

1 **Title:** SRSF2 is required for mRNA splicing and spermatogenesis

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8 **Running title:** SRSF2 and spermatogenesis

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51

52 **Abstract**

53 RNA splicing plays significant roles in fundamental biological activities.
54 However, our knowledge about the roles of alternative splicing and underlying
55 mechanisms during spermatogenesis is limited. Here, we report that
56 Serine/arginine-rich splicing factor 2 (SRSF2), also known as SC35, plays
57 critical roles in alternative splicing and male reproduction. Male germ
58 cell-specific deletion of *Srsf2* by *Stra8-Cre* caused complete infertility and
59 defective spermatogenesis. Further analyses revealed that deletion of *Srsf2*
60 disrupted differentiation and meiosis initiation of spermatogonia.
61 Mechanistically, by combining RNA-seq data with LACE-seq data, we showed
62 that SRSF2 regulatory networks play critical roles in several major events
63 including reproductive development, spermatogenesis, meiotic cell cycle,

64 synapse organization, DNA recombination, chromosome segregation, and
65 male sex differentiation. Furthermore, SRSF2 affected expression and
66 alternative splicing of *Stra8*, *Stag3* and *Atr* encoding critical factors for
67 spermatogenesis in a direct manner. Taken together, our results demonstrate
68 that SRSF2 has important functions in spermatogenesis and male fertility by
69 regulating alternative splicing.

70

71 **Keywords**

72 SRSF2, male infertility, spermatogenesis, alternative splicing, LACE-seq

73

74 **Introduction**

75 Spermatogenesis is a consistent and highly organized developmental process
76 by which male germline stem cells divide and differentiate to produce mature
77 spermatozoa. In mammalian testes, this process consists of three phases:
78 mitosis, meiosis and spermiogenesis (1). In the first phase of spermatogenesis,
79 mitosis is characterized by the self-renewal and differentiation of
80 spermatogonial stem cells (SSCs), which are also known as A_{single} (A_s)
81 spermatogonia. There are two outlets for A_s spermatogonia, self-renewal to
82 maintain the germline stem cell pool and differentiation to enter meiosis after
83 multiple rounds of mitotic divisions of undifferentiated spermatogonia (2). A_s
84 spermatogonia undergo unconventional mitotic processes to produce A_{paired}

85 (A_{pr}) spermatogonia and $A_{aligned}$ (A_{al}) spermatogonia(3). These spermatogonial
86 progenitors including committed A_s , A_{pr} , and A_{al} spermatogonia, are uniformly
87 identified as undifferentiated spermatogonia. Then, A_{al} spermatogonia
88 transform into type A1 spermatogonia and further go through a series of
89 mitoses to form A2, A3, A4, intermediate (In) and B spermatogonia. These
90 germ cells are called differentiating spermatogonia(4). Next, B spermatogonia
91 will divide into the pre-leptotene stage to prepare for entering meiosis which is
92 initiated by retinoic acid (RA) and STRA8(5, 6). Any mistake in the proliferation
93 and differentiation of SSCs can lead to failure of spermatogenesis, further
94 resulting in severe consequences including infertility (7).

95 Alternative splicing (AS) is one of the most important transcriptional and
96 post-transcriptional regulatory mechanisms to enrich the amount of mRNA and
97 protein isoforms from a single gene, and these different protein isoforms
98 always have different structural characteristics and functions(8-10). Generally,
99 AS occurs more frequently in highly complex organs and organisms(11-13).
100 There are numerous AS events during many developmental processes.
101 Recently, it has been shown that several proteins including RAN-Binding
102 Protein 9 (RANBP9), PTB protein 2 (Ptpb2), MORF-related gene on
103 chromosome 15 (MRG15) and Breast carcinoma amplified sequence 2
104 (BCAS2) play important roles in AS events during spermatogenesis (14-17) ,
105 indicating the importance of AS events during spermatogenesis, however, the

106 functional significance of AS in the testis remains ambiguous, and the roles
107 and regulation of AS in spermatogenesis are very limited.

108 The serine/arginine-rich splicing factors (SRs) have an exceedingly critical role
109 in the alternative splicing process of precursor RNAs. The SRs can identify the
110 splicing components of precursor RNA, then recruit and assemble
111 spliceosomes to promote or inhibit the occurrence of alternative splicing
112 events(18). There is a substantial amount of researches indicating that SRs
113 are involved in nearly every step of spliceosome assembly, genomic stability,
114 mRNA export, mRNA stability and translation(19, 20). Serine/arginine-rich
115 splicing factor 2 (SRSF2), also known as SC35, is a member of the SRs
116 protein family. It is an essential element of the nuclear structure, speckles(21).

117 Recently, several studies have suggested that SRSF2 plays important roles in
118 regulating gene transcription, mRNA stability, genomic stability, and
119 translation(22-25). Also, some findings suggested that SRSF2 may serve as a
120 therapeutic target for various diseases(26-29). SRSF2 is also expressed in
121 testis, however, its functions in male germ cells are still completely unknown.

122 Here, by crossing *Srsf2^{Floxed/Floxed}* (*Srsf2^{F/F}*) mice with *Stra8-Cre* mice to
123 generate mutant mice with specific deletion of the *Srsf2* gene in male germ
124 cells, we found that the SRSF2 knockout caused complete infertility and germ
125 cells were drastically lost during spermatogenesis. Further investigation
126 revealed that deletion of the *Srsf2* gene in germ cells affected the

127 differentiation of spermatogonia and meiosis initiation. By combining advanced
128 linear amplification of complementary DNA ends and sequencing (LACE-seq)
129 and RNA-seq with bioinformatics analysis, we unbiasedly mapped the binding
130 sites of SRSF2 at single-nucleotide resolution and revealed the changes of the
131 transcriptome and transcripts splicing in SRSF2-null testes. Our results
132 showed that SRSF2 deletion caused abnormal alternative splicing during
133 spermatogenesis. In particular, we found that SRSF2 directly regulated the
134 expressions of *Stra8*, *Stag3* and *Atr* via AS, which have pivotal roles during
135 spermatogenesis.

136

137 **Results**

138 **SRSF2 is essential for male fertility**

139 To investigate the function of SRSF2 in spermatogenesis, we first analyzed the
140 expression of SRSF2 in the testis by using the anti-SRSF2 antibody. As a
141 well-known marker of nuclear speckles, staining of cross-sections of
142 seminiferous tubules in the adult mouse testis showed that SRSF2 was
143 expressed in both germ cells and somatic cells of the testis (Figure 1A),
144 suggesting that SRSF2 may play a potential role in spermatogenesis.

145 Then, we generated *Srsf2* conditional knockout mice (referred to as *Srsf2*^{CKO})
146 by crossing *Srsf2*^{Floxed/Floxed} (*Srsf2*^{F/F}) mice in which the first and second exons
147 were floxed (30), and *Stra8-Cre* mice in which cre activity is initiated at 3 days

148 after birth(31). *Srsf2* was specifically deleted (Figure 1B), and the knockout
149 efficiency of SRSF2 was confirmed by using Western blotting. The protein
150 level of SRSF2 was significantly decreased in testes of *Srsf2*^{ckO} mice (Figure
151 1C). Thus, we successfully established male germ cell-specific knockout mice
152 for SRSF2. The breeding assays showed that the *Srsf2*^{ckO} male mice were
153 completely infertile (Figure 1D and Figure 1E). Although copulatory plugs were
154 routinely observed, no pups were obtained when adult *Srsf2*^{ckO} males were
155 mated with normal fertile females.

156 ***Srsf2* depletion causes abnormal spermatogenesis in cKO mice**

157 To determine the reasons of infertility in *Srsf2*^{ckO} male mice, we firstly
158 performed histological analyses. Compared with controls, the testes of
159 *Srsf2*^{ckO} mice were much smaller (Figure 2A). The testis weight and the testis
160 weight to body weight ratio of *Srsf2*^{ckO} mice was significantly lower (Figure 2B
161 and 2C). Then we analyzed the histology of the epididymes and testes by
162 Hematoxylin and Eosin (H&E) staining. The results showed that no mature
163 spermatozoa were found in the epididymal lumens of *Srsf2*^{ckO} mice (Figure
164 2D). The seminiferous tubules of *Srsf2*^{WT} testes contained a basal population
165 of spermatogonia, several types of spermatocytes and spermatids. However,
166 germ cells were severely reduced in number, spermatocytes and spermatids
167 were absent in the seminiferous tubules of *Srsf2*^{ckO} testes (Figure 2E). These
168 results indicated that germ cell-specific *Srsf2* knockout results in

169 spermatogenesis failure and thus male infertility.

170 To validate the above results, we performed immunofluorescent staining by
171 using lectin peanut agglutinin (PNA) and antibodies against SOX9 and MVH,
172 markers for the acrosomes of spermatids, Sertoli cells, and germ cells,
173 respectively. Immunofluorescence results indicated that there were no
174 PNA-positive signals in the seminiferous tubules of *Srsf2^{ckO}* testes and the
175 number of MVH positive signals was significantly reduced in cKO testicular
176 sections compared with those in the control (Figure 3A). Sertoli cells marker
177 SOX9 staining showed that the number and location of Sertoli cells did not
178 show an obvious change (Figure 3A).

179 Meiotic recombination and homologous chromosome synapsis are two pivotal
180 events in meiotic progression. Next we examined meiotic progression by
181 immunostaining the axial element component of the synaptonemal complex
182 with SYCP3 and double-strand break (DSB) marker γ H2AX. Similarly,
183 immunofluorescence results indicated that there were no SYCP3 positive
184 signals in the seminiferous tubules of *Srsf2^{ckO}* testes at 8-week-old and P12,
185 suggesting that meiosis initiation is disrupted after SRSF2 cKO (Figure 3B and
186 Figure 3- figure supplement 1).

