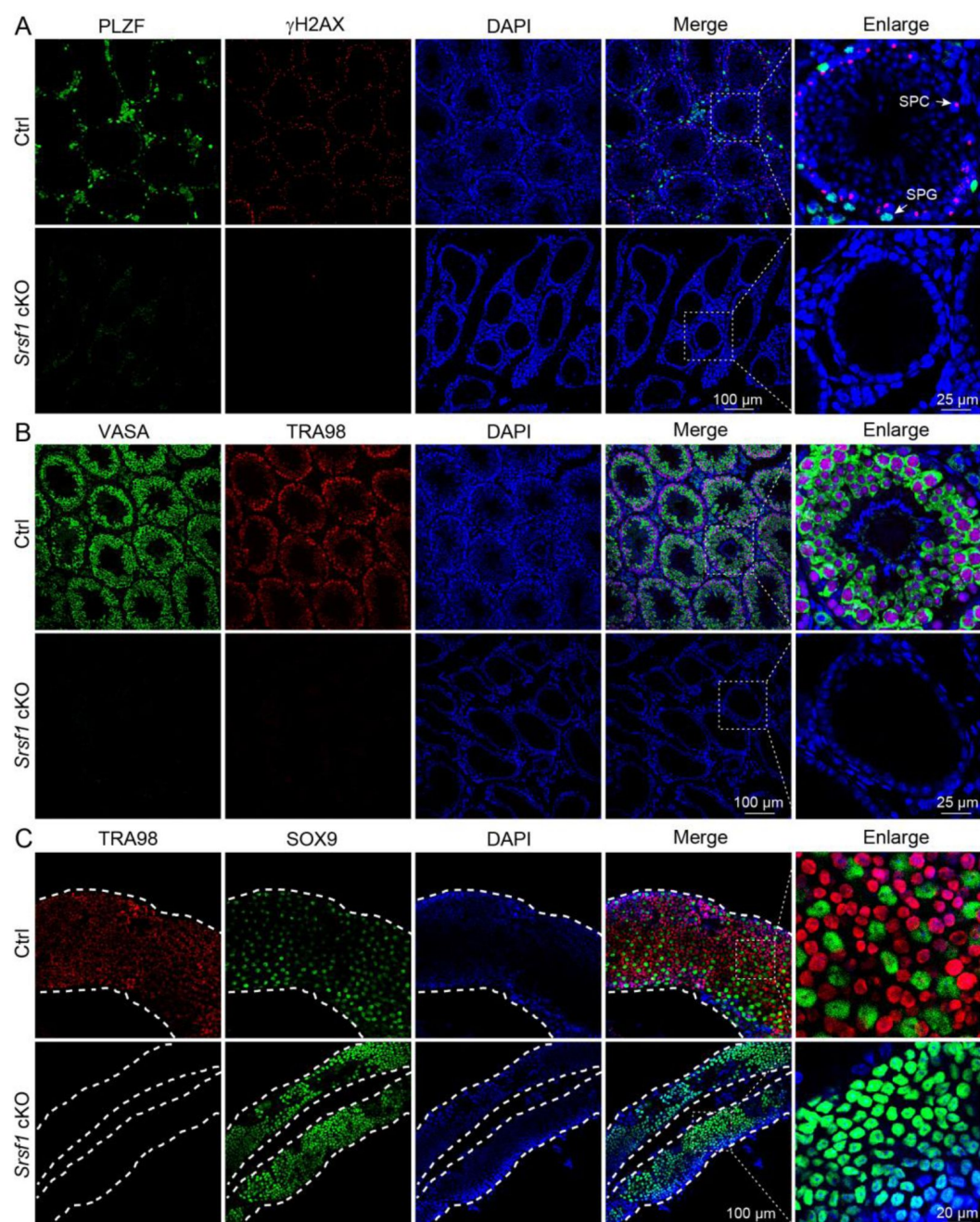


Figure 4

Loss of germ cells in adult cKO mouse testes.

(A) Co-immunostaining of PLZF and γ H2AX in adult Ctrl and cKO testes. DNA was stained with DAPI. Scale bar, right panel: 25 μ m, other panels: 100 μ m.

(B) Co-immunostaining of VASA and TRA98 in adult Ctrl and cKO testes. DNA was stained with DAPI. Scale bar, right panel: 25 μ m, other panels: 100 μ m.

(C) Whole-mount co-immunostaining of TRA98 and SOX9 in adult Ctrl and cKO testes. DNA was stained with DAPI. White dashed lines, boundary of the tubule. Scale bar, right panel: 20 μ m, other panels: 100 μ m.

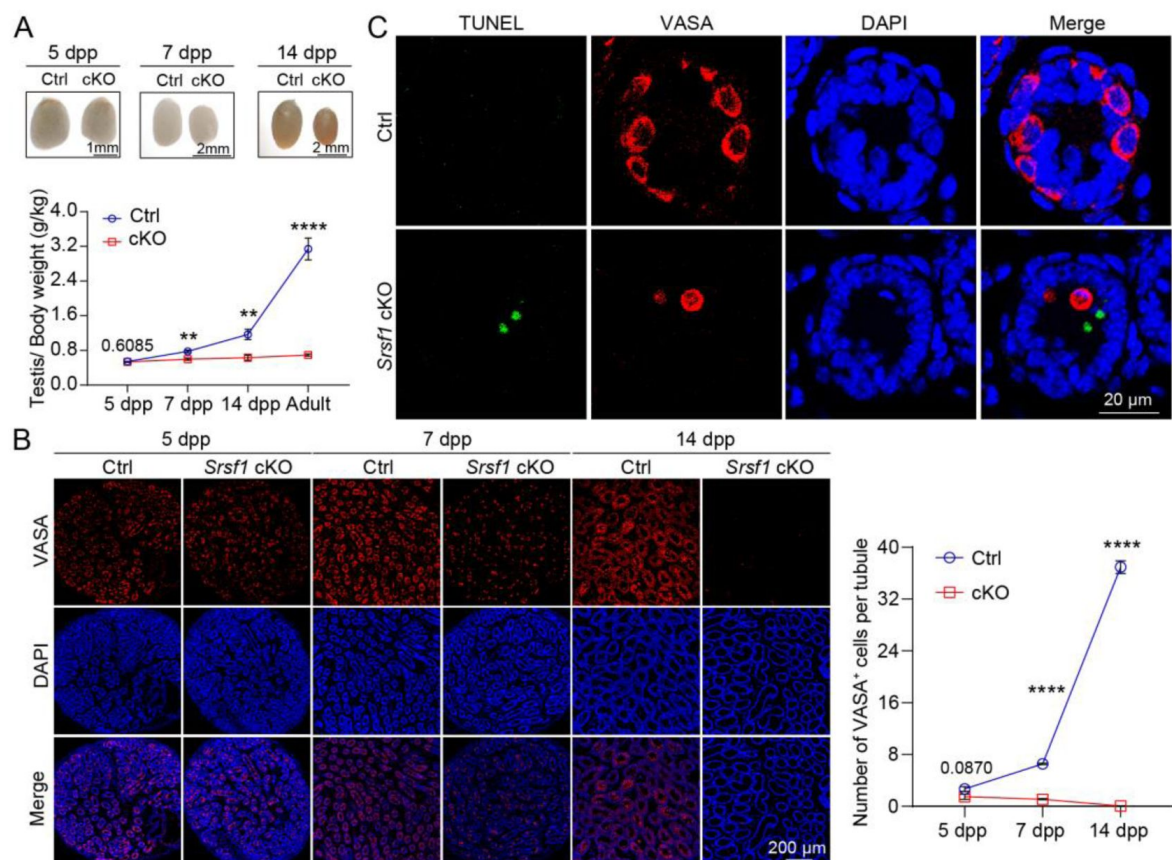


Figure 5

SRSF1 is required for spermatogonia survival.

(A) Testis sizes of 5 dpp, 7 dpp, and 14 dpp Ctrl and cKO mice are shown. The testis/body weight ratios (g/kg) of 5 dpp, 7 dpp, 14 dpp, and adult Ctrl and cKO mice are shown as the mean $\pm$ SEM. $n=4$.

(B) Immunostaining of VASA in 5 dpp, 7 dpp, and 14 dpp Ctrl and cKO testes. DNA was stained with DAPI. Scale bar, 200 μ m. Number of VASA-positive cells per tubule is the mean $\pm$ SEM. $n=3$.

(C) TUNEL apoptosis assay was performed on sections from 7 dpp Ctrl and cKO testes. DNA was stained with DAPI. Scale bar, 20 μ m.

Unpaired Student's t test determined significance; exact P value $P \geq 0.05$, $**P < 0.01$, $****P < 0.0001$. The points and error bars represent the mean $\pm$ SEM.

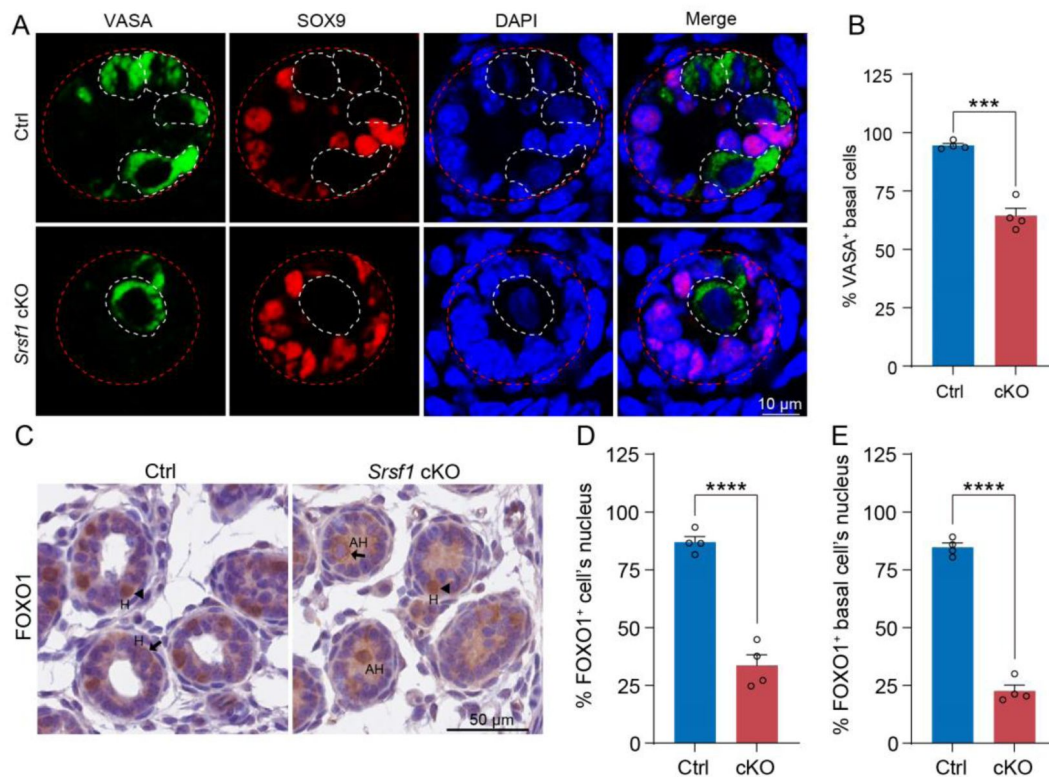


Figure 6

SRSF1 is required for SSC homing of precursor SSCs.