187 To further identify which stage of spermatogenesis was impaired in
188 SRSF2-deficient mice, we performed immunofluorescence staining of the
189 undifferentiated spermatogonia marker promyelocytic leukaemia zincfinger

190 protein (PLZF; also known as Zbtb16) and the germ cell marker MVH (mouse
191 vasa homologue) to characterize the first wave of spermatogenesis in mice at
192 postnatal day 6 (P6), P8, P10, and P12. The results showed that nearly all the
193 germ cells were undifferentiated spermatogonia in both the *Srsf2*^{WT} and
194 *Srsf2*^{CKO} group at P6 (Figure 3C). Then the undifferentiated spermatogonia
195 proliferated to self-renew or divided into differentiating spermatogonia from P8
196 to P12 in the *Srsf2*^{WT} group. However, MVH positive signals and PLZF positive
197 signals were always nearly co-localized in the *Srsf2*^{CKO} group from P6 to P12
198 (Figure 3C). Altogether, these results indicated that the differentiation of
199 spermatogonia was affected in *Srsf2*^{CKO} mice, which may further cause the
200 failure of meiosis initiation.

201 **Changes in transcriptome and splicing of transcripts in SRSF2-null** 202 **testes**

203 According to the above-presented data, SRSF2 cKO mice displayed defects in
204 spermatogenesis. To investigate a comprehensive perspective of the
205 mechanisms of SRSF2 deletion in male germ cells, we isolated mRNA from
206 *Srsf2*^{WT} and *Srsf2*^{CKO} testes at P10 and then performed RNA sequencing
207 (RNA-seq). RNA-seq results firstly showed the reduction of *Srsf2* RNA in
208 *Srsf2*^{CKO} mice testes (Figure 4A). Clustering and principal component analysis
209 (PCA) clearly distinguished the gene expression patterns of *Srsf2*^{CKO} mice
210 testes from the *Srsf2*^{WT} mice testes (Figure 4B). A total of 977 genes were

211 upregulated, and 1742 genes were downregulated in *Srsf2*^{ckO} testes (P value
212 of <0.05, |log₂FoldChange| ≥ 0.6) (Figure 4C). Heatmap showed hierarchical
213 clustering of differential expression genes (DEGs) of *Srsf2*^{WT} and *Srsf2*^{ckO}
214 testes (Figure 4D). To obtain more comprehensive information, we then
215 performed Gene Ontology (GO) annotation. GO analysis showed that these
216 upregulated genes were involved in reproductive development, sex
217 differentiation, and gonad development (Figure 4E). Meiotic cell cycle,
218 chromosome segregation, DNA repair, DNA recombination, and cellular
219 processes involved in reproduction in multicellular organisms were significantly
220 enriched among these downregulated genes (Figure 4E). In short, these
221 differential expression genes may account for the SRSF2-null phenotypes in
222 spermatogenesis.

223 Because SRSF2 played critical roles in regulating RNA splicing, we then
224 analyzed the five different types of AS events between *Srsf2*^{WT} and *Srsf2*^{ckO}
225 testes by using the rMATS computational tool. Compared with the *Srsf2*^{WT}
226 group, a total of 320 AS events were identified as significantly changed in the
227 *Srsf2*^{ckO} group (|Diff| > 0.05, FDR < 0.001). Among these 320 changed AS
228 events, most (267) of AS events were skipped exons (SE). Moreover, there
229 were 17 alternative 3' splice sites (A3SS), 15 alternative 5' splice sites (A5SS),
230 16 mutually exclusive exons (MXE), and 5 retained introns (RI) (Figure 4F and
231 Figure 4- figure supplement 1). Together, these results suggested that SRSF2

232 is essential for RNA splicing during spermatogenesis.

233 **Binding landscape of SRSF2 proteins analysis in mouse testes**

234 To further investigate the molecular mechanisms by which SRSF2 causes the
235 failure of spermatogenesis, we performed LACE-seq analysis by using testes
236 at P10 to profile SRSF2-binding sites in testes (Figure 5A). Two independent
237 replicates with a high correlation in read counts were pooled together for the
238 following analysis (Figure 5B). Among these SRSF2 clusters, more than half of
239 them were derived from intergenic regions, while others were aligned to intron,
240 CDS (coding sequence), UTR3 (3' untranslated region), and UTR5 (5'
241 untranslated region) (Figure 5C). We also found that SRSF2 “preferentially”
242 bound to exons and enriched between 0 and 100 nt of the 5' and 3' exonic
243 sequences flanking the constitutive splice sites as revealed by analyzing the
244 distributions of SRSF2-binding peaks within 500 nucleotides (nt) upstream or
245 downstream of the constitutive splice site (Figure 5D). Among these SRSF2
246 peaks, most of them had at least one CG-rich hexamer, and more than half of
247 the peaks contained at least one top-10 motif (Figure 5E and Supplementary
248 file 2). GO analysis showed that these SRSF2-binding genes were involved in
249 the regulation of RNA splicing, reproductive development, male sex
250 differentiation, regulation of synapse organization, and regulation of
251 chromosome segregation (Figure 5F and Figure 5G). Together, these analyses
252 suggested that SRSF2 is essential for reproductive development.

253 **SRSF2 affects expression and AS of *Stra8*, *Stag3* and *Atr* in a direct**
254 **manner**

255 By combining RNA-seq data with LACE-seq identified peaks, we identified 262
256 downregulated, and 187 upregulated transcripts as direct targets of SRSF2 in
257 testes (Supplementary file 3). To obtain more comprehensive information,
258 similarly, we then performed GO annotation. GO analysis showed that both
259 significantly upregulated genes and SRSF2-binding genes were involved in
260 reproductive development, male sex differentiation, and germ cell
261 development (Figure 6A and Figure 6B). And spermatogenesis, meiotic cell
262 cycle, male gamete generation, chromosome segregation, DNA repair, and
263 DNA recombination were significantly enriched among these both significantly
264 downregulated genes and SRSF2-binding genes (Figure 6C and Figure 6D).
265 We next validated these both significantly DEGs and SRSF2-binding genes
266 which were involved in spermatogenesis by using quantitative polymerase
267 chain reaction (qPCR) to check the mRNA abundance (Figure 6E and Figure
268 6F). These data reflected that deletion of SRSF2 directly affects the
269 expression levels of critical genes involved in spermatogenesis.

270 Furthermore, we investigated the relationship of SRSF2-binding genes, DEGs,
271 and AS genes to confirm the direct targets that account for the abnormal
272 spermatogenesis after SRSF2 cKO. Venn diagram showed that 14 SRSF2
273 directly binding genes were differentially down-regulated and spliced (Figure

274 7A). These genes included *Stra8*, *Stag3*, *Atr*, *Hmga1*, and *Setx*, and all of
275 them were necessary for the male germ cell development (Figure 7B). We
276 then researched SRSF2 regulatory mechanism on the expression of *Stra8*,
277 *Stag3* and *Atr* by combining the RNA-seq with LACE-seq. The data showed
278 that the abundance of *Stra8* mRNA was decreased and the ratio of exon 2
279 skipping was increased after SRSF2 cKO. Similarly, the abundance of *Stag3*
280 mRNA was decreased and the ratio of exon 19 and 20 skipping was increased
281 after SRSF2 cKO. The abundance of *Atr* mRNA was decreased and the ratio
282 of exon 34 skipping was increased after SRSF2 cKO (Figure 7C). We also
283 performed RT-PCR and semiquantitative reverse transcription PCR to confirm
284 the above results (Figure 7D and Figure 7E). These experiments indicated that
285 SRSF2 affects the expression levels and AS of *Stra8*, *Stag3* and *Atr* in a direct
286 manner, which were critical for male germ cell differentiation and development.
287

288 **Discussion**

289 As members of the serine arginine-rich protein family, SRs which include 12
290 members in mammalian (SRSF1–12) are well-known for their regulatory
291 function of splicing(32). The first SRs identified were SRSF1 (previously known
292 as SF2/ASF) and SRSF2 (previously known as SC35) (33). SRs consist of one
293 or two RNA-recognition motifs (RRM) in the N-terminus and arginine/serine
294 amino acid sequences (RS domain) in the C-terminus(34). In general, RRM

295 can recognize RNA and determine the binding of SRs to RNA, while the RS
296 domain can regulate diverse protein-RNA and protein-protein interactions(33).
297 Like other SR splicing factors, several studies in recent years have suggested
298 that SRSF2 have important roles in regulating gene transcription, mRNA
299 stability, genomic stability, and translation (22-25). Also, some findings
300 suggested that SRSF2 may serve as a therapeutic target for various diseases
301 (26-29).

302 Recently, it has been found that RNA-binding proteins (RBPs) have important
303 functions during germline and early embryo development. As a RBP, SRSF2 is
304 also expressed in testis, however, its functions in male germ cells is still
305 completely unknown. In this study, by crossing *Srsf2*^{FF} mice with *Stra8-Cre*
306 mice to generate mutant mice, we found that SRSF2 is essential for
307 spermatogenesis and fertility in males.

308 The RBPs could serve post-transcriptional functions to determine cellular RNA
309 and protein levels. For the past few years, high throughput sequencing
310 techniques have become an increasingly essential tool for biological research.
311 RNA immunoprecipitation with sequencing (RIP-seq) and crosslinking
312 immunoprecipitation coupled with high-throughput sequencing (CLIP-seq or
313 HITS-CLIP) are two major methods to identify RBPs targets from millions of
314 cells(35, 36). There are also some modified versions, such as iCLIP, irCLIP
315 and eCLIP(37-39). Up to now, LACE-seq is the latest method developed by us,

316 which can unbiasedly map the binding sites of these RBPs at single-nucleotide
317 resolution in low-input cells (40). To gain a comprehensive perspective of the
318 mechanisms of SRSF2 depletion in male germ cells, we isolated testes from
319 wildtype mouse at P10 and systematically profiled binding landscape of
320 SRSF2 proteins by using LACE-seq. The results showed that SRSF2 proteins
321 could bind numerous genes in a direct manner. Then, our analysis showed that
322 these SRSF2-binding genes were closely involved in the regulation of RNA
323 splicing, reproductive development, male sex differentiation, regulation of
324 synapse organization, and regulation of chromosome segregation. In addition,
325 RNA-seq analysis further showed that transcriptome and splicing of transcripts
326 change in SRSF2-null testes. By combining RNA-seq and LACE-seq data, we
327 identified 262 downregulated, and 187 upregulated transcripts as direct targets
328 of SRSF2 in testes. The two omics data reflected that deletion of SRSF2
329 directly affects the expression levels of critical genes involved in
330 spermatogenesis, such as *Sycp1*, *Rnf114*, *Setx*, *Hmgb2*, *Gata4*, *Sox8*, *Amh*,
331 *Kitl*, and *Axl*.

332 Retinoic acid (RA) is an important factor of spermatogenesis, with functions on
333 spermatogonial differentiation and subsequently initiation of meiosis (41, 42).
334 The two certain targets for RA are *Stra8* and *Kit*. Several surveys indicated that
335 *Stra8* has two different roles during spermatogenesis. On one hand, under the
336 influence of RA, *Stra8* functions as a transcriptional repressor of the

337 pluripotency program during differentiation of spermatogonia. When
338 differentiating spermatogonia are near the end of their mitotic phase, *Stra8*
339 switches to the second role and acts as a transcription activator of genes
340 involved in meiosis initiation(43-45). In addition to RA signaling, *Dazl* is also
341 regarded as a regulator of meiotic initiation(46). Of particular note, in *Srsf2*^{CKO}
342 mice, the differentiation of spermatogonia and meiosis initiation were disrupted.
343 Except for *Stra8*, *Stag3* and *Atr* are crucial regulators of meiotic processes
344 during spermatogenesis(47-51). The two omics data also indicated that
345 SRSF2 affects the expression levels and AS of *Stra8*, *Stag3* and *Atr* in a direct
346 manner, which are critical for the male germ cell development process. Also,
347 we found that the reduced expression and abnormal AS of *Dazl* were indirectly
348 caused by SRSF2 deletion (Figure 7- figure supplement 1A, B, C).

349 In summary, our study has demonstrated for the first time that SRSF2 has
350 important functions in male fertility and spermatogenesis, especially in the
351 differentiation of spermatogonia and meiosis initiation. Mechanistic analyses
352 reveal that SRSF2 is essential for posttranscriptional regulation by specifically
353 adjusting the gene expression and AS in direct or indirect manners during
354 spermatogenesis. These abnormally expressed genes, such as *Stra8*, *Stag3*,
355 *Atr* and *Dazl*, caused by SRSF2 deletion finally result in the failure of
356 spermatogenesis and male infertility.

357

358

359 **Materials and Methods**

360 **Mice**

361 Mice lacking *Srsf2* in male germ cells (referred to as *Srsf2*^{CKO}) were generated

362 by crossing *Srsf2*^{Floxed/Floxed} (*Srsf2*^{F/F}) mice with *Stra8-Cre* mice. All transgenic

363 mouse lines have C57BL/6J genomic background. Genotyping PCR for *Srsf2*

364 was performed using the following primers: forward:

365 GTTATTTGGCCAAGAATCACA, and reverse: TAGCCAGTTGCTTGTCCAA.

366 The PCR conditions were as follows: 94 °C for 5 min; 35 rounds of 94 °C for

367 30 sec, 60 °C for 30 sec, and 72 °C for 30 sec; and 72 °C for 5 min.

368 Genotyping PCR for *Stra8-Cre* was performed using the following primers:

369 forward: ACTCCAAGCACTGGGCAGAA, wildtype reverse:

370 GCCACCATAGCAGCATCAAA and reverse: CGTTTACGTCGCCGTCCAG.

371 The PCR conditions were as follows: 94 °C for 5 min; 35 rounds of 94 °C for

372 30 sec, 60 °C for 30 sec, and 72 °C for 30 sec; and 72 °C for 5 min. Four

373 genotypes in the progeny, including *Srsf2*^{F/+}, *Srsf2*^{F/-}, *Srsf2*^{F/+}; *Stra8-Cre* and

374 *Srsf2*^{F/-}; *Stra8-Cre* were identified. The *Srsf2*^{F/+} male mice were used as

375 control group.

376 The mice were maintained under specific-pathogen-free (SPF) conditions and

377 housed under controlled environmental conditions with free access to water

378 and food. All animal operations were approved by the Animal Care and Use

379 Committee of the Institute of Zoology, Chinese Academy of Sciences (CAS).

380 **Antibodies**

381 β -actin antibody (mouse, sc-47778; Santa Cruz); SYCP3 (mouse, sc-74569;
382 Santa Cruz); γ H2AX (rabbit, 9718; Cell Signaling Technology, Inc.); MVH
383 (mouse, ab27591; Abcam); SOX9 antibody (rabbit, AB5535, Sigma-Aldrich);
384 PLZF antibody (goat, AF2944, R&D Systems); SFRS2 polyclonal antibody
385 (rabbit, 20371-1-AP, Proteintech); SC35 antibody (mouse, S4045,
386 Sigma-Aldrich); green-fluorescent Alexa Fluor® 488 conjugate of lectin PNA
387 (L21409, Thermo). Horseradish peroxidase–conjugated secondary antibodies
388 were purchased from Zhongshan Golden Bridge Biotechnology Co, LTD
389 (Beijing). Alexa Fluor 488–conjugated antibody, 594–conjugated antibody and
390 Alexa Fluor 647–conjugated antibody were purchased from Life Technologies.

391 **Breeding assay**

392 Males of different genotypes (8 weeks) were used for the breeding assay.
393 Each male mouse was caged with two wild-type ICR (Institute of Cancer
394 Research) females (7 weeks), and their vaginal plugs were checked every
395 morning. The number of pups in each cage was counted within a week of birth.
396 Each male underwent six cycles of the above breeding assay.

397 **Immunoblotting**

398 To prepare protein extracts, testes were homogenized in RIPA lysis buffer
399 supplemented with protease and phosphatase inhibitor cocktail (Roche

400 Diagnostics). After transient ultrasound treatment, the testis lysates were
401 incubated on ice for 30 min and then centrifuged at 4 °C, 12000 rpm for 20 min.
402 The supernatant was transferred to a new tube and quantified using a BCA
403 reagent kit (Beyotime, P0012-1). Then equal volume loading buffer was added.
404 After being boiled at 95 °C for 10 min, the protein lysates were used for
405 immunoblotting analysis. Immunoblotting was performed as described
406 previously(52). Briefly, the separated proteins in SDS-PAGE were electrically
407 transferred to a polyvinylidene fluoride membrane. After incubation with
408 primary and secondary antibodies, the membranes were scanned with
409 Bio-Rad ChemiDoc XRS+.

410 **Tissue collection and histological analysis**

411 For histological analysis, at least three adult mice for each genotype were
412 analyzed. Testes and caudal epididymides were dissected immediately
413 following euthanasia. The tissues were then fixed in Bouin's fixative (saturated
414 picric acid: 37% formaldehyde: glacial acetic acid= 15: 5: 1) overnight at room
415 temperature, dehydrated in an ethanol series, and embedded in paraffin wax.
416 Then, 5µm sections were cut with a microtome. After 48 °C overnight drying,
417 the sections were deparaffinized in xylene, hydrated by a graded alcohol
418 series and stained with Hematoxylin and Eosin for histological analysis.
419 Images were collected with a Nikon inverted microscope with a charge
420 coupled device (CCD) (Nikon, Eclipse Ti-S, Tokyo, Japan).

421 **Immunofluorescence**

422 Testes used for immunostaining were fixed in 4% paraformaldehyde (pH 7.4)
423 overnight at 4 °C, dehydrated, and embedded in paraffin. Paraffin-embedded
424 testes were cut into sections of 5µm thickness. Then, the sections were
425 deparaffinized, immersed in sodium citrate buffer (pH 6.0) and heated for 15
426 min in a microwave for antigen retrieval. After blocking with 5% donkey serum
427 albumin, sections were incubated with primary antibodies at 4 °C overnight.
428 Then the sections were incubated with an appropriate FITC-conjugated
429 secondary antibody. The nuclei were stained with DAPI. Images were captured
430 using a laser scanning confocal microscope LSM880 (Carl Zeiss, Germany).

431 **RNA extraction and gene expression analysis**

432 Total RNA was extracted from whole testes using TRNzol Universal Reagent
433 (cat. # DP424, Tiangen, China) according to the manufacturer's instructions.
434 Then reverse transcription (RT) was performed using the 5X All-In-One RT
435 MasterMix (cat. # G490, Abm, Canada). RT-PCR was performed using the
436 UltraSYBR Mixture (cat. # CW0957, Cowin Bio, China) on a LightCycler 480
437 instrument (Roche). The results were analyzed based on the $2^{-\Delta\Delta C_t}$ method to
438 calculate the fold changes. *β-actin* was used as an internal control. At least
439 three independent experiments were analyzed. All primer sequences are listed
440 in the Supplementary file 1.
441 Semiquantitative PCR experiment was carried out with primers (listed in

442 Supplementary file 1) amplifying endogenous transcripts. Then the PCR
443 products were detected on 2% agarose gels. *Gapdh* was used as an internal
444 control.

445 **RNA sequencing and data analysis**

446 Total testes samples were used from P10 *Srsf2*^{WT} and *Srsf2*^{cKO} male mice
447 according to three individual collections. One Total RNA was extracted from
448 whole testes using TRNzol Universal Reagent (cat. # DP424, Tiangen, China)
449 according to the manufacturer's instructions. The quality of RNA samples was
450 examined by NanoDrop 2000&8000 and Agilent 2100 Bioanalyzer , Agilent
451 RNA 6000 Nano Kit. The high-quality RNAs were used to prepare the libraries,
452 followed by high-throughput sequencing on an Illumina NovaSeq 6000. The
453 RNA sequencing experiment was supported by Annoroad BioLabs.

454 After trimming adaptor sequence and rRNA, the retained reads from *Srsf2*
455 control and cKO samples were aligned to mouse genome (mm9) using
456 HISAT2 with default parameters. Only non-RCR duplicate and uniquely
457 mapped reads were used for subsequent analysis. Significantly changed
458 genes were screened using DESeq2 with $|\log_2FC| > 1$ and $FDR < 0.05$.
459 Alternative splicing events were identified by rMATS with default parameters.
460 Only events with $FDR < 0.001$ and splicing difference > 0.05 were regarded as
461 significant.