(A) Co-immunostaining of VASA and SOX9 in 5 dpp Ctrl and cKO testes. DNA was stained with DAPI. Scale bar, 10 μ m. Red dashed circles, tubule. White dashed circles, germ cell.

(B) The percentage of VASA positive basal cells is shown as the mean $\pm$ SEM. $n=4$.

(C) Immunohistochemistry staining of FOXO1 in 5 dpp Ctrl and cKO testes. The nuclei were stained with haematoxylin. Scale bar, 10 μ m. Arrowheads, FOXO1 in the nucleus. Arrows, FOXO1 in the cytoplasm. H, homing. AH, abnormal homing.

(D) The percentage of FOXO1 positive cell's nucleus is shown as the mean $\pm$ SEM. $n=4$.

(E) The percentage of FOXO1 positive basal cell's nucleus is shown as the mean $\pm$ SEM. $n=4$.

Unpaired Student's t test determined significance; *** $P < 0.001$, **** $P < 0.0001$. The points and error bars represent the mean $\pm$ SEM.

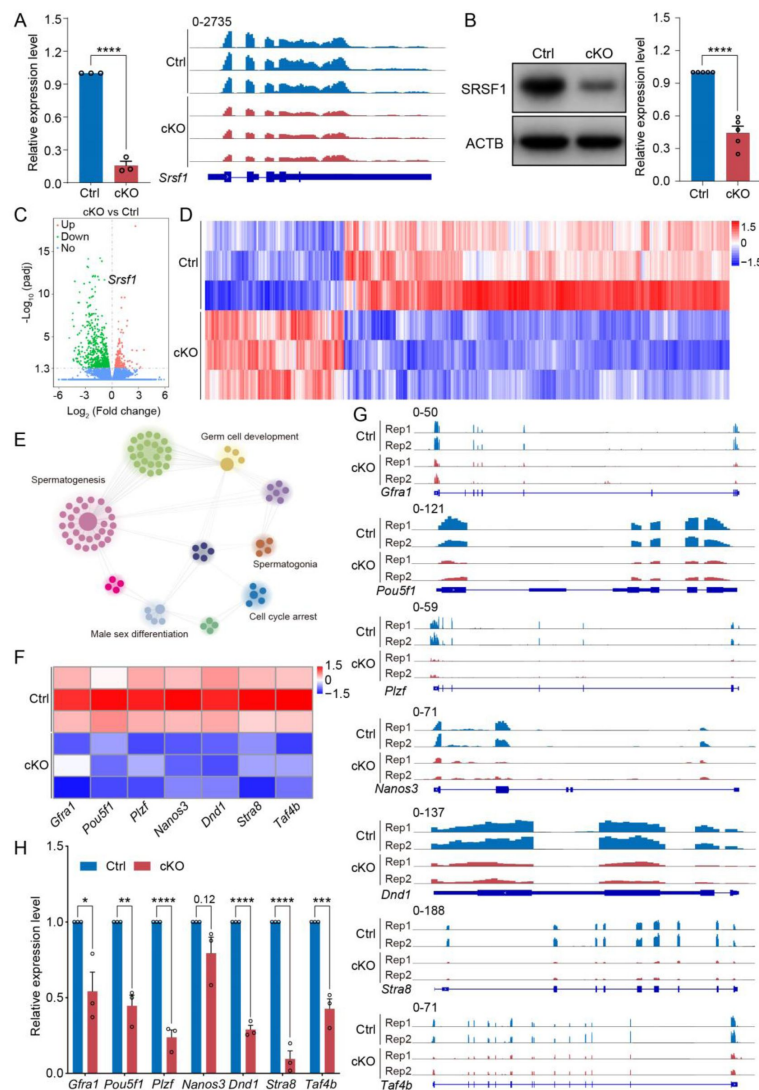


Figure 7

SRSF1 regulates the expression of spermatogonia-related genes.

(A) Expression of *Srsf1* in 5 dpp Ctrl and cKO mouse testes. The RT-qPCR data were normalized to *Gapdh*. $n=5$. The expression of *Srsf1* is shown as reading coverage in 5 dpp mouse testes.

(B) Western blotting of SRSF1 expression in 5 dpp mouse testes. ACTB served as a loading control. The value in Ctrl testes was set as 1.0, and the relative values in cKO testes are indicated. $n=5$.

(C) Volcano map displaying the distribution of differentially expressed genes from RNA-seq data. The abscissa in the figure represents the gene fold change in 5 dpp cKO and Ctrl mouse testes. $|\log_2\text{FoldChange}| \geq 0$. The ordinate indicates the significance of gene expression differences between 5 dpp cKO and Ctrl mouse testes. $\text{padj} \leq 0.05$. Upregulated genes are shown as red dots, and downregulated genes are shown as green dots.

(D) Cluster heatmap of differentially expressed genes. The ordinate is the genotype, and the abscissa is the normalized FPKM (fragments per kilobase million) value of the differentially expressed gene. Red indicates a higher expression level, while blue indicates a lower expression level.

(E) Network showing GO enrichment analyses of differentially expressed genes.

(F) Heatmap of spermatogonia-related gene expression.

(G) The expression of spermatogonia-related genes is shown as read coverage.

(H) The expression of spermatogonia-related genes in 5 dpp cKO and Ctrl mouse testes. The RT-qPCR data were normalized to *Gapdh*. The value in the Ctrl group was set as 1.0, and the relative value in the cKO group is indicated. $n=3$. Unpaired Student's *t* test determined significance; exact P value $P \geq 0.05$, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$. The points and error bars represent the mean $\pm$ SEM.

715 down-regulated genes were bound by SRSF1 and underwent abnormal AS (**Figure 8A**). And one out of 258 upregulated genes was bound by SRSF1 and underwent abnormal AS (**Figure 8A**). Interestingly, we found that 39 stabilized genes were bound by SRSF1 and underwent abnormal AS (**Figure 8A**). The AS genes were subsequently investigated in 5 dpp cKO mouse testes using transcriptomic analyses. RNA-seq analyses showed that 162 AS events were significantly affected (FDR<0.05) in cKO mouse testes (**Figure 8B**, **8C** and Table S3). Most of the 133 affected AS events (162) were classified as skipped exons (SEs), with ten AS events categorized as retained introns (RIs), thirteen as mutually exclusive exons (MXEs), four as alternative 5' splice sites (A5SSs), and two as alternative 3' splice sites (A3SSs) (**Figure 8C**). Additionally, the overall analysis of aberrant AS events showed that SRSF1 effectively promotes the occurrence of SE and MXE events and inhibits the occurrence of RI events. (**Figure 8C**). Then, GO enrichment analyses of AS genes revealed that four genes concerning germ cell development were altered in AS forms (**Figure 8D**). It has been shown that *Tial1/Tiar* affects the survival of primordial germ cells (Beck et al., 1998). Moreover, *Tial1/Tiar* is one of 39 stabilizing genes that are bound by SRSF1 and undergo abnormal AS. Thus, multiomics analyses suggested that *Tial1/Tiar* were posttranscriptionally regulated by SRSF1. Next, we investigated the mechanism by which SRSF1 regulates the AS of *Tial1/Tiar*, RT-PCR results showed that the pre-mRNA of *Tial1/Tiar* in 5 dpp cKO mouse testes exhibited abnormal AS (**Figure 8E**). We then visualized the different types of AS based on RNA-seq data by using IGV (**Figure 8F**). The results of RIP-qPCR showed that SRSF1 could bind to the pre-mRNA of *Tial1/Tiar* (**Figure 8G**). Interestingly, RNA-seq analyses showed that the FPKM of *Tial1/Tiar* was stabilized in 5 dpp cKO mouse testes (**Figure 8H**). RT-qPCR results showed that *Tial1/Tiar* transcript levels were not inhibited (**Figure 8I**). However, Western blotting showed that expression levels of TIAL1/TIAR isoform X2 were significantly suppressed (**Figure 8J**). In summary, the data indicate that SRSF1 is required for TIAL1/TIAR expression and splicing in spermatogonia survival.