462 **LACE-sequencing and data analysis**

463 Total testes samples were used from P10 WT male mice for LACE-seq.
464 LACE-seq method was performed as described recently by us (40). Briefly, the
465 samples were firstly irradiated twice with UV-C light on ice at 400 mJ. Then
466 RNA immunoprecipitation of the samples was performed. The
467 immunoprecipitated RNAs were then fragmented by MNase and
468 dephosphorylated. Then a series of steps were performed to include, reverse
469 transcription, first-strand cDNA capture by streptavidin beads, poly(A) tailing,
470 pre-PCR, IVT, RNA purification, RT, PCR barcoding and deep sequencing.
471 The adapter sequences and poly(A) tails at the 3' end of raw reads were
472 removed using Cutadapt (v.1.15) with two parameters: -f fastq -q 30,0 -a
473 ATCTCGTATGCCGTCTTCTGCTT -m 18 --max-n 0.25 --trim-n., and -f fastq -a
474 A -m 18 -n 2. Clean reads were first aligned to mouse pre-rRNA using Bowtie,
475 and the remaining unmapped reads were then aligned to the human (hg19) or
476 mouse (mm9) reference genome. For LACE-seq data mapping, two
477 mismatches were allowed (Bowtie parameters: -v 2 -m 10 --best -strata; -v 2 -k
478 10 --best -strata). Peaks were identified by Piranha with parameters: -s -b 20
479 -p 0.01. Peaks without IgG signal were selected for further usage. For motif
480 analysis, LACE-seq peaks/clusters were first extended 30 nt to 5' upstream,
481 and overrepresented hexamers in the extended sequences were identified as
482 previously described(53). The consensus motifs were generated from the
483 top-10 enriched hexamers using WebLogo.

484 **Statistical analysis**

485 All of the experiments were performed at least three times independently.
486 Paired two-tailed Student's t-test was used for statistical analysis. Data
487 analyses were carried out via GraphPad Prism 8.00 (GraphPad Software, Inc.)
488 and presented as mean \pm SEM and $P < 0.05$ (*), 0.01(**) or 0.001(***) was
489 considered statistically significant.

490 **Data availability**

491 The data sets from this study have been submitted to the NCBI Gene
492 Expression Omnibus (GEO; <https://www.ncbi.nlm.nih.gov/geo/>) under
493 accession number: GSE 206537.

494

495 **Acknowledgements**

496 We thank Dr Xiang-Dong Fu for providing the *Srsf2*^{F/F} mice. We appreciate
497 and acknowledge Shiwen Li and Xili Zhu for their technical assistance. We
498 thank all members of the Sun lab for their help and discussion. This study was
499 supported by National Key R&D Program of China (2018YFA0107701,
500 2019YFA0109900), Guangdong Basic and Applied Basic Research
501 Foundation (2021A1515111118) and the Shenzhen High-level Hospital
502 Construction Fund.

503

504 **Conflict of Interest**

505 The authors declare no conflict of interest.

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625

626 **Figure Legends**

627 **Figure 1 SRSF2 is essential for male fertility**

628 (A) Representative images of localization of SRSF2 in the control testes of
629 8-week-old mice. The DNA was stained with DAPI (Scale bar: 20 μ m). (B)
630 Schematic diagram of deletion of *Srsf2* exons 1 and 2 and generation of *Srsf2*
631 Δ allele by *Stra8-GFP Cre*-mediated recombination in male germ cells. (C)
632 Western blotting analysis of SRSF2 protein in *Srsf2*^{WT} and *Srsf2*^{ckO} total testes
633 of 8-week-old mice. β -actin was detected as an internal control. (D) Pregnancy
634 rates (%) of plugged wild-type females after mating with *Srsf2*^{WT} and *Srsf2*^{ckO}
635 8-week-old males. (E) Average litter size of plugged wild-type females after
636 mating with *Srsf2*^{WT} and *Srsf2*^{ckO} 8-week-old males. For this part, at least 3
637 mice (8-week-old) of each genotype were used for the analysis. Data are
638 presented as the mean \pm SEM. $P < 0.05$ (*), 0.01(**) or 0.001(***)).

639

640 **Figure 2 SRSF2 is required for spermatogenesis**

641 (A) The testes of *Srsf2*^{ckO} were smaller than those of the control (8-week-old,
642 the same as below). (B) Testis weight of *Srsf2*^{WT} and *Srsf2*^{ckO} 8-week-old male
643 mice (n=3). (C) Testis weight to body weight ratio of *Srsf2*^{WT} and *Srsf2*^{ckO}
644 8-week-old male mice (n=3). Data are presented as the mean \pm SEM.
645 $P < 0.05$ (*), 0.01(**) or 0.001(***). (D) Histological analysis of the caudal
646 epididymes of the *Srsf2*^{WT} and *Srsf2*^{ckO} mice. (Scale bar: 50 μ m) (E)
647 Histological analysis of the seminiferous tubules of the *Srsf2*^{WT} and *Srsf2*^{ckO}

648 mice. Scale bar: (top) 100 μm ; (bottom) 50 μm .

649

650 **Figure 3 *Srsf2* deficient germ cells fail to progress into meiosis**

651 (A) PNA-lectin histochemistry (green), SOX9 (a marker of Sertoli cells, white)

652 and MVH (a marker of germ cells, red) immunofluorescence analysis of the

653 *Srsf2*^{WT} and *Srsf2*^{CKO} 8-week-old male mice. Scale bar: (top) 50 μm ; (bottom)

654 20 μm . (B) γ H2AX (green) and SYCP3 (red) immunofluorescence analysis of

655 the *Srsf2*^{WT} and *Srsf2*^{CKO} 8-week-old male mice. Scale bar: (top) 50 μm ;

656 (bottom) 20 μm . (C) PLZF (green) and MVH (red) immunofluorescence

657 analysis of the *Srsf2*^{WT} and *Srsf2*^{CKO} male mice at P6, P8, P10 and P12. Scale

658 bar, 20 μm . In this part, at least 3 mice of each genotype were used for the

659 analysis.

660

661 **Figure 4 Transcriptome and splicing of transcripts changes in**

662 **SRSF2-null testes**

663 (A) RNA-seq results showing the reduction of *Srsf2* RNA in *Srsf2*^{CKO} mice

664 testes. Three independent RNA-seq experiments are shown. (B) *Srsf2*^{CKO}

665 groups rather than to *Srsf2*^{WT} groups are clustered together by PCA. (C)

666 Volcano plot showing transcriptome changes between *Srsf2*^{WT} and *Srsf2*^{CKO}

667 testes. (D) Heatmap showing hierarchical clustering of differential expression

668 genes of *Srsf2*^{WT} and *Srsf2*^{CKO} male mice testes. (E) GO term enrichment

669 analysis of upregulated genes. (F) GO term enrichment analysis of
670 downregulated genes. (G) The five different types of alternative splicing (AS)
671 events. The numbers of abnormal AS events were counted between *Srsf2*^{WT}
672 and *Srsf2*^{CKO} testes by rMATS software.

673

674 **Figure 5 Global landscape of SRSF2-binding sites in mouse testes as**
675 **revealed by using LACE-seq**

676 (A) Flowchart of the LACE-seq method. RBP, represents RNA-binding protein.
677 A circled B represents biotin modification. N, represents random nucleotide; V
678 represents A, G or C. IVT, represents in vitro transcription. (B) Spearman
679 correlation plot between SRSF2 LACE-seq replicates in total testes for
680 assessing the reproducibility of the data. Spearman correlation for the reads
681 counts of each sample was calculated from two replicates. (C) Genomic
682 distribution of SRSF2 binding sites in testes. CDS, coding sequence. UTR3, 3'
683 untranslated region. UTR5, 5' untranslated region. (D) Schematic analysis
684 showing the distribution of SRSF2-binding sites in the vicinity of the 5'
685 exon-intron and the 3' intron-exon boundaries (500 nt upstream and 500 nt
686 downstream of 3'SS; 500 nt upstream and 500 nt downstream of 5'SS). (E)
687 SRSF2-binding motifs identified by LACE-seq in mouse testes. (F) GO
688 enrichment map of SRSF2-binding genes. (G) Network analysis of the
689 enriched GO terms of SRSF2-specific targets.

690

691 **Figure 6 The expressions of key SRSF2-binding genes involved in the**
692 **spermatogenesis change after *Srsf2* KO**

693 (A) Correlation analysis between the RNA-seq and LACE-seq. GO analysis of
694 the significantly upregulated genes and SRSF2-binding genes. (B) Network
695 analysis of the enriched GO terms of the significantly upregulated genes and
696 SRSF2-specific targets. (C) Correlation analysis between the RNA-seq and
697 LACE-seq. GO analysis of the significantly downregulated genes and
698 SRSF2-binding genes. (D) Network analysis of the enriched GO terms of the
699 significantly downregulated genes and SRSF2-specific targets. (E)
700 Quantitative RT-PCR validation of the expression of genes involved in (B). β
701 -actin was used as the internal control. Data are presented as the mean \pm SEM.
702 $P < 0.05$ (*), 0.01 (**) or 0.001 (***). (F) Quantitative RT-PCR validation of the
703 expression of genes involved in (D). β -actin was used as the internal control.
704 Data are presented as the mean \pm SEM. $P < 0.05$ (*), 0.01 (**) or 0.001 (***).

705

706 **Figure 7 SRSF2 affects expression and alternative splicing of *Stra8*,**
707 ***Stag3* and *Atr* in a direct manner**

708 (A) Venn diagram shows the correlation among SRSF2-binding genes, DEGs,
709 and AS genes. (B) The detailed genes of SRSF2-binding, differentially
710 expressed, and AS. (C) A magnified view showing RNA-seq and LACE-seq

711 signals of the selected candidate genes. IgG, immunoglobulin G. (D)
712 Quantitative RT-PCR validation of the expression of *Stra8*, *Stag3*, and *Atr*. (E)
713 Semiquantitative RT-PCR analysis of AS patterns of the changed spliced
714 genes in *Srsf2*^{WT} and *Srsf2*^{cKO} testes at P10. PCR primers are listed in
715 Supplementary file 1. The scheme and cumulative data on percentage of the
716 indicated fragments are shown accordingly.

717

718 **Figure 3- figure supplement 1 Spermatogenesis fails to progress into**
719 **meiosis in *Srsf2* deficient germ cells at P12**

720 γ H2AX (green) and SYCP3 (red) immunofluorescence analysis of the *Srsf2*^{WT}
721 and *Srsf2*^{cKO} male mice at P12. Scale bar: (top) 50 μ m; (bottom) 20 μ m.

722

723 **Figure 4- figure supplement 1 SRSF2 regulates mRNA alternative**
724 **splicing in testes**

725 Five AS events significantly affected by deletion of SRSF2 in the testes at P10.
726 The different types of alternatively spliced events were shown.

727

728 **Figure 7- figure supplement 1 SRSF2 indirectly regulates splicing and**
729 **expression of *Dazl***

730 (A) A magnified view showing RNA-seq signals of the *Dazl* gene. (B)
731 Quantitative RT-PCR validation of the expression of *Dazl*. (C) Semiquantitative

732 RT-PCR analysis of AS patterns of the changed spliced genes in *Srsf2*^{WT} and
733 *Srsf2*^{CKO} testes at P10. PCR primers are listed in Supplementary file 1. The
734 scheme and cumulative data on percentage of the indicated fragment are
735 shown accordingly.

736

737 **Figure 1-source data 1 Actin and SRSF2 protein levels**

738 **Figure 1-source data 2 The fertility of *Srsf2*^{CKO} male mice.**

739 **Figure 2-source data 1 The testis weight of adult male mice.**

740 **Figure 6 source data 1 Quantitative RT-PCR validation of the expression**
741 **of genes.**

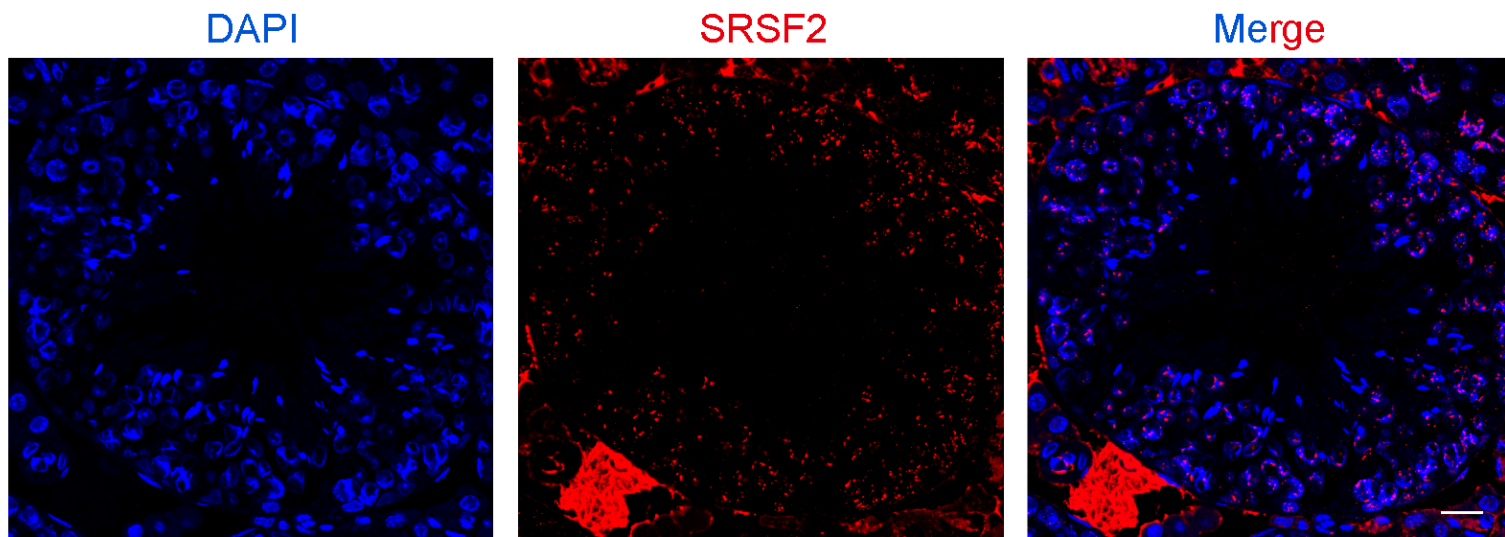
742 **Figure 7-source data 1 Quantitative RT-PCR validation of the expression**
743 **of *Stra8*, *Stag3*, and *Atr*.**

744 **Figure 7-source data 2 Semiquantitative RT-PCR analysis of AS patterns**
745 **of the changed spliced genes.**

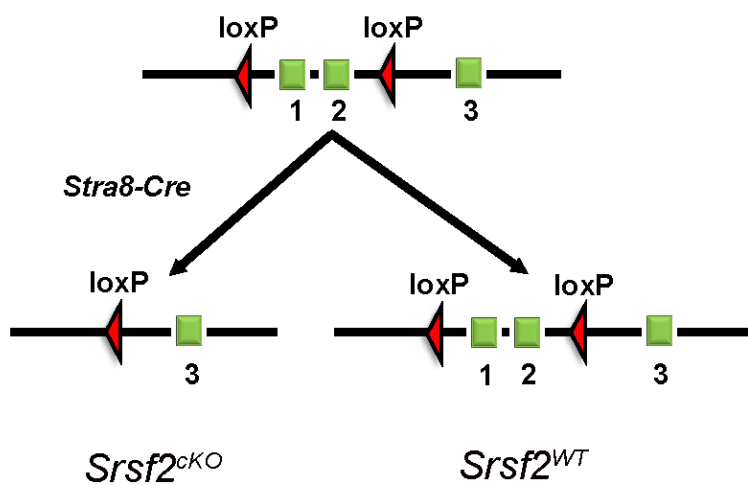
746 **Figure 7-figure supplement 1-source data1 Quantitative RT-PCR**
747 **validation of the expression of *Dazl*.**

748 **Figure 7-figure supplement 1-source data2 Semiquantitative RT-PCR**
749 **analysis of AS patterns of the changed spliced genes.**