SRSF1 recruits AS-related proteins to modulate AS in testes

To identify the interacting proteins for which SRSF1 exerts its AS role, we performed MS analyses of IP samples from 5 dpp mouse testis extracts. The silver-stained gel of SRSF1 and normal IgG showed several SRSF1-interacting proteins from 5 dpp mouse testis extracts (**Figure 9A**). The IP results indicated that SRSF1 was able to effectively IP the testis extracts of 5 dpp mice (**Figure 9B**). IP-MS data demonstrated the efficient enrichment of SRSF1 (**Figure 9C** and Table S4). These data showed that the two samples were highly reproducible, especially for SRSF1 (**Figure 9D**). Then, GO enrichment analyses of the IP proteins revealed that AS-related proteins could interact with SRSF1 (**Figure 8E**). A circular heatmap showed that SRSF1 could interact with AS-related proteins (e.g., SRSF10, SART1, RBM15, SRRM2, SF3B6, and SF3A2) (**Figure 9F**). The Co-IP results indicated that FLAG-SRSF1 interacted with HA-SART1, HA-RBM15 and HA-SRSF10 in 293T cells (**Figure 9G**). In addition, the fluorescence results showed complete co-localization of mCherry-SRSF1 with eGFP-SART1, eGFP-RBM15 and eGFP-SRSF10 in 293T cells (**Figure 9H**). Co-IP suggested that the RRM1 domain of SRSF1 interacted with both HA-TRA2B and HA-U2AF2 in 293T cells (**Figure 9I**). Determining the complex structures of these interactions is valuable, in which molecular docking has played an important role (Yan et al., 2017). HDOCK is a novel web server of our hybrid docking algorithm of template-based modelling and free docking (Yan et al., 2017). The HDOCK analysis results depicted the RRM1 domain of SRSF1 with SRSF10, SART1, and RBM15 docking based on a hybrid strategy (**Figure 9J**). Together, the above data show that SRSF1 may interact with SRSF10, SART1, and RBM15 to regulate AS in 5 dpp mouse testes.

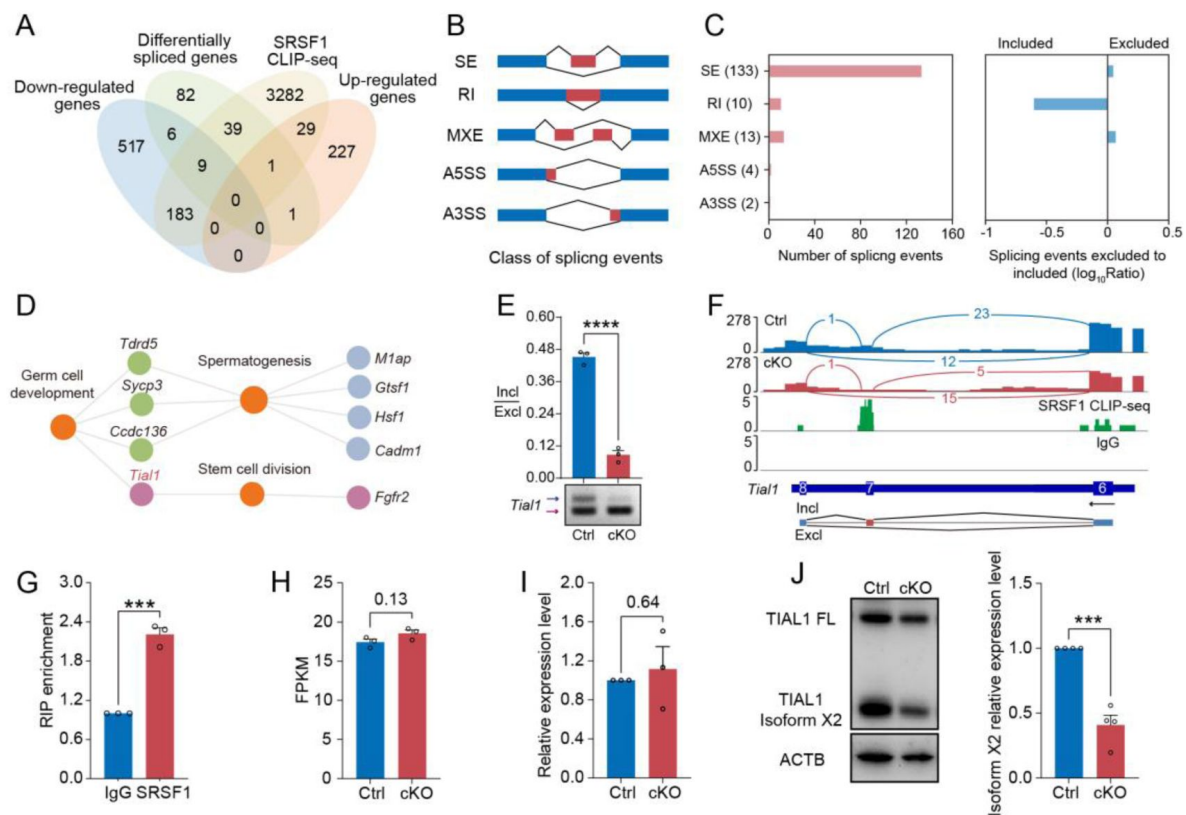


Figure 8

SRSF1 directly binds and regulates the expression and AS of *Tial1/Tiar*.

(A) Venn diagram showing the correlation among down-regulated, up-regulated, alternatively spliced, and SRSF1-binding genes.

(B) Schematic diagram showing the classes of splicing events.

(C) Splicing events were analysed by number, exclusion, and inclusion.

(D) Network showing GO enrichment analyses of AS genes.

(E) The ectopic splicing of *Tial1/Tiar* in 5 dpp cKO and Ctrl mouse testes was analysed by RT-PCR. $n=3$. The ratio of inclusion (Incl) to exclusion (Excl) is shown accordingly.

(F) Analyses of *Tial1/Tiar* expression and exon-exon junctions were performed.

(G) SRSF1 directly binds the pre-mRNA of *Tial1/Tiar* by RIP-qPCR in 5 dpp mouse testes. $n=3$.

(H) The FPKM of *Tial1/Tiar* in 5 dpp cKO and Ctrl mouse testes.

(I) The expression of *Tial1/Tiar* in 5 dpp cKO and Ctrl mouse testes. The RT-qPCR data were normalized to *Gapdh*. The value in the Ctrl group was set as 1.0, and the relative value in the cKO group is indicated. $n=3$.

(J) Western blotting of TIAL1/TIAR expression in 5 dpp mouse testes. ACTB served as a loading control. The isoform X2/FL value of TIAL1/TIAR in the Ctrl group was set as 1.0, and the relative value in the cKO group is indicated. $n=3$. FL, Full length. Unpaired Student's *t* test determined significance; exact P value $P \geq 0.05$, $***P < 0.001$, $****P < 0.0001$. The points and error bars represent the mean $\pm$ SEM.

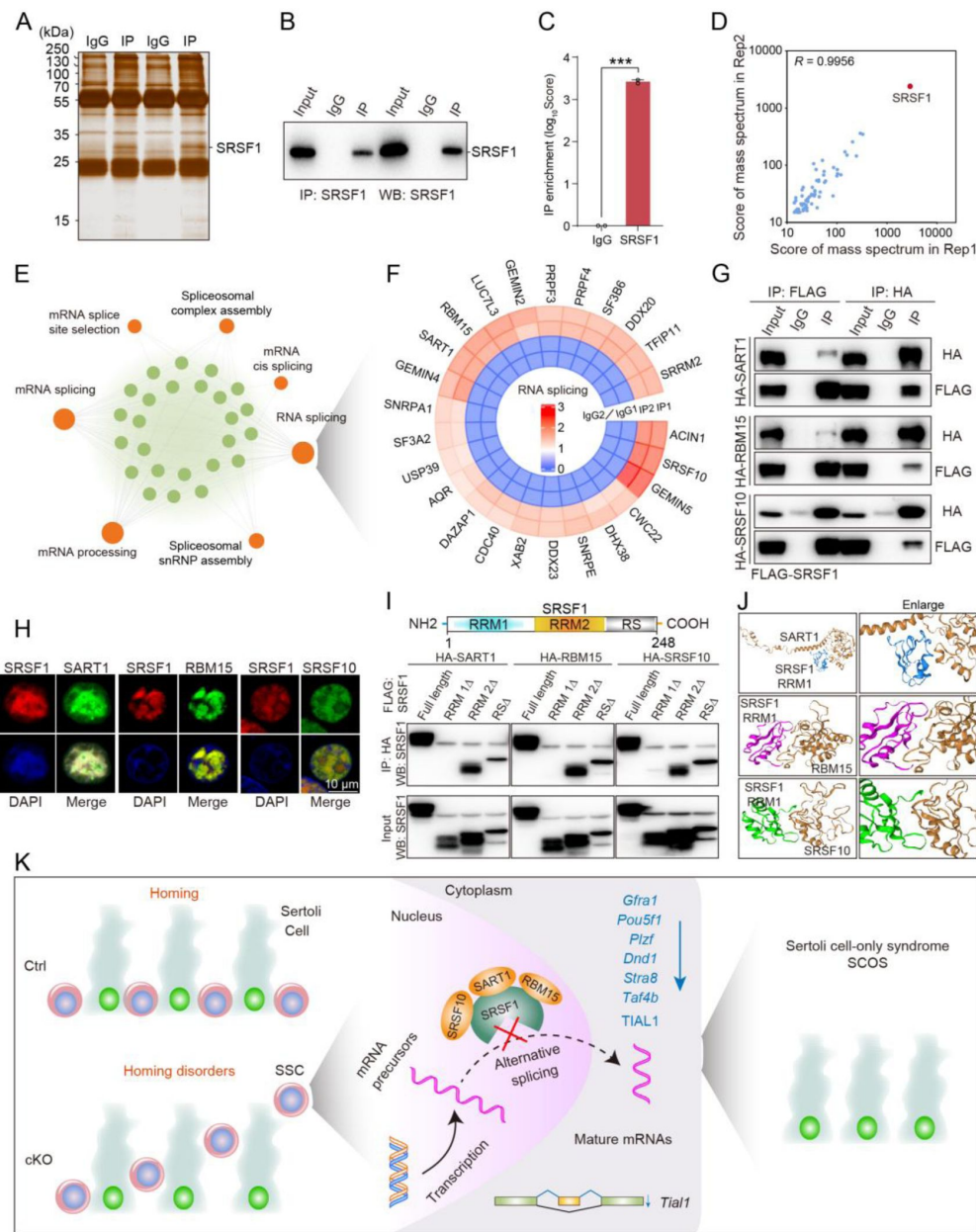


Figure 9

SRSF1 recruits AS-related proteins to modulate AS in testes.