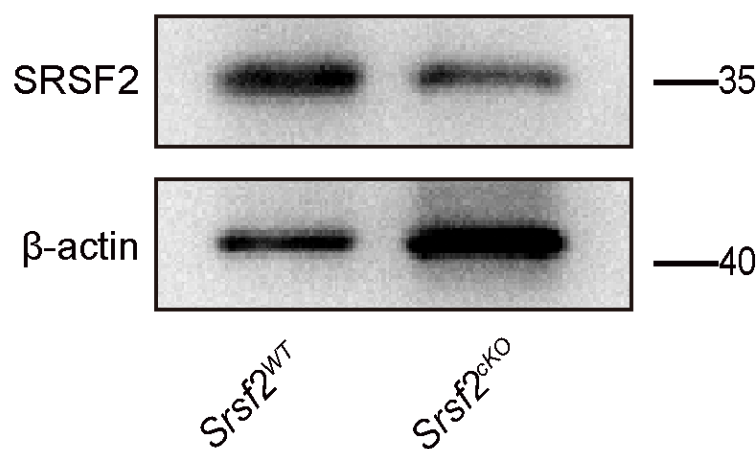
A



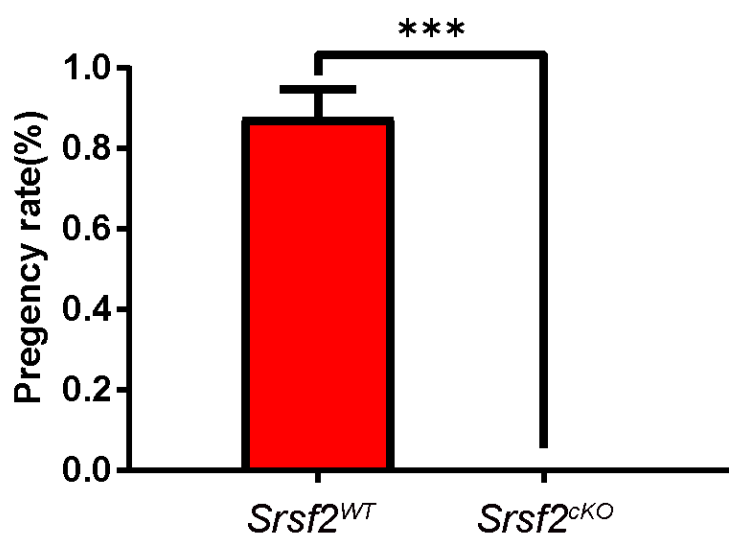
B



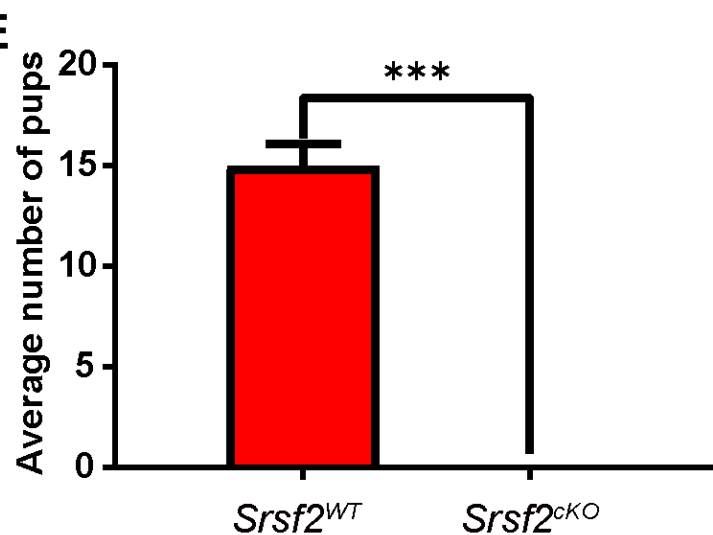
C

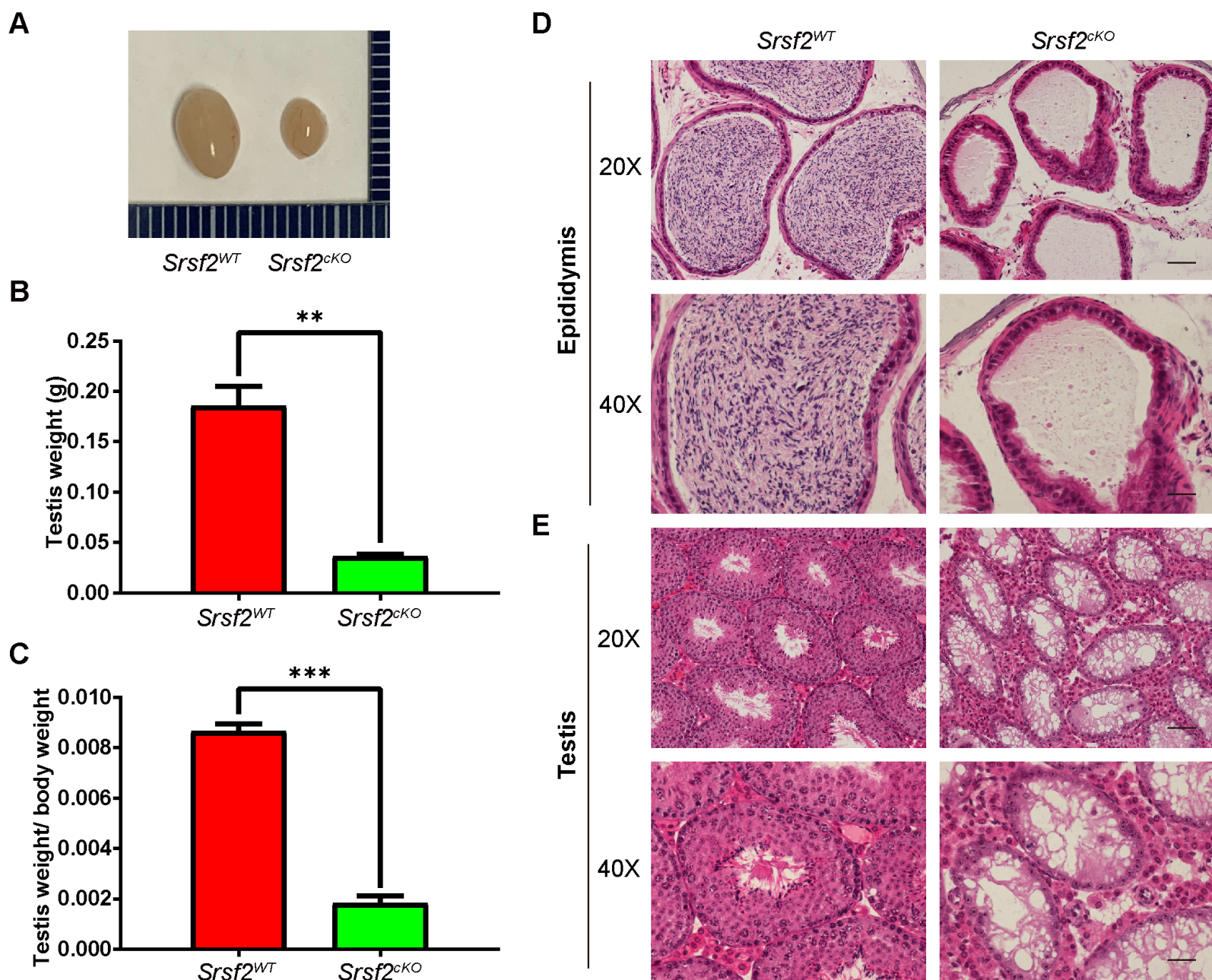


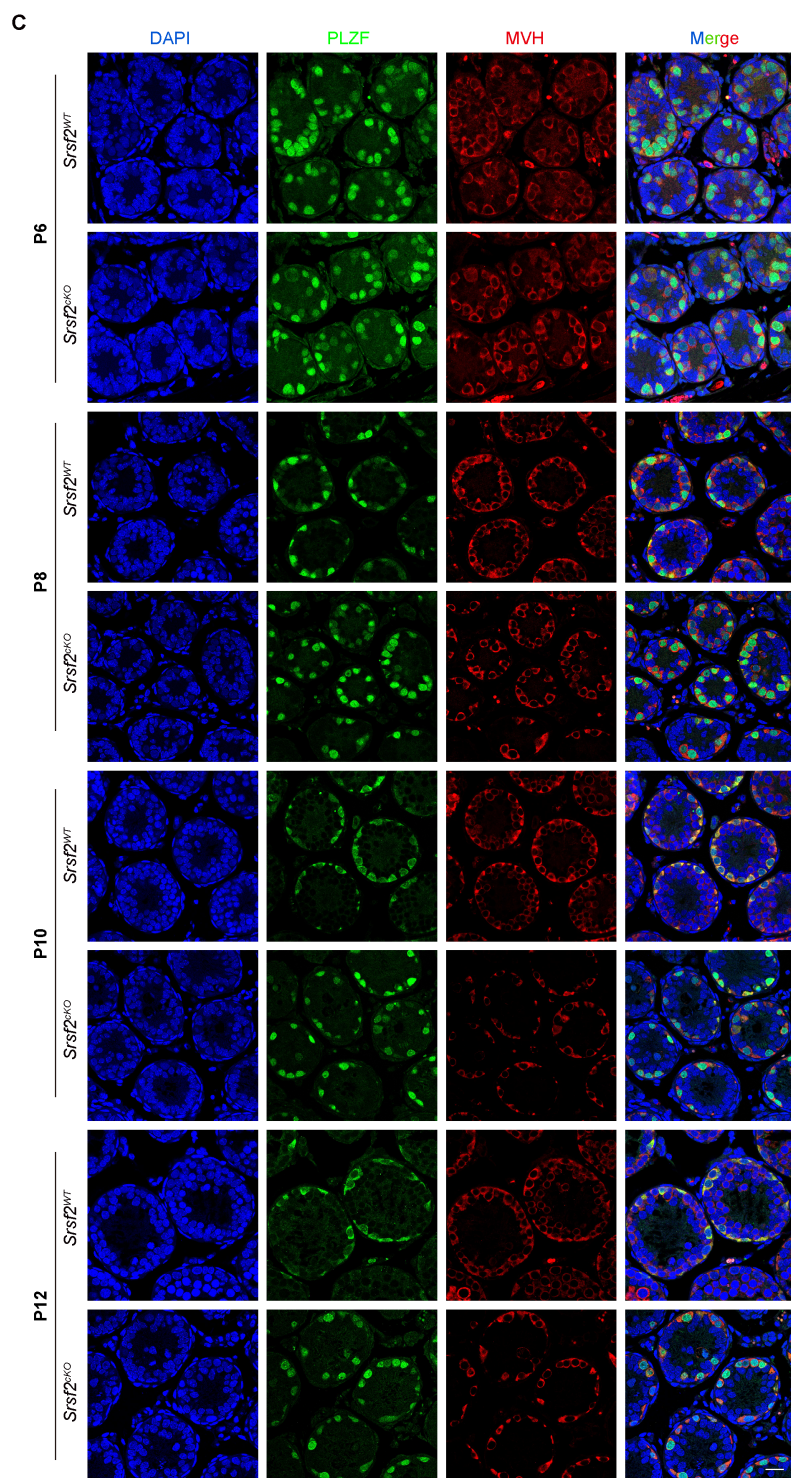
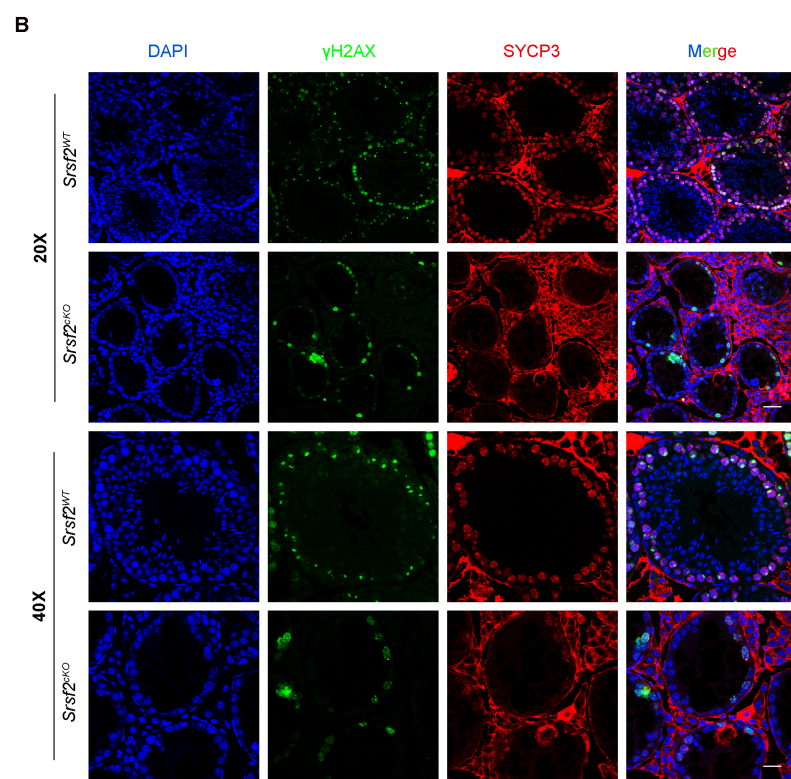
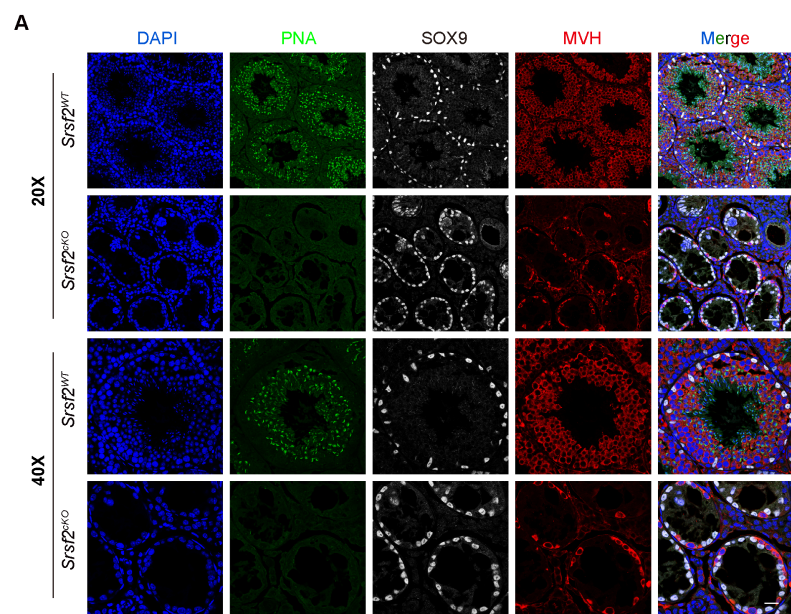
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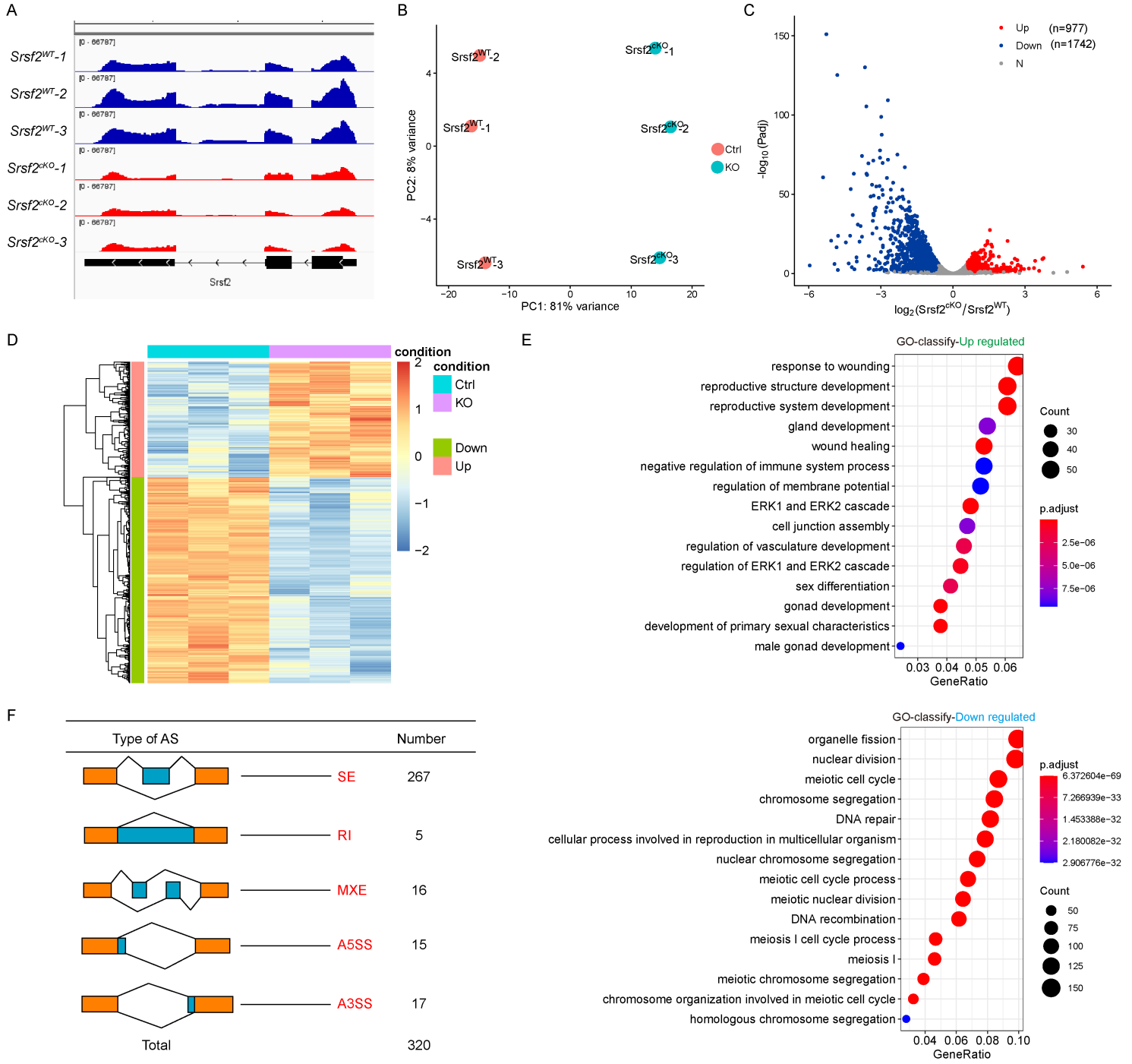