- (A) Silver-stained gel of SRSF1 and control immunoprecipitates from 5 dpp mouse testis extracts.
- (B) IP experiment was performed in 5 dpp mouse testis extracts.
- (C) IP of SRSF1 from IP-MS data.
- (D) Pearson's correlation analysis showed the coefficient between the two samples for IP-MS data.
- (E) Network showing GO enrichment analyses of SRSF1-binding proteins.
- (F) Circular heatmap of AS-related proteins.
- (G) Co-IP experiment for detecting the SRSF1 association between SART1, RBM15, and SRSF10 in 293T cells.
- (H) SRSF1-mCherry cotransfected with SART1-eGFP, RBM15-eGFP, and SRSF10-eGFP 293T cells is shown. DNA was stained with DAPI. Scale bar, 10 μ m.
- (I) Co-IP experiment for detecting the SRSF1 truncated protein association between SART1, RBM15, and SRSF10 in 293T cells.
- (J) A schematic diagram of protein interactions is shown.
- (K) Schematic illustration of the molecular mechanisms by which SRSF1 regulates homing of mouse precursor SSCs.

Failure of spermatogonia survival led to SCOS

Disturbed spermatogenesis can cause SCOS and ultimately male sterility (Jiao et al., 2021 [↗](#)). In recent years, it has been reported that many spermatogonia-related gene deletions have disrupted SSC self-renewal and differentiation in patient and mouse models (La and Hobbs, 2019a [↗](#); Tan and Wilkinson, 2020 [↗](#); Wang et al., 2021 [↗](#)). SCOS was observed in *Ddx5*, *Tial1/Tiar*, *Uhrf1*, *Pramef12*, *Dot1l*, and *Rad51* deletion mouse models (Beck et al., 1998 [↗](#); Legrand et al., 2019 [↗](#); Lin et al., 2022 [↗](#); Qin et al., 2022 [↗](#); Wang et al., 2019 [↗](#); Zhou et al., 2022 [↗](#)). Mouse models are still of great significance and reference for human SCOS studies, and they will provide a better understanding of how SCOS occurs and develops over time. Interestingly, our mouse model had SCOS (Figure 3D-I [↗](#) and Figure 4 [↗](#)). The absence of germ cells represents classical SCOS in adult mouse testes (Figure 4 [↗](#)) (Wang et al., 2023 [↗](#)). In addition, we found abnormal expression of spermatogonia-related genes (*Gfra1*, *Pou5f1*, *Plzf*, *Dnd1*, *Stra8*, and *Taf4b*) in cKO mouse testes (Figure 7F-H [↗](#)). These differentially expressed genes regulate SSC self-renewal and differentiation in mouse testes (Kanatsu-Shinohara and Shinohara, 2013 [↗](#); La and Hobbs, 2019a [↗](#); Tan and Wilkinson, 2020 [↗](#)). Thus, this provided an opportunity for us to better study the underlying molecular mechanisms. These data indicate that SRSF1 deficiency impairs spermatogonia survival, leading to SCOS in male mice.

The formation of SSC pools and the establishment of niches are essential for spermatogenesis

The earliest event in the development of the SSC population is the migration of prospermatogonia from the centre of seminiferous cords where they have resided since sex determination of the embryonic gonad to the basement membrane (Oatley and Brinster, 2012 [↗](#)). In mice, this process is also known as homing of precursor SSCs, which occurs in the first 3 dpp and then develops into SSCs at 4 to 6 dpp for continuous self-renewal and differentiation (Lee and Shinohara, 2011 [↗](#); McLean et al., 2003 [↗](#); Oatley and Brinster, 2012 [↗](#); Tan and Wilkinson, 2020 [↗](#)). Therefore, homing analysis of precursor SSCs was performed in 5 dpp cKO mouse testes. Interestingly, the VASA and SOX9 co-staining results demonstrated that partial germ cells could not complete homing in 5 dpp cKO testes (Figure 6A [↗](#) and 6B [↗](#)). Further, immunohistochemical staining for FOXO1 and statistical results indicated that germ cells in which FOXO1 is expressed in the nucleus similarly undergo abnormal homing (Figure 6C-E [↗](#)). Germ cells that do not migrate to the basement membrane are unable to form SSC pools and establish niches (McLean et al., 2003 [↗](#)). These SSCs that lose their ecological niche will cease to exist. In our data, TUNEL results showed that apoptosis significantly increased in 7 dpp cKO mouse testes. At once, the germ cell count per tube showed a significant reduction in 7 dpp and 14 dpp cKO testes, especially 14 dpp cKO testes (Figure 5B [↗](#)). In conclusion, SRSF1 is crucial for the formation of SSC pools and the establishment of niches through homing of precursor SSCs.

Abnormal AS of *Tial1/Tiar* impaired the survival of spermatogonia

AS is commonly found in mammals, especially in the brain and testes (Mazin et al., 2021 [↗](#); Merkin et al., 2012 [↗](#); Wang et al., 2008 [↗](#)). AS plays essential roles in the posttranscriptional regulation of gene expression during many developmental processes, such as SSC self-renewal and differentiation (Chen et al., 2018 [↗](#); Song et al., 2020 [↗](#)). Recently, BUD31-mediated AS of *Cdk2* was shown to be required for SSC self-renewal and differentiation (Qin et al., 2023 [↗](#)). *Srsf10* depletion disturbed the AS of genes, including *Nasp*, *Bclaf1*, *Rif1*, *Dazl*, *Kit*, *Ret*, and *Sycp1* (Liu et al., 2022 [↗](#)). UHRF1 interacts with snRNAs and regulates AS of *Tle3* in mouse SSCs (Zhou et al., 2022 [↗](#)). *Mettl3*-mediated m6A regulates AS of *Sohlh1* and *Dazl* (Xu et al., 2017 [↗](#)). We found that SRSF1 acts as an alternative RNA splicing regulator and directly interacts with *Tial1/Tiar* transcripts to regulate splicing events in spermatogonia (Figure 8E-G [↗](#)). Additionally, expression levels of TIAL1/TIAR isoform X2 were significantly suppressed (Figure 8J [↗](#)). Interestingly, *Tial1/Tiar* transcript levels

were not inhibited (**Figure 8H** and **8I**). These results suggested that SRSF1 explicitly regulates the expression of *Tial1/Tiar* via AS. Studies have reported that TIAL1/TIAR is essential for primordial germ cell development in mouse testes (Beck *et al.*, 1998). *Tial1/Tiar* deletion impairs spermatogonia survival leading to SCOS, consistent with our phenotype (**Figure 3E-I** and **4A-C**) (Beck *et al.*, 1998). Taken together, SRSF1 may affect spermatogonia survival by directly binding and regulating *Tial1/Tiar* expression through AS.

We found that SRSF1 could interact with AS-related proteins (e.g., SRSF10, SART1, RBM15, SRRM2, SF3B6, and SF3A2) (**Figure 9F**). A recent study reported that SRSF10 deficiency impaired spermatogonia differentiation but did not affect homing of precursor SSCs (Liu *et al.*, 2022). However, our data showed that SRSF1 is essential for homing of mouse precursor SSCs. Therefore, this suggests that SRSF1 has a specific function in the homing of precursor SSCs that are not bound by SRSF10.

SRSF1-mediated posttranscriptional regulation during homing of precursor SSCs provides new insights into the treatment of human reproductive diseases

Aberrant homing of precursor SSCs often lead to gametogenic failure or produce aneuploid gametes, resulting in subfertility or infertility, miscarriage, or congenital disabilities (Jiao *et al.*, 2021; Kanatsu-Shinohara and Shinohara, 2013; La and Hobbs, 2019a; Song and Wilkinson, 2014). Loss-of-function mutations in humans and corresponding knockout/mutated mice have been extensively researched (Jiao *et al.*, 2021). However, AS-related posttranscriptional regulation during meiosis has not been well studied. In recent years, there have been reports that the RNA-binding proteins SRSF10, UHRF1, BUD31, and BCAS2 regulate AS in mouse SSCs (Liu *et al.*, 2022; Liu *et al.*, 2017; Qin *et al.*, 2023; Zhou *et al.*, 2022). This study used a multiomics approach to perform in-depth analyses of SRSF1-mediated posttranscriptional regulatory mechanisms to enrich the field. It also provides new ideas and insights for clinical diagnosis and treatment.

In summary, this study demonstrates that SRSF1 plays a critical role in posttranscriptional regulation to implement homing of precursor SSCs (**Figure 9K**). Thus, the posttranscriptional regulation of SRSF1-mediated splicing is resolved during the formation of SSC pools and the establishment of niches.

Materials and Methods

Mouse strains

C57BL/6N and ICR mice were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. *Srsf1*^{F1/F1} mice were generated in the laboratory of Prof. Xiangdong Fu (University of California, San Diego, USA) and were kindly provided by Prof. Yuanchao Xue (Institute of Biophysics, Chinese Academy of Sciences, Beijing, China) (Xu *et al.*, 2005). *Vasa*-Cre mice were obtained from The Jackson Laboratory (Gallardo *et al.*, 2007). To generate *Srsf1* cKO mice, *Vasa*-Cre mice were crossed with *Srsf1*^{F1/F1} mice. The primers used for PCR to genotype *Srsf1*^{F1/F1} and *Vasa*-Cre mice are shown in Table S5. All mice were bred and housed under specific pathogen-free conditions with a controlled temperature (22 ± 1°C) and exposed to a constant 12-hour light-dark cycle in the animal facilities of China Agricultural University. All experiments were conducted according to the guidelines and with the approval of the Institutional Animal Care and Use Committee of China Agricultural University (No. AW80401202-3-3).

Fertility test

For 15 days, two 8-week-old ICR female mice were caged with one 8-week-old male control (Ctrl) or cKO mouse. The mice were kept individually after the appearance of the vaginal plug, and the dates were recorded. Male mice continue to be caged after two days. The number of pups from each female was recorded each day, and the date of parturition was recorded.

Immunostaining and histological analyses

Mouse testes were fixed with 4% paraformaldehyde (PFA, P6148-500G, Sigma–Aldrich) in PBS (pH 7.4) at 4°C overnight, dehydrated in graded ethanol solutions, vitrified with xylene, and embedded in paraffin. Testis sections were cut at a 5- μ m thickness for immunostaining and histologic analyses. For histological analyses, sections were dewaxed in xylene, rehydrated in a graded ethanol solution, and stained with haematoxylin-eosin. After sealing the slides with neutral resin, a Ventana DP200 system was used for imaging. For immunofluorescence analyses, antigen retrieval was performed by microwaving the sections with sodium citrate buffer (pH 6.0). After blocking with 10% normal goat serum at room temperature for 1 hour, the sections were incubated with primary antibodies (Table S6) in 5% normal goat serum overnight at 4°C. After washing with PBS, the sections were incubated with secondary antibodies (Table S6) at room temperature in the dark for 1 hour. The slides were mounted in an antifade mounting medium with DAPI (P0131, Beyotime). Photographs were taken with a Nikon A1 laser scanning confocal microscope and a Zeiss OPTOME fluorescence microscope. For immunohistochemistry analyses, antigen retrieval was performed by microwaving the sections with sodium citrate buffer (pH 6.0). 5 dpp testis sections were prepared as described in the instructions for Immunohistochemistry Kit (PV-9001, ZSGB-BIO). After sealing the slides with neutral resin, a Ventana DP200 system was used for imaging.

Whole-mount immunostaining

The testes were collected and dispersed with 5ml syringes. Blown-out tubules were fixed in PFA at 4°C for 4 hours. The tubules were washed three times with PBS (pH 7.4) for 5 min each and stored at 4°C. The tubules were permeated with 0.3% Triton X-100 for 1 h at 4°C. Then, whole-mount staining followed the immunostaining protocol.

TUNEL apoptosis analyses

7 dpp testis sections were prepared as described in the instructions for the TUNEL Apoptosis Assay Kit (C1088, Beyotime). Photographs were taken with a Nikon A1 laser scanning confocal microscope and a Zeiss OPTOME fluorescence microscope.

RT-PCR and RT-qPCR

Total RNA was extracted by using RNAiso Plus (9109, Takara), and the concentration was measured with a Nano-300 ultramicro spectrophotometer (Allsheng). cDNA was obtained according to the instructions of a TIANScript II RT kit (KR107, TIANGEN). The expression of transcripts of the target gene was measured by using a LightCycle® 96 instrument (Roche) with Hieff UNICON SYBR green master mix (11198ES08, Yeasen). AS analyses were performed on a RePure-A PCR instrument (BIO-GENER). Primers were synthesized by Sangon Biotech (Table S5). The expression level of *Gapdh* or *Actb* was used as the control, and this value was set as 1. Other samples' relative transcript expression levels were obtained by comparing them with the control results.

RNA-seq

Total RNA was extracted from mouse testes according to the above protocol at 5 dpp. Briefly, mRNA was purified from total RNA using poly-T oligo-attached magnetic beads. After fragmentation, we established a transcriptome sequencing library and assessed library quality on

an Agilent Bioanalyzer 2100 system. The clustering of the index-coded samples was performed on a cBot Cluster Generation System using a TruSeq PE Cluster kit v3-cBot-HS (Illumina) according to the manufacturer's instructions. After cluster generation, the library preparations were sequenced on the Illumina NovaSeq platform, and 150 bp paired-end reads were generated. After quality control, all downstream analyses were performed on clean, high-quality data. The reference genome index was built, and paired-end clean reads were aligned to the reference genome using HISAT2 software (version 2.0.5). FeatureCounts (version 1.5.0) counted the reads mapped to each gene. Then, the fragments per kilobase million (FPKM) value of each gene was calculated based on the length of the gene and the read count mapped to this gene. Differential expression analyses of cKO/Ctrl mouse testes (three biological replicates per condition) were performed using the DESeq2 R package (version 1.20.0). Genes with a $\text{padj} \leq 0.05$ identified by DESeq2 were considered differentially expressed.

AS analyses

rMATS software (version 3.2.5) was used to analyse the AS events in cKO mouse germ cells based on RNA-seq data. Five types of AS events (SE, RI, MXE, A5SS, and A3SS) were revealed by rMATS software. Our threshold for screening differentially significant AS events was a false discovery rate (FDR) of less than 0.05. Splicing events with an FDR less than 0.05 and an inclusion-level difference with a significance of at least 5% (0.05) were considered statistically significant. Integrative Genomics Viewer (IGV, 2.16.0) software was used to visualize and confirm AS events based on RNA-seq data.

Gene Ontology (GO) enrichment analyses

The GO enrichment analyses of differentially expressed genes and AS genes were implemented with the clusterProfiler R package (version 3.4.4), in which gene length bias was corrected. All expressed genes (TPM sum of all samples ≥ 1) are listed background. GRCm38/mm10 was used as a mouse reference genome, and the Benjamini–Hochberg multiple methods was applied to adjust for multiple testing. GO enrichment analyses with corrected P values of less than 0.05 were significantly enriched for differentially expressed genes and AS genes.

Western blotting

Total protein was extracted with cell lysis buffer (P0013, Beyotime) containing PMSF (1:100, ST506, Beyotime) and a protease inhibitor cocktail (1:100, P1005, Beyotime). A BCA protein assay kit (CW0014S, CWBiotech) measured the protein concentration. The protein lysates were electrophoretically separated on sodium dodecyl sulfate-polyacrylamide gels and electrically transferred to polyvinylidene fluoride membranes (IPVH00010, Millipore). The membranes were blocked in 5% skimmed milk for 1 hour and incubated with the primary antibodies (Table S6) for one night at 4°C. Then, the membranes were incubated with secondary antibodies (Table S6) at room temperature for 1 hour. The proteins were visualized using a Tanon 5200 chemiluminescence imaging system following incubation with BeyoECL Plus (P0018S, Beyotime).

IP, IP-MS, Co-IP

Total protein was extracted with cell lysis buffer (P0013, Beyotime) containing PMSF (1:100, ST506, Beyotime) and a protease inhibitor cocktail (1:100, P1005, Beyotime). After incubation on ice for 20 min, the lysate was added to 20 μl of protein A agarose (P2051-2 ml, Beyotime) for pre-clearing at 4°C for 1 hour. Two micrograms of an SRSF1 antibody (sc-33652, Santa Cruz Biotechnology) and a normal mouse IgG (sc-3879, Santa Cruz Biotechnology) were added to the lysate the mixture was incubated overnight incubation at 4°C. The next day, 60 μl of protein A agarose was added to the lysate, which was then incubated at 4°C for 4 hours. The agarose complexes containing antibodies and target proteins were washed 3 times for 5 min at 4°C. IP and Co-IP were performed according to the above Western blotting protocol. The complex was sent to the protein mass spectrometry laboratory for IP-MS analyses using a Thermo Q-Exactive high-resolution mass spectrometer

(Thermo Scientific, Waltham). Raw data from the mass spectrometer were preprocessed with Mascot Distiller 2.4 for peak picking. The resulting peak lists were searched against the uniprot mouse database using Mascot 2.5 search engine.

RNA immunoprecipitation (RIP) and RIP-qPCR

As described previously (Gagliardi and Matarazzo, 2016 [↗](#)), RIP was performed using 5 dpp mouse testes. The testes were lysed in cell lysis buffer (P0013, Beyotime) containing PMSF (1:100, ST506, Beyotime), a proteinase inhibitor cocktail (1:100, P1005, Beyotime), DTT (1:50, ST041-2 ml, Beyotime), and an RNase inhibitor (1:20, R0102-10 kU, Beyotime). After incubation on ice for 20 min, the lysate was added to 20 μ l of protein A agarose (P2051-2 ml, Beyotime) for pre-clearing at 4°C for 1 hour. Two micrograms of an SRSF1 antibody (sc-33652, Santa Cruz Biotechnology) and a normal mouse IgG (sc-3879, Santa Cruz Biotechnology) were added to the lysate, which was then incubated overnight at 4°C. The next day, 60 μ l of protein A agarose was added to the lysate, and the mixture was incubated at 4°C for 4 hours. The agarose complexes containing antibodies, target proteins, and RNA were washed 3 times for 5 min at 4°C and repeated. Protein-bound RNA was extracted using RNAiso Plus and a Direct-zol RNA MicroPrep Kit. RIP-qPCR was performed according to the above RT-qPCR protocol.

Statistical analyses

Pearson's correlation coefficients (R) were calculated by using the scores of the two samples for MS. GraphPad Prism software (version 9.0.0) was used for the statistical analyses, and the values and error bars represent the mean $\pm$ SEM. Significant differences between the two groups were analysed using Student's *t* test. Statistical significance is indicated as follows: exact *P* value $P \geq 0.05$; **P* < 0.05; ***P* < 0.01; ****P* < 0.001; *****P* < 0.0001).

Data availability

All data generated or analysed during this study are included in this published article, its supplementary information files and publicly available repositories. Mass spec data are included in supplementary information files (Table S7). The RNA-seq data were deposited in GEO (<https://www.ncbi.nlm.nih.gov/geo/> [↗](#)) under accession number GSE227575. The CLIP-seq data were deposited in GEO under accession number GSE227422 (Sun *et al.*, 2023a [↗](#)).

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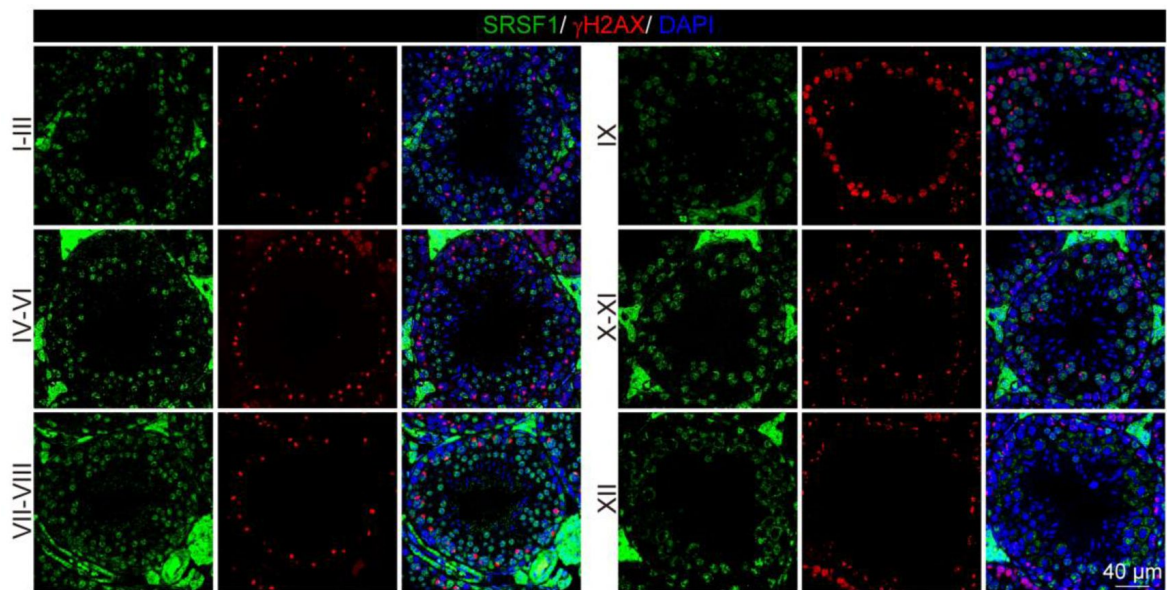


Figure S1.