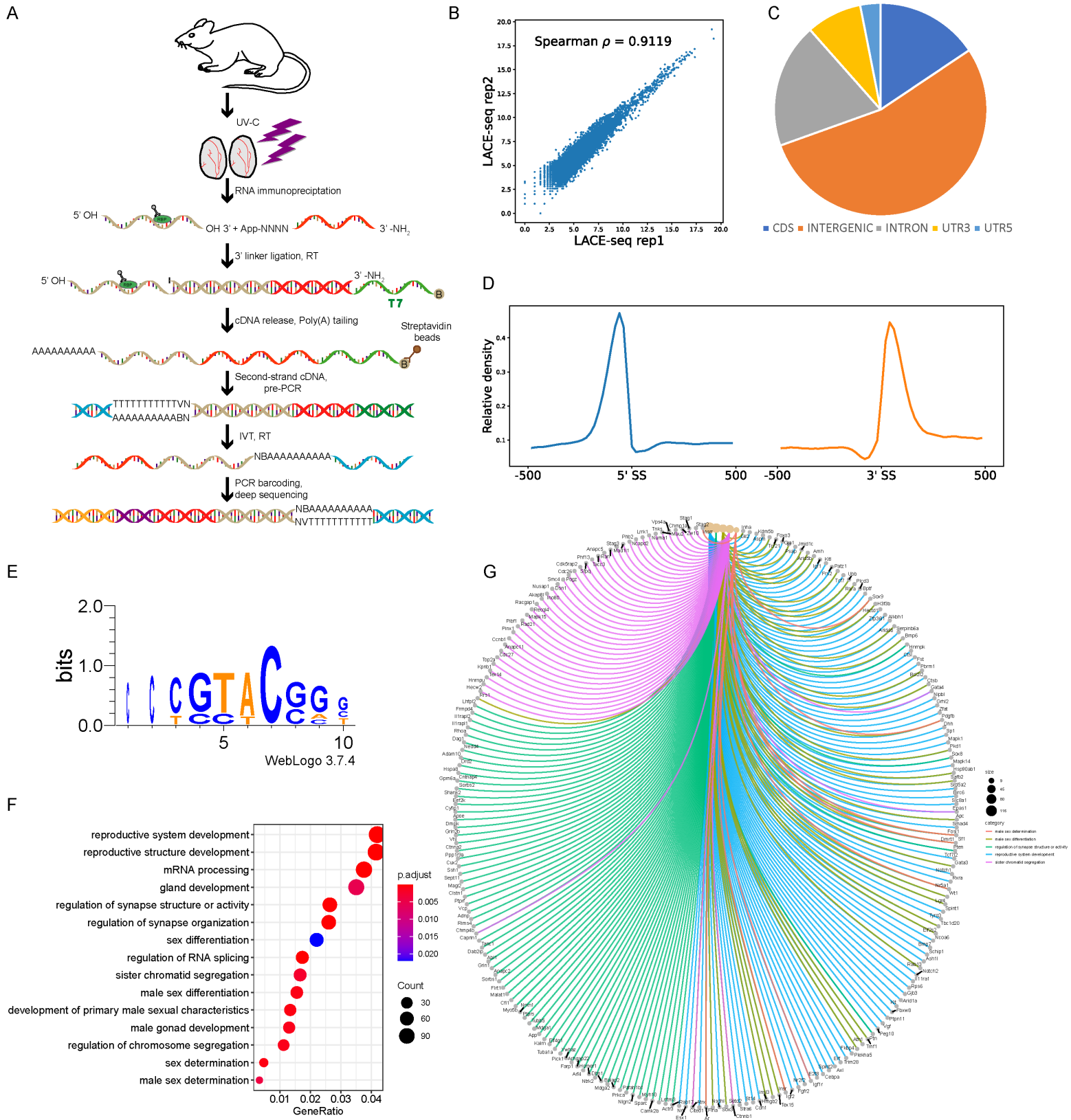
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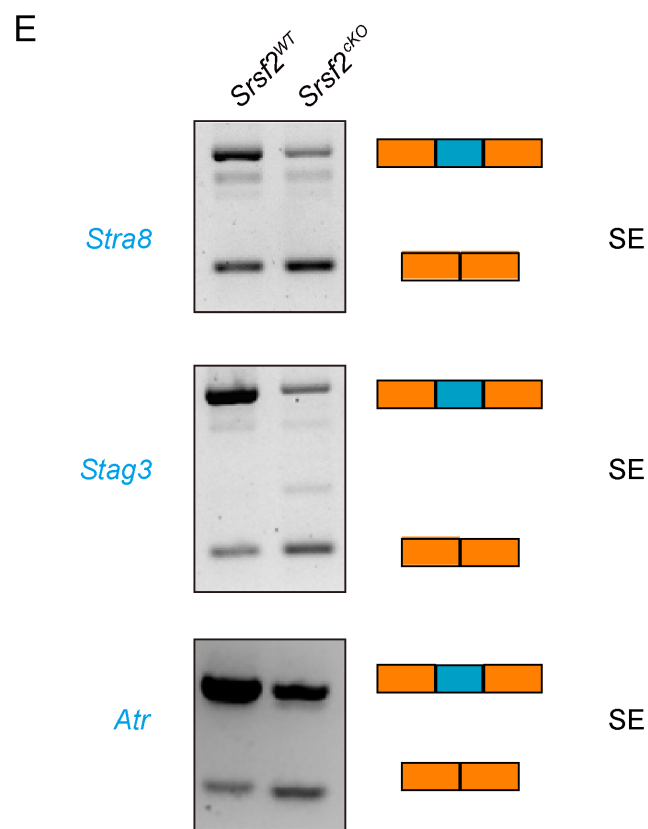
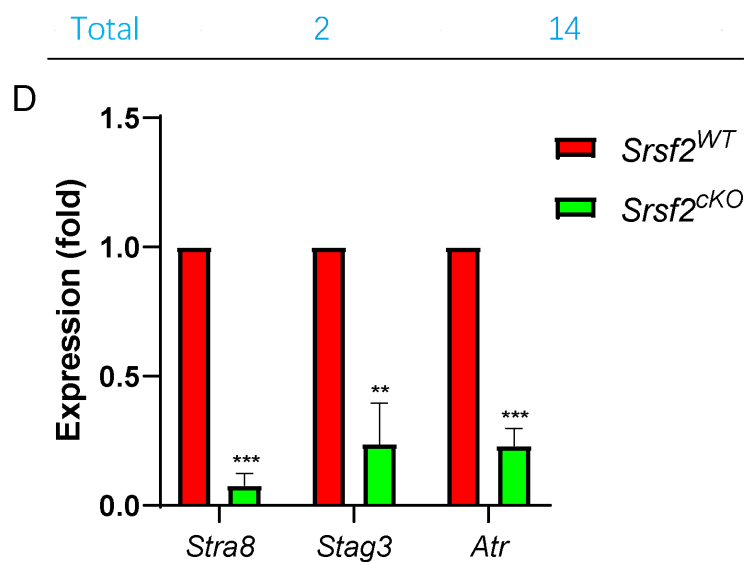
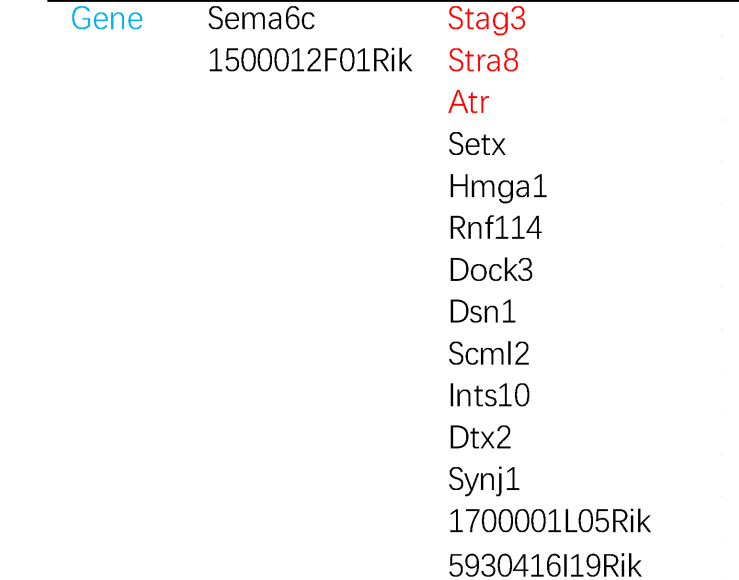
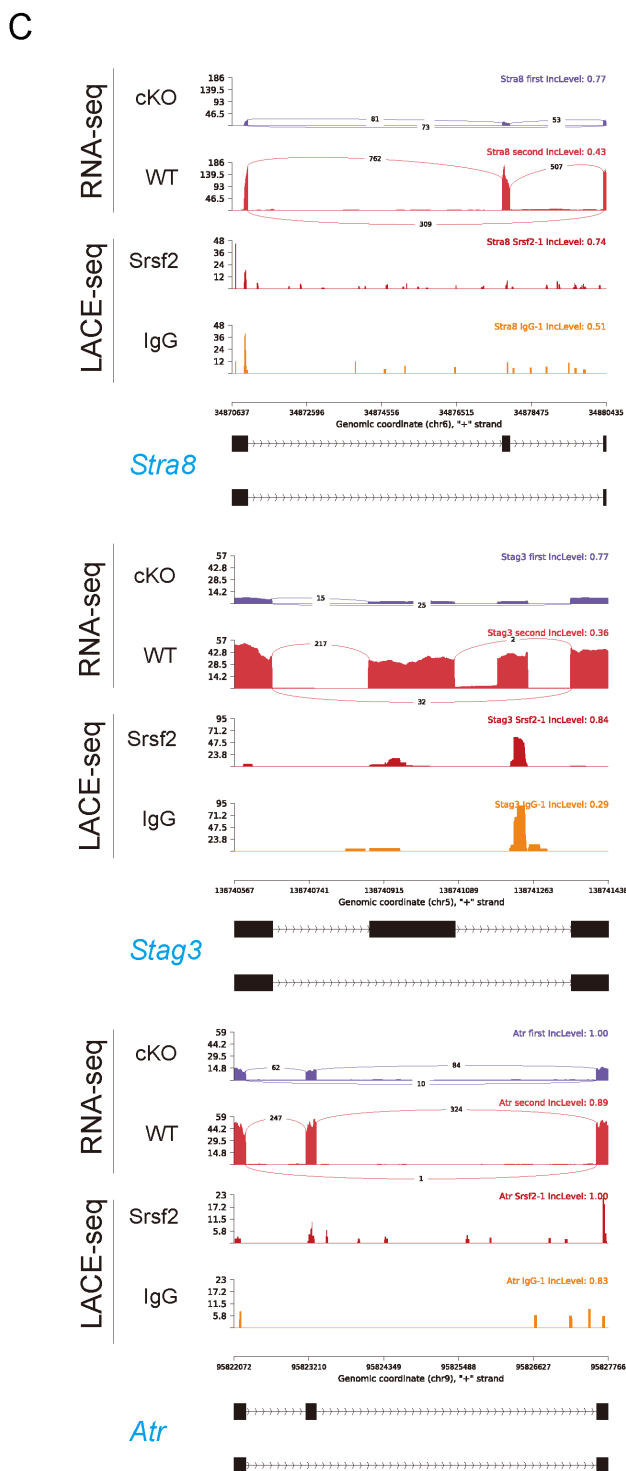
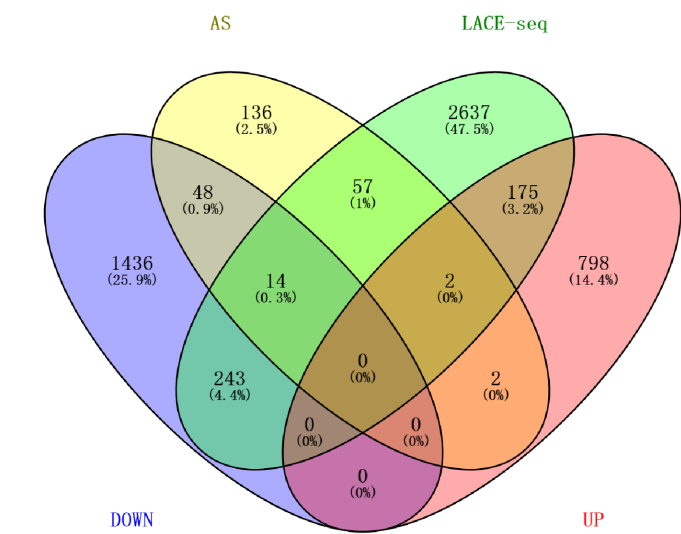