Dynamic localization of SRSF1 during spermatogenesis.

Co-immunostaining was performed using SRSF1 and γH2AX antibodies in adult mouse testes. DNA was stained with DAPI. Scale bar, 40 μm.

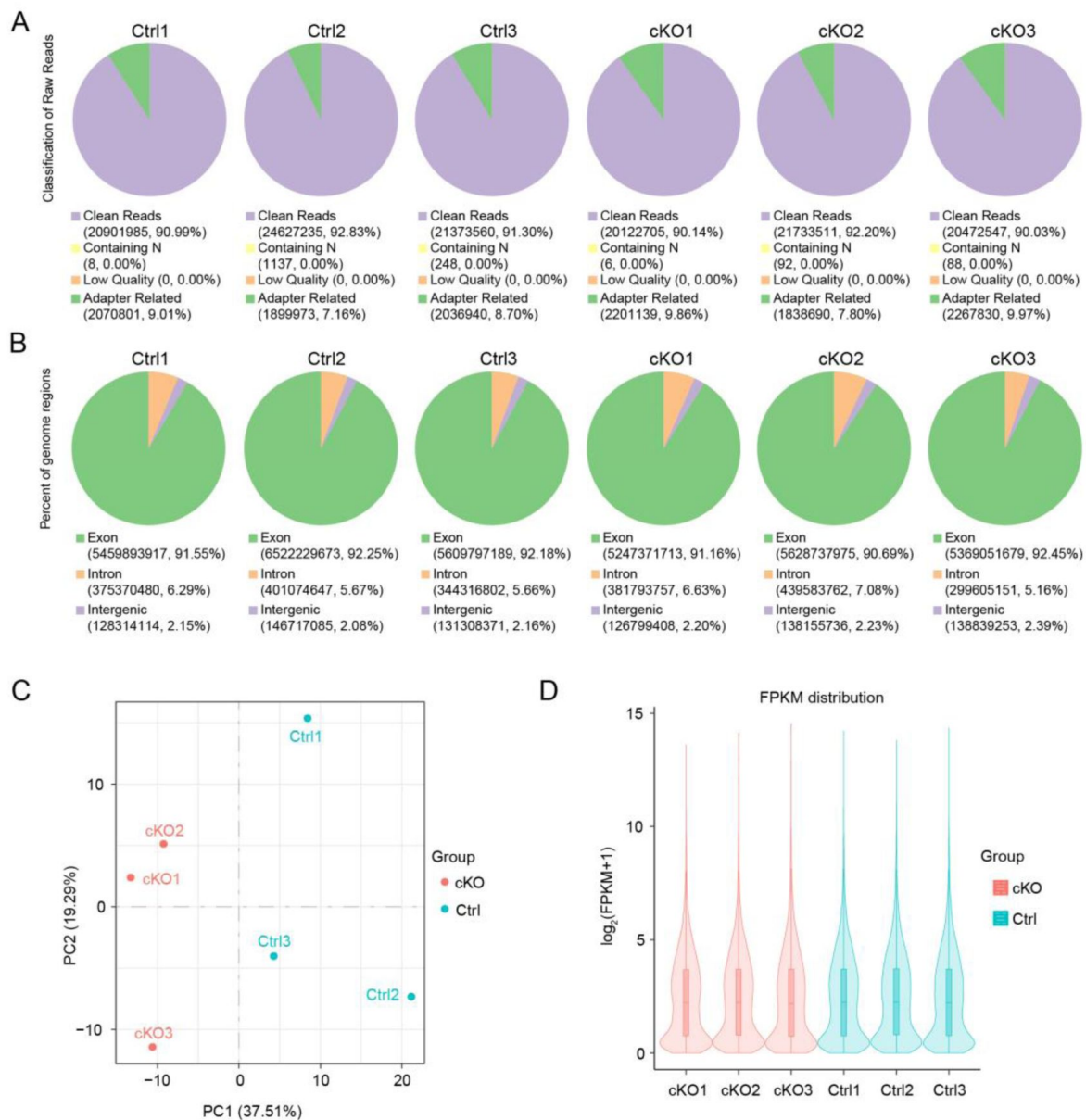


Figure S2.

The libraries of RNA-seq are of good quality.

(A) Filtering of sequencing data per sample.

(B) Distribution of sequenced reads across genomic regions for all samples.

(C) Gene expression values (FPKM) of all samples were subjected to principal component analysis (PCA).

(D) The distribution of gene expression levels of different samples is demonstrated by violin plots as shown below.

The horizontal coordinate of the graph is the name of the sample, and the vertical coordinate is $\log_2(\text{FPKM}+1)$.

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Editors

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Joint Public Review:**Summary:**

In this study, the authors seek to characterize the role of splicing factor SRSF1. Using a conditional deletion of *Srsf1* in germ cells, they find that SRSF1 is required for male fertility. Via immunostaining and RNA-seq analysis of the *Srsf1* conditional knockout (cKO) testes, combined with SRSF1 CLIP-seq and IP-MS data from the testis, they conclude that *Srsf1* is required for homing of precursor spermatogonial stem cells (SCCs) due to alternative splicing of *Tial1*. They further show that spermatogonia-related genes (*Plzf*, *Id4*, *Setdb1*, *Stra8*, *Tial1/Tiar*, *Bcas2*, *Ddx5*, *Srsf10*, *Uhrf1*, and *Bud31*) were bound by SRSF1 in the mouse testes by CLIP-seq. They show that SRSF1 coordinates with other RNA splicing-related proteins to directly bind and regulate the expression of several spermatogonia-related genes, including *Tial1/Tiar*, via alternative splicing. Ultimately, the study shows that SRSF1's effects on alternative splicing are required to establish spermatogenesis. In the absence of *Srsf1*, the postnatal gonocytes do not properly mature into spermatogonia and consequently never initiate spermatogenesis.

Strengths:

This study shows a role of SRSF1-mediated alternative splicing in establishment and survival of precursor SCCs, which may provide a framework to elucidate the molecular mechanisms of

the posttranscriptional network underlying the formation of SSC pools. The histological analysis of the Srsf1 cKO traces the origins of the fertility defect to the postnatal testis, and the authors have generated interesting CLIP-seq, IP-MS, and RNA-seq datasets characterizing SRSF1's RNA targets and interacting proteins specifically in the testis. Together, this study provides detailed phenotyping of the Srsf1 cKO, which convincingly supports the Sertoli Cell Only phenotype, establishes the timing of the first appearance of the spermatogonial defect, and provides new insight into the role of splicing factors and SRSF1 specifically in spermatogenesis. The experiments are well-designed and conducted, the overall methods and results are robust and convincing.

Weaknesses:

This study does not provide a full mechanistic explanation connecting altered splicing with defects in SSC precursors. The claim that altered splicing of the Tial1 transcript mediates the effect of SRSF1 loss is not convincingly supported. In addition, some regions of the text suggest that misregulated splicing of Tial1 disrupts spermatogonial survival; while Tial1 is required for primordial germ cell survival in embryonic gonads (E11.5-13.5; Beck et al 1998), it is unclear if Tial1 is required for germline development beyond this embryonic stage.

Author Response

The following is the authors' response to the original reviews.

Reviewer #1 (Recommendations For The Authors):

The conclusions of this paper are mostly well supported by data, but some aspects need to be corrected.

1. Line 99. The title is not suitable for summarizing this part of the results. In this paragraph, the results mainly describe SRSF1 expression pattern and binding of spermatogonia-associated gene's transcripts in testes. There is no functional assay to conclude SRSF1 has an essential role in mouse testes. The data only indicate that SRSF1 may have a vital role in posttranscriptional regulation in the testes.

Thank you for the professional suggestions. Following this advice, we have corrected the text in this revised version (Page 4, Line 98 and 112).

1. Line 141. In the mating scheme, Vasa-Cre Srsf1^{Fl/del} mice should be obtained instead of Vasa-Cre Srsf1^{Fl/Fl} mice.

Thank you for the professional suggestions. Following this advice, we have corrected the text in this revised version (Page 4, Line 118).

1. Fig 2 C, "PZLF" should be corrected to "PLZF".

Thank you very much for the helpful comments. We have corrected this in Figure 2C.

1. Fig 5 B, "VASA" and "Merge" should be interchanged.

Thank you very much for the helpful comments. We have interchanged "VASA" and "Merge" in Figure 5B.

1. Fig 5 D, "Ctrl" should be added in the up panel.

Thank you very much for the helpful suggestions. We have added "Ctrl" in Figure 5C.

1. *The legend for Figure 6 D should be revised.*

Thank you very much for the helpful suggestions. We have revised the legend for Figure 7D

1. *The legend for Figure 7 G should be revised.*

Thank you very much for the helpful suggestions. We have revised the legend for Figure 8D

1. *Immunoprecipitation mass spectrometry (IP-MS) data showed that t SRSF1 interacts with other RNA splicing-related proteins (e.g., SRSF10, SART1, RBM15, SRRM2, SF3B6, and SF3A2). The authors should verify the interactions in testis or cells.*

We thank the reviewer for the professional comments and suggestions. Following this advice, we performed co-transfection and co-IP to verify the protein-protein interactions in 293T cells, the results showed that the RRM1 domain of SRSF1 interacted with SART1, RBM15 and SRSF10 in 293T cells. In addition, the fluorescence results showed complete co-localization of mCherry-SRSF1 with eGFP-SART1, eGFP-RBM15 and eGFP-SRSF10 in 293T cells. Therefore, we have incorporated the data into the Figure 9G-J. Meanwhile, these have been incorporated into the text, given descriptions, and highlighted (Page 17, Lines 338-347).