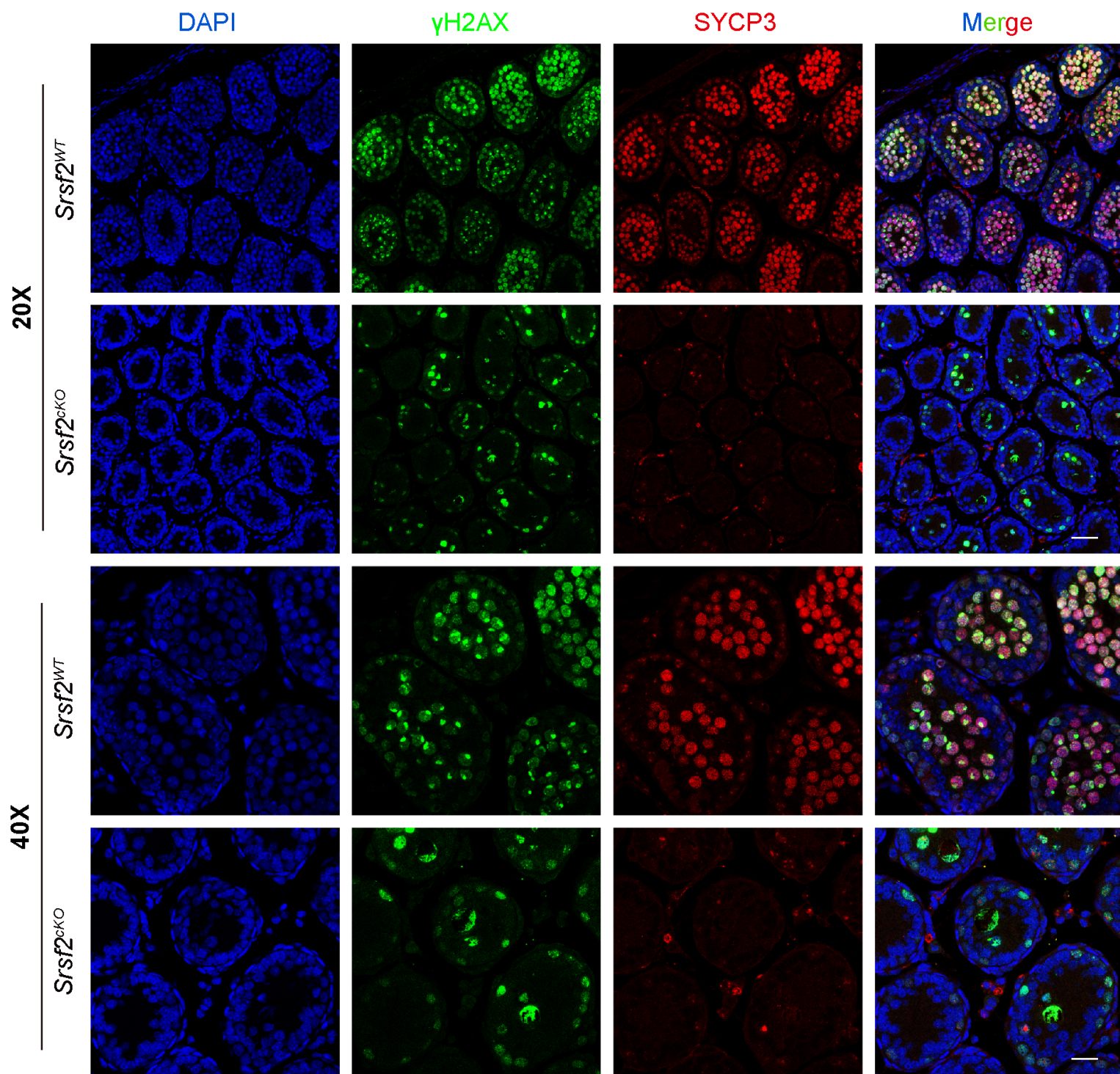








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