1. *To avoid overstatement, the authors should pay attention to the use of adjectives and adverbs in the article, especially when drawing conclusions about the role of Tail1.*

We thank the reviewer for the professional comments and suggestions. To avoid overstatement, we have revised the entire text (Page 4, Lines 98, and 112; Page 16, Lines 308; Page 17, Lines 346-347; Page 20, Lines 413-414; Page 21, Lines 432-433).

Reviewer #2 (Recommendations For The Authors):

Major

1. *I find the use of "SSC homing" misleading/confusing because this "homing" or relocation of postnatal gonocytes/nascent spermatogonia to the basement membrane precedes the maturation of the nascent spermatogonia into SSCs. In addition, "SSC homing" is commonly used in the SSC transplantation field to describe a transplanted SSC's ability to find and colonize its niche within the seminiferous tubules. I appreciate that "postnatal gonocytes/nascent spermatogonia homing" is not easily grasped by a broader audience. Perhaps "homing of precursor SSCs" is more appropriate.*

Thank you very much for the helpful comments and suggestions. Following this advice, we have corrected the text in this revised version (Line 1-2, 39, 44, 49, 54-55, 68, 70, 72-73, 77, 84, 93-95, 191, 201, 240, 384-387, 397, 417-422, and 433)

1. *If I am misunderstanding the description of the Srsf1 cKO phenotype, and the authors truly believe SSCs have formed in the Srsf1 cKO testis, I strongly recommend immunostaining to show that the cKO germ cells robustly express SSC markers, not just markers of undifferentiated spermatogonia.*

We thank the reviewer for the professional suggestions. We fully agree with the reviewer. Immunohistochemical staining for FOXO1 and statistical results indicated a reduced number of prospermatogonia (Figure 6C-E). So, we have corrected the text in this revised version (Line 1-2, 39, 44, 49, 54-55, 68, 70, 72-73, 77, 84, 93-95, 191, 201, 240, 384-387, 397, 417-422, and 433).

1. If the authors have the available resources, the significance of this report would be enhanced by additional characterization of the cKO phenotype at the transition from gonocyte to nascent spermatogonia. Do any cKO germ cells exhibit defects in maturing from gonocytes to nascent spermatogonia at the molecular level? I.e., by P5-7, do all cKO germ cells express PLZF and localize FOXO1 to cytoplasm, as expected of nascent spermatogonia? If the cKO germ cells are actually a heterogenous population of gonocytes and nascent spermatogonia, what is the distribution of each subpopulation in the lumen vs basement membrane?

Thank you for the professional suggestions. Following this advice, immunohistochemical staining for FOXO1 was performed on 5 dpp mouse testis sections (Figure 6C). Further, germ cell statistics of FOXO1 expression in the nucleus showed a reduced number of prospermatogonia in cKO mice (Figure 6D). And germ cells in which FOXO1 is expressed in the nucleus similarly undergo abnormal homing (Figure 6E). Thus, all the above data indicated that SRSF1 has an essential role in the homing of precursor SSCs. we have incorporated the data into the Figure 6C-E. Meanwhile, these have been incorporated into the text, given descriptions, and highlighted (Page 9, Lines 191-201; Page 20, Lines 389-391).

Minor

1. Could the authors clarify why Tial1 exon exclusion in the cKO results in reduced protein expression? Is it creating a transcript isoform that undergoes nonsense-mediated decay?

Thank you for the professional suggestions. Following this advice, we analyzed Tial1 transcripts again, and we found that Tial1 exon exclusion resulted in reduced expression of protein isoform X2 (Figure 8J). Since this region is not in the CDS, no clear evidence of nonsense-mediated decay was found in the analysis.

1. Could the authors confirm that the TIAL1 antibody is not detecting the portion of the protein encoded by the alternatively spliced exon?

Thank you for the helpful comments. The TIAL1 monoclonal antibody is produced by Proteintech Group under the product number 66907-1-Ig. Immunogen is TIAL1 fusion protein Ag11981. The sequence is as follows.
MDARVVKDMATGKSKGYGFVSFYNKLDAENAIVHMGQWLGGGRQIRTNWATRKPPAPKSTQENNTKQLRFED
The homology was 99% in mice and all TIAL1 isoforms were detected. So, TIAL1 antibody is detecting the portion of the protein encoded by the alternatively spliced exon.

1. Lines 143: should "cKO" actually be "control"?

Thank you for the helpful suggestions. There is a real problem in the text description. we have corrected the text in this revised version (Page 6, Line 138-139).

1. Lines 272-3 "visual analysis using IGV showed the peak of Tial1/Tiar was stabilized in 5 dpp cKO mouse testes (Figure 7H)": "peak stabilization" is not evident to me from the figure nor do I see Tial1 listed as differentially expressed in the supplemental. I would refrain from using IGV visualization as the basis for the differential abundance of a transcript.

Thank you very much for the helpful comments and suggestions. Tial1/Tiar is one of 39 stabilizing genes that are bound by SRSF1 and undergo abnormal AS. Following this advice, we have substituted Tial1/Tiar's FPKM for his peaks (Figure 8H). Meanwhile, we have corrected the text in this revised version (Page 15, Line 296-300; Page 16, Line 303-304).

1. Lines 468-473: please clarify the background list used for GO enrichment analyses. By default, the genes expressed in the testis are enriched for spermatogenesis-related genes. To control for this and test whether a gene list is enriched for spermatogenesis-related genes beyond what is already seen in the testis, I recommend using a list of all expressed genes (for example, defined by $TPM \geq 1$) as the background list.

We thank the reviewer for the professional comments and suggestions. Following this advice, all expressed genes (TPM sum of all samples ≥ 1) are listed background for GO enrichment analyses. The results of GO enrichment analysis of the AS gene turned out to be the same. The results of GO enrichment analysis of the SRSF1 peak-containing genes, differential genes, and IP proteins-associated genes have corrected in the figure (Figure 2A, 7E, and 9E)

1. Figure 2B: Could the authors mark where the statistically significant peaks appear on the tracks? There are many small peaks and it's unclear if they are significant or not.

Thank you for the helpful suggestions. Following this advice, we have marked the areas of higher peaks in the figure (Figure 2B). We generally believe that any region above the peaks of IgG is likely to be a binding region, and of course, the higher the peak value, the more pre-mRNA is bound by SRSF1 in that region.

1. Figure 7A: I assume the SRSF1 CLIP-seq genes are all the genes from the adult testis experiments. I would suggest limiting the CLIP-seq gene set to only those expressed in the P5 RNA-seq data, as if the target is not expressed at P5, there's no way it will be differentially expressed or differentially spliced in at P5.

Thank you very much for the helpful comments and suggestions. Following this advice, we found that 3543 of the 4824 genes bound by SRSF1 were expressed in testes at 5 dpp. we have corrected in the figure (Figure 8A). these have been incorporated into the text, given descriptions, and highlighted (Page 14, Lines 274-277).

1. Figure 7F: Could the authors clarify where the alternatively spliced exon is relative to the total transcript, shown in 7H?

Thank you for the helpful suggestions. Following this advice, we have labeled the number of exons where variable splicing occurs. (Figure 8F).

1. Please include where the sequencing and mass spec data will be publicly available.

Thank you very much for the helpful comments and suggestions. Following this advice, these have been incorporated into the text, given descriptions, and highlighted (Page 25, Lines 560-

Reviewer #3 (Recommendations For The Authors):

Suggestions for improving the data and analysis

- 1. The claim that TIAL1 mediates SRSF1 effects is not well supported; this claim should be adjusted or additional supporting data should be provided. To support a claim that alternative splicing of Tial1 mediates the effects of SRSF1, at least two additional pieces of data are needed: first, a demonstration that the two alternative protein isoforms have different molecular functions, either in vitro or in vivo; and second, a better quantitation of the levels and ratios of expression of the two different isoforms in vivo.*

Thank you for the helpful comments and suggestions. Following this advice, we quantified the expression levels and ratios of two different isoforms in vivo, and we found that Tial1 exon exclusion resulted in reduced expression of protein isoform X2 (Figure 8J). However, it is not possible to prove that the two alternative protein isoforms have different molecular functions. So, this claim has been adjusted in the text. these have been incorporated into the text, given descriptions, and highlighted (Lines 1-2, 43-45, 95, 306, 323-325, 408, 413-414).

- 1. Likewise, the claim that "SRSF1 is required for "homing and self-renewal" of SSCs should be adjusted or better supported. As of now, the data supports a claim that SRSF1 is required for the establishment of the SSC population in the testis after birth. This could be due to defects in homing, self-renewal, or survival. To support claims about homing and self-renewal, these phenotypes should be tested more directly, for example by quantitating numbers of spermatogonia at the basal membrane in juvenile testes (homing) and expression of SSC markers in addition to the pan-germ cell marker VASA across early postnatal time points.*

Thank you very much for the helpful comments and suggestions. Immunohistochemical staining for FOXO1 was performed on 5 dpp mouse testis sections (Figure 6C). Further, germ cell statistics of FOXO1 expression in the nucleus showed a reduced number of prospermatogonia in cKO mice (Figure 6D). And germ cells in which FOXO1 is expressed in the nucleus similarly undergo abnormal homing (Figure 6E). Thus, all the above data indicated that SRSF1 has an essential role in the homing of precursor SSCs. we have incorporated the data into the Figure 6C-E. These have been incorporated into the text, given descriptions, and highlighted (Page 9, Lines 191-201; Page 20, Lines 387-389). Meanwhile, "homing and self-renewal" of SSCs have corrected the text in this revised version (Line 1-2, 39, 44, 49, 54-55, 68, 70, 72-73, 77, 84, 93-95, 191, 201, 240, 384-387, 397, 417-422, and 433).

- 1. Additional, more detailed analyses of CLIP-seq and RNA-seq data at least showing that the libraries are of good quality should be provided.*

Thank you very much for suggestions. Following this advice, detailed analyses of RNA-seq data have been incorporated the data into the figures (Figure S2). But detailed analyses of CLIP-seq have already been used in another paper (Sun et al., 2023), and we have not provided it in order to avoid multiple uses of one figure. Meanwhile, we made a citation in the article (Page 4, Lines 105; Page 25, Lines 564-565).

- 1. Gene Ontology analyses should be redone with a more appropriate background gene set.*

Thank you for the helpful suggestions. All expressed genes (TPM sum of all samples ≥ 1) are listed background for GO enrichment analyses. The results of GO enrichment analysis of the AS gene turned out to be the same. The results of GO enrichment analysis of the SRSF1 peak-containing genes, differential genes, and IP proteins-associated genes have been corrected in the figure (Figure 2A, 7E, and 9E)

Minor points about the text and figures

1. The species (mouse) should be stated earlier in the Introduction.

Thank you for the professional suggestions. Following this advice, the mouse has been stated earlier in the Introduction (Page 3, Line 65).

1. In Fig. 1C (Western blot), the results would be more convincing if quantitation of band intensities normalized to the loading control was added.

Thank you very much for comments and suggestions. Following this advice, ACTB served as a loading control. The value in 16.5 dpc testes were set as 1.0, and the relative values of testes in other developmental periods are indicated. Therefore, we have incorporated the data into the figures (Figure 1C).

1. In Fig 5D, TUNEL signal in the single-channel image is difficult to see; please adjust the contrast.

Thank you for the professional suggestions. Following this advice, the images of the channels have been replaced by enlarged images for better visibility (Figure 5C).

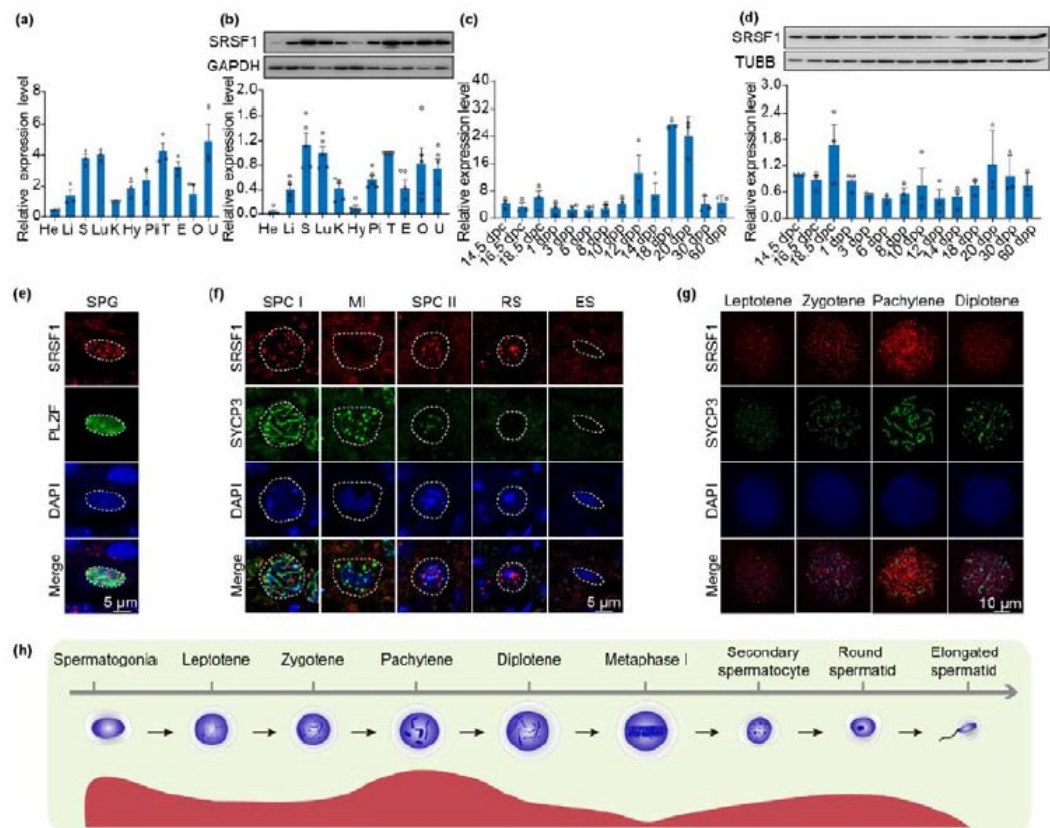
Major comments

1. In Fig 1D, it appears that SRSF1 is expressed most strongly in spermatogonia by immunofluorescence, but this is inconsistent with the sharp rise in expression detected by RT-qPCR at 20 days post partum (dpp) (Fig. 1B), which is when round spermatids are first added; this discrepancy should be explained or addressed.

We appreciate the important comments from the reviewer. In another of our studies, we showed that SRSF1 expression is higher in pachytene spermatocytes and round spermatids (Sun et al., 2023). So, it is normal for the sharp rise in expression detected by RT-qPCR at 20 days post partum (dpp).

Author response image 1.

Dynamic localization of SRSF1 in male mouse germ cells. (Sun et al., 2023)



1. It is important to provide a more comprehensive basic description of the CLIP-seq datasets beyond what is shown in the tracks shown in Fig. 2B. This would allow a better assessment of the data quality and would also provide information about the transcriptome-wide patterns of SRSF1 binding. No information or quality metrics are provided about the libraries, and it is not stated how replicates are handled to maximize the robustness of the analysis. The distribution of peaks across exons, introns, and other genomic elements should also be shown.

Thank you very much for the helpful comments and suggestions. In fact, detailed analyses of CLIP-seq have already been presented in another paper (Sun et al., 2023), and we have not provided it in order to avoid multiple uses of one figure. Meanwhile, we made a citation in the article (Page 4, Lines 105; Page 25, Lines 564-565). In addition, the distribution of peaks in exons, introns, and other genomic elements is shown in Figure 2B.

1. The claim that SRSF1 is required for "homing and self-renewal" of SSCs is made in multiple places in the manuscript. However, neither homing nor self-renewal is ever directly tested. A single image is shown in Fig. 5E of a spermatogonium at 5dpp that does not appropriately sit on the basal membrane, potentially indicating a homing defect, but this is not quantified or followed up. There is good evidence for depletion of spermatogonia starting at 7 dpp, but no further explanation of how homing and/or self-renewal fit into the phenotype.

Thank you very much for the helpful comments and suggestions. Following this advice, immunohistochemical staining for FOXO1 was performed on 5 dpp mouse testis sections (Figure 6C). Further, germ cell statistics of FOXO1 expression in the nucleus showed a

reduced number of prospermatogonia in cKO mice (Figure 6D). And germ cells in which FOXO1 is expressed in the nucleus similarly undergo abnormal homing (Figure 6E). Thus, all the above data indicated that SRSF1 has an essential role in the homing of precursor SSCs. we have incorporated the data into the Figure 6C-E. These have been incorporated into the text, given descriptions, and highlighted (Page 9, Lines 191-201; Page 20, Lines 387-389). Meanwhile, "homing and self-renewal" of SSCs have corrected the text in this revised version (Line 1-2, 39, 44, 49, 54-55, 68, 70, 72-73, 77, 84, 93-95, 191, 201, 240, 384-387, 397, 417-422, and 433).

1. In Fig. 6A (lines 258-260) very few genes downregulated in the cKO are bound by SRSF1 and undergo abnormal splicing. The small handful that falls into this overlap could simply be noise. A much larger fraction of differentially spliced genes are CLIP-seq targets (~33%), which is potentially interesting, but this set of genes is not explored.

Thank you for the helpful comments. Following this advice, this was specifically indicated by the fact that 39 stabilizing genes were bound by SRSF1 and underwent abnormal AS. In our study, Tial1/Tiar is one of 39 stabilizing genes that are bound by SRSF1 and undergo abnormal AS. Therefore, we fully agree with the reviewers' comments. These have been added in this revised version (Page 14, Lines 279-280; Page 15, Lines 296-300).

1. The background gene set for Gene Ontology analyses is not specified. If these were done with the whole transcriptome as background, one would expect enrichment of spermatogenesis genes simply because they are expressed in testes. The more appropriate set of genes to use as background in these analyses is the total set of genes that are expressed in testis.

We thank the reviewer for the professional comments and suggestions. All expressed genes (TPM sum of all samples ≥ 1) are listed background for GO enrichment analyses. The results of GO enrichment analysis of the AS gene turned out to be the same. The results of GO enrichment analysis of the SRSF1 peak-containing genes, differential genes, and IP proteins-associated genes have been corrected in the figure (Figure 2A, 7E, and 9E)

1. In general, the model is over-claimed: aside from interactions by IP-MS, little is demonstrated in this study about how SRSF1 affects alternative splicing in spermatogenesis, or how alternative splicing of TIAL1 specifically would result in the phenotype shown. It is not clear why Tial1/Tiar is selected as a candidate mediator of SRSF1 function from among the nine genes that are downregulated in the cKO, are bound by SRSF1, and undergo abnormal splicing. Although TIAL1 levels are reduced in cKO testes by Western blot (Fig. 7J), this could be due just be due to a depletion of germ cells from whole testis. The reported splicing difference for Tial1 seems very subtle and the ratio of isoforms does not look different in the Western blot image.

Thank you very much for the helpful comments and suggestions. In our study, Tial1/Tiar is one of 39 stabilizing genes that are bound by SRSF1 and undergo abnormal AS. However, Western blotting showed that expression levels of TIAL1/TIAR isoform X2 were significantly suppressed (Figure 8J). So, the data indicate that SRSF1 is required for TIAL1/TIAR expression and splicing.

Sun, L., Chen, J., Ye, R., Lv, Z., Chen, X., Xie, X., Li, Y., Wang, C., Lv, P., Yan, L., et al. (2023). SRSF1 is crucial for male meiosis through alternative splicing during homologous pairing and synapsis in mice. *Sci Bull* 68, 1100-1104. [10.1016/j.scib.2023.04.030](https://doi.org/10.1016/j.scib.2023.04.030